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AROMATASE EXPRESSION AND ENZYME ACTIVITY
IN BREAST CANCER

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in the Graduate School of The Ohio State University

By
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ABSTRACT

This research has focused on the examination of CYP19 gene expression and enzymatic activity in ovary and breast tissue. This important cytochrome P450 enzyme exhibits a complex tissue specific mechanism of CYP19 gene expression. There are multiple promoters, which allow for differential gene expression in ovary, placenta and in stromal fibroblasts. Understanding the regulation of this enzyme is critical to understanding pathological process with which it may be involved, such as estrogen dependent breast cancer.

Ovarian CYP19 gene expression was evaluated using a kinetic RT-PCR method and also Northern analysis. The aromatase inhibitor 7α-APTADD was effective in suppressing ovarian aromatase activity in normal animals, supporting the role of aromatase inhibitors as therapeutic agents in the treatment of diseases where estrogen are involved.

Evaluation of aromatase activity was performed in stromal fibroblasts grown on a collagen I matrix. Growing fibroblasts on this ECM protein altered aromatase activity while retaining a hormone responsive state. Further experiments suggest integrin VLA-2 receptor-mediated interactions, specific to stromal fibroblasts. This system is an improvement over conventional culturing systems as it takes into account any affect of the ECM on gene expression.

Patient tumor samples were evaluated for CYP19 gene expression. Comparison of CYP19 levels were made with COX-2 expression and histopathology. A correlation was observed towards increased CYP19 gene expression with increasing quartile ranking based on histopathology. This appears to correlate with COX-2 expression in these same patient samples. CYP19 expression can be regulated by prostaglandins, suggesting COX-2 expression may influence CYP19 expression in breast tumors.
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Induced Expression of Vascular Permeability Factor mRNA by Human Vascular

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgment</td>
<td>iii</td>
</tr>
<tr>
<td>Vita</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>Chapter I: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Cytochrome P450\textsuperscript{aromatase} : General Background</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Tissue Specific Expression of CYP19 Gene</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1 CYP19 Gene Expression in Placenta</td>
<td>5</td>
</tr>
<tr>
<td>1.2.2 CYP19 Gene Expression in Ovary</td>
<td>8</td>
</tr>
<tr>
<td>1.2.3 CYP19 Gene Expression in Adipose Tissue</td>
<td>10</td>
</tr>
<tr>
<td>1.2.4 Role of the Estrogen Receptor</td>
<td>13</td>
</tr>
<tr>
<td>1.3 Role of Estrogens and Aromatase in Normal Physiology</td>
<td>19</td>
</tr>
<tr>
<td>1.4 General Breast Cancer Information</td>
<td>20</td>
</tr>
<tr>
<td>1.4.1 Role of Estrogens in Breast Cancer</td>
<td>23</td>
</tr>
<tr>
<td>1.5 Clinical Management of Breast Cancer</td>
<td>26</td>
</tr>
<tr>
<td>1.5.1 Antiestrogens</td>
<td>27</td>
</tr>
</tbody>
</table>

V
Chapter 2 Evaluation of 7α-APTADD as an \textit{in vivo} Inhibitor of Aromatase

2.1 Statement of Problems and Objectives

\hspace{1cm} 2.1.1 DMBA Model of Mammary Carcinogenesis

2.2 Experimental Methods

\hspace{1cm} 2.2.1 Animal Studies

\hspace{1cm} 2.2.2 RT-PCR Kinetic Method

\hspace{1cm} 2.2.3 cDNA Probes

\hspace{1cm} 2.2.4 Production of Probes using PCR

\hspace{1cm} 2.2.5 Northern Analysis

\hspace{1cm} 2.2.6 Southern Analysis

\hspace{1cm} 2.2.7 Aromatase Activity Assay

\hspace{1cm} 2.2.8 Ovarian Homogenates

2.3 Results and Discussion

\hspace{1cm} 2.3.1 Tumor Volume of DMBA Rat Model

\hspace{1cm} 2.3.2 Ovarian Expression of CYP19 Gene

2.4 Further Evaluation of 7α-APTADD: Normal Animals

\hspace{1cm} 2.4.1 Effect of 7α-APTADD on Estrous State

\hspace{1cm} 2.4.2 Measurement of Ovarian Aromatase Activity

\hspace{1cm} 2.4.3 Measurement of Ovarian mRNA levels

\hspace{1cm} 2.4.4 Measurement of Serum Estradiol Levels

2.5 Conclusions

2.6 Cited References
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proposed Mechanism of Aromatization of Androstenedione</td>
</tr>
<tr>
<td>2</td>
<td>Structure of the Human CYP19 Gene</td>
</tr>
<tr>
<td>3</td>
<td>The Genomic Sequence Recognized by the Estrogen Receptor</td>
</tr>
<tr>
<td>4</td>
<td>Domains of the Human Estrogen Receptor</td>
</tr>
<tr>
<td>5</td>
<td>Inhibition of Tumors Growth by 7α-APTADD</td>
</tr>
<tr>
<td>6</td>
<td>Southern Blot: RT-PCR Amplification of Rat Ovary CYP19</td>
</tr>
<tr>
<td>7</td>
<td>Linear Regression of RT-PCR Kinetic Method: CYP19 Primers</td>
</tr>
<tr>
<td>8</td>
<td>Linear Regression of RT-PCR Kinetic Method: β actin Primers</td>
</tr>
<tr>
<td>9</td>
<td>Ovarian Aromatase Activity of 7α-APTADD Treated Animals</td>
</tr>
<tr>
<td>10</td>
<td>Northern Analysis of CYP19 gene expression in Rat Ovary</td>
</tr>
<tr>
<td>11</td>
<td>RIA Determination of Serum Estradiol Levels</td>
</tr>
<tr>
<td>12</td>
<td>Orientation of Cells Types and ECM in the Breast</td>
</tr>
<tr>
<td>13</td>
<td>Association of the ECM and Cytoskeleton</td>
</tr>
<tr>
<td>14</td>
<td>Comparison of Aromatase Activity using Collagen I Plates</td>
</tr>
<tr>
<td>15</td>
<td>Aromatase Activity of Individual Patients</td>
</tr>
<tr>
<td>16</td>
<td>Aromatase Activity of MCF-7 and MCF-7ADR Cells</td>
</tr>
<tr>
<td>Chapter</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>17</td>
<td>Vimentin Expression of Primary Fibroblasts and MCF-7&lt;sub&gt;ADR&lt;/sub&gt; cells</td>
</tr>
<tr>
<td>18</td>
<td>Constitutive and Induced pS2 Expression in MCF-7 cells</td>
</tr>
<tr>
<td>19</td>
<td>Thymidine Incorporation of Primary Fibroblasts</td>
</tr>
<tr>
<td>20</td>
<td>Graph of Conditioned Media Using Androstenedione</td>
</tr>
<tr>
<td>21</td>
<td>Northern Blot of Conditioned Media using Androstenedione</td>
</tr>
<tr>
<td>22</td>
<td>Northern Blot of Conditioned Media using Testosterone</td>
</tr>
<tr>
<td>23</td>
<td>Graph of Conditioned Media Using Testosterone</td>
</tr>
<tr>
<td>24</td>
<td>Effect of Conditioned Media on Individual Patients</td>
</tr>
<tr>
<td>25</td>
<td>pGEM-T transcription vector</td>
</tr>
<tr>
<td>26</td>
<td>Image from Gel Quantitation System: RT-PCR: 36B4 primers</td>
</tr>
<tr>
<td>27</td>
<td>Comparison of CYP19 to COX-2 Expression</td>
</tr>
<tr>
<td>28</td>
<td>Southern Analysis: CYP19 primers</td>
</tr>
<tr>
<td>29</td>
<td>Correlation of CYP19 Expression to Quartile Ranking</td>
</tr>
<tr>
<td>30</td>
<td>Graph of JAr cells Treated with 50nM 7α-APTADD</td>
</tr>
<tr>
<td>31</td>
<td>Graph of JAr cells Treated with 250nM 7α-APTADD</td>
</tr>
<tr>
<td>32</td>
<td>Northern Analysis of JAr cells Treated with 7α-APTADD</td>
</tr>
<tr>
<td>33</td>
<td>Northern Analysis of JAr cells grown in 10% fetal calf serum</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proteins known to be Regulated By Estrogens</td>
</tr>
<tr>
<td>2</td>
<td>RT-PCR Data from Rat Ovaries: Treated and Control Animals</td>
</tr>
<tr>
<td>3</td>
<td>Characteristics of the Rat Estrous Stage</td>
</tr>
<tr>
<td>4</td>
<td>Ovarian Aromatase Activity form 7α-APTADD Treated Animals</td>
</tr>
<tr>
<td>5</td>
<td>Data from Northern Blot of Conditioned Media using Androstenedione</td>
</tr>
<tr>
<td>6</td>
<td>Data from Northern Blot of Conditioned Media using Testosterone</td>
</tr>
<tr>
<td>7</td>
<td>Data from Patient Samples: RT-PCR: CYP19 Expression-1</td>
</tr>
<tr>
<td>8</td>
<td>Data from Patient Samples: RT-PCR: 36B4 Expression-1</td>
</tr>
<tr>
<td>9</td>
<td>Data from Patient Samples: RT-PCR: CYP19 Expression-2</td>
</tr>
<tr>
<td>10</td>
<td>Data from Patient Samples: RT-PCR: 36B4 Expression-2</td>
</tr>
<tr>
<td>11</td>
<td>Combined RT-PCR Data from 20 Breast Cancer Patients</td>
</tr>
<tr>
<td>12</td>
<td>Comparison of Quartile Ranking, CYP19 and COX-2 Expression</td>
</tr>
<tr>
<td>13</td>
<td>Estrogen Receptor and Progesterone Receptor Status</td>
</tr>
</tbody>
</table>
Chapter 1

INTRODUCTION

1.1: Cytochrome P450<sub>aromatase</sub>: General Background

Estrogen synthase (aromatase) belongs to the cytochrome P450 superfamily of enzymes and catalyzes the conversion of C<sub>19</sub> androgens to C<sub>18</sub> estrogens in the final step of the steroid biosynthetic pathway.¹ The aromatase complex is a membrane bound monoxygenase consisting of two proteins, the aromatase cytochrome P450<sub>arom</sub> and a NADPH-cytochrome P450 reductase. The aromatase portion is a heme containing protein which binds either androstenedione, testosterone or 16α-hydroxyandrostendione substrate, converting it to the respective estrogen with subsequent aromatization of the A-ring of the steroid and loss of the C-19 methyl group.² The reductase portion is a ubiquitous flavoprotein which is responsible for transferring reducing equivalents from NADPH to P450<sub>arom</sub>. Aromatase is a membrane bound enzyme, localized to the endoplasmic reticulum of the cells in which it is expressed.
The mechanism of estrogen formation occurs via three sequential steps, utilizing three moles of molecular oxygen and three moles of NADPH for each mole of substrate used (Fig. 1). The first two steps are typical cytochrome P450 oxidations with the exact mechanism of the last oxidation step still being debated. The overall conversion results in aromatization of the A-ring on the steroid with the production of formic acid and water.

Cytochrome P450rare cDNAs have been sequenced for a wide variety of vertebrates, including chicken, rat, mouse, and bovine. Human aromatase shows the highest homology to the bovine with 86% sequence identity at the amino acid level. The cytochrome P450rare shares a number of structural features common to all P450 isoforms. There is a heme binding region located near the carboxy terminus, which contains a totally conserved cysteine residue that functions as the fifth coordinating ligand of the heme iron. Very little structural information has been obtained since there are no crystal structures available for this membrane bound enzyme. Studies were based on the structure of the soluble P450cam and core conserved regions were aligned using the heme region as a point of attachment. The active site is thought to be composed of helices G, F, B and I, providing a hydrophobic pocket for substrate binding. Site directed mutagenesis studies utilizing different aromatase inhibitors have identified key residues which are necessary for full function. Mutation of these residues within the proposed binding pocket results in less effective binding of various
aromatase inhibitors. Interesting leads have been made at making an N-terminal deletion of residues 1-38. This results in a soluble isoform, which has been expressed in baculovirus with the proper 450nM UV spectrum. Although expression is low, future work may result in a crystallized mutant. Additionally, Osawa has been able to crystallize a FAB fragment from a monoclonal antibody which has good inhibitory characteristics. This may provide information which will further our understanding of the active site and general three dimensional structure of this critical P450 enzyme.

Figure 1: Proposed Mechanism of Aromatization of Androstenedione
1.2: Tissue specific expression of the CYP19 gene:

Aromatase activity has been localized to a number of human tissues and cell lines, including the syncytiotrophoblasts of the placenta, adipocytes, ovarian granulosa cells, Sertoli and Leydig cells in the male and several sites in the brain including regions of the preoptic area, hypothalamus and the limbic system. The majority of breast tumors (~60%) also express aromatase to varying degrees which has been localized to the epithelial component and also the surrounding stromal fibroblasts. The amount and type of estrogens produced at these various sites varies considerably with age, reproductive state and availability of C19 precursor. For example, placenta primarily produces large amounts of estriol by the conversion of fetally derived 16α-hydroxyandrostenedione, while granulosa cells convert locally produced testosterone to estradiol.

The regulation of aromatase activity in ovary, placenta and adipose tissue is subject to complex multifactorial regulation. It has been shown by Simpson et al. that changes in aromatase activity are closely correlated with changes at the transcriptional level. The aromatase enzyme is a product of the CYP19 gene which has been localized to chromosome 15. The gene is >70kB containing 9 translatable exons numbered 2-10. Alternative transcriptional start sites arise from the use of tissue specific promoters. Cytochrome P450 arom is unique in the P450 superfamily in that it has an untranslated exon 1 which varies depending from what tissue the transcript was
isolated. Primer extension analysis has revealed at least six distinct 5' termini corresponding to the untranslated exon 1 which share a common splice site 36bp upstream of the translational initiation site. Regardless of which promoter region was utilized, an identical protein is produced in all tissues (Fig 2).

1.2.1: Expression of P450 arom in Placenta:

Humans show a distribution of aromatase not seen with other mammals. Placentally derived aromatase is not seen in the rat or mouse. The physiological significance of extragonadal estrogen production is unknown at this time. The placenta does produce large quantities of estrogens, mostly estriol, but lack of this hormone is not catastrophic to the development of the fetus. Cases of placental sulfatase deficiencies, an X-linked metabolic disease where patients cannot hydrolyze DHAS or 16α-DHAS, results in extremely low levels of estriol. Parturition is delayed but the births are uncomplicated and children normal.

Initial screening of a human cDNA placental library revealed clones with 2 distinct 5' termini, which correspond to two alternatively expressed exons I. These have been designated exons I.1 and I.2. The major regulator in placenta is promoter PI.1 as transcripts containing exon I.1 were the major product. Exon I.1 is located at least 35kB upstream of exon II where as exon I.2 is located 9.0kB upstream of exon II which contains the translation start site. An additional exon has recently been
isolated which represent a small fraction (3%) of placental transcripts. This exon has been designated exon 2a and characterization of its promoter region has not been completed. The mRNA transcripts isolated from placenta and placentally derived cell lines is 2.9kB. A 2.5kB transcripts has been detected in ovary. These two transcripts are derived from usage of alternative polyadenylation sites.

The CYP 19 gene

Figure 2: Structure of the human CYP19 gene upstream the translational start site showing the major alternative splicing patterns. (Adapted from Simpson et.al 1995)
Factors which alter regulation of placental CYP19 expression are being uncovered. Both insulin and insulin-like growth factor-1 (IGF-1) are known to inhibit placental aromatase activity. A mechanism proposed by Nester is that the inhibitory action of insulin is a result of inositolglycan mediators acting as second messengers. This mechanism could explain how insulin results in reduction of aromatase activity even in disease states characterized by insulin resistance such as Type II diabetes mellitus. Cytokines have also been found to increase aromatase activity. Both human interleukin 1-α and β stimulate aromatase activity in culture cytotrophoblasts. Interleukin-1 of either decidual or placental origin may play a role in placental steroidogenesis.

Phorbol esters can also markedly enhance placental CYP19 expression. The JAr choriocarcinoma cell line shows a time dependent increase in both aromatase mRNA levels and aromatase activity in response to TPA (phorbol 12-myristate, 13-acetate). The stimulation by phorbol esters is usually protein kinase C dependent activation of c-fos and c-jun transcriptional factors interacting with an AP-1 site. An AP-1 like site has been found in the promoter region of the aromatase gene (PI.1). Interestingly, chloramphenicol acetyltransferase expression (CAT) functional assays have shown that the major stimulating effect of phorbol esters on aromatase expression in this human placenta choriocarcinoma cell lines is mediated through regions other than the AP-1 site. An alternative mechanism for phorbol ester action is a down regulation of protein kinase C function. With longer treatment with phorbol esters, the cell may become
relived of a negative feedback due to a growth factor, such as transforming growth factor β. It is suggested that this maybe the primary effect of phorbol ester since the increase in aromatase message and activity required treatment with TPA for 24-48 hours to see maximal effect. This is temporally consistent with degradation of protein kinase C in a cell.

1.2.2: CYP19 Gene Expression in the Ovary:

Ovarian production of estradiol is the physiological regulator of several reproductive functions. These include coordinating the ovulatory surge of luteinizing hormone (LH) and priming the endometrium for implantation. For premenopausal women, the ovary is the primary source of estrogens. The regulation of aromatase in ovary is significantly different to that seen in the placenta. A unique CYP19 transcript has been isolated from the human ovary. Utilizing primer extension and S1 nuclease protection assays, Simpson et al. has determined that the transcripts in the corpus luteum and from cultured granulosa cells are generated from promoter II, which is located about 23bp downstream of the TATA element (Fig. 2). This is in contrast to placental transcripts, which fail to hybridize to a probe specific to a region upstream of exon II. Likewise, transcripts from ovarian sources were not detected using a probe specific to exon I.1, which was used to detect placental transcripts. Extensive characterization of transcripts from multiple ovarian sources concludes that the same promoter II is used for aromatase expression throughout the ovarian cycle. It has been previously reported
by Harada et al.\textsuperscript{29, 23} that there could be a promoter switching that occurred during the follicular and luteal phases of the ovarian cycle. This does not appear to be the case. A study evaluating aromatase expression in testicular and ovarian sex cord tumors derived from pre-pubertal children with Peutz-Jeghers Syndrome show that only ovarian promoter II is utilized.\textsuperscript{28} This is consistent with expression of aromatase from normal fetal through adult ovary and testes. Thus the promoter II is a universal gonadal promoter controlling aromatase transcription from fetal to adult and throughout the neoplastic transformation. This is in contrast to breast adipose aromatase expression which shows a marked switch associated with malignant transformation. This will be discussed in detail in section 1.2.3.

Ovarian estrogen synthesis is induced by FSH resulting in aromatization of theca-derived testosterone. Binding of FSH to its receptor results in activation of adenyl cyclase and increases in intracellular cAMP.\textsuperscript{30} Mapping of the 5' region of promoter II has revealed a variety of cis-acting regulatory sequences. Most significant are two potential cAMP response element binding protein-binding sites identified at -522bp and -292bp.\textsuperscript{31} A potential site for steroidigenic factor 1 (SF-1), an orphan member of the steroid receptor family, has also been located in promoter II region, specifically, -82bp from the start site.\textsuperscript{13, 32} SF-1 mRNA is expressed in both small and large granulosa cells. Increased expression of SF-1 by FSH and cAMP correlate well with increased
P450<sub>arom</sub> expression in the developing follicle, suggesting a role of this transcriptional factor in the regulation of aromatase.<sup>33</sup>

Analysis of rat ovarian aromatase has revealed that there are multiple transcripts which are derived from promoter II usage but are alternatively spliced at the 3' end. Northern analysis identified transcripts of three sizes, 2.5, 2.2 and 1.7kB. Sequencing of the cDNA clones identified the presence of a 3' intron present in the smaller two transcripts. These smaller variants are lacking the appropriate exon and heme binding region. These nonfunctional aromatase mRNA transcripts represent a significant fraction of the total aromatase mRNA population.<sup>34</sup> The significance of these splice variants is not known.

