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Conserved Molecular and Epigenetic Determinants of Aromatase Gene Induction by the Herbicide Atrazine in Human and Rat Cellular Models Relevant to Breast Cancer Risk

By

Theresa Ryan Stueve

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular Toxicology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Gary L. Firestone, Co-Chair
Professor Dale Leitman, Co-Chair
Professor Chris Vulpe
Professor Daniella Kauffer

Fall 2011

Abstract

Conserved Molecular and Epigenetic Determinants of Aromatase Gene Induction by the Herbicide Atrazine in Human and Rat Cellular Models Relevant to Breast Cancer Risk

By

Theresa Ryan Stueve

Doctor of Philosophy in Molecular Toxicology

University of California, Berkeley

Professor Gary Firestone, Co-Chair

Professor Dale Leitman, Co-Chair

The widely-applied herbicide atrazine (ATR) is a potent endocrine disruptor that elicits anti-androgenic and estrogenic effects, often at concentrations below the current drinking water standard (3 ppb), in wildlife and laboratory models of every vertebrate class studied to date. In oncogenicity studies conducted more than 15 years ago by its principle registrant, Ciby-Giecy (formerly Novartis Crop Protection, currently Syngenta), ATR increased plasma estradiol levels and mammary tumor incidence in ovary-intact but not ovariectomized (OVX) Sprague-Dawley (SD) rats. ATR inhibits phosphodiesterases and increases cAMP-signaling and steroidogenesis in several cell lines. Most relevant to rat and human mammary tumor risk, ATR induces expression of the enzyme aromatase (CYP19), which irreversibly converts androgens to estrogens, in several human and rat steroidogenic cell lines; however, ATR does not affect CYP19 in human estrogen-sensitive MCF-7 breast cancer cells. The significance of these findings to human health is debated and ATR is classified as 'not likely to be carcinogenic to humans' by the EPA due to 'lack of human and mechanistic data".

The mechanisms of rat mammary tumor induction by ATR have not been robustly interrogated for human relevance. Molecular determinants of ATR sensitivity in cell lines relevant to breast cancer (BC) risk, particularly fibroblast stromal cells, are poorly defined. In the present work, we assessed the effect of ATR on *cyp19* promoter activity, mRNA levels, and trans-acting factors with consensus binding sites in the highly conserved cAMP-responsive gonadal region of c*yp19,* and identify conserved molecular and epigenetic determinants of CYP19 induction by ATR in human estrogen growth-sensitive BC cells (T-47D cell line), human adipose fibroblasts (SGBS cell line), and rat ovarian granulosa cells (SIRGC cell line).

ATR increased human gonadal *cyp19* promoter activity in gene promoter-reporter studies by GATA, LRH-1, PKA- and PI3K-mediated mechanisms in human T-47D, SGBS, and significantly, rat SIRGC cells. ATR also increased endogenous Cyp19 mRNA levels in SGBS fibroblast and SIRGC rat granulosa cells, but only increased transcripts in T-47D cells pre-treated with the DNA-methylation inhibitor, 5-aza-dC.

Attempts to reconcile robust and conserved cAMP-responsive gonadal *cyp19* promoter activation with varied mRNA-level effects in three alternately-responsive cell lines led us to the discovery that previously unknown epigenetic determinants are critical in conferring susceptibility to aberrant aromatase induction by ATR. We propose sensitivity to this widespread estrogenic herbicide varies inversely with *Cyp19* gonadal promoter methylation, and directly with expression of GATA-3/4 and LRH-1/SF-1 transcription factors. Interrogating in other models the determinants of responsiveness we here define may enhance our understanding of how reported inter -strain, -species, and -laboratory differences in sensitivity can best inform risk assessments that account for human inter-individual susceptibility to gene-environment -related estrogen disorders.