1.2.3: <i>P450<sub>arom</sub></i> Expression in Adipose Tissue:

The principal site of estrogen production in postmenopausal women and men is adipose tissue, due to conversion of adrenal androgens. McDonald and others have observed an increase in the efficiency of extraglandular conversion with increasing age and obesity.<sup>28</sup> Adipose tissue is composed of mature adipocytes, stromal fibroblasts and a small amount of vascular endothelial cells. The aromatase activity is localized primarily within the fibroblast population,<sup>35</sup> although cultured human microvascular endothelial cells (HMVEC) can turnover androgens at low levels.<sup>36</sup>
Analysis of the stromal fibroblast transcripts revealed two previously unknown 5' termini which are unique to fibroblasts, designated I.4 and I.3. Experiments utilizing breast tissue show exclusive use of exon I.4 as the major transcripts and exon I.3 as the minor. However, primary fibroblasts grown in culture show additional 5' termini depending upon the hormonal environment to which they are exposed. Cultured fibroblasts show a marked increase in aromatase activity when grown in the presence of glucocorticoids and serum. In the absence of serum factors, dibutyl cAMP and other cAMP analogs result in an 20-50 fold increase in aromatase activity. Phorbol esters used in conjuncti on with cAMP analogs results in a further 10 fold increase aromatase activity. Epidermal growth factor (EGF) inhibits cAMP mediated induction with or without phorbol esters. Transforming growth factor beta (TGF-β) also suppresses aromatase expression in human stromal cells.

Increases in aromatase activity generally correlate with increases in levels of mRNA due to increased rate of transcription. The distribution of unique 5' termini in fibroblasts depends upon the hormonal environment of the cell. Exon I.4 is present in normal breast adipose tissue as well as in cultured fibroblasts treated with dexamethasone (9α-fluoro-16α-methyl-prednisolone) in the presence of serum. The region of the CYP19 gene upstream of exon I.4 has been shown to contain a TATA-less promoter, a glucocorticoid response element (GRE), and an interferon-gamma activating sequence (GAS). A number of cytokines, IL-11 and IL-6, in the presence
of its soluble receptor, oncostatin, (OSM) and leukemia inhibitory factor (LIF) are potent inducers of P450arom in the presence of dexamethasone. Zhao et al.\textsuperscript{39} reported the rapid phosphorylation of JAK-1 kinase following treatment with IL-11. JAK-2 kinase is constitutively phosphorylated and JAK-3 is not affected by IL-11. STAT-3 was also phosphorylated by IL-11 treatment. Nuclear extracts were capable of shifting a specific GAS sequence when stromal cells were treated with IL-11. The cytokines and the JAK/STAT signaling pathway appears to play an important role in the regulation of estrogen biosynthesis in normal breast adipose tissue.

Exon II specific transcripts are present in cultured cells treated with (Bt)$_2$cAMP ± PMA in the absence of serum. Exon I.3 is present in adipose tissue and cultured cells to a different degree under all conditions.\textsuperscript{19}

Recently several reports have observed higher levels of aromatase activity and P450arom mRNA levels in breast tumor tissue and in quadrants adjacent compared to normal tissue from unaffected quadrants of the breast.\textsuperscript{91, 92} This increased expression is associated with a switch in the major promoter that is utilized in adipose tissue. In normal tissue, promoter I.4 predominates, resulting in an exon I initiated from exon I.V sites, where as in tumor tissue, the predominant promoter used is Promoter II which is the promoter used primarily in ovary.\textsuperscript{40} In the transformation associated with
neoplasia the regulation of estrogen biosynthesis switches from one controlled primarily by glucocorticoids and cytokines to one regulated through cAMP mediated pathways.\textsuperscript{40}

1.2.4: Role of the Estrogen Receptor:

Once estrogens are synthesized by the action of aromatase, they are secreted into the bloodstream where it exists in two forms, either bound or free. The majority of estrogens are bound to a carrier protein, such as sex steroid binding globulin or albumin. When in this bound state the estrogen is inactive, and binding serves to prevent rapid metabolism and to maintain a steady state of free estradiol in the blood (about 1% free).\textsuperscript{41}

Estrogens have many physiological roles, mediated through the interaction of the estrogen receptor which is primarily nuclear in location. This receptor exists in an inactive state in the absence of ligand, associated with a variety of heat shock proteins, such as HSP90 and HSP27.\textsuperscript{42} These small proteins have highly charged helical regions that can interact with the DNA binding site on the estrogen receptor. Heat shock proteins serve to stabilize the unbound receptor and maintain an inactive state. Upon hormone binding there are a series of conformational changes that result in displacement of the heat shock proteins and in exposure of regions necessary for transcriptional activity, including dimerization regions, phosphorylation sites and the DNA binding region. Recent studies have highlighted the importance of
phosphorylation for steroid receptor function. The estrogen receptor has two transcriptional activation functions, AF-1 and AF-2, which are located in the NH$_2$ terminal A/B region and the E region (ligand binding) respectively.$^{43}$ Most phosphorylation sites are serine or threonine residues, but a few are phosphorylated on tyrosine residues. The estrogen receptor when associated with heat shock proteins is basally phosphorylated and exhibits further phosphorylation upon ligand binding.$^{44}$ Serine$^{118}$ is the main residue in the A/B region to be specifically phosphorylated and is required for full activation.$^{43}$

Most phosphorylation sites have a serine-proline motif and there is evidence that cyclin dependent kinases and mitogen-activated protein kinases are responsible for estrogen receptor phosphorylation$^{44}$ Additionally, there are a variety of splice variants of the estrogen receptor whose sequences have been characterized. These variant estrogen receptors are identical in the 5' region but shown divergence as alternatively spliced transcripts at points which mark either the exon/intron or exon 3/intron boundaries.$^{45}$ The majority of these variants are missing the E/F coding region which contains the ligand binding region. The role of these splice variants is unknown at this time but they have been observed in both normal and tumorigenic tissue.

The estrogen receptor complex recognizes regions of the DNA referred to as enhancers or response elements. These are cis-acting, orientation independent regions that
regulate transcription. In the case of the steroid family these enhancers are usually located 5' to the gene being regulated. These hormone responsive elements are specific for different steroids. The ERE is depicted in Figure 3, where N = any nucleotide.

Wilde type ERE: 5' -NNN GGTC A NNN TGACC NNN - 3'  

Figure 3: The genomic sequence recognized by the estrogen receptor complex.

The number of genes which are regulated by estrogen is rather extensive (Table 1). The general trend is upregulation of growth factors, with the ultimate consequence being cell division. In addition to the genomic affects of estrogen, reports of non-genomic affects of estrogens exist but are not clearly defined. Estrogen specifically regulates the expression of the retinoblastoma gene product by a posttranscriptional mechanism that occurs in the nucleus. Again, the result enhances growth by the negative regulation of retinoblastoma protein. In addition to regulation of transcription,
it has become evident that steroid action also involves post-transcriptional regulation of mRNA stability.\textsuperscript{47} Schoenberg has identified an estrogen inducible, Mg\textsuperscript{2+} independent ribonuclease activity from Xenopus liver which may mediate mRNA stability.\textsuperscript{48} There are additional reports of nongenomic actions of estrogen which result in increases in intracellular cAMP levels.\textsuperscript{49} In addition to the classical effect of the estrogen receptor in stimulating gene expression, the estrogen receptor is able to modulate transcription of AP-1 responsive genes without interacting directly with the DNA. Cross talk between the estrogen receptor and members of the Fos/Jun family occurs through protein-protein interactions.\textsuperscript{50}

<table>
<thead>
<tr>
<th>Proteins Known to Be Regulated by Estrogens</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Induced</strong></td>
</tr>
<tr>
<td>TGF-α</td>
</tr>
<tr>
<td>EGF</td>
</tr>
<tr>
<td>EGF receptor</td>
</tr>
<tr>
<td>pS2</td>
</tr>
<tr>
<td>IGF-II</td>
</tr>
<tr>
<td><strong>Inhibited</strong></td>
</tr>
<tr>
<td>c-erbB-2</td>
</tr>
<tr>
<td>TGF-β\textsubscript{1}</td>
</tr>
<tr>
<td>TGF-β\textsubscript{2}</td>
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</tbody>
</table>

\textbf{Table 1}: A list of some of the proteins known to be regulated by estrogens.\textsuperscript{51}
The estrogen receptor has been cloned and sequenced and it is a member of the steroid superfamily of receptors, which includes the thyroid hormones, vitamin D$_3$ and retinoic acid receptors.$^{52}$ This receptor superfamily shares areas of structural and functional homology. The 66kD estrogen receptor protein was originally divided into domains A through F based upon sequence homology of other steroid receptors (Fig 4). The least well conserved is the N-terminal A/B region which is variable in length between the family. This region has a modulatory effect on the activation of transcription.$^{53}$ The domain designated region C is a short, well conserved region which corresponds to the DNA binding region. Two DNA binding zinc fingers are formed when the cysteines tetrahedrally coordinate and interact with the major groove of DNA.$^{54}$ Specificity of different receptors is conferred by three amino acids at the base of the first zinc finger which interacts specifically with the response element. Region E is the hormone binding region which is extensively hydrophobic and offers specificity of ligand binding between the receptor family.$^{53}$ The C-terminus of the DNA binding region contains the nuclear localization sequence, necessary for the nuclear location of the receptor. Region D is thought to be a hinge region between the hormone binding and DNA binding regions. In the absence of ligand the DNA binding region is masked by the hormone binding region. It has also been suggested that region D mediates the inhibitory effects of the estrogen receptor on gene transcription.$^{55}$
Recently, a novel estrogen receptor has been isolated in human tissues, designated estrogen receptor β. The novel receptor is highly homologous with the classical estrogen receptor α and has an overlapping but non-identical tissue distribution. The DNA binding region is 98% conserved and the ligand binding region is 58% homologous. This receptor interacts with estrogens and antiestrogens and is able to modulate estrogen responsive reporter gene expression. The implications of this alternative estrogen receptor gene awaits further characterization.

Figure 4: Domain structure of the estrogen receptor. Numbers refer to genomic exons. Letters refer to functional domains.
1.3: Role of Aromatase and Estrogens in Normal Physiology

Normal reproductive development is critically regulated by a variety of hormones, including estrogens. Estrogen levels begin to rise during puberty, at around 10-12 years. The female menstrual cycle is dependent upon essential changes in estradiol levels at key moments. The beginning of the cycle is initiated by a rise in FSH which occurs in response to a decline in estradiol and progesterone in the proceeding luteal phase. Estradiol maintains follicular sensitivity to FSH by increasing the follicular content of FSH receptor. During ovulation, the dominant follicle is under synergistic influence of estradiol and FSH. The rise in estradiol is necessary for normal development of secondary sex characteristics. Estradiol is necessary for maintenance of normal pregnancy. In the ovary follicular steroidogenesis is regulated by a two cell system. The theca cells are characterized by steroidogenic activity resulting in the production of androgens in response to LH. Androgens produced by the theca cells must diffuse into the granulosa cells where they are converted into estrogens by the action of aromatase, which is under the control of FSH induced increases in cAMP.

Estrogens are also involved in regulation of other cellular functions. Estrogens have been shown to be cardioprotective, with women having markedly reduced incidence of cardiac disease until the cessation of ovarian function occurs. Estradiol reduces the level of the plasma enzyme, hepatic lipase. This enzyme catalyzes the hydrolysis of
phospholipids on the surface of lipoproteins. This results in a decrease in LDL levels
and overall increase in HLD/LDL ratios, offering cardioprotective effects.\textsuperscript{58}

Estradiol also has an effect on bone. The sex steroids have an important impact on
normal bone morphology. Estradiol participates in sexual dimorphism of the skeleton
and maintenance of mineral homeostasis.\textsuperscript{59} Withdrawal of estrogens at menopause is
associated with loss of cancellous bone and compromised trabecular architecture.
Estrogens affect bone turnover and possibly bone remodeling. The molecular targets of
estrogens have not been identified, however, estradiol's inhibitory action on expression
of certain cytokines, e.g. IL-1 and TNF-\(\alpha\) may be important.\textsuperscript{60}

1.4. General Breast Cancer Information:

Diseases of the breast constitute a wide variety of pathological states, of which only a
small fraction are malignant neoplasms. 186,000 new cases of breast cancer were
reported in 1996.\textsuperscript{61} Malignant tumors of the breast typically are of two different
origins: ductal or lobular carcinomas. The ductal tumors, being either of the non-
infiltrating type or the more aggressive invasive tumors that have progressed beyond
the basement membrane, are the single largest group of malignant tumors, constituting
65-80\% of mammary carcinomas.\textsuperscript{62} The lobular carcinomas probably arise from the
terminal ductules of the breast lobule. These type of tumors are less common than
infiltrating ductal carcinoma but patients are especially prone to bilateral occurrence.\textsuperscript{62}
These types of tumors tend to have a higher incidence of estrogen receptors. Tumors of the breast can readily be distinguished from benign lesions however, the presence of hyperplasia, papilloma or sclerosing adenosis are considered to have a 1.5 to 2 fold increase in the risk of breast cancer. Atypical hyperplasia results in a four to five fold lifelong increase in risk. The presence of fibrocystic changes are not felt to reflect an increased breast cancer risk.

Breast cancer is characterized by dysregulated proliferation, sometimes with associated loss of epithelial characteristics (epithelial-mesenchymal transition), by genomic instability (mutations, deletions, amplifications and chromosomal rearrangements) and by loss of normal compartmentalization (invasion and metastasis). Tumor progression is due to genetic instability, a characteristic central to the carcinogenic process. It is proposed that genetic instability of transformed cells is due to compromise of cell cycle checkpoint function. Breast cancer is the most prevalent malignancy in women of Western industrialized nations. Although men do contract this malignancy, it is only 1% the rate of women, leading to the conclusion that there is a profound influence of the female sex hormones. Breast cancer usually presents as a postmenopausal disease, but it is of particularly poor prognosis when detected in the premenopausal woman. The risk of developing breast cancer depends upon a variety of familial, hormonal and environmental risk factors. Epidemiological analysis has shown that early puberty and late menopause are risk factors, while early loss of
ovarian function is protective. Pregnancy early in life appears to be protective, but becomes a risk factor if delayed until 35 years or more. A diet high in fat, low in fiber and including significant alcohol consumption are felt to put a women at risk for developing breast cancer. It is important to note, however, that less than 30% of all breast cancer cases can be linked to any specific risk factor. The exceptions to this are individuals carrying the breast cancer susceptibility genes BRCA-1 and BRCA-2 which have been cloned and localized to a variety of mutations on chromosome 17 and chromosome 13, respectively. This hereditary form of breast cancer accounts for only a small percentage of all reported cases.

The incidence of breast cancer has increased slowly while the mortality rate has held steady over the last 6 decades. Currently one in eight women will develop breast cancer within her lifetime. Improved diagnostic techniques have contributed to the increase in cancer incidence, but have also helped lead to improved chances of a cure due to earlier detection. In addition to the clinical criteria for disease classification, e.g. involvement of lymph nodes, tumor size and grade, the prognosis of breast cancer depends upon the molecular characteristics of the individual tumor. Expression of estrogen and progesterone receptors are not only of good prognostic value but also predict good response to endocrine therapy. Other molecular markers can be of value when classifying tumors. Expression of the estrogen inducible pS2 protein, and expression of cathepsin D, a lysosomal protease, may more accurately reflect the
population of tumors which will respond to endocrine treatment. Additionally, overexpression of the EGFR-like tyrosine kinase erbB-2 due to gene amplification is usually associated with poor prognosis. 68 About 20-30% of invasive tumors possess amplified copies of the erbB-2 gene which result in abnormally high levels of this protein. Additionally, 10% of tumors showed over-expression not as a result of amplification but due to transcriptional dysregulation. 71 Overexpression of c-erbB-2 is rarely seen in benign breast disease.

1.4.1: Role of Estrogens in Breast Cancer:
The regulation of normal breast development and the carcinogenesis, growth and progression of tumors of the breast are dependent upon hormonal factors. A wide range of growth factors and steroid hormones have a putative role in these processes. Clinical and epidemiological data indicate that prolonged unopposed estrogenic stimulation increases the risk of both breast and endometrial cancers. The exact mechanism by which estrogens can have tumorigenic properties is unclear nor is it known whether it is a direct or indirect effect. Because aromatase is the biosynthetic enzyme producing estrogens, an aberrant expression of aromatase in the breast cancer cell or surrounding stroma may significantly influence tumor growth. 72 In the breast there appears to be a correlation between aromatase activity and the location of the tumor. The quadrant bearing the tumor tends to have the highest levels of aromatase transcripts and also shows a higher ratio of stromal fibroblasts to adipocytes. 35 Due to
higher levels of aromatase near the tumor it is likely that increases in locally produced estrogens can directly stimulate cellular proliferation and increase neoplastic growth. Although plasma levels of estrogens in postmenopausal women are very low, estrogen concentration within tumors has been shown to be significantly higher. Additionally, *in vitro* studies have shown that certain cancer cell lines (MCF-7) can be hypersensitive to very low levels (10^-12 M) of estradiol. This suggests a very important role for stromal and epithelial derived aromatase activity with regard to maintaining locally high levels of estrogen in the breast.

There are two possible non-exclusive mechanisms to account for increased aromatase transcripts in quadrants associated with the tumor: 1) inherently higher production of estrogen which encourages tumor growth or 2) tumor derived factors regulating aromatase activity in the surrounding stroma. As mentioned previously, stromal fibroblasts have a high level of aromatase activity which can *in vitro* be regulated by tumor derived factors. This suggests that there is cross-talk between breast tumor cells and the proximal adipose cells. A study evaluating aromatase expression in normal premenopausal women showed the highest level was consistently observed in the outer quadrant, the most common site of breast tumors. Coincidentally, the quadrant with the highest level of expression also had the highest local ratio of fibroblasts/adipocytes. Recently, overexpression of the aromatase gene in the mammary gland of transgenic mice resulted in the induction of hyperplasia, including
hyperplastic alveolar nodules, ductal and glandular dysplasia and nuclear abnormalities such as multinucleation and karyomegaly. These results strongly suggest that under normal circumstances, overexpression of aromatase predisposes mammary tissue topreneoplastic changes which may increase the risk of developing breast cancer.

Paracrine interactions between the transformed epithelial cells of the tumor and the normal stromal fibroblasts have been widely documented. Growth of a tumor beyond a microscopic size requires stromal-epithelial interactions in order to form an environment favorable for tumor growth. *In vitro* evaluation of breast cancer cell lines showed that they produce a variety of factors which are capable of stimulating aromatase activity in the surrounding stroma. The converse is also true, as conditioned media from stromal fibroblasts shows a marked stimulatory affect on epithelial cells. Recently, it has been determined that estrogen biosynthesis proximal to tumors is stimulated by the prostaglandin PGE$_2$. This prostaglandin is produced by tumors and is known to result in increases in cAMP levels. As mentioned previously, the primary adipose promoter found adjacent to breast tumors is promoter II, which is highly responsive to cAMP levels. Elevated aromatase levels can result in increases in estrogens which are utilized by the tumor cells to further enhance growth. This paracrine feedback loop is advantageous to the tumor until it progresses to a state of estrogen independence, at which time aromatase expression ceases to be of paramount importance.
1.5: Clinical Management of Breast Cancer:

The treatment of breast cancer falls into four major categories. Surgery, which includes mastectomy (complete removal of the breast) or lumpectomy (local removal of the tumor), remains the primary approach. Surgery is most useful when the tumors have not metastasized. Adjuvant therapy usually follows surgery to combat undetected metastasis. This can include radiation of the localized tumor margins or cytotoxic chemotherapeutic drugs (e.g. adriamycin) which are useful in the treatment of both hormone dependent and independent disease. An additional approach to treatment of the hormone dependent tumor is endocrine treatment.

Breast cancer as a disease can be divided into two major categories: hormone dependent and hormone independent. About 50% of breast tumors at the time of clinical presentation are characterized as estrogen dependent. This classification is based upon expression of estrogen receptor and estrogen regulated progesterone receptor. Hormonal therapy has been found to be useful in the majority of cases with the tumor cells have significant estrogen receptor. During the 1890's, Beatson established that there is a direct link between hormones and breast cancer by showing the oophorectomy of pre-menopausal women with breast cancer resulted in regression of secondary metastasis. This observation has lead to the development of three major types of endocrine therapy, which all have a common endpoint, to limit estrogen action.
1.5.1: Antiestrogens:

The most common form of endocrine therapy is the use of antiestrogens. Antiestrogens function by competing with estradiol for binding to the estrogen receptor. Binding of antiestrogens to the estrogen receptor interrupts the normal biological response of estradiol. It is clear that estrogens have an important role in some hormonally dependent pathological processes such as endometrial and breast cancer. Of the antiestrogens available, the most widely studied and used is a nonsteroidal compound, tamoxifen (Nolvadex®). This compound shows both antiestrogenic and partial estrogenic activity depending upon the target tissue but the exact mechanism of action of tamoxifen is poorly defined. Clinically, tamoxifen shows low toxicity and the drug is well tolerated. It has been successful in decreasing mortality by 17% in postmenopausal women and increasing disease free survival for patient with estrogen dependent breast cancer, with a clear benefit for women over the age of 50.79

One problem associated with the use of antiestrogens is that drug resistance is an inevitable consequence. This resistance occurs over time and the mechanism is unknown. The tumor progresses to a hormone independent state perhaps by constitutive growth factor expression which circumvents the need for estrogen. In MCF-7 cells, treatment for six months with tamoxifen show a switch from growth inhibition to growth stimulation. This appears to be a receptor mediated event which
depends upon the slight estrogenic activity of tamoxifen, since pure antiestrogens (ICI-164384) are capable of blocking this growth stimulation. Compounds which show pure antiestrogen activity may be more useful in the treatment of breast cancer.

1.5.2: Aromatase Inhibitors:

An additional treatment to antiestrogen therapy is the use of aromatase inhibitors. These compounds inhibit the biosynthesis of estrogens at all sites of conversion. Research to develop very potent and selective inhibitors of the cytochrome P450 enzyme has been carried out since the early 1970's. Aromatase inhibitors, both steroidal and nonsteroidal have been shown to be useful in the treatment of hormone dependent breast cancer. As with antiestrogen use, this approach of this adjuvant therapy has been found to be most useful in postmenopausal women. Postmenopausal disease is more likely to respond to endocrine therapy as more tumors express the estrogen receptor. Also since the ovary ceases to produce steroids, the major source of estrogen production is peripheral conversion of adrenal androgens in the adipose tissue of postmenopausal women.

Two classes of aromatase inhibitors have been developed, steroidal and nonsteroidal. Both classes have been effective in vitro and in vivo at reducing tumor volume in animal models and reducing serum estradiol levels. Anastrozole (Arimidex®) is a nonsteroidal aromatase inhibitor recently approved by the FDA for use in the treatment
of breast cancer in patients for whom tamoxifen has failed. This drug has very high potency, inhibiting human placental aromatase with an IC$_{50}$ of 15 nM.$^8$ Steroidal aromatase inhibitors have also been developed which have very high potency and selectivity. The majority of steroidal aromatase inhibitors are competitive inhibitors of the natural substrate, androstenedione or testosterone. Additionally, inhibitors based on the steroidal ring structure can offer information on the structural aspects of the active site of this cytochrome P450 enzyme. Many modifications of the steroid ring have been screened for activity, resulting in several lead compounds which have been further developed.$^{85, 86, 84}$ There are several extensive reviews on the subject of A and B ring modifications of steroidal inhibitors and steroidal and nonsteroidal aromatase inhibitors in general.$^{87-88}$

1.5.3: GnRH agonist:

An earlier approach to the treatment of breast cancer was the use of gonadotropin releasing hormone (GnRH) agonists. The administration of these compounds resulted in a biphasic response. There is an initial increase in the release of gonadotropins LH and FSH. With chronic treatment this increase is followed by a complete suppression of gonadotropin release. This is a result of down regulation of the GnRH receptors in the anterior pituitary gland resulting in a state of medical castration.$^{89}$ Although initially tried in both post and premenopausal women, this treatment was only effective in premenopausal women with hormone responsive tumors. This is due to the fact that
the ovary is not the major source of estrogen in postmenopausal women. The clinical response in premenopausal women was around 30-40%, similar to that seen with surgical castration.  

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Chapter 2

EVALUATION OF 7α-APTADD AS AN in vivo INHIBITOR OF AROMATASE

2.1: Statement of Problems and Objectives:

Estrogens have been determined to play a role in the progression and growth of hormone dependent breast tumors. The inhibition of estrogen biosynthesis by the use of aromatase inhibitors is one approach to lowering levels of steroid available to the tumor. This form of endocrine therapy has been successful in the treatment of hormone dependent breast cancer.

One class of aromatase inhibitors is the steroidal 7α-substituted compounds which have high affinity and show high specificity towards the aromatase enzyme. Brueggemeier et. al synthesized a competitive inhibitor with a 7α-aminophenylthiol group, 7α-APTA (1). Insertion of a double bond between the C-1 and C-2 positions of the A-ring of the steroid makes this compound an enzyme activated irreversible inhibitor of aromatase, designated 7α-(aminothiophenyl)androsta-1,4-ene-3,17-dione (7α-APTADD, 2). This compound offers a type of inhibition which involves specific
enzymatic conversion of the inhibitor to a chemically reactive species which is then capable of covalent binding to the active site of the enzyme. This inactivation is irreversible and is overcome only with concomitant protein synthesis. The parent compound, 7α-(aminothiophenyl)androst-4-ene-3,17-dione (7α-APTA, 1) has previously been shown to be effective at reducing hormone dependent tumors in the DMBA rat model.3

The objective of this research was to evaluate the ability of the aromatase inhibitor 7α-APTADD (2) to inhibit tumor growth. Additionally, the effect of this compound on the endocrine system of the animal will be determined by direct measurement of ovarian aromatase mRNA levels, measurement of protein activity and determination of blood levels of estradiol.

\[
\text{7α-APTA} \quad (1)
\]

\[
\text{7α-APTADD} \quad (2)
\]
2.1.1: DMBA Model of Mammary Carcinogenesis:

7,12-Dimethylbenz(a)anthracene (DMBA) induced mammary carcinoma in the rat is a widely used model of hormone sensitive breast cancer. The development and growth of these tumors are particularly sensitive to the stimulatory actions of estrogens and prolactin. DMBA (3) is a compound of the polyaromatic hydrocarbon family. The parent compound DMBA is relatively inactive, however activation occurs once this compound is metabolized by liver P450 enzymes. There are many sites of oxidation, with hydroxylation of the 7,12 methyl groups and the formation of epoxides being most important. Oxidation of this compound makes it an electrophilic reactant which can interact with DNA. The predominant genomic modification results in transformation of bases A:T to T:A. This mutation occurs as a consequence of interaction of DNA with the carcinogen predominantly as the N^6-7,12 dimethylbenzanthrace diol epoxide-deoxyadenosine adduct. Treatment of one dose of DMBA (15mg/kg) to 50 day old female rats results in nearly 100% tumor incidence within 3-6 months which have been characterized as adenocarcinomas. These tumors are inhibited by ovariectomy and require supplementation of estrogen to develop. The role of progestins in the development of these tumors is complex. Initial reports showed that pregnancy promotes the growth of DMBA induced tumors and co-administration of progestins and carcinogen increases the number of tumors and augments the growth rates of developing tumors. However, progestins given prior to carcinogen appear to be protective.
2.2: Experimental Methods

The majority of chemicals and regents used were from Gibco BRL, (Gaithersburg, Maryland), unless otherwise noted. 7α-APTADD used was prepared as previously described. Purity was determined by melting point and mass spectrometry. Drug was dissolved in sesame seed oil.

2.2.1: Animal Study

Sprague Dawley female rats used in the tumor study were dosed at day 50 with a single 15 mg/Kg dose of carcinogen by gastric lavage in a 1mg/ml solution of corn oil. Animals were maintained in an American Association for Accreditation of laboratory Animal Care accredited animal facility, on a 14 hour diurnal, 10 hour nocturnal photoperiod. They were fed Puriena laboratory rat chow and water ad libitum. Sprague Dawley female rats, were dosed with aromatase inhibitor or vehicle only on
alternating flanks daily. Animals were briefly anesthetized using ether during handling for vaginal cytology and injection of drug. Animals were sacrificed using CO₂ asphyxiation at the end of the study. Staining of vaginal swabs was done using Wright's stain. Briefly, 100mg of Wright's Stain powder was dissolved in 60ml methanol and then added to 940ml of Wright's Buffer (0.1M HNa₂PO₄ and 0.1M KH₂PO₄). Cycle stage was determined by careful microscopic evaluation.

2.2.2: RT-PCR Kinetic Method

The determination of ovarian CYP19 mRNA levels employed a semi-quantitative method of RT-PCR which was a modification of the kinetic method. This method is a common method of comparative analysis between two samples. This method is well suited to making relative comparisons between two samples such as was present in the DMBA rat study. Pairs of control and treated animals were evaluated for the differences in starting levels of CYP19 transcripts. There are two criteria which must be met in order for this type of analysis to be accurate. The amplification efficiency which is determined as the number of transcripts which serve as a template in each round of amplification, must be equivalent in each tube so that a constant value of E is assumed. Secondly, all data must be obtained prior to the reaction approaching the plateau phase of the reaction. In a typical PCR reaction the following relationship is present:

\[ N = N_0 (1 + E)^n \]
where \( N \) = the number of amplified molecules

\( N_0 \) = the initial number of molecules

\( n \) = The number of amplification cycles

\( E \) = The amplification efficiency

By taking the Log values of this equation the following equation can be derived:

\[
\log N = \left[ \log (1+E) \right] \times n + \log N_0
\]

If the following conditions are met, i.e., \( E \) is constant and the reaction is in the early exponential phase, then there is a linear relationship which exists when cycle number is plotted versus the log of cpm/mm\(^2\), as quantitated by the Ambis 100 proportional counter. Tube to tube efficiency of the amplification reaction is constant when the plotted lines are parallel; represented by equal slopes. The \( y \) intercept values are extrapolated from the linear regression and represent relative differences in the amount of starting template prior to amplification. This method has been shown to accurately detect 2 to 10 fold differences in the amount of starting material between two samples.\(^7,8\)

Ovaries from control animals were used to determine the range of cycles which would reproducibly be in the early exponential phase. Initial titration studies determined that cycles 18-25 were still producing exponential amplification and there was no plateau
effect observed. At cycles 26-28, a slow down was beginning to appear. Therefore, all reactions were halted after cycle 22 to ensure exponential amplification.

mRNA from one control animal and one animal treated with the aromatase inhibitor were evaluated each time. A master mix of reagents was used for the reverse transcriptase step and amplification step in order to reduce pipetting errors. The following mixture was made for the reverse transcription step: Random Primers (100pmol); dNTPs (1.0mM); MgCl₂ (2.5mM); RNase Inhibitor (40 units) 5 x RT buffer (250mM Tris-HCl, pH 8.3; 375mM KCl) and 200 units of Superscript RNase H Reverse Transcriptase (Gibco BRL) which was combined with 5μg total RNA in a final volume of 20μl. First strand cDNA synthesis was performed with a 15 minutes incubation at 23°C to allow annealing of random primers followed by incubation at 42°C for 60 minutes. A final incubation at 95°C for 5 minutes inactivates the reverse transcriptase.

An amplification master mix was prepared as follows: P450 arom specific primers (sense and anti) (100pmol each); MgCl₂ (2.5mM); 10 x PCR Buffer (200mM Tris-HCl, pH 8.4; 500mM KCl) and Taq polymerase (2.5units). 5μl of the previous described reverse transcription reaction was added to a final volume of 60μl. Samples were overlaid with mineral oil and amplified by repeated cycles at 93°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec. After cycle 18 and for subsequent cycles through 22, 10μl aliquots were removed.
and quenched immediately on ice. Samples were separated by 2% agarose electrophoresis and analyzed by Southern blotting. Quantitation of the cpm/mm² of the signal was done using the Ambis 100 Proportional Counter. Log values were used to plot linear regressions of the data. Slopes from parallel lines were used to extrapolate the y intercept, or the starting level of transcripts.

2.2.3: cDNA Probes

The sequences listed below represent the cDNA sequences that were used either as cDNA probes for Southern or Northern analysis. β Actin (#65129) was purchased from ATCC stocks. The human CYP19 full length sequence was constructed by Y. Sugimoto and was used with permission. The nearly full length P450<sub>arom</sub> from the rat was a gift from Dr. E. Lephert, Brigham Young University.

PCR primers were synthesized by Oligo’s Etc (Wilsonville, OR) which would amplify regions of each of these genes to be used as probes or for use in RT-PCR. The following were the sequences synthesized.

**CYP19**

* sense 5' - ATA CCA GGT CCT GGC TAC TG - 3'  
* anti 5' - TTG TTG TTA AAT ATG ATG CC - 3'

PCR amplification of either rat or human CYP19 cDNA utilized the same primer pair. In both cases this produced a PCR product which was 0.273kB in length. The sense primer corresponds to bases 230-249 in the human and 737-756 in the rat. The anti
sense corresponds to bases 483-503 in the human and 989-1009 in the rat. These primers were chosen to be of high homology between the human and rat and also to span two exon/intron junctions. This makes amplification of genomic DNA impossible. The human sequence was provided subcloned into a pCMV transfection vector and the provided rat sequence was subcloned into a Bluescript vector.

2.2.4: Production of Probes using PCR:

The production of probes for use in Southern and Northern analysis was carried out in the following manner. Typically, a 100μl reaction was used and the following master mix would be made up in a 500μl polypropylene PCR tube: H₂O - 47.5μl; MgCl₂ (25mM) - 12.0μl; dNTPs (250μM) - 10.0μl; primers (250nM) - 10μl; 10x PCR buffer (200mM Tris-HCl, pH 8.4; 500mM KCl) - 10μl; template plasmid in water (0.1ng/μl) - 10μl; Taq polymerase (5U/μl: Gibco BRL) - 0.5μl. The mixture was overlaid with mineral oil and amplified for 35 cycles under the following conditions: denaturation at 94°C for 1 minute, annealing at 55°C for 30 seconds followed by extension at 72°C for 30 seconds. This annealing temperature was used for amplification of β- actin and rat aromatase; temperature was not optimized for individual primer pairs. A 10μl portion of the reaction was run on a 1.0 agarose TAE gel and the band visualized by ethidium bromide staining. Typically, the reaction yielded only one band representing the sequence of interest. This DNA was ethanol precipitated and quantitated spectrophotometrically or by estimation using a mass ladder (Gibco BRL). Stock vials
of probes were aliquoted at a concentration of 2ng/μl and stored at -20°C until further use.

2.2.5: Northern Analysis

Cellular RNA was isolated by the following method, which is a modification of the method of Chomczynski and Sacchi. Media was removed and cells washed with PBS two times. Cells were lysed using a guanidine isothiocyanate buffer (guanidine isothiocyanate, 4M; sodium acetate, 30mM, pH 5.3; dithiothreitol, 0.1mM; and N-laurylsarcosine, 0.5%). Cell lysate was transferred to phase lock gels (PLG, 5 Prime-3 Prime, Boulder, CO) and DNA was sheared using a 28 gauge needle. Mixing after each addition, 3M sodium acetate, pH 5.3 (1/10 volume), water saturated phenol (equal volume) and a mixture of chloroform:isoamyl alcohol (24:1)(1/2 volume) were added and tubes were centrifuged 12 000 xg for 5 minutes. The supernatant was transferred to a fresh PLG tube and equal volumes of water saturated phenol and chloroform were added. Samples were inverted and centrifuged at 12 000 xg for 5 minutes. A final extraction was carried out using an equal volume of chloroform to remove trace amounts of phenol. Purified RNA was isolated by ethanol precipitation. Pellets were washed with 70% ethanol (3x) and resuspended in RNase-DNase free water. Quantitation of the isolated RNA was determined spectrophotometrically at 260nm and protein contamination by measurement at 280. Samples having a 260nm/280nm ratio of at least 1.7 were used in Northern analysis. Samples below 1.7
ratios were re-extracted with phenol and chloroform: isoamyl alcohol and RNA samples were stored at -70°C.

RNA formaldehyde gels (0.66M): 1.2% agarose gels were prepared using 70ml of 1 x MOPS buffer (20mM MOPS; 5mM NaOAc, pH 5.3; 1mM EDTA; pH buffer to 7.0 and filter sterilized), 5.5ml of formaldehyde. RNA (10-40μg) was dried down on a speedvac, resuspended in sample buffer (50% formamide; 2.2M formaldehyde and 1x MOPS) and run at 90V for 120 minutes. Formaldehyde gels were transferred under neutral conditions using the Turbo Blotter (Schleicher and Schuell, Keene, NH) overnight. Neutral nylon membranes were used and baked for 60 minutes at 80°C after transfer.

Nylon membranes were prehybridized at 42°C for 2-6 hours using a buffer of 50% formamide, 2.5 SSPE, 2.5x Denhart’s solution and 1% SDS and sheared salmon sperm DNA (100μg/ml). Membranes were hybridized using a similar buffer as the prehybridization with the addition of 10% polyethylene glycol. Membranes were hybridized using radiolabelled probes specific to the gene of interest, which were prepared by the random priming method (Rad Prime Labeling Kit, Gibco BRL) using 50μCi of [α-32P] dCTP (NEN). After 15-18 hours of hybridization at 42°C, the blots were washed with a final stringency of 0.1xSSPE, 1% SDS at 60°C. The Northern blots were scanned using a Phospholmager direct counter. The levels of gene expression were normalized by the expression of a control gene, β actin.
2.2.6: Southern Analysis

Southern analysis was used to confirm the identity of PCR products produced by using the previously mentioned primers. Agarose gels (1 x TAE) ranging from 0.8% to 2.5% were used to separate PCR products. Portions of the PCR reactions were mixed with loading dye and loaded directly onto the gels. Size of PCR product was determined using a 123bp DNA ladder (Gibco BRL). Gels were soaked in denaturing buffer (0.5M NaOH, 1.5M NaCl) for 30 minutes followed by neutralization buffer (0.5M Tris-HCl, pH 7.0; 1.5M NaCl) for 30 minutes followed by 20xSSPE. Overnight transfer to neutral membranes was done with the TurboBlotter apparatus. Blots were baked for 1 hour at 80°C. Prehybridization and hybridization of membranes was as detailed for Northern blots with the following changes; the temperature for both pre- and hybridization was 45°C and the final washes were done at 65°C using 0.1 xSSPE and 1% SDS. Signals were detected using the PhosphoImager (Molecular Dynamics).

2.2.7: Aromatase Assay

The ability to aromatize androgens to estrogens was measured in a variety of whole cell and homogenate systems. The rationale was the same in each case with modifications specific to each system. Generally, [1β-^3H]androst-4-ene-3,17-dione is provided as the substrate for aromatase. The conversion of this compound by the cytochrome P450 enzyme results in the production of formic acid and water. The radiolabel at the 1β position on androstenedione is incorporated into the water molecule. Thus isolation
of the aqueous fraction and subsequent scintillation counting allows for quantitation of
the amount of tritiated water produced. The tritiated water formed has been shown to
be stochiometrically equivalent to the amount of estrogen formed.\textsuperscript{10}

\subsection*{2.2.8: Ovarian Homogenates}

Ovaries from animals were procured at the time of death, snap frozen in liquid nitrogen
and were maintained at -70°C until used. Ovaries were homogenized using a Dounce
homogenizer in 0.1M Tris-HCl, pH 7.4. Reaction mixtures were 1ml in volume and
consisted of 700μl of ovarian homogenates, 80nM androst-4-ene-3,17-dione
(including 0.5μCi of [1\textsuperscript{3}H]androst-4-ene-3,17-dione (specific activity 24.1Ci/mmol),
and a regenerating system for NADPH which included NADP (1.8mM), glucose-6-
phosphate (2.85mM) and glucose-6-phosphate dehydrogenase (1 unit). Reactions were
incubated at 37°C with rapid shaking for 30 minutes. The reactions were quenched
using 5 ml of chloroform. Samples were vortexed (15 s) and centrifuged for 10 min.
The aqueous layer was removed and extracted a further two times with chloroform
(5ml). Three 0.5ml aliquots were mixed with 0.5ml of dextran coated charcoal and
placed at 4°C for 30 minutes followed by centrifugation for 10 minutes. Aliquots of
water (0.5ml) were mixed with scintillation cocktail (5.5ml) and the amount of
radioactivity determined using scintillation counting. Protein determination was done
using the BioRad protein assay with bovine serum albumin as the standard, based on
the method of the Bradford assay.\textsuperscript{11} Blanks were run simultaneously which had an

51
equivalent amount of boiled ovarian homogenate. Aromatase activity was expressed as moles $[^3]$H$_2$O formed/hour/mg protein.