This work is dedicated to my grandmother

Darla Eugene Stueve

who departed with
breast, lung, and bone cancer
in my mother's strong arms
in Winter Garden, Florida
December 19th, 2004
while I was ashamed and away at school
terrified and senselessly trying to save us all

You taught me more that day
than I learned all the days before or after
and I won't forget
no matter what changes
and I won't relent
living everyday to earn your forgiveness

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LIST OF ABBREVIATIONS AND DEFINITIONS

5’-aza-dC, 5-aza-2’-deoxycytosine- a DNA methylation inhibitor;
ATR, atrazine;
BAF, breast adipose fibroblasts;
BC, breast cancer;
cAMP, 3'5'-cyclic adenosine monophosphate;
ChIP, chromatin immunoprecipitation;
CLS, cAMP-responsive element-like binding site;
CREB, cAMP-response element binding protein;
CYP19- aromatase;
ERE, Estrogen response element;
F344- Fisher 344 rat strain; progesterone-dominant in senescence; tumor-insensitive;
GAPDH, glyceraldehyde-6-phosphate dehydrogenase;
GATA-family of structurally related and conserved transcription factors that bind the consensus (A/T)GATA(A/G) motif;
H89, N-[2-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide, PKA inhibitor;
LRH-1, Liver Receptor Homolog-1;
NR5A, Nuclear receptor family to which LRH-1 and SF-1 belong;
NR5A1, Steroidogenic Factor-1 (SF-1);
NR5A2, Liver Receptor Homolog-1 (LRH-1);
OVX- ovariectomized;
P450arom, aromatase;
pII, proximal cAMP-responsive gonadal promoter II of the human *cyp19* gene;
PCR, polymerase chain reaction; qPCR, quantitative PCR
PKA, protein kinase A;
PM, postmenopausal;
ppb, part per billion; ppm, part per million;
RE, DNA response element;
RT-PCR, reverse transcriptase polymerase chain reaction;
SF-1 steroidogenic factor-1;
WBA, Western Blot Analysis.

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GENERAL INTRODUCTION

Endocrine Axes are Necessarily Highly Responsive to Environmental Signals

Hormones orchestrate nearly every biological process in the human body while maintaining homeostasis in the face of constant external and internal environmental flux. As such, components of the endocrine system, including endocrine glands, their target tissues, and the cognate hormone receptors that modulate both, are highly responsive to environmental stimuli.

In the most simplistic view of an endocrine axis, the stunning degree of phenotypic plasticity required to maintain homeostasis from a fixed genotype is governed by endocrine glands that respond to environmental influences by modulating release of specific hormones in the bloodstream. These changes in circulating hormones evoke responses only in target tissues that express cognate membrane or nuclear hormone receptors, and specific hormone-receptor interactions permit initiation, amplification, or refinement of distinct signal transduction and transcriptional programs that ultimately facilitate acute or sustained adaptations to defined environmental stimuli- minimally depending on the greater developmental and endocrine context in which a particular hormone receptor type is activated.

Thus, in governing adaptive end-organ responsiveness requisite for maintaining homeostatic balance in developmental and environmental flux, endocrine axes must function critically and coordinately at four levels: pertinent, proper synthesis, and timely secretion of hormones; maintenance of effective and bioavailable levels of hormones in the bloodstream; functional cognate receptor expression at appropriate levels in responsive tissues; negative and positive feedback ‘loops’; degradative and elimination mechanisms that can terminate hormone action when an initiating environmental stimulus or the need to respond to it has passed. A general scheme of an endocrine axis is provided in Figure 1.