2.3: Results and Discussion:

2.3.1: Tumor Volume of Animals treated with 7α-APTADD

An initial six week study was done to evaluate the ability of the steroidal aromatase inhibitor 7α-APTADD to inhibit estrogen dependent tumors in the DMBA induced rat mammary cancer model. Animals entered into the study when they had developed 1-3 tumors of 0.8-1.2 cm diameter. The drug was injected daily for six weeks at doses of 25mg/kg and 50mg/kg in sesame seed oil with control animals receiving only vehicle. Figure 5 represents the effects of 7α-APTADD (2) on the growth of tumors. (This data was collected in collaboration with Pat Ward, Ph.D.) The tumors in the control group grew steadily throughout the study, reaching an increase in total tumor volume of 400% of the original volume. Administration of 7α-APTADD (2) resulted in a marked reduction within the first week. Doses of 50mg/kg were more effective at reducing tumor size than was the 25mg/kg dose. In order to ensure that this reduction in tumor volume was a result of inhibition of estradiol production, one group of animals receiving 50mg/kg/day were co-administered 0.3µg/kg of 17β-estradiol after 3 weeks of treatment with aromatase inhibitor only. There was an increase in tumor growth once animals started to receive estradiol. It was previously demonstrated that 7α-
APTADD (2) is an effective aromatase inhibitor under *in vitro* conditions, using placental microsomal preparations. These studies show that it is also an effective inhibitor *in vivo* using the DMBA induced rat mammary cancer model.

### 2.3.2: Ovarian CYP19 Gene Expression

In addition to evaluating the effect of this aromatase inhibitor on tumor volume, it was of interest to determine what effect this drug would have on ovarian CYP19 expression. Since intact rats were used throughout the study it was likely that there would be endocrine consequences due to inhibition of estrogen biosynthesis. Ovaries from animals in the 50mg/kg/day group and control animals were harvested at the time the animals were sacrificed. Measurement of steady state mRNA levels was achieved using a semi-quantitative RT-PCR method which was a variation of the kinetic method.⁸
Figure 5: Reduction in tumor volume in DMBA rats treated with (■) 25mg/kg/day, (▲) 50mg/kg/day, (●) 50mg/kg/day 7α-APTADD +0.3μg E₂ and (♦) control animals.
Initially, eight animal pairs (control and treated) were randomly selected and were compared for levels of CYP19 transcripts. Six-week treated animals showed a consistent and significant increase in CYP19 expression over control animals. (Table 2) There was a relatively large amount of animal to animal variation within the control group of animals as well as the treatment animals, although the general trend showed an increase in CYP19 transcripts with drug treatment. To make a more accurate assessment of relative levels, RNA from four additional control and four treated (50mg/kg/day) animals was isolated and used to make an RNA master mix which was then reverse transcribed and subjected to PCR amplification. This master mix of four animals showed a 3.4 (n=4) fold increase in CYP19 transcripts. (Figure 6) Identical RT reactions were evaluated for β actin expression using the same method. The levels of β actin from this master mix of RNA showed that the expression was essentially the same from both groups. This confirms that the difference in CYP19 transcripts measured in the treatment group is not a consequence of inadvertent differences in starting levels of total RNA.
Figure 6: Representative Southern Blot of RT-PCR experiment. Aliquots of PCR mixture are removed at successive cycle numbers and run out on a gel. A radiolabeled probe specific to rat CYP19 was used to visualize the bands. Lane numbers refer to cycles number, 1) 18, 2) 19, 3) 20, 4) 21 and 5) 22. A) refers to control animals and B) refers to treated animals. (RT-PCR.TIF)
<table>
<thead>
<tr>
<th>Animal pair</th>
<th>Control (a)</th>
<th>Treated (a)</th>
<th>Relative Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.59x10⁻⁵</td>
<td>9.53x10⁻⁵</td>
<td>3.7</td>
</tr>
<tr>
<td>2</td>
<td>3.52x10⁻⁶</td>
<td>1.81x10⁻⁵</td>
<td>5.2</td>
</tr>
<tr>
<td>3</td>
<td>2.58x10⁻³</td>
<td>2.81x10⁻²</td>
<td>10.9</td>
</tr>
<tr>
<td>4</td>
<td>4.49x10⁻⁵</td>
<td>1.49x10⁻⁴</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>5.08x10⁻⁸</td>
<td>1.067x10⁻⁷</td>
<td>2.1</td>
</tr>
<tr>
<td>6</td>
<td>1.5x10⁻⁶</td>
<td>2.10x10⁻⁵</td>
<td>14.0</td>
</tr>
<tr>
<td>7</td>
<td>2.6x10⁻⁶</td>
<td>4.01x10⁻⁵</td>
<td>15.4</td>
</tr>
<tr>
<td>8</td>
<td>2.67x10⁻⁷</td>
<td>8.12x10⁻⁷</td>
<td>3.04</td>
</tr>
</tbody>
</table>

**Table 2:** RT-PCR analysis of 8 pairs of control and treated animals. Relative levels of CYP19 specific transcripts were compared using the Kinetic Method. (a) refers to the extrapolated y-intercept which corresponds to starting levels of transcripts.
Figure 7: RT-PCR analysis of rat ovary master mix RNA. Primers were specific to rat CYP19.
Figure 8: RT-PCR analysis of rat ovary master mix RNA. Primers were specific to β-actin.
One possible mechanism for the observed increase in CYP19 transcripts involves an endocrine feedback loop. In intact rats, there are critical ovarian-hypothalamus-anterior pituitary interactions. A decrease in blood levels of estradiol due to inhibition of aromatase results in increases in gonadotropin (FSH and LH) secretion due to GnRH action on the anterior pituitary. This increase in gonadotropins results in increased expression of ovarian aromatase through the utilization of promoter II which is induced by increases in cAMP. The net affect of aromatase inhibition appears to be increased expression of the aromatase message in an effort to overcome reduced estradiol levels.

There is literature precedent for this regulatory reflex occurring in rat using other aromatase inhibitors although no clear mechanism is available at this time. Reports in the literature suggest that compounds having slight androgenic activity, such as 4-hydroxyandrostenedione, do not result in increased aromatase expression. This is probably due to the negative effect of androgens on gonadotropin secretion. This DMBA induced rat mammary cancer model is more representative of premenopausal disease since estradiol production is regulated primarily by gonadotropins. Since actual aromatase enzymatic activity was not simultaneously measured in these animals it is impossible to determine if this increase in ovarian CYP19 expression was translated into increased protein and more estradiol produced. It is possible that, even if there was increased protein produced, it was immediately inhibited by the action of the 7α-
APTADD (2), thus having no real overall effect. There were no animals which showed total regression with the use of this aromatase inhibitor. It is possible that this is due to the fact that aromatase may be markedly reduced but the ovaries continually try to overcome this inhibition with the production of new protein.

2.4: Further Evaluation of 7α-APTADD (2):

Based upon the results of this preliminary study, a more detailed examination of the endocrine effects of 7α-APTADD was necessary. In addition to looking at mRNA expression, it was necessary to measure enzymatic activity, serum blood levels and evaluate the estrus state of the animals at the time of treatment.

2.4.1 Effect of 7α-APTADD (2) on the estrus stage of animals:

The estrus stage of 60 day old normal animals was evaluated. Female cyclic activity is manifested by changes in the vaginal cytology and behavioral changes. The regular cycle is four days in length, consisting of proestrus, estrus, metestrus and diestrus. The four cycles are summarized in Table 3.

Vaginal cytology was stained using Wright’s Stain and stage determined using microscopic evaluation. It was possible to stage animals and group them according to cycle. Animals were evaluated for 3 successive cycles prior to the administration of drug. Although there was an obvious 4 day pattern associated with this cycle, only two
of the four days were associated with distinct cytology that we could determine. Therefore, identifying the exact cycle for a particular animal could be problematic. This is a critical parameter since aromatase activity and mRNA levels are known to change with cyclic pattern. In normal rats, the highest level of aromatase is found in proestrus and this decreases sharply after estrus at ovulation. Aromatase activity was lowest at metestrus. This level of activity was closely correlated with mRNA levels also showing a cyclic pattern of expression. It was possible that administration of the aromatase inhibitor would interrupt the normal cyclic pattern of the animal. Therefore, animals were dosed in a manner that control and treated animals would be sacrificed while in diestrus. This would minimize the possibility that treatment of the animals would send them into artificial diestrus which would have chronic low levels of aromatase mRNA levels and activity.

Normal animals were treated for 14 days with 50mg/kg/day of 7α-APTADD (2) and vaginal cytology was monitored throughout the study. There was no apparent effect on vaginal cytology due to 7α-APTADD administration and animals continued to cycle normally throughout the 14 day study. However, it was not possible to have the animals exactly synchronized at the time of tissue procurement.
<table>
<thead>
<tr>
<th>Cycle Stage</th>
<th>Characteristic Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>proestrus</td>
<td>Vagina has increasing amounts of cornified superficial epithelial cells. The rounded cytoplasmic borders become replaced by straight edges. Nuclei become pyknotic and may disappear.</td>
</tr>
<tr>
<td>estrus</td>
<td>Vagina has more cornified epithelial cells, straight cytoplasmic borders and late pyknotic nuclei. Various bacteria are present. Cellular debris.</td>
</tr>
<tr>
<td>metestrus</td>
<td>Neutrophils are abundant and small round noncornified, epithelial cells reappear.</td>
</tr>
<tr>
<td>diestrus</td>
<td>Non-cornified round epithelial, few neutrophils and minimal debris</td>
</tr>
</tbody>
</table>

Table 3: Characteristics of Rat Estrous Stage

2.4.2: Measurement of Ovarian Aromatase Activity:
Ovarian homogenates were prepared from frozen ovaries from control and treated animals from time points 1,3,5,7 and 14 days. The activity of the ovarian homogenates
was determined by a radiometric assay developed by Sitteri and Thompson in which the tritium from $[1\beta^3\text{H}]$androst-4-ene-3,17-dione was released as tritiated water during aromatization.$^{10}$ Measurement of activity was compared to blanks which contained no ovarian homogenates. Table 4 represents the mean ($n=2$) measured ovarian aromatase activity for control and treated animals for 1,3,5,7 and 14 days. Values are represented as picomoles of tritiated water produced/milligram of protein/hour of incubation. Protein determination was carried out using the Biorad Protein Determination Reagent.$^{11}$

A marked and nearly complete suppression (compared to background values) of ovarian aromatase activity was observed after 24 hours of 50mg/kg/day dosing of 7α-APTADD which was sustained throughout the duration of the study. This animal data is consistent with the in vitro microsomal studies with this compound which showed excellent inhibition of placental aromatase. Additionally, this is the type of inhibition we would expect to see based on the reduction in tumor volume that was observed in the DMBA rat model system.
<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Animal 1</th>
<th>Animal 2</th>
<th>Animal 3</th>
<th>mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol/mg/hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>0.322</td>
<td>0.985</td>
<td>1.76</td>
<td>1.022</td>
<td>0.415</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>0.054</td>
<td>0.071</td>
<td>0.110</td>
<td>0.078</td>
<td>0.017</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>0.422</td>
<td>0.623</td>
<td>0.373</td>
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<td>0.172</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>0.0804</td>
<td>0.0388</td>
<td>0.0361</td>
<td>0.0515</td>
<td>0.014</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>0.565</td>
<td>0.595</td>
<td>1.282</td>
<td>0.814</td>
<td>0.234</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>0.054</td>
<td>0.046</td>
<td>0.040</td>
<td>0.047</td>
<td>0.004</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>0.120</td>
<td>0.298</td>
<td>0.425</td>
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<tr>
<td></td>
<td>Treated</td>
<td>0.048</td>
<td>0.048</td>
<td>Sample Lost</td>
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<td>N.A.</td>
</tr>
<tr>
<td>14</td>
<td>Control</td>
<td>0.939</td>
<td>1.02</td>
<td>0.557</td>
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<td>Treated</td>
<td>0.048</td>
<td>0.041</td>
<td>0.087</td>
<td>0.059</td>
<td>0.0143</td>
</tr>
</tbody>
</table>

Control = vehicle only

Treated = 50mg 7α-APTADD/mg/day

**Table 4:** Ovarian Aromatase Activity from 7α-APTADD Treated and Control Rats
Figure 9: Bar Graph representing ovarian aromatase activity inhibited by 7α-APTADD (2). Control animals (vehicle only) from treatment groups 1, 3, 5, 7 and 14 days are represented by the solid black bars. Treatment (50mg/kg/day) animals are represented by the hatched bars. Aromatase activity is expressed as picomoles of tritiated water formed/mg protein/hour of incubation. (ova.spw)
2.4.3: Measurement of Ovarian mRNA levels by Northern Analysis:

Measurement of ovarian CYP19 mRNA levels was done using the remaining ovary from control and treated animals (50mg/kg/day 7α-APTADD for 1, 3, 5, 7 and 14 days.) It was known that measurement of CYP19 expression was difficult due to low levels of expression. As stated previously, RT-PCR has been used successfully to accurately quantitate relative amounts of CYP19. However, it has recently been shown by Lephert et al. that there are multiple transcripts present in the ovary of the rat. Three major species have been identified corresponding to 1.7, 2.2 and 2.5kB and they all hybridize to probes generated using the 5’ sequence. Sequence analysis of these three transcripts showed that the smaller transcripts are splice fragments which are missing portions of the 3’ end which contains the heme binding region. Therefore, if these transcripts are translated they result in a functionless protein which would not be able to turnover substrate. The primers utilized in the previous DMBA study span a region in the rat 5’ to these deletions. As a consequence, amplification of all three transcripts in the ovary was probable and would not necessarily correlate to aromatase activity since a portion of the measured transcripts code for non-functional protein. Additionally, it has been suggested that these individual transcripts may be differentially regulated due to the complexity of CYP19 regulation. If this is the case, Northern analysis is a superior method of quantitation because detection of individual size transcripts would be possible. Initial problems with low levels of expression were overcome by subcloning a much larger fragment of the P450<sub> arom</sub> cDNA from a
sequence given to us by E. Lephart. A nearly 1kB fragment was used to make probes of much higher specific activity, compared to the 273bp PCR product which had previously been used.

Isolated RNA from these animals was separated under denaturing conditions and then sequentially probed using cDNA sequences specific to CYP19 and β-actin. Samples were run in duplicate and the relative CYP19/β-actin was averaged. Using 40μg of total RNA it was possible to quantitate the largest of the three transcripts from the ovary. This message appears at 2.5kB and was readily detected in most animal samples. The smaller transcripts were evident in some lanes but were not significantly above background to accurately quantitate. It was therefore impossible to determine if these transcripts were differentially regulated. When treated animals were compared to control animals there was no real significant difference in the amount of message produced. (Figure 12) Due to the cyclic and transient nature of aromatase expression in the rat, subtle differences in the stage of the animal when sacrificed could lead to erroneous results. Animals were also evaluated based upon exact stage of the cycle as determined by cytology, and grouped accordingly. Again the data suggests that there is no significant difference in the levels of the 2.5kB transcript.
Figure 10: Northern analysis of the 2.5kB transcripts of ovarian CYP19 from control and treated animals grouped by estrous stage at the time of tissue procurement.

2.4.4: Serum estradiol levels:

Serum was isolated from blood drawn at the time of death and serum estradiol levels were measured using an RIA method. (Done in collaboration with Y.C. Lin) Although
there was a trend towards a reduction in estradiol levels in the animals which were treated with the aromatase inhibitor, this never achieved statistical significance.

Figure 11: Serum estradiol levels as determined by RIA. (Collaboration with Y.C. Lin) (ria.spw)
2.5: Conclusions:

The objective of these animal studies was to evaluate the steroidal aromatase inhibitor, 7α-APTADD (2) in its ability to reduce hormone dependent tumors in the DMBA rat model. Additionally, the effect this drug would have on the endocrine system of the animals was also evaluated in part by measurement of ovarian mRNA levels, ovarian aromatase enzymatic activity and serum estradiol levels.

As shown by Figure 1, this drug is effective at reducing tumor volume when given at two doses, either 25mg/kg/day or 50mg/kg/day with the higher dose showing the more sustained reduction in tumor volume. This reduction in tumor volume is a direct consequence of inhibition of aromatase since co-administration of 0.3μg estradiol with inhibitor after 4 weeks of treatment resulted in stimulation and further growth of tumors with rates comparable to control animals.

Initial evaluation of mRNA levels from ovaries of control and treated animals utilized a semi-quantitative RT-PCR method to determine relative differences between control and treated pairs of animals. Individual animals showed a trend towards an increase in CYP19 mRNA levels with treatment of the aromatase inhibitor. Due to experimental inconsistencies and animal variations, this trend was evaluated further using a mixture of RNA from 4 animals from the control and treated groups. This mixture of RNA showed on average a 3.4 fold increase in CYP19 specific transcripts.
Normal animals were used to further characterize the effect of this compound on the endocrine system of the rat. The estrous stage of the animals was determined using vaginal cytology and administration of this drug did not result in any obvious disruptions to this 4 day cycle. Animals continued to cycle normally throughout the 14 day study. Only two of the four days showed very obvious cytology, making very specific assignments as to the state of estrous at the time of tissue procurement impossible. Very careful microscopic evaluation of each animal over the time course allowed for grouping of animals which showed similar stages.

Ovarian homogenates from these animals were used to measure ovarian aromatase activity. A nearly complete elimination of aromatase activity was observed in animals that had received 50mg/kg/day for even 24 hour. This inhibition was sustained throughout the course of the study. This loss of protein activity was not reflected in a sustained decrease in serum levels of estradiol as would be expected. It has been suggested that there may be contaminating phytoestrogens in the soy chow given to animals used in this study which is cross reacting with the commercially available antibody towards estradiol used in this study (personal communication). This is one explanation for the higher levels of immunoreactive estradiol observed in these animals. With levels of ovarian aromatase being nearly completely eliminated, it does not seem likely that true estradiol levels would be sustained at the levels measured throughout the 14 days. If the overall basal level of measured estradiol was artificially elevated,
then the reduction due to aromatase inhibition would not appear so dramatic. Our data shows a trend towards a reduction, which did not achieve statistical significance. The possibility that a phytoestrogen from the food source may be cross reacting will be tested in the future. Ovariectomized rats will be given a variety of chow and estradiol levels compared to normal animals treated similarly.

The more recent discovery of multiple transcripts in rat ovary led to the utilization of Northern analysis for measurement of ovarian CYP19 mRNA expression. Measurement of the largest transcript (2.5kB) which codes for a functional protein was carried out. There was no significant elevation of this particular transcript in treated animals. In the DMBA tumor study, all three transcripts were amplified by the chosen primers. Even though all three transcripts originate from utilization of the universal gonadal promoter PII, factors which regulate production of the splice variants are unknown. Increased expression of one of the other two smaller splice variants, leads to proteins which are not able to turn over substrate due to loss of the heme binding region. These transcripts have no role in estrogen biosynthesis in either the DMBA induced rat mammary cancer model or in normal animals.

Additionally, there were other differences in these two studies. Initially, the DMBA induced rat mammary cancer model treated animals for six weeks as compared to the 14 day treatment in normal animals. However, inhibition of ovarian aromatase activity
was immediate, clearly after 24 hours. If there were going to be endocrine effects it is likely we would have observed them by day 14. It is not known what effect treatment with DMBA or having significant tumor burden has on the endocrine system or liver function of these animals. In the normal animals, one animal treated for 5 days and all 6 animals from the 7 day and 14 day treatment groups, presented at autopsy with liver enlargement and marked fatty changes in the liver and spleen. There was involvement of the entire abdominal area in some animals. This was not observed in any of the animals treated for 6 weeks. This may be an indication of temporary altered liver function and/or metabolism which adds another obstacle to drawing very clear conclusions as to the endocrine effects of this aromatase inhibitor.

2.6: Reference List


Chapter 3

REGULATION OF BREAST AROMATASE ACTIVITY

Interactions between the Epithelial and Stromal Components and the ECM

3.1 Statement of Problems and Objectives

Estrogen is felt to be important for the local stimulation of growing malignancies in the breast. However, this steroid has many functions which include controlling growth, proliferation and differentiation as well as metabolic processes. Many of these events are mediated through the regulation of key genes which are expressed in a well controlled temporal and tissue specific pattern. However, the factors which control this expression and also the utilization of this steroid are complex. Paracrine interactions, referring to cell-cell mediated communication, are an important physiological process by which surrounding cells can modulate other cells’ activity and maintain proper tissue integrity. Normal tissue communication can become dysregulated in the presence of malignancies. Tumor cells exhibit an insensitivity to regular cellular cues and setup an environment which will maximize tumor growth. Tumor derived factors may stimulate expression of factors which may normally not be
expressed and also tumors cells fail to respond to contact inhibition, normally a signal to halt cellular growth.\(^2\) An additional level of organization is provided by the surrounding extracellular matrix which provides the three dimensional framework upon which the tissue is structured. These extracellular matrix proteins interact via specific receptor mediated events and alter transcriptional activity of the cell.\(^3\)

It is unclear at this time the exact paracrine interactions between normal epithelial and stromal cells, and what happens when these interactions become dysregulated due to carcinogenesis. Initial studies have focused upon the role of the extracellular matrix protein, collagen I on aromatase activity. Further experimentation evaluates the direct paracrine interactions of soluble factors produced by the stroma which can support tumor growth.

### 3.2: The Role of the Extracellular Matrix Proteins: (ECM)

The extracellular matrix generally consists of at least 50 different proteins, which provide a framework of tissue throughout the body.\(^4\) There are two general classifications of ECM. In the breast, the *basement membrane*, which is a specialized form of the extracellular matrix, surrounds epithelial cells and other cell types and is primarily composed of laminin, type IV collagen and proteoglycans. The *interstitial tissue* matrix is made up of structural ECM proteins, in addition to the stromal fibroblasts and the adipocytes. The interstitial matrix of breast tissue is composed
primarily of collagen I and some fibronectin. The role of the ECM appears to be more complex than simply providing physical structure to the tissue. Expression of specific genes is highly dependent on receiving coordinated extracellular signals. These signals represent a cooperation of extracellular matrix signals with other regulatory molecules such as hormones and growth factors.

The extracellular matrix proteins generally exert their effect through transmembrane receptors, usually from the integrin family. The integrin receptors are made up of an α and β chain; a wide variety of specific receptors can be formed through variable association of these α and β subunits. These receptors generally have short cytoplasmic domains which interact directly with cytoskeletal α-actinin and talin. Generally, the cytoskeleton controls the shape of the cell, the motility and gene expression. The major mechanism responsible for re-organizing the cytoskeleton is the polymerization and depolymerization of the microtubules. It has been hypothesized that the alterations in the cytoskeleton may be central to the acquisition or loss of a differentiated phenotype. The exact link between ECM interaction with the integrin receptor and alterations in gene expression is tentative. In one model study, laminin provided an environment where cultured mammary epithelial cells produced β casein when stimulated with prolactin. The subsequent addition of colchicine was able to inhibit the production of β casein and also the transcription of the mRNA. This supports the link between the ECM, cytoskeletal rearrangement, and gene expression.
Figure 12: Diagram showing the orientation of the two major cell types in the breast in relation to their ECM environment. E=epithelial cell; S=stromal fibroblasts. (Adapted from M. J. Bissell et. al.)

Jackson and Cook showed in the early 1980’s that the nuclear matrix was commonly associated with DNA being replicated and transcribed. They suggested that the nuclear
matrix was one part of the active site of the transcription complex. The nuclear matrix could help organize and stabilize the DNA template with which soluble transcription factor could then interact (Figure 13).

"A Model of Reciprocity" between the ECM, the transmembrane receptors, the cytoskeleton and the nuclear matrix was proposed in 1982. This concept of integrated signaling has been reinforced and must be taken into consideration when evaluating in vitro models of gene expression.

Figure 13: Schematic showing the association of extracellular ECM proteins and the cytoskeleton. (Adapted from M.J. Bissell et.al.)
3.2.1: Collagen I: The Role of the Extracellular Matrix

The collagen family of proteins is composed of 14 different types representing homo- and heterotrimeric complexes showing a wide range of structures and functions. Collagen I is a heterotrimeric helix of \( \alpha_1(\text{I}),\alpha_2(\text{I})_1 \). These complexes are synthesized as propeptides and assembled extracellularly into crosslinked fibrils. The collagen I receptor is the integrin VLA-2 \((\alpha2,\beta1)\) transmembrane receptor. This receptor is commonly expressed on the surface of several cell types, such as fibroblasts, breast epithelial cells, and endothelial cells. Collagen I has been shown to be a very potent modulator of cellular function. In vitro studies have shown that growing cells on a collagen I substratum can lead to alterations in rates of proliferation, adhesion, and migration, not only in fibroblasts but in other cell types too. Peripheral blood monocytes which migrate through the interstitium come into contact with collagen I. In vitro studies have showed that collagen I markedly increases interleukin-8 expression compared to cells grown on plastic. In all species, the milk proteins are specifically expressed in the mammary gland under the control of lactogenic hormones and the extracellular matrix. A different integrin, \( \alpha3\beta1 \), is expressed on colon carcinoma cells. Differentiation of these cells on collagen gels has been observed. Additionally, the monoclonal antibody against the \( \beta1 \) subunit interrupts this differentiation. This suggests that the differentiated state is dependent on collagen-integrin interactions. The breast stromal fibroblasts normally deposit collagen, but this becomes excessive in the presence of an invasive tumor. There is usually an intense stromal reaction
(desmoplasia) to invasive tumors which results in excessive collagen deposition and fibroblast proliferation. The significance of this stromal reaction is unclear. It has been suggested that it provides a barrier to metastasis and might be a protective response.

3.3: Experimental Methods

3.3.1: Cell Culture

The following cell lines were obtained from The American Type Culture Collection (ATCC) and were stored in liquid nitrogen (-196°C) until needed. MCF-7 (#22-HTB; breast adenocarcinoma, ER+), MCF-7_{ADR} (# an adriamycin resistant epithelial cell line, ER-). The MCF-7 and MCF-7_{ADR} were maintained using a supplemented DMEM media (Gibco), without phenol red having 1.5x essential amino acids, 1.5x vitamin and 2x nonessential amino acids. The sterilized liquid media was prepared by the OSU Comprehensive Cancer Center by dissolving the powder into water containing sodium chloride (8.3mM), pyruvic acid (1.25mM) and sodium bicarbonate (17.5mM). Cells were maintained at 37°C, 5% CO₂ and 85-95% humidity (Forma model 3052) using Corning culture flasks and plates. Cells were grown to 80% confluence and split as needed for experiments using a typsin (0.5%) EDTA mixture (Gibco). The passage number for the MCF-7 cell line was monitored and cells discarded after 10 serial passages (155-165) when used in studies looking at gene expression.
Primary breast fibroblasts were isolated from patients at The Ohio State University Hospital through the Tissue Procurement facility. Five 0.5 gram aliquots were obtained from patients undergoing reduction mammoplasty or mastectomy. (see patient listing). Tissue was stored in DMEM without serum at 4°C until used. Samples were then aseptically minced and extraneous fat and connective tissue removed. Minced tissue was incubated with 1% collagenase type III (Gibco) with shaking at 37°C for 12-15 hours or until all of the pieces were digested. Samples sat for 30 minutes to let any undigested fragments settle. The supernatant was recovered and centrifuged at 1500 xg for 5 minutes. Lipid accumulated at the top and was removed with the supernatant. Cells were resuspended in media and centrifuged for 1 minutes at 300-500 xg. Under these conditions epithelial cells will pellet and the resulting fibroblast enriched supernatant was plated in T-75 culture flasks in DMEM/F-12 Ham’s mixture (Gibco). 10% fetal calf serum was added, in addition to L-glutamine (5mM) and gentamicin (0.025%). Cultures were grown until they reached near confluence and then used in experiments. Under the conditions described, any contaminating epithelial component will not plate and will be washed away with regular media changes.

Serum was obtained from Gibco and was used at 10% in all media unless noted. Serum was stripped of endogenous steroids by two treatments with a dextran coated charcoal mixture. A suspension (5ml) of dextran coated charcoal (0.5% charcoal, 0.05% dextran and 0.14M NaCl) was added to 100ml of serum and incubated at 50°C for 30 minutes. This method has been shown to remove 98% of endogenous steroids from the serum.16
This stripped serum was used in experiments where cells could not be exposed to endogenous estrogens. Collagen I coated plates were prepared in the following way. Rat tail collagen I was purchased from Collaborative Biomedical Products at a concentration of 4mg/ml. The final collagen concentration in the gel is 1mg/ml in PBS. This concentration provides a gel of sufficient strength to allow media to be removed and added without the losing integrity of the gel. The following mixture was used for 1ml of collagen I mixture (85μl 10x PBS; 210μl 1N NaOH; 450μl H2O and 250μl of collagen I). A volume of 0.8ml of the collagen mixture was added to 6x well plates (Corning) and placed at 37°C for 30 minutes to allow gel to form. Cells were plated directly on top of the gel.

3.3.2: cDNA Probes

The sequences listed below represent the cDNA sequences that were used as cDNA probes for Northern analysis. Plasmids for pS2 (#57136), 36B4 phosphoriboprotein PO (#65917) and vimentin (#59161) were purchased from ATCC stocks. PCR primers were synthesized by Oligo's Etc (Wilsonville, OR) which would amplify regions of each of these genes to be used as probes or for use in RT-PCR. The following were the sequences synthesized.

pS2

sense 5' - ATC CCT GAC TCG GGG TCG CCT TTG - 3'

anti 5' - CAA TCT GTG TTG TGA GCC GAG GCA CAG -3'
The plasmid vector, pBR322 contained a 0.559kB fragment of human pS2 cDNA. The sense primer corresponds to bases 1-25 and the antisense corresponds to bases 408-434. Amplification using these primers produces a 0.434kB cDNA product.

36B4

<table>
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<th>Sense</th>
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</thead>
<tbody>
<tr>
<td>Anti</td>
<td>5' - TTT CAG CAA GTG GGA AGG TG - 3'</td>
</tr>
</tbody>
</table>

The plasmid vector, pBR322 contained a 1.068 fragment of human cDNA corresponding to the ribosomal phosphoprotein P0. The sense primer corresponds to bases 306-325 and the antisense corresponds to bases 848-867. Amplification using these primers produces a 0.562kB cDNA product.

**Vimentin**  
The ATCC plasmid was transformed into competent DH5α cells (Gibco BRL) and plated onto LB plates with ampicillin. Positive colonies were isolated for large scale midi-preps and plasmids were isolated. Plasmids were digested with EcoRl and the 1.1kB fragment corresponding to 331-1431bp was isolated using the Quiagen band prep system.

### 3.3.3: Cell Studies: Aromatase Activity in MCF-7 cells and Primary Fibroblasts

**MCF-7/MCF-7_{ADR} cells:** Cells were plated at a concentration of 5x10^5 cells/well and grown to 60-70% confluence in 6x well plates using media containing 10% charcoal stripped serum. Due to the very low levels of aromatase in these cell lines it was necessary to make the following changes to the general aromatase activity assay.
described in Chapter 2. The amount of radiolabelled androgen used was increased to 2μCi/well and the incubation was increased to 24 hours. Cell number was determined either by counting using a Coulter Counter or by hemocytometer. Cells were counted a total of 6 times/well.

**Primary Fibroblasts:** Cells were plated at a concentration of 5x10^5 cells/well in 6 x well plates and incubated with 2μCi/well of [1β-^3^H]androst-4-ene-3,17-dione (80nM total androgen concentration) with an incubation time of 12 hours. Fibroblasts were grown on either collagen I gels or plastic. A separate blank sample was used for the plastic and collagen wells which had no cells plated. These values were used as background values.

3.3.4: Thymidine Incorporation

The measurement of incorporated tritiated thymidine allows for a correlation to growth of cells. The incorporation of thymidine was carried out using primary breast fibroblasts which were grown on either plastic or collagen I. Cells were plated at 5x10^5 cells/well and separate flasks were used for each of the seven days. Thymidine (1μCi/well) was added to each plate in triplicate for each time point. Incubation was performed for eight hours at 37°C. After the incubation, cells were washed two times and then lifted using either trypsin:EDTA or collagenase type III depending on which type of plate was used. Cells were centrifuged and washed twice using PBS. The supernatent was decanted and 1ml of 5% TCA solution was added and left at 4°C
overnight. The mixture was centrifuged at 3000 xg for 10 minutes at 4°C and the supernatant decanted. A 1ml mixture of 0.1N NaOH and 1% Triton N101 was added to each pellet and vortexed vigorously. An aliquot of 270μl of mixture was added to 5ml of cocktail which had been acidified with 31μl of 1N HCl. Amount of radioactive thymidine incorporated into the cellular DNA was counted using a scintillation counter.

3.4: Results and Discussion:

3.4.1: Aromatase Activity of Primary Fibroblasts:

Primary normal stromal fibroblasts were obtained from breast tissue samples and were cultured as described in the section 3.3.1. Cells from twelve female patients of varying age (16-80 years) were evaluated for aromatase activity when grown under different culturing conditions. The effect of the extracellular matrix protein, collagen I, on aromatase activity was investigated. Aromatase plays a critical role in regulating levels of the estrogen in the breast and collagen I is the major ECM protein in the breast stroma. It is important to determine if this ECM protein alters regulation of this enzyme. Although several studies have examined fibroblasts aromatase activity, no study has used an appropriate substratum which mimics in vivo conditions. The results of this twelve patient study are presented in Figure 14. Constitutive aromatase activity was measured in addition to the ability of these cells to respond to dexamethasone. Normal fibroblasts utilize promoter I.4 which has a glucocorticoid responsive element
Figure 14: Comparison of aromatase activity in primary fibroblasts cultured on plastic or a collagen I matrix. (fibaa.spw)
located upstream of the untranslated exon 1.4, making them responsive to treatment with 100nM dexamethasone. This data show that growing primary fibroblasts on a collagen I matrix results in a decrease in constitutive aromatase activity. This decrease corresponds to a six fold reduction in activity. These values were corrected for the number of cells so this reduction is not a reflection of differences in the number of cells.

Significant amount of variability within the levels of dexamethasone induction exists within this patient group. Figure 15 represents this data as individual patients rather than as a group. The level of induction was variable which has also been observed in the literature and loosely correlates with age.\textsuperscript{17,18}
Figure 15: Aromatase activity of Primary fibroblasts represented as individual patients, showing a comparison of plastic and Collagen I matrix with constitutive and dexamethasone induced activity. (fibaa2.pre)
3.4.2: Aromatase Activity of MCF-7 and MCF-7_{ADR} Cells

MCF-7 cells have been shown to have low, yet measurable aromatase activity. Additionally, it has been shown that they can convert significant amounts estradiol to produce an estrogen mediated response, such as pS2 expression.\(^{19}\)

The previous experiments with the primary fibroblasts determined that there is a reduction in aromatase activity when these cells were cultured on a collagen I substratum. The first issue that was addressed was whether this was a response specific to primary fibroblasts or a general cellular response to exposure to collagen I. Epithelial cells also express the specific collagen I receptor, the VLA-2 integrin subtype. Similar experiments were carried out to determine the aromatase activity of both the hormone responsive (MCF-7) and hormone insensitive (MCF-7_{ADR}) on plastic and collagen I substratum. Experiments were setup using both 10% charcoal stripped media and also 10% normal serum to evaluate the effect of estradiol on their response.

Neither MCF-7 or MCF-7_{ADR} cells showed any decrease in aromatase activity as a consequence of growing cells on a collagen I matrix, regardless of the type of serum used (Figure 16). The MCF-7 cell line showed an overall reduction in aromatase activity (both on plastic and collagen I) when the cells were grown using the 10% charcoal stripped serum. These cells could be indirectly modulated by estradiol since
the presence of 10% normal serum showed a higher level of aromatase activity than under conditions of charcoal stripped serum.

Figure 16: Comparison of Aromatase activity of MCF-7 and MCF-7_{ADR} cells grown on either plastic or collagen I matrix in the presence of normal or charcoal stripped serum. (MCFaa.spw)
Since it is thought that there are no estrogen responsive elements present in the promoters region of the epithelial cells, it is possible that estradiol stimulated growth factors are having some effect. However, serum is a complex mixture and charcoal stripping can remove other small molecules in addition to estrogens and other steroids. It should be noted that the MCF-7<sub>adr</sub> cells were not altered by the type of serum used and that these cells do not express the estrogen receptor. This suggests an indirect role for estrogen through the induction of growth factors.

3.4.3: Expression of Vimentin in Fibroblasts Grown on Collagen:

In an attempt to evaluate the effect of collagen I on fibroblasts, the expression of vimentin was measured. Vimentin is an intermediate filament protein which is expressed by fibroblasts and cells of mesenchymal origin. It is also expressed by the MCF-7<sub>adr</sub> cell line which has progressed through the epithelial-mesenchymal transition. This cell line has acquired fibroblast-like properties through the exposure to adriamycin. Expression of vimentin is associated with an increased metastatic potential. MCF-7 cells which express vimentin show a diminished keratin 19 expression and have lost expression of the cell adhesion molecule, uvomorulin. Transfecting MCF-7 cells with the full length cDNA for vimentin does not affect the phenotype of the cells. Vimentin is a marker for an invasive phenotype, however it does not by itself give rise to this phenotype. As vimentin is a structural protein, there was no functional assay to measure; therefore mRNA levels were measured by Northern analysis. Fibroblasts
were grown on collagen I for 4 days and the levels of steady state mRNA were measured using Northern analysis and a cDNA probe specific to vimentin.

Figure 17: Steady State mRNA levels of Vimentin expressed in fibroblasts and MCF-7_{ADR} cells grown on either plastic or collagen I matrix. (vim.spw)
There was no significant difference in the expression of vimentin when fibroblasts were grown on collagen I compared to plastic. MCF-7\textsubscript{ADR} cells were also grown in this manner and vimentin also measured. These cells showed no alterations in vimentin expression (Figure 17).

MCF-7 cells are known to express a small trefoil protein, known as pS2. This is a small 84 residue protein, containing at its amino terminus a signal peptide characteristic of secreted proteins, which was isolated from a human breast carcinoma cell line. Transcriptional activation of the pS2 gene is a primary response to estrogens in the MCF-7 cell line.\textsuperscript{22, 23} The proximal 5' flanking region of the pS2 gene contains a transcriptional enhancer estrogen responsive element, a 13 base pair imperfect palindromic sequence. Cells which are estrogen deprived for 48 hours show a low level of this message and it becomes markedly stimulated by the addition of estradiol. The increase in pS2 message is quick, with an increase in mRNA expression after 12 hours of treatment, reaching a maximum after 48 hours.

MCF-7 cells were also evaluated for differences in pS2 expression when these cells were grown on collagen I (Figure 18). Both constitutive and induced pS2 expression was measured. Constitutive pS2 expression is observed when cells are grown using 10% serum for 48 hours. Induced pS2 expression is observed when cells are grown using a media containing 10% charcoal stripped serum for 48 hours to deprive the cells
estrogen, and then adding 10nM estradiol for 48 hours. The levels of induction observed in my hands are typically a five fold increase over the estrogen deprived (control) level.

Figure 18: Northern Analysis of constitutive and induced pS2 expression in MCF-7 cells grown on collagen and plastic. Lane 1) Control (48 hours defined media); Lane 2) 10nM estradiol (48 hour treatment); Lane 3) MCF-7 cells grown on plastic in media containing 10% FCS: constitutive pS2 expression. Lane 4) MCF-7 cells grown on collagen in media containing 10% FCS: constitutive pS2 expression. Lane 5) MCF-7 cells grown plastic (48 hours defined media prior to 10nM estradiol treatment); Lane 6) MCF-7 cells grown on plastic (48 hours defined media prior to 10nM estradiol treatment)
3.4.4: Thymidine Incorporation

To evaluate the relative growth rates of fibroblasts grown on plastic and collagen I, the incorporation of radiolabeled thymidine was used. It has been stated by Dr. Mina Bissell that even in very rapidly growing tumors, only 4% of the cells are growing at any given time. Historically cell culture systems have been optimized for maximal growth. Since growth and differentiation are two mutually exclusive cellular functions, a cell culture system which offers a maintenance of cells rather than supporting maximal proliferation would be more representative of in vivo conditions. The rationale of growing cells on a collagen I substratum was that this would provide a more relevant model of what was occurring in the breast. With the importance of the stromal fibroblasts to the growing tumor, it is critical that measurement of gene expression and enzyme function be carried out under circumstances seen in the normal breast.

Fibroblasts were grown on plastic or collagen I for 1 to 7 days. Radiolabelled thymidine was added to separate plates every day. The incorporation of thymidine can be related to the growth rates of these cells (Figure 19). The fibroblasts which were grown on collagen I showed a significant reduction in the incorporation of thymidine from day 1 to day 5. The cells that were grown on plastic had a faster growth rate and reached confluence at around day 5. Due to contact inhibition, these cells halted growth, resulting in the drastic decrease seen after 5 days for the cells grown on plastic. During
the time frame that the aromatase activity measurements were taken (day 4), the
difference in thymidine incorporation is substantial.