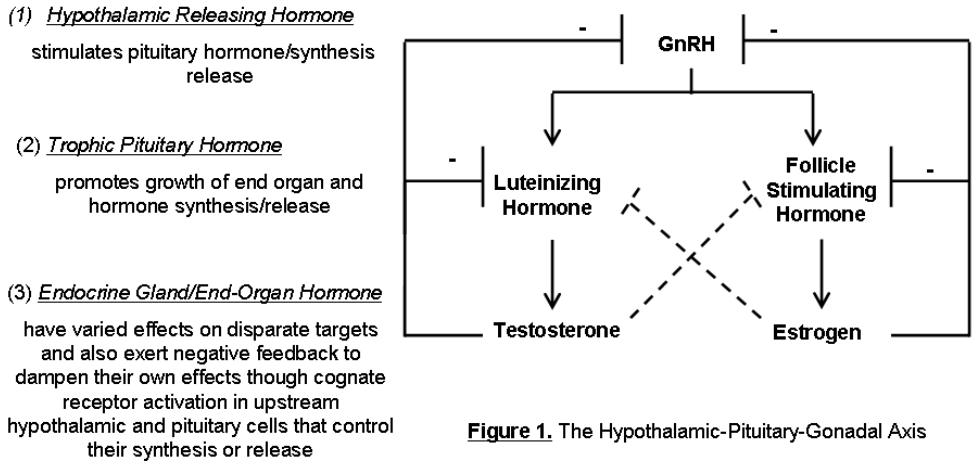


Figure 1. The Hypothalamic-Pituitary-Gonadal Axis

Figure 1. The Canonical Endocrine 'Hormone Axis'. The hypothalamic-pituitary-gonadal (HPG) axis is shown as an example of an endocrine axis. Releasing hormones secreted into the hypophyseal pituitary portal system stimulate *de novo* biosynthesis and/or release of stored pituitary hormones, which have trophic effects on end organs. When released in the bloodstream trophic pituitary hormones cause both end-organ growth and *de novo* hormone biosynthesis where their cognate receptors are expressed (gonadal tissue in this example). End-organ hormones (T and E in this example) can dampen their own signaling in a 'negative feedback loop' when these travel through the blood to the hypothalamus and pituitary gland and bind their cognate receptors (androgen and estrogen receptor in this example). In the HPG, hypothalamic gondotropin releasing hormone (GnRH) stimulates release of LH and FSH from the pituitary, which respectively, cause thecal/or leydig cell growth, and granulosa/or sertoli cell growth/proliferation, and also synthesis of testosterone and estradiol. Subsequently, estradiol and testosterone can dampen their own rate of synthesis after traveling in the blood and binding their central cognate receptors- which results in downregulation of the upstream hormones (GnRH, LH and FSH) that positively regulate their production.

Incidence of Endocrine-Sensitive Disorders and Cancers is Increasing Annually in Industrialized Countries; Twin and Immigrant Studies Underscore the Significance of Gene-Environment Interactions in BC Risk

When hormonal control of ion balance or body temperature homeostasis is compromised, immediate death may result. Endocrine hormones also steward differentiation, development, and growth of tissues and organs from fetal programming and developmental periods through reproductive senescence and death. Malfunctioning of hormone action at any level of an axis can result in initiation or progression of chronic endocrine-sensitive disorders that are on the rise all over the world, including but not limited to: obesity; metabolic disorders; altered pubertal timing; infertility; breast and male and female reproductive cancers (Fenton, S, 2007; Milkian R, 2004).

Incidence of testicular and breast cancer (BC) varies widely by geography, but has increased globally over the last 40 years- disproportionately in industrialized countries. In the U.S. specifically, incidence of BC has risen about one percent per year over the last 60 years, and while certain inherited risk factors, most notably mutations in the gene BRCA1, significantly contribute to BC risk, genetic factors alone cannot account for annual increases in BC incidence in the U.S. and other westernized societies (Millikan, R, 2004).

Studies of monozygotic twins indicate inherited factors only account for 5-27% of BC cases (Millikan, R, 2004). Epidemiological studies of immigrant populations, notably a study of descendants of Asian women who migrated to the northern California bay area (Table 1), indicate immigrants inherit the cancer risk of their host country within two generations (Thomas and Karagas, 1996). These twin and immigrant studies strongly underscore the significance of gene-environment interactions in BC incidence and promotion, but also the promise of disease prevention and reversibility through education, lifestyle interventions, and hazardous chemical exposure reduction.

Table 1. Geographic Variation in Breast Cancer Incidence Rates Among Women. Breast cancer incidence rates in southeast Asian countries are on average significantly lower relative to rates in the United States, implicating both genetics and geography as significant contributors to BC incidence. Descendants of migrants tend to inherit the cancer incidence of their host country within two generations. Daughters and granddaughters of southeast Asian women who migrated to the Northern California Bay Area have higher BC incidence rates than do women in countries their elders migrated from, underscoring the importance of gene-environment interactions in breast cancer risk and prevention strategies.