Figure 19: Thymidine Incorporation of Fibroblasts grown on Plastic or Collagen over
7 days.
3.5: Paracrine Interactions: Conditioned Media Experiments

In order to further characterize stromal interactions with the epithelial cells, conditioned media experiments were used as an indirect measure of aromatase activity. Fibroblasts were cultured under a variety of conditions and then the media was used to treat MCF-7. The soluble factors produced by the fibroblasts would be able to interact with the epithelial cells. Additionally, if provided to the fibroblasts, turnover of androgen precursor would elicit an estrogen mediated response in the epithelial cells if it were of sufficient magnitude.

If the fibroblasts were producing estrogens, then the estrogen responsive MCF-7 cells would respond by an increase in pS2 gene expression. This is easily measured by Northern analysis and is an indirect measure of the estrogen produced. It indicates whether fibroblasts produce estrogens not at an absolute value, but at levels which are able to elicit a response in a neighboring cell. This type of experiment is limited in that it allows for only one way communication. In the breast there is a dynamic, two-way communication which can respond to changes in the microenvironment. However, it is very difficult to distinguish actual cause and effect relationships when both cells types are present in a transwell type of experiment. If the cultured fibroblasts produce sufficient estradiol in the media there will be a measurable pS2 signal from the epithelial cells. These experiments posed the following question: Is sufficient fibroblast-derived...
aromatase activity present to convert exogenous androgen and result in a concomitant increase in estrogen mediated gene expression?

Both testosterone and androstenedione were used as substrate for the fibroblasts. The most common group of women to get breast cancer are postmenopausal and androstenedione is the relevant androgen due to loss of ovarian function. Aromatization of this steroid results in the production of estrone, a very weak estrogen, which binds to the receptor with 1/100 the affinity of estradiol. Initially, this was the androgen used, with the rationale being that 17β-hydroxysteroid dehydrogenase (17β-HSD) would be able to convert this to the more potent estradiol. The 17β-HSD enzyme is actually several isoforms having reductive or oxidative capability. Breast epithelial cells are known to have a very active reductive dehydrogenase, converting peripherally formed estrone into locally active estradiol. Based on previous experiments it was suspected that the subline of MCF-7 cells used in our lab is deficient in the reductive isoform of this 17β-HSD. Without conversion of estrone to the more potent estradiol, there would be no increase in pS2 expression. Therefore, testosterone was also used as a substrate, circumventing the need for the 17β-hydroxysteroid dehydrogenase enzyme.

3.5.1: Conditioned Media Supplemented with Androstenedione

Conditioned media from fibroblasts supplemented with androstenedione or vehicle only
(EtOH) was given to MCF-7 cells which had been estrogen deprived for 48 hours. Initially, fibroblasts were also grown on either collagen I or plastic to see if inherent levels of aromatase would alter the levels of estrone produced to the extent that there would be differences in pS2 expression. Figure 20 shows the results of these experiments in a graphical manner. The 10nM estradiol response is normalized to 100%. The level of pS2 expression in MCF-7 cells which received fresh DMEM/F12 media and were estrogen deprived throughout the study was 19.14% (SEM 1.74) of the E2 value. MCF-7 cells received conditioned media from fibroblasts which were grown on plastic or collagen and also received 100nM androstenedione or ethanol as a control. Conditioned media from fibroblasts grown on plastic showed an increase of pS2 expression of 54.98% of E2 control. Fibroblasts grown on collagen showed an increase of 51.37%. When these fibroblasts were supplemented with substrate, ie. 100nM androstenedione, the levels of expression were plastic 46.7%, and collagen 40.56%.
Figure 20: Effect of fibroblast derived conditioned media on pS2 expression in MCF-7 cells. ■: 10nM estradiol, (n=10); □: EtOH Control, (n=7); □: Conditioned Media, (n=7); □: Conditioned Media supplemented with 100nM androstenedione (n=4). Error bars derived from S.E.M. and letter represent statistical differences by unpaired students t-test (p<0.01). (conda.spw)
Figure 21: Northern Blot illustrating pS2 (0.6kB) and 36B4 (1.8kB) gene expression. MCF-7 cells were steroid deprived for 48 hours. Conditioned media from primary fibroblasts was subsequently added. 1) Control: After 48 hours, fresh DMEM/F12 media was added. 2) 10nM estradiol: positive control: After initial 48 hours, fresh DMEM/F12 was added supplemented with 10nM estradiol. 3) Conditioned media from fibroblast grown on plastic with 100nM androstenedione. 4) Conditioned media from fibroblasts grown on collagen I matrix supplemented with 100nM androstenedione. 5) Conditioned media only from fibroblasts grown on plastic. 6) Conditioned media only from fibroblasts grown on collagen I matrix. (conda.tif)
Table 5: Data from Northern Analysis of A Representative Conditioned Media Experiment. The ratio of pS2/36B4 (cpm/mm^2 - background) was used to determine the relative expression of pS2; the maximal response of pS2 by 10nM E_2, was normalized to 100% and experimental values compared to that. Samples were done in duplicate with the exception of conditioned media from fibroblasts grown on plastic.
An unexpected observation was the induction of pS2 message in MCF-7 cells when they were treated with conditioned media without androstenedione substrate. These cells were initially used as a negative control. It was anticipated that in this system, pS2 expression would primarily be an estrogen mediated response. These samples should show a pS2 expression similar to that seen with the control. The lack of androgen substrate for the fibroblasts to convert suggests that this increase in pS2 expression must be an estrogen independent response. There are reports of growth factors which when added to MCF-7 cells result in increases in pS2 expression through the activation of an SP1 site in the promoter. This is an example of an increase in pS2 expression resulting from conditioned media which was proven to be independent of estrogens. Additionally, the samples which had androstenedione added to the fibroblasts, showed no additional increase in pS2 expression. There are two possible explanations for these results: (1) The fibroblasts, in the presence of substrate, do not produce sufficient estrone to elicit an increase in pS2 expression or (2) this subline of MCF-7 cells is incapable of converting estrone into the more potent estradiol through the action of the enzyme 17β-hydroxysteroid dehydrogenase. If the latter is the case, using testosterone as a substrate would result in the production of estradiol, which would immediately be able to alter pS2 expression if produced at sufficient levels.
3.5.2: Conditioned Media using Testosterone

Identical experiments were performed using 100nM testosterone as the provided androgen. Also, some flasks were given dexamethasone in addition to testosterone which is known to increase aromatase activity. Finally other flasks had testosterone, dexamethasone and 7α-APTADD which would inhibit conversion of testosterone.

Figure 22: Northern blot showing pS2 and 36B4 (ribosomal phosphoprotein P0) expression: MCF-7 cells were steroid deprived for 48 hours. Conditioned media from primary fibroblasts was added after 48 hours. 1) Negative Control: after 48 hours, fresh DMEM/F12 media was added; 2)10nM E₂-Positive control; after 48 hours fresh DMEM/F12 media was added supplemented with 10nM E₂; 3)Conditioned Media from fibroblasts grown on plastic; 4)Conditioned Media with 100nM testosterone added 5) Conditioned Media with 100nM testosterone and 100nM dexamethasone 6) Conditioned Media with 100nM testosterone, 100nM dexamethasone and 100nM 7α-APTADD. (condt.tif)
Figure 23: Effect of fibroblast derived conditioned media on pS2 expression in MCF-7 cells. ■) 10nM estradiol, (n=4); □) EtOH Control, (n=4); Conditioned Media, (n=4); ▲) Conditioned Media supplemented with 100nM testosterone (n=4). ▼) C.M. + testosterone + dexamethasone (n=4). □□) C.M. + testosterone + dexamethasone + 7α-APTADD (n=4). Error bars derived from S.E.M. and letter represent statistical differences by unpaired students t-test (p<0.01). (condt.spw)
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<th>Sample</th>
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<th>36B4 (cpm/mm²)</th>
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<th>%10nM E₂</th>
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**Table 6**: Data from Northern Analysis of A Representative Conditioned Media Experiment. The ratio of pS2/36B4 (cpm/mm² -background) was used to determine the relative expression of pS2; the maximal response of pS2 by 10nM E₂, was normalized to 100% and experimental values compared to that. Samples were done in duplicate.

109
Figure 23 shows the combined results of these experiments using testosterone as a substrate. The control flasks, which were deprived of estrogen for the course of the experiment, showed a pS2 expression which was 21.52% (SEM 4.41) of the maximal response of pS2 (normalized to 100%). Conditioned media from fibroblasts showed an increase in pS2 expression corresponding to 67.36% of the 10nM E2 value. The addition of 100nM testosterone resulted in a further increase in pS2 expression over the conditioned media alone, equaling 87.46% (SEM 16.86). It was anticipated that the addition of dexamethasone and testosterone would result in a further enhancement of the pS2 expression. Dexamethasone is known to increase aromatase activity in fibroblasts as determined in the earlier part of this chapter. In an attempt to maximize the amount of estrogen formed, the dexamethasone was added at the beginning of the 3 day treatment, whereas in the previous experiments treatment was for 24 hours only. Figure 23 shows that in all cases, this resulted in a reduction of pS2 expression, 50.4% (SEM 4.45) over that seen for 100nM testosterone alone. A report evaluating the result of longer term dexamethasone treatment determined that long term treatment >36 hours alters aromatase activity and decreases glucocorticoid receptor levels. It is possible that in order to see the stimulatory affects of dexamethasone, treatment should have started 24 hours prior to the end of the study rather than dosing all drugs, testosterone, dexamethasone and the aromatase inhibitor at the beginning. There is no statistical difference in the pS2 expression between conditioned media alone and the testosterone and dexamethasone treatment. This supports the hypothesis that long term
treatment of dexamethasone reduces aromatase activity in addition to decreasing glucocorticoid receptor levels. The addition of 7α-APTADD, a potent aromatase inhibitor, results in a further reduction in pS2 expression, as would be expected.

This series of experiments provide further insight into the importance of the conversion of testosterone to estradiol in these cells. The estrogen independent increase in pS2 expression was observed throughout the study. If the fibroblasts are supplemented with testosterone (100nM) there is a further increase in pS2 expression. This suggests that the failure of androstenedione to elicit a further increase in pS2 expression was not a consequence of low fibroblast turnover rate, but an inability of the MCF-7 cells to convert estrone into estradiol. In vivo, the 17β-hydroxysteroid dehydrogenase is an ubiquitous enzyme and has been shown to be active in breast tissue. This cell line is probably incapable of converting estrone to estradiol and thus is a further step away from in vivo conditions. It does however, remain estrogen responsive and the estrogen response produced by the stromal derived factors is what was being measured.

The factor(s) produced by the fibroblasts that cause increased pS2 expression in these experiments is not known at this time. There have been literature citations suggesting that cytokine IL-6 and also growth factors TGF-α and insulin growth factor I, when added to flasks of astrocytes and MCF-7 cells, were able to induce a pS2 increase. Insulin-like growth factor is the only factor observed so far to have an effect on pS2
expression which is also produced by primary breast fibroblasts. Interestingly, normal fibroblasts have been shown to express large amounts of IGF-1. Fibroblasts isolated directly from the tumor seem to express large amounts of IGF-2 and no IGF-1. The fibroblasts themselves do not become genetically altered in breast tumors but the factors secreted are markedly affected by the presence of the tumor. IGF-2 has not been shown to increase pS2 expression.

All of the conditioned media data was combined, looking only at the estrogen independent increase in pS2 expression in both normal and tissue from tumor patients which was taken from an unaffected quadrant (Figure 24). There is a consistent increase between all of the patients within the range that they all have variability as to starting levels. The slopes of these lines are similar with one exception. The only fibroblasts which were unable to generate an increase in pS2 expression, were derived from a patient with breast cancer, not a reduction mammoplasty. It is possible that this patient’s fibroblasts were producing IGF-2 and not IGF-1. This supports the hypothesis for a role for IGF-1 in this unexpected increase in pS2 expression in the absence of estrogens.
Figure 24: Effect of conditioned media from fibroblasts on pS2 expression as individual patients. Values are compared to 10nM estradiol which has been normalized to 100%. (cond.pre)
3.6. Conclusions

A collagen I matrix has a measurable affect on the aromatase activity of primary breast fibroblasts. The levels of aromatase activity were significantly increased by the presence of 100nM dexamethasone, showing that these primary fibroblast grown on a collagen I matrix retain their hormone responsive state. The specific nature of this interaction is supported by the fact that there were no alterations in the aromatase activity of MCF-7 cells or MCF-7\textsubscript{ADR} cells grown on collagen I. The epithelial cells express the appropriate integrin receptor, but are not generally exposed to collagen I to any great extent, being more closely confined to the basement membrane which is primarily composed of laminin. Additionally, there was no alteration in the intermediate filament protein vimentin in either primary fibroblasts or MCF-7\textsubscript{ADR} cells. There was no difference in the MCF-7 constitutive expression of pS2 or the ability of 10nM estradiol to induce this transcript. Collagen I reduced incorporation of radiolabelled thymidine and may allow for a more differentiated phenotype as a consequence of slowed growth. Although preliminary, this data supports the specific interaction of the fibroblasts VLA-2 integrin receptor with a collagen I matrix, possibly resulting in chromatin alterations which affect gene transcription. These experiments highlight the need for evaluation of gene expression in cell culture systems which accurately reflect the tissue environment. In the debate over local production of estrogens in the breast, it seems necessary to take into consideration the relative activity of the two major cell types. The fibroblasts, based on previous cell culture data, were thought to have the majority of the estrogen
producing capabilities. If in fact however, *in vivo* they have lower levels constitutively, their role may not be singularly paramount. Their potential for high aromatase activity may depend solely on tumor derived factors.

In an effort to examine paracrine interactions between these two cell types, conditioned media experiments were used. These experiments demonstrated that there are two factors produced by fibroblasts which can induce pS2 expression in MCF-7 cells. There is an estrogen independent factor which is capable of stimulating pS2 expression to a large extent which is further increased when testosterone is provided to the fibroblasts. There was only one patient for whom there was no increase, suggesting that the factor produced by the fibroblasts is IGF-I. This needs further evaluation. Additionally, the MCF-7 cell line utilized in our lab apparently is lacking in the reductive isoform of the 17β-hydroxysteroid dehydrogenase enzyme. The addition of androstenedione did not result in a further stimulation of pS2 expression. When testosterone was used, the increase was observed as expected. Co-incubation of MCF-7 cells with conditioned media supplemented with testosterone and dexamethasone resulted in a decrease in pS2 expression over a 3 day treatment. The addition of an aromatase inhibitor resulted in a complete suppression of fibroblast derived estrogens and showed only a modest increase in pS2 expression.
3.7: Cited References


Chapter 4

MEASUREMENT OF CYP19 GENE EXPRESSION IN BREAST CANCER SAMPLES

4.1 Statement of Problem and Objectives

As discussed in the Introduction, the regulation of CYP19 gene expression is complex. Local production of aromatase in breast is thought to be important to the maintenance of developing tumors.\textsuperscript{1, 2, 3} Adipose expression of aromatase can utilize one of three promoters, designated I.4, I.3 or II, all of which share a common splice site (Figure 2). Simpson and others have demonstrated that patients bearing tumors exhibit a higher level of aromatase activity in the quadrant of the breast with the tumor.\textsuperscript{4-5} This suggests that tumor derived factors stimulate aromatase expression in the surrounding mesenchymal tissue. In normal tissue, the predominant promoter used is the distal promoter I.4, resulting in aromatase protein. Transcripts originating from usage of promoter I.3 or II are present at very low copy number. Conversely, breast tissue from women with breast cancer have higher levels of aromatase, but also the promoter used is predominantly promoter II. Levels of transcripts derived from promoter I.4 are not
present. This increased expression is associated with a promoter switch, such that promoter II predominates at sites proximal to the tumor as well as in the tumor itself. This observation means that factors which can regulate aromatase in normal tissue (such as class I cytokines, IL-6 through the JAK-STAT signal transduction pathway) are superseded by regulation by cAMP through promoter II. Promoter II is stimulated by cAMP and this is potentiated by phorbol esters, suggesting that both the PKA and PKC pathways are important for full expression.

4.1.1: Production of Prostaglandins in Breast Tissue

Malignant breast tumors contain high levels of the prostaglandin E₂. Prostaglandins belong to a family of related eicosanoids (C₂₀) compounds. They are produced exclusively within the plasma membrane of the cell and are derived mainly from arachidonic acid; released from the phospholipids by the action of phospholipases. Cyclooxygenase 1 and 2 (prostaglandin endoperoxide synthase) are two isoforms of the enzyme which is the rate limiting step in the conversion of arachidonic acid to prostaglandins. Prostaglandins are one of the three products derived from arachidonic acid, with production of leukotrienes and thromboxanes being the other possibilities. Cyclooxygenase produces cyclic endoperoxide intermediates (PGG₂) which are further converted into PGE₂. The two isoforms of COX have been characterized; COX-1 is constitutively expressed in most tissues and is responsible for the physiological production of prostaglandins. COX-2 which is induced by cytokines, mitogens and
endotoxins in inflammatory cells is responsible for the elevated production of prostaglandins during inflammation. This induction is rapid but transient. The regulation of COX-1 and COX-2 in breast tissue is under investigation and the significance of PGE$_2$ in the breast is unclear. A recent report showed that the estrogen receptor positive cell line MCF-7 expresses relatively high levels of COX-1 and COX-2 which can be induced transiently by treatment with phorbol esters. PGE$_2$ is also produced by tumor fibroblasts, stromal fibroblasts and macrophages which commonly infiltrate tumor sites. PGE$_2$ is a potent activator of intracellular cyclic AMP. The increase in cAMP results through the interaction of PGE$_2$ with one of the four receptor subtypes, EP$_2$, which activates Go$s$, resulting in the stimulation of adenylate cyclase.

Simpson recently showed that tumor derived prostaglandin PGE$_2$ is the major regulator of aromatase in the surrounding breast tissue. Exogenous PGE$_2$ was added to cultured stromal cells from reduction mammoplasty samples. This resulted in a 50 fold increase in aromatase activity.

The connection between prostaglandins and aromatase activity in the breast is further strengthened by an epidemiological study of 511 breast cancer patients and 1534 population control patients, which suggests that non-steroidal anti-inflammatory drugs (NSAID) have a chemopreventative effect for breast cancer. Since the mode of action of many NSAIDS is the inhibition of prostaglandin production, it is possible that...
these compounds are then limiting the amount of aromatase activity in the breast of these patients.

The purpose of this study was to determine if there was any correlation between COX-2 levels and aromatase in women with breast cancer.

4.2: Experimental Methods

4.2.1: Semi-quantitative RT-PCR using cRNA as an External Control

The study involving patient samples required the measurement of CYP19 gene expression from breast tissue where levels are known to be very low. Since multiple samples were to be compared, it was not possible to utilize the previous described kinetic method. A semi-quantitative method was developed which utilized cRNA as an external control. In separate tubes, primers for CYP19 and primers for 36B4 control gene were amplified. For each group of samples done within a single experimental group, a known amount of cRNA was also amplified from the same master mix of reagents. Ten picomoles of cRNA theoretically should produce the same amount of template at the end of 30 cycles. However, practically speaking there are multiple sources of error which will alter the actual amount of template formed. Primarily this can be due to minute differences in amount of starting enzyme (both RT and Taq) which can alter amplification efficiency. By running a control tube with the same
amount of cRNA, differences in efficiency in both the RT reactions and PCR reactions will be accounted for by differences in cRNA signals. The signals of the patient samples can be normalized to the signal obtained for the cRNA control. This allows direct comparison of patients which were done on different days from different master mixes. The levels of CYP19 expression were then normalized for the amount of 36B4 control gene which was amplified separately, also utilizing a cRNA external control. This method is not without error and was used as a preliminary screen to look for correlations with the COX-2 gene expression. This method reflects improvements in the amplification of CYP19 previously used. It was necessary to make alterations to the method as ovarian CYP19 levels are relatively abundant compared to the breast. There were significant problems observed in the breast samples not previously observed in the ovarian samples, eg. amplification of extraneous bands with the target signals at times being the minor band and smearing of signals due to higher levels of RNA needed to detect the CYP19 signal. This was unacceptable, so an alternate method was developed.

A separate RT reaction was set up for each patient samples and then a separate RT for both CYP19 and 36B4. Ten picograms of cRNA previously prepared was used as the template for the CYP19 and 36B4. A negative RT control was prepared using patient RNA but without the addition of reverse transcriptase. This control is to protect against amplification of contaminating genomic DNA in the RNA sample. A PCR control was made up using all the reagents used in the master mix using water instead
of the RT reaction. This control determines if there is sporadic contamination of the PCR reagents. In all cases, the negative control lanes were devoid of extraneous bands.

The following reverse transcription master mix was made for patient samples and the cRNA controls with a final volume of 10μl: 1μg total RNA; H2O, 3.44μl; DTT (10mM), Random Primers (100ng); 1 x RT buffer (250mM Tris-HCl, pH 8.3; 375mM KCl); dNTPs (0.5mM); RNase Inhibitor (5 units); and 200 units of Superscript RNase H Reverse Transcriptase (Gibco BRL). The reaction was incubated at 23°C for 15 minutes, 42°C for 50 minutes and 95°C for 5 minutes.

The master mix for the CYP19 gene was made in the following way: 10x PCR buffer II (670mM Tris-HCl, pH 8.3; 67 mM MgCl2; 166mM (NH4)2SO4; add β-mercaptoethanol to 28mM before using), DMSO, 10% final volume; CYP19 primer mix (1μM in each); dNTPs (200μM); H2O 37.8μl; Taq polymerase (1 unit). The total volume was 50μl and the reaction cycles were as follows: 95°C for 1 min; 50°C for 30 sec and 72°C for 30 sec. 35 cycles were run under these conditions. The presence of the DMSO markedly increases the specificity of the Taq but by doing so reduces the efficiency of the amplification. Under these conditions, 35 cycles is still in the exponential phase. Prior to cycle 34 there are no bands present on the gel based on ethidium bromide staining.
The 36B4 tubes were amplified using the Gibco PCR buffer by using the method described in the kinetic method. Reaction volume was 50μl and reaction went for 28 cycles: 95°C for 1 min, 60°C for 30 sec, and 72°C for 30 sec.

Initial detection of PCR products was done using Southern analysis as described previously. Subsequent documentation and quantitation was carried out using the gel documentation system. Images were saved as TIFF files and quantitated using the PhosphorImager software, Molecular Dynamics.

4.2.2: pGEM-T Subcloning of PCR Products

The production of single stranded cRNA sequences was used as an external control for PCR and also for use as a riboprobe in Northern analysis. Riboprobes provide much higher incorporation of radiolabel into a probe resulting in a higher specific activity. There are also advantages in that the specificity of the RNA:RNA hybrid is higher than the DNA:RNA hybrid and washing can be carried out under more stringent conditions.

cDNA sequences for 36B4, pS2 and human CYP19 were amplified by PCR off of the corresponding plasmids. The PCR product was run out on an agarose gel and the band purified using the Quiagen kit for isolation of DNA from agarose gels. The recovered DNA was quantitated using 260nm/280nm ratios. The PCR products were subcloned into a commercially available transcription vector from Promega pGEM-T vector. This vector has a SP6 bacteriophage promoter and a T7 bacteriophage promoter in opposite
orientations of a polycloning region (Figure 25). Ligation of PCR products was carried out using 1µl T4 ligase buffer, 1µl (50ng/µl) PGEM-T vector (Promega), 1µl T4 ligase, and a range of insert ratios from (0.5:1) to (2: 1) insert to pGEM-T vector, with a final volume of 10µl. Reaction mixtures were incubated at 15°C overnight. Transformation was carried out using competent DH5α cells. Competent cells were thawed quickly and 100µl was used for each ligation reaction. 5µl of ligation reaction was mixed gently with the competent cells. The mixture was placed on ice for 20 minutes, heat shocked at 37°C for 1.5 minutes, and placed on ice again for 20 min. 850µl of LB broth (1 liter: 10g typtone, 5gm yeast extract, and 10gm NaCl; pH 7.0) was added to the transformation reaction and incubated at 37°C for 30 minutes to allow expression of ampicillin resistance. 100µl of a 1x and a 10 x concentration were plated onto LB plates (15gm Bacto agar to 1 liter of LB broth; ampicillin, 100µg/ml) which had X-gal (20mg/ml in DMF, use 1ml /500ml agar) and IPTG (20mg/ml, use 1.8ml/500ml agar) spread over the top. Plasmids containing the insert were detected by insertional inactivation of the β-galactosidase gene which turns the X-gal substrate from white to blue in the presence of the inducer IPTG. Plates were placed at 37°C overnight and positive colonies (white) were selected for further evaluation.

4.2.3: Large Scale-Prep of Positive Colonies:

Large amounts of plasmid were obtained by using an alkaline method. Plasmid preps were purified by a lithium chloride precipitation followed by 13% polyethylene glycol
treatment. The following regents were used in this procedure: TPG (100mM Tris-
phosphate, pH 8.0; 10mM EDTA, pH 8.0 and 50mM glucose), TPG Complete (TPG
with 5mg/ml lysozyeme and 50μg/ml RNase A solution added), Solution B: (0.2N
NaOH, 1.0% SDS), Solution C: (3M potassium acetate, pH 5.2), TE (10mM Tris-HCl,
1mM EDTA, pH 8.0).

Positive colonies were grown up in 200ml of LB broth containing ampicillin for 12-15
hours at 37°C with vigorous shaking. Cells were pelleted at 4000 xg for 15 minutes.
The supernatant was discarded and the pellet drained. 2ml of TPG Complete was used
to resuspend the pellet. Samples were incubated at 37°C for 10 minutes. Solution B
(4ml) was added to the mixture and mixed by gentle swirling then incubated at room
temperature for 5 minutes. 3ml of ice cold Solution C was added to the sample and
mixed by careful inversion. After a 5 minute incubation on ice, 0.6X volume of
isopropanol was added and pellets were recovered by centrifugation for 15 minutes at
5000 xg at room temperature. The pellet was dissolved in 3ml of TE buffer and
transferred to a 15ml tube. 3ml of ice cold 5M LiCl solution was added. Samples were
centrifuged at 10,000 rpm at 4°C for 10 minutes. Supernatant was removed and
samples transferred to 50ml tube where an equal volume of isopropanol was added.
Samples were mixed well and centrifuged at 10,000rpm room temperature for 10
minutes. The supernatant was decanted and pellet rinsed with 70% ethanol (room
temperature). The pellet was then dissolved in 500μl of TE buffer (pH 8.0) which had
20μg/ml DNase free-RNase added. Samples were transferred to 1.6ml microfuge tubes and incubated at room temperature for 30 minutes. 500μl of a 1.6M NaCl solution with 13% w/v polyethylene glycol 8000 was added to the mixture and centrifuged at 12 000xg for 5 minutes at 4°C. The supernatant was removed and pellet resuspended in 500μl TE buffer. Samples were then sequentially extracted with 1x Tris buffered phenol, 1x Tris-buffered phenol: chloroform:isoamyl alcohol (25:24:1) and 1 x chloroform:isoamyl alcohol (24:1). 100μl of 10M ammonium acetate was added to the samples and mixed well. 2x volume of ethanol was added and samples were incubated at room temperature for 10 minutes. Plasmid DNA was recovered by centrifugation at 12 000xg at 4°C for 5 minutes. Pellet was washed two times with 70% ethanol. Pellet was dried and redissolved in TE buffer. Samples were quantitated by 260nm/280nm.

4.2.4: Determination of Orientation

The orientation of the inserts was determined using restriction analysis. This was necessary since the choice of the polymerase used to transcribe the appropriate template strand required knowing into which orientation the insert was ligated.
Figure 25: pGEM-T transcription vector used to subclone PCR products.
**CYP19 Human:** Based upon the sequence of the insert and the vector it was possible to determine orientation by using Pst I restriction enzyme. This enzyme cuts the vector at base pair 73 and cuts the insert at base pair 73. The two possible size fragments produced by this digestion are either 50bp or 247 bp. When the digested fragments were run on an agarose gel, clone #3 had a band at 247bp which was in the SP6 orientation. This was selected for use in transcription of single stranded RNA which was identical to the natural sequence for use as an external control in PCR. This plasmid was linearized by using the Sph I and run out on a 0.8% low melting point agarose gel. The appropriate band was excised and purified using a PCR Select column. Linear plasmid was quantitated using 260nm/280nm. Single stranded RNA was produced using SP6 polymerase.

**pS2:** The orientation of the 431bp insert was determined by digestion using Sst I (Sac I). This enzyme cuts the vector at base pair 94 and cuts the insert at base pair 365. The two possible sized fragments produced by this digestion are either 105bp or 404bp. The insert selected showed a band of 404bp corresponding to an orientation in the SP6 direction. Since production of a riboprobe was the goal of this method, synthesis of the antisense strand will be necessary so that the synthesized RNA will be complementary to mRNA on the membrane. Therefore, utilization of the T7 polymerase will yield antisense RNA. Plasmid was linearized using Not I, which cuts the vector at base pair 92 but does not cut the insert. The plasmid was purified using low melting point agarose and spin columns.
36B4: The orientation of this 562bp insert was determined by digestion using Pst I. This enzyme cuts the vector at base pair 73 and cuts the insert at base pair 451. The two possible sized fragments produced by this digestion are 129bp and 469bp. The selected insert showed a band at 469bp corresponding to an orientation in the SP6 direction. Half of this plasmid prep was digested with Sph I, which would be used with the SP6 polymerase for the synthesis of RNA to be used in RT-PCR. The remaining plasmid was digested with Not I for synthesis of complementary RNA for use as a riboprobe in Northern analysis.

4.2.5: Production of cRNA transcripts

SP6: The functional assay for the production of single stranded RNA was as follows: 5x SP6 buffer (0.2M Tris-HCl, pH 7.9; 30mM MgCl₂, 10mM spermidine); 0.5mM each ATP, CTP, UTP and GTP; 1mM DTT; 0.2μg linearized template and 15 units of SP6 polymerase were dissolved in a reaction volume of 10μl. The solution was incubated at 37°C for 60 minutes. Two microliters/tube DNase (RNA free) was added and incubated at 37°C for 10 minutes to remove contaminating plasmid DNA. Synthesized RNA was quantitated spectrophotometrically and verified by using a mass ladder. Samples were diluted to a concentration of CYP19 = 4.7pg/μl and 36B4 = 5.6pg/μl. Samples were aliquoted and 5 units of RNase Inhibitor was added. Samples were stored at -70°C until used as an external control in the RT-PCR reaction.
T7: The synthesis of complementary cRNA for use as riboprobes was carried out using the T7 polymerase under the following reaction conditions. 5x T7 buffer (0.2M Tris-HCl, pH 7.8; 40mM MgCl2, 10mM spermadine, 125mM NaCl); 0.5mM each ATP, UTP and GTP; 5.0μM [α-32P] CTP (10μCi at 800 Ci/mmol); 10μM cold CTP, 5mM DTT; 0.2μg linearized template and 15 units of T7 polymerase were dissolved in a reaction volume of 10μl. The solution was incubated at 37°C for 60 minutes. Two microliters/tube DNase (RNA free) was added and incubated at 37°C for 10 minutes to remove contaminating plasmid DNA. 2μl of glycogen, 5μl of 3M NaOAc, pH 5.2 and 2.5 times 95% ethanol were added sequentially with mixing after each addition. Labeled RNA was precipitated for 2 hours at -20°C. RNA was recovered by centrifugation at 16 000 xg for 30 minutes at 4°C. Pellet was washed 3 times with 70% ethanol and an aliquot counted. Pellet was resuspended in hybridization buffer and heated at 70°C for 5 minutes prior to the addition to the hybridization reaction as described in section on Southern analysis Chapter 2.

4.2.6: Histopathology of Tissue Samples:

Adult human breast tumor tissue was obtained from the cooperative human tissue network (CHTN) at the Ohio State University. Tissue arrived frozen in liquid nitrogen. A portion of the tissue was fixed with 10% formalin and embedded in paraffin. The remainder of tissue was used to isolate RNA. This was done in the lab of Dr. F. Robertson and RNA samples were used as provided. Pathological evaluation of stained
and sectioned tissue was performed and tissue specimens were ranked for tumor cell density and tumor grade. Tissue sections were also evaluated for the presence and extent of inflammatory cell infiltration on a scale of 0-4, and for evidence of invasion across the basement membrane. This independent analysis was performed by Dr. K. Clausen, Department of Pathology, College of Medicine, O.S.U. Quartiles were based upon the percentage of cells which exhibited a malignant phenotype.

4.3: Results and Discussion

This preliminary evaluation of aromatase expression was done utilizing RNA from 20 patients whom had undergone surgery for breast cancer. Twenty samples of human breast RNA were purified and quantitated by the lab of Dr. Robertson, Comprehensive Cancer Center, The Ohio State University. 1μg of total RNA was used for each reverse transcription reaction. For every sample at least two separate reverse transcription reactions were done. The cDNA produced was further used in between 2-4 separate PCR reactions. For each RT reaction done, the PCR reactions were averaged for each primer pair, CYP19 and 36B4. The ratio of CYP19/36B4 was calculated using this average. The mean of the ratios of CYP19/36B4 for the two RT reactions was used to determine relative levels of CYP19 gene expression. This value was compared to the levels of COX-2 expression from the same RNA sample previously carried out in Dr. Robertson's laboratory.
### Table 7: Data from Reverse Transcriptase Reaction 1; Samples 1-21 CYP19 primers.

Two or three separate PCR reactions were carried out using 10 picograms of cRNA as an external control.

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<td>111.36</td>
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<td>103.97</td>
</tr>
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<td>111.00</td>
<td>143.07</td>
<td>135.80</td>
</tr>
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<td>114.84</td>
<td>122.16</td>
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<tr>
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<td>99.57</td>
<td>140.42</td>
</tr>
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<td>181.01</td>
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<td>128.24</td>
<td>175.27</td>
</tr>
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</tr>
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<td>96.58</td>
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</tr>
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<td>106.21</td>
<td>142.15</td>
</tr>
<tr>
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<td>154.10</td>
<td>60.31</td>
<td>128.63</td>
</tr>
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<td>103.13</td>
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<td>39.45</td>
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</table>

Table 8: Data from Reverse Transcriptase Reaction 1; Samples 1-21 36B4 primers. Three separate PCR reactions were carried out using 10 picograms of cRNA as an external control.
### Reverse Transcriptase Reaction 2: CYP19 Primers

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>PCR-1</th>
<th>PCR-2</th>
<th>mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>9393A</td>
<td>4.821</td>
<td>4.76</td>
<td>4.79</td>
</tr>
<tr>
<td>9259B</td>
<td>25.19</td>
<td>23.09</td>
<td>24.15</td>
</tr>
<tr>
<td>9245B</td>
<td>36.23</td>
<td>33.35</td>
<td>34.79</td>
</tr>
<tr>
<td>9094A</td>
<td>41.33</td>
<td>45.49</td>
<td>43.41</td>
</tr>
<tr>
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<td>59.82</td>
<td>52.63</td>
</tr>
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<td>34.90</td>
<td>32.15</td>
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<td>50.88</td>
<td>49.25</td>
</tr>
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<td>20.19</td>
<td>22.08</td>
<td>21.13</td>
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<td>36.44</td>
<td>37.91</td>
</tr>
<tr>
<td>9717A</td>
<td>134.41</td>
<td>111.30</td>
<td>122.86</td>
</tr>
<tr>
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<td>24.86</td>
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<td>40.38</td>
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<td>42.65</td>
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<td>9675A</td>
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<td>9519A</td>
<td>52.88</td>
<td>51.54</td>
<td>52.21</td>
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<td>60.07</td>
<td>57.44</td>
<td>58.76</td>
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<tr>
<td>cRNA-CYP19</td>
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<td>100</td>
<td>100</td>
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</table>

**Table 9:** Data from Reverse Transcriptase Reaction 1; Samples 1-21 CYP19 primers. Two separate PCR reactions were carried out using 10 picograms of cRNA as an external control.
### Reverse Transcriptase Reaction 2: 36B4 Primers

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>PCR-1</th>
<th>PCR-2</th>
<th>PCR-3</th>
<th>PCR-4</th>
<th>mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>9393A</td>
<td>65.22</td>
<td>63.69</td>
<td></td>
<td></td>
<td>64.45</td>
</tr>
<tr>
<td>9259B</td>
<td>57.83</td>
<td>54.91</td>
<td></td>
<td></td>
<td>56.37</td>
</tr>
<tr>
<td>9245B</td>
<td>85.50</td>
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<td></td>
<td></td>
<td>91.39</td>
</tr>
<tr>
<td>9094A</td>
<td>105.47</td>
<td>97.69</td>
<td></td>
<td></td>
<td>101.58</td>
</tr>
<tr>
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<td>103.96</td>
<td>98.18</td>
<td></td>
<td></td>
<td>101.07</td>
</tr>
<tr>
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<td>103.77</td>
<td>106.65</td>
<td></td>
<td></td>
<td>105.21</td>
</tr>
<tr>
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<td>109.25</td>
<td></td>
<td></td>
<td>108.88</td>
</tr>
<tr>
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<td>101.18</td>
<td>84.44</td>
<td></td>
<td></td>
<td>92.81</td>
</tr>
<tr>
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<td>54.97</td>
<td></td>
<td></td>
<td>60.15</td>
</tr>
<tr>
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<td>87.35</td>
</tr>
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<td>112.91</td>
<td>89.57</td>
<td>103.42</td>
</tr>
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<td>175.30</td>
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<td>195.78</td>
<td>157.76</td>
<td>188.19</td>
</tr>
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<td>268.21</td>
<td>126.47</td>
<td>160.38</td>
</tr>
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<td>182.94</td>
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<td>212.71</td>
<td>264.02</td>
<td>181.76</td>
<td>210.11</td>
</tr>
<tr>
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<td>161.59</td>
<td>218.41</td>
<td>164.13</td>
<td>176.86</td>
</tr>
<tr>
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<td>221.45</td>
<td>237.27</td>
<td>188.18</td>
<td>207.24</td>
</tr>
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<td>257.65</td>
</tr>
<tr>
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<td>144.02</td>
<td>176.93</td>
</tr>
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<td>170.25</td>
<td>270.90</td>
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<td>198.74</td>
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<td>174.17</td>
<td>250.05</td>
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<td>195.13</td>
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<td>cRNA-36B4</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</table>

**Table 10:** An example of data from Reverse Transcriptase Reaction 1; Samples 1-21 36B4 primers. Multiple PCR reactions were carried out using 10 picograms of cRNA as an external control.
<table>
<thead>
<tr>
<th>Patient #</th>
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<th>CYP19/36B4</th>
<th>mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>9393A</td>
<td>0.359</td>
<td>0.074</td>
<td>0.217</td>
</tr>
<tr>
<td>9259B</td>
<td>0.354</td>
<td>0.428</td>
<td>0.391</td>
</tr>
<tr>
<td>9245B</td>
<td>0.615</td>
<td>0.381</td>
<td>0.498</td>
</tr>
<tr>
<td>9094A</td>
<td>0.669</td>
<td>0.427</td>
<td>0.548</td>
</tr>
<tr>
<td>9181</td>
<td>0.677</td>
<td>0.521</td>
<td>0.599</td>
</tr>
<tr>
<td>9020</td>
<td>0.975</td>
<td>0.923</td>
<td>0.949</td>
</tr>
<tr>
<td>9065</td>
<td>0.59</td>
<td>0.295</td>
<td>0.443</td>
</tr>
<tr>
<td>9476</td>
<td>0.675</td>
<td>0.531</td>
<td>0.603</td>
</tr>
<tr>
<td>9196</td>
<td>0.349</td>
<td>0.153</td>
<td>0.251</td>
</tr>
<tr>
<td>9035</td>
<td>0.333</td>
<td>0.242</td>
<td>0.288</td>
</tr>
<tr>
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<td>0.233</td>
<td>0.367</td>
<td>0.300</td>
</tr>
<tr>
<td>9717A</td>
<td>0.550</td>
<td>0.653</td>
<td>0.602</td>
</tr>
<tr>
<td>9263A</td>
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<td>0.173</td>
<td>0.115</td>
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<td>0.266</td>
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<td>0.237</td>
</tr>
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<td>9271</td>
<td>0.122</td>
<td>0.269</td>
<td>0.196</td>
</tr>
<tr>
<td>9213</td>
<td>0.092</td>
<td>0.195</td>
<td>0.144</td>
</tr>
<tr>
<td>9371B</td>
<td>0.162</td>
<td>0.166</td>
<td>0.164</td>
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<td>0.160</td>
</tr>
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<td>0.153</td>
<td>0.263</td>
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<tr>
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<td>0.301</td>
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</table>

**Table 11:** Combined RT-PCR Data from 21 Breast Cancer Patients: Data for CYP19 and 36B4 is the average of 2-4 PCR reactions for each reverse transcription reaction. Values are normalized to an external control which was set to 100% for each separate PCR reaction.
Figure 26: Representative Image generated from RT-PCR analysis using 36B4 primers. Quantitation was carried out using the Image Quant Software. Lane numbers refer to patient samples. Values were normalized to cRNA control values. In all experiments the RT negative control lane was devoid of bands. (36B4.tif)
Figure 27: Comparison of CYP19 expression to COX-2 expression from breast cancer tissue from 21 patients.
(cox-2.spw)
Figure 28: Representative Image generated from RT-PCR analysis using CYP19 primers. Southern analysis was performed using a radiolabelled probe specific to human CYP19 gene. Quantification was done using Image Quant software, Molecular Dynamics. Lane numbers refer to patient samples. Values are normalized to cRNA control values. In all experiments the RT negative control lane was devoid of bands. (cyp.tif)
<table>
<thead>
<tr>
<th>Quartile</th>
<th>Patient #</th>
<th>CYP19/36B4</th>
<th>COX-2/hprt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9272</td>
<td>Not done</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>9880A</td>
<td>0.300</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>9424</td>
<td>Not done</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>9140</td>
<td>Not done</td>
<td>0.587</td>
</tr>
<tr>
<td>1 (well differentiated Ca)</td>
<td>9065</td>
<td>0.443</td>
<td>0.199</td>
</tr>
<tr>
<td>1</td>
<td>9196</td>
<td>0.251</td>
<td>0.055</td>
</tr>
<tr>
<td>1</td>
<td>9307B</td>
<td>0.208</td>
<td>0.175</td>
</tr>
<tr>
<td>2</td>
<td>9259B</td>
<td>0.391</td>
<td>0.165</td>
</tr>
<tr>
<td>2</td>
<td>9064A</td>
<td>Not done</td>
<td>0.295</td>
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<tr>
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<td>0.237</td>
<td>0.385</td>
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<tr>
<td>2</td>
<td>9213</td>
<td>0.144</td>
<td>0.197</td>
</tr>
<tr>
<td>3 (50% inflammation)</td>
<td>9393A</td>
<td>0.359</td>
<td>1.13</td>
</tr>
<tr>
<td>3</td>
<td>9181</td>
<td>0.599</td>
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</tr>
<tr>
<td>3</td>
<td>9476</td>
<td>0.603</td>
<td>0.86</td>
</tr>
<tr>
<td>3</td>
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<td>0.288</td>
<td>0.268</td>
</tr>
<tr>
<td>3</td>
<td>9675</td>
<td>0.160</td>
<td>0.523</td>
</tr>
<tr>
<td>3 (50% inflammation)</td>
<td>9519A</td>
<td>0.208</td>
<td>1.88</td>
</tr>
<tr>
<td>3 (inflammation)</td>
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<td>0.196</td>
<td>2.16</td>
</tr>
<tr>
<td>3 (50% inflammation)</td>
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<td>1.11</td>
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<tr>
<td>4 (invasive cancer)</td>
<td>9245B</td>
<td>0.498</td>
<td>1.08</td>
</tr>
<tr>
<td>4 (invasive cancer)</td>
<td>9020</td>
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<td>4 (invasive cancer)</td>
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<td>1.06</td>
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<tr>
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<td>9678</td>
<td>0.257</td>
<td>0.667</td>
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</table>

**Table 12:** Comparison of Quartile Ranking based on tissue cellularity, CYP19 and COX-2 levels.
Figure 29: Comparison of CYP19 expression of patients grouped into five quartiles based upon histopathology of samples.
Table 13: Estrogen receptor and progesterone receptor status of 10 of the patients evaluated in this study.

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>ER</th>
<th>PR</th>
</tr>
</thead>
<tbody>
<tr>
<td>9393A</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>9259B</td>
<td>86</td>
<td>Negative</td>
</tr>
<tr>
<td>9245B</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>9094A</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>9181</td>
<td>345</td>
<td>59</td>
</tr>
<tr>
<td>9020</td>
<td>Negative</td>
<td>Unknown</td>
</tr>
<tr>
<td>9065</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>9476</td>
<td>Negative</td>
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<td>38</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
Using this method it was possible to reproducibly amplify CYP19 transcripts from breast tissue from a large sample of patients. This method is semi-quantitative and provides information about the relative levels of message. The use of an external control of cRNA allows for comparison of samples which were done using separate master mixes. There was a range of aromatase message which was the highest level of expression observed in patient # 9020, which had a level of 0.949. This measurement is an arbitrary unitless number determined from cpm/mm² of CYP19/cpm/mm² of 36B4. Sample #9213 expressed the lowest level of CYP19 transcripts with a value of 0.144. A sample of tissue derived from human liver was used as a negative control. #9263A is RNA from liver and this sample consistently showed the lowest level of expression. This is a good control as liver contains large amounts of other related cytochrome P450 enzymes. There is no aberrant amplification of related P450 enzymes which confirms that the amplification seen in the breast tissue is from the CYP19 gene only and not a related cytochrome P450 enzyme.

This data is awaiting further statistical analysis by Dr. Randall Harris and his colleagues, to determine the error within each measurement and also if there is a correlation of aromatase expression and COX-2 expression. Based upon independent analysis of histopathology of these samples patients were divided into five quartiles, based on the level of disease present in the sample. There was a positive correlation found between COX-2 expression and the level of pathology in each sample. The three
samples which showed no evidence of disease also showed no expression of COX-2. The highest levels of COX-2 expression were observed in fourth quartile patients with invasive carcinoma. The levels of COX-2 expression also showed a much larger range of expression than aromatase. This is because the COX-2 isoform is the inducible form and levels will be low until stimulated, then the induction is large. Aromatase is constitutively expressed in normal patients and shows an increase in tumor tissue. A variety of factors can regulate aromatase expression, as detailed previously. Higher levels of COX-2 expression could result in higher levels of PGE$_2$, which in turn could increase CYP19 expression through increases in intracellular cAMP levels and activation of promoter II.

A trend was observed in CYP19 expression when relative levels of expression were plotted versus quartile (Figure 29). There is an increase in aromatase expression with the highest levels in patients ranked in quartile 4. Only one patient from quartile 0 was evaluated, showing a mean value of 0.300. The average expression of quartile 4 patients was 0.577(SEM 0.143). The three patients in quartile 4 which were designated as invasive carcinoma from the histopathology also showed very high levels of aromatase expression. The is in agreement with literature reports showing that aromatase levels were higher in tumors than in normal tissue.
4.4: Conclusions

These experiments detail the development of a PCR method which allows for semi-quantitative measurement of CYP19 transcripts. The use of cRNA as an external control allows for normalization of signals for separate experiments. This provides a rapid method by which to screen large groups of patient samples for correlations between disease state and gene expression. A more thorough method of quantitation involving an internal control of cRNA could be used to confirm any interesting findings.

The production of cRNA for human CYP19 and 36B4 control gene was carried out utilizing a pGEM-T vector containing bacteriophage promoter regions. The addition of 10% DMSO allowed for very clean amplification of CYP19 in breast tissue, although it resulted in a less efficient amplification overall. An increase in MgCl₂ concentration (6.0mM) was used to increase activity of Taq. The PCR protocol which worked well using ovary mRNA was ineffective using breast tissue mRNA. This is probably due to the large differences in starting number of transcripts, with ovary having very high levels and breast tissue having relatively low levels.

The level of CYP19 gene expression for the 20 patients examined ranged from 0.144 to 0.949. This reflects a 6.6 fold increase of CYP19 gene expression within this group of patients. An increase in CYP19 expression with an increase in quartile level was
observed. It is not known at this time whether there is positive correlation between COX-2 expression and CYP19 gene expression. This is awaiting further statistical analysis.

4.5: Cited References


Chapter 5

AROMATASE ACTIVITY IN PLACENTALLY DERIVED JAR CELLS

5.1: Statement of Problems and Objectives

The choriocarcinoma cell line, JAr is known to have a high level of aromatase activity. This is a placentally derived cell line; therefore, the major regulator of aromatase expression is utilization of promoter I.1. Previous work with the Sprague Dawley rats showed that when animals were treated with the aromatase inhibitor there was a subsequent elevation of ovarian aromatase mRNA when compared to control animals. The effect of the aromatase inhibitor, 7α-APTADD, provides a type of inhibition which is irreversible. Unlike competitive inhibitors, this type of inhibition is overcome only with new protein synthesis.

It was of interest to see if this inhibitor could directly affect the level of gene expression using a cell line. An alternative explanation for the increase in aromatase expression was that the cell could recognize that the protein was dysfunctional and compensated by producing more protein. The effect of this irreversible aromatase inhibitor was
evaluated using Northern analysis and also the effect of serum on the expression of CYP19 would be considered.

5.2: Experimental Methods

5.2.1: Cell Culture

JAr (HTB 144, a choriocarcinoma cell line, ER-) were purchased from The American Type Culture Collection (ATCC, Rockville, MD) and maintained on RPMI 1640 media (Gibco, BRL) supplemented with 10% fetal calf serum and gentamicin (20mg/L). Cells were grown on 75cm² plastic flasks (Corning Glass Works, Corning, NY). For experiments calling for a defined media, cells were grown using DMEM/F12 containing albumin, insulin and transferrin.

5.2.2: Northern Analysis

Northern analysis was done as previously described, with the following modifications. 40µg of total RNA isolated from JAr cells was separated out on a 1.2% formaldehyde gels. A 273bp cDNA probe specific to human CYP19 was radiolabelled and used to hybridize Nylon membranes. Placentally derived aromatase message produces a 2.9kB transcript due to an alternative polyadenylation signal. Signals were normalized using the ribosomal phosphoprotein PO, as stated previously.
5.2.3: Aromatase Activity

Aromatase activity was measured by the release of tritiated water due to the conversion of $[1\beta-\text{H}]$androstenedione to $[^3\text{H}]\text{H}_2\text{O}$. $^1$ JAr cells were grown to 80% confluence in T-25 flasks in triplicate. One microCurie of androstenedione (50nM total) was added and after four hours, the media was removed and extracted as previously described. DNA content of each flask was determined using the diphenylamine assay. Aromatase activity is expressed as picomoles of $^3\text{H}_2\text{O}$ produced/10$^6$ cells.

5.3: Results and Discussion

The purpose of this study was to determine if there was a direct effect of the aromatase inhibitor 7α-APTADD on aromatase expression. Initial experiments were performed using JAr cells which were grown on a defined media system 48 prior to the addition of aromatase inhibitor. Two concentrations of the aromatase inhibitor was used, 50nM and 250nM for several time periods, from 6, 24 and 48 hours of treatment (Figure 30 and 31). The 50nM treatment showed a 1.8 fold increase in aromatase expression at 24 hours of treatment. This increase was transient and after 48 hours was reduced to the control levels. The 250nM treatment also showed an increase in aromatase expression at 24 hours and was reduced by 48 hours. However, there appeared to be no dose response with the higher concentration of drug. If this increase was a consequence of the aromatase inhibitor, then 250nM should show a higher level of induction, assuming
the inhibitor was not at saturating concentration. Placental microsomal assays have shown an effective range of aromatase inhibitor concentration ranging up to 500nM.\(^2\)

Figure 30: Northern Analysis of JAr cells treated with 50nM 7α-APTADD
Figure 31: Northern Analysis of JAr cells treated with 250nM 7α-APTADD.
One thing that was noted was that the control flasks which were put on defined media for 48 hours showed a significantly higher level of aromatase expression than the control flasks which had media containing 10% fetal calf serum. At this time it was not expected that serum would have any effect on aromatase expression. The time frame of the drug dosing was such that all flasks were placed on defined media for 48 hours and then all drug was added. The flasks which were treated for 6 hours were on defined media 42 hours less than the flasks treated for 48 hours. It is possible that there is an inhibitory factor which is present in serum which is reducing the constitutive expression of aromatase in this cell line. This is apparent by comparison of lanes 3 and 4 from Figure 32. Both lanes are control lanes with no inhibitor added, however, lane 4 was placed on defined media for 48 hours and lane 3 was grown in media containing 10% serum. There is a significant 2 fold difference in aromatase expression. It is very likely that the difference that was initially observed with treatment of aromatase inhibitor was a consequence of this serum effect. The levels at 48 hours were equal to that seen in control levels of defined media but this was still an elevation over the value obtained with serum. It is unknown what this inhibitory factor is. It is not an estrogen since JAr cells do not have estrogen receptors. Figure 33 shows the expression of aromatase over the 48 hour treatment when cells were maintained on 10% serum, while running a simultaneous control for each time point. This data shows that there is no effect of this drug on aromatase expression.
Figure 32: Northern analysis: Effect of 7α-APTADD on CYP19 expression in JAr cells. Lane 1) dexamethasone (250nM, 24 hours, 10% FCS); Lane 2) PMA (10μM, 48 hours, 10% FCS); Lane 3) Control Flask (10% FCS); Lane 4) Control Flask (Defined media); Lane 5) 50nM 7α-APTADD (6 hours, defined media); Lane 6) 250nM 7α-APTADD (6 hours, defined media); Lane 7) 500nM 7α-APTADD (6 hours, defined media); Lane 8) 50nM 7α-APTADD (24 hours, defined media); Lane 9) 250nM 7α-APTADD (24 hours, defined media); Lane 10) 500nM 7α-APTADD (24 hours, defined media); Lane 11) 50nM 7α-APTADD (48 hours, defined media); Lane 12) 250nM 7α-APTADD (48 hours, defined media); Lane 13) 500nM 7α-APTADD (648 hours, defined media).
It was observed that the addition of dexamethasone, which has been shown to increase CYP19 message in stromal fibroblasts, showed no effect in this placental cell line. The addition of a phorbol ester, in the presence of serum, showed an increase in CYP19 expression of 4.67 fold. Neither compounds showed any effect while these cells were grown using defined media.

Phorbol esters, such as PMA, are known to upregulate and down regulate many cellular processes. Short term treatment results in an initial increase of PKC activation, while chronic exposure to high concentrations of phorbol esters results in a down regulation of most of the protein kinase C isoforms. (PKCy is independent to phorbol esters). PKC is protease insensitive when in the inactive state. PMA activates this kinase and mobilizes it to the plasma membrane. At this point it can be degraded by intracellular proteases. After 24 hours, this results in a depletion of PKC isoforms and a loss of PKC signal transduction pathways. It is possible that chronic exposure to high concentrations of PMA (48 hours, 10μM) cells are relieved from the negative control of a growth factor, such as TGF-β, which is found in serum and is known to inhibit aromatase expression. The effect of PMA is only observed after 24-48 hours. This is consistent with the data from the inhibitor study. Simply having the cells on defined for an increasing amount of time resulted in an increase in CYP19 expression. Over time, the lack of this inhibitory molecule allows for a rebound of this gene expression.
Figure 33: Northern Analysis of CYP19 gene expression of JAr cells grown using 10% fetal calf serum treated with 7α-APTADD. Lane 1) Control; Lane 2) 6 hour treatment; Lane 3) 6 hour control; Lane 4) 12 hour treatment; Lane 5) 12 hour control; Lane 6) 24 hour treatment; Lane 7) 24 hour control; Lane 8) 48 hour treatment; Lane 9) 48 hour control.
5.4: Conclusions

This study was undertaken to determine if the aromatase inhibitor 7α-APTADD had a direct effect on the gene expression of cytochrome CYP19 in the choriocarcinoma cell line JAr. Treatment of three concentrations, 50, 250nM and 500nM 7α-APTADD over several time periods, 6, 24 and 48 hours, resulted in no significant increase in CYP19 expression. The effect of serum on the expression of this gene was noted. Removing JAr cells from media containing serum and growing them on a defined media results in the increase in expression of CYP19 gene over 72 hours. The increase is about 3.6 fold increase throughout the 96 hour experiment. This was observed simply by comparing two control flasks, one grown in the presence of serum and one in defined which resulted in a 2 fold increase in aromatase expression with the first 48 hours. A further increase was observed when cells were treated with drug, for a further 24 hours. The maximal increase was observed at 72 hours after being placed on defined media. This effect was independent of the aromatase inhibitor. No increase in CYP19 gene expression was observed when cells were treated with 7α-APTADD in the presence of serum.

Chapter 6

CONCLUSIONS

6.1: General Summary

The underlying theme of this research is the examination of CYP19 gene expression and enzymatic activity in a variety of tissues. This cytochrome P450 enzyme is responsible for the biosynthesis of estrogen production. Estrogens have a critical role in both physiological and pathological processes; estrogen dependent breast tumors are one example. Therefore, understanding the factors and mechanisms by which aromatase is regulated is critical to understanding these pathological processes.

The regulation of the CYP19 gene in a variety of tissue exhibits an intricately regulated mechanism which allows for tissue specific expression. This provides an additional level of transcriptional control, allowing for expression of aromatase only when required. In pathological processes like estrogen dependent breast cancer, inappropriate expression of CYP19 results in higher levels of estrogen production and enhances tumor growth. The three sites of CYP19 expression which were examined in this research are the ovary, placenta and breast tissue: stromal fibroblasts and epithelial components. The
simultaneous measurement of aromatase enzymatic activity allows for an evaluation of the transcriptional and translational processes.

6.1.1: Ovarian CYP19 Expression and Enzyme Activity

Initial studies evaluated the *in vivo* effect of a mechanism based aromatase inhibitor, 7α-APTADD. This potent and selective inhibitor was effective at reducing tumor volume in the DMBA rat model system. Ovarian CYP19 gene expression showed a 3.4 fold increase in treated animals (50mg/kg/day) over that of controls evaluated using a kinetic RT-PCR method. Measurement of ovarian aromatase activity was also carried out using normal mature rats. Treatment with 7α-APTADD at 50mg/kg/day from 1 to 14 days resulted in a nearly complete suppression of enzymatic activity.

Northern analysis of ovarian transcripts was carried out to evaluate specific splice variants of the CYP19 message. Only the largest (2.5kB) transcript is translated into a functional protein. Quantification of only this transcripts showed no increase in CYP19 gene expression. The two smaller fragments, which could be differentially regulated showed weak signals at 1.7kB and 2.2kB, but were impossible to quantitate.

The steroidal aromatase inhibitor, 7α-APTADD, shows *in vivo* effectiveness at reducing tumors and ovarian enzymatic activity is abolished. The effect of 7α-APTADD on the level of transcription is less clear. The presence of the splice variants
is a complicating variable. Nevertheless, the desired end point of effective inhibition of enzymatic activity and reducing tumor volume by administering this compound were met.

6.1.2: Stromal Aromatase Activity from Breast Tissue

The levels of aromatase activity in the breast are primarily provided by the stromal fibroblasts. An evaluation of the effect of extracellular matrix protein, collagen I, on aromatase activity was performed. Culturing primary fibroblasts on a collagen I matrix reduced constitutive aromatase activity while retaining a highly hormone responsive state. This effect was not seen using an epithelial cell line (MCF-7 or MCF-7_adr). The growth rates of primary fibroblasts grown on collagen I were significantly less than conventional culture systems, as measured by thymidine incorporation. This system seems to provide an improvement over conventional culture conditions, as it addresses the role of the extracellular matrix as a regulator of gene expression.

6.1.3: Breast Cancer Patient Samples

Twenty patient tumor samples were evaluated for CYP19 gene expression. This required the development of a RT-PCR methodology which utilized an external control of cRNA for CYP19 and the control gene. The mean expression of the CYP19 gene was compared to COX-2 gene expression and to the independently determined histopathology of the sample. A trend towards increasing levels of CYP19 with increasing quartile ranking was observed. This is consistent with literature reports of
higher levels of aromatase in and around tumors. Prostaglandins may have a role in the regulation of CYP19 in breast tumors. A strong positive correlation with COX-2 expression was observed. This is consistent with the hypothesis that in tumors, CYP19 gene expression is regulated by cAMP mediate pathways utilizing promoter II.

6.1.4: Placental CYP19 Expression

JAr cells express high levels of CYP19 and have correspondingly higher levels of activity. The possible effect of 7α-APTADD on CYP19 gene expression was evaluated using this cell line. There was no increase in CYP19 expression when cells were treated with this compound. It was possible that the irreversible nature of the inhibition would stimulate the cell to produce more protein. This possibility was ruled out using the conditions described in Chapter 5. There was a significant effect of the presence of serum on this cell line. It is postulated that there is an inhibitory factor present in serum, which results in a suppression of CYP19 expression. Simply removing this factor, by placing the cells on a defined media results in an increase in constitutive CYP19 expression over several days. Then nature of this negative regulating factor is unknown. Phorbol esters in the presence of serum were able to induce CYP expression after 48 hours, suggesting that the depletion of protein kinase C is critical to the de-repression of this gene. Dexamethasone, which markedly increases CYP19 gene expression in stromal fibroblasts, had no effect on placentally derived cells, either in the presence or absence of serum factors.
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