Table 1: Geographic Variation in Breast Cancer Incidence Rates Among Women[a]

Group and place	Cases	Rate[b]
Chinese		
China, Shanghai	6084	26.5
US, San Francisco: Chinese	459	55.2
US, Hawaii: Chinese	159	57.6
Japanese		
Japan, Osaka	7544	24.3
US, Los Angeles: Japanese	319	63.0
US, San Francisco: Japanese	138	68.4
US, Hawaii: Japanese	903	72.9

[a]Source: Parkin *et al.*, 1997.
[b]Per 100,000 women.

‘Endocrine-Disrupting Chemicals’ are Contaminants in the Environment and Consumer Goods Capable of Perturbing Hormonal Activity

As suggested previously, hormone axes, by definition, must be highly responsive to environmental stimuli- be those signals endogenous or exogenous- to maintain homeostatic balance. The majority of BCs initially diagnosed in industrialized countries are hormone-sensitive. Diet and lifestyle, and increasingly, exposure to ‘endocrine-disrupting chemicals’ (EDCs) in foods, consumer/personal care products, and the environment, are gaining significance in our understanding of demographic and geographic disparities in BC incidence and promotion (Millikan, R, 2004). Many of these factors have recently been discovered to alter the epigenome- which partitions the DNA one inherits into transcriptionally active an inactive regions- and may pivotally govern adaptive responses of the expressed genome to each individual’s unique environment.

Indeed, as many as thirteen large epidemiological studies, including the Long Island Breast Cancer Study (LIBCS)- the largest of its kind conducted through collaborative efforts of New York Area epidemiologists affiliated with Mount Sinai, Columbia, Sloan-Kettering, Cornell, CUNY, and Albert Einstein College- indicate risk of BC with high exposure to polycyclic aromatic hydrocarbons (PAH)- products of incomplete combustion contained in tobacco smoke, diesel, air pollution, and grilled and smoked meat- is increased by as much as fifty percent (Millikan, R, 2004). This increased BC risk owing to PAH exposure observed by several LIBCS investigative groups was significantly higher than that reported associated with progesterone plus estrogen hormone-replacement therapy (HRT) in the Women’s Health Initiative (WHI) conducted by the NIH and NCI in 2002. Five to six percent increases in BC risk among post-menopausal (PM) HRT users led to termination of this arm of the trial, as in the estimation of researchers the risks of estrogen and progesterone use by PM women outweigh much-advertised benefits (Chlebowski, *et al*; 2003).

Lifetime Estrogen Exposure is Strongly Correlated with BC Risk

The most recognized risk factors for BC are being a woman in advancing years. BC rates are 100 times higher in women than men, and risk for every woman increases dramatically every year she grows older. Less obviously, number of years on this earth as a female is a reliable proxy of cumulative ovarian estradiol production, and lifetime estrogen exposure is the major non-genetic determinant of BC risk (Millikan, R, 2004). As such, stature; parity; post-menopausal obesity; breast density; and age at menarche and menopause- clinical indices of estrogen exposure, are also predictive of BC risk. Estrogens drive proliferation of breast epithelial cells in critical stages of normal mammary gland differentiation and development via binding their cognate nuclear receptors- typically estrogen receptor alpha (ERα). ERα binds to estrogen response elements (EREs) in regulatory elements of target genes, such as *cyclin*

d1 and *cyclin e*, whose products stimulate progression through the stages of mitotic cell cycle. This proliferative action of ERα is also critically involved in BC risk, as an excess of estrogen or ligand-independent ERα activity may progress mammary epithelial cells that have sustained mutations thorough the cell cycle without time enough for DNA repair. Further, a general increase in mammary epithelial proliferation by estrogens simply translates into a higher density of cells susceptible to mutagenenic assault in a tissue not particularly enriched in detoxification enzymes for contending with chemical hazards. Elements of the mitotic cell cycle (A, top panel) and ERα-mediated mammary epithelial cell cycle progression (B, bottom panel) are depicted in Figure 2.

Figure 2:

Estrogen receptor-mediated cell cycle progression. (A) The mitotic cell cycle proceeds in complexly-regulated discrete stages that result in high-fidelity DNA replication in parent cells and faithful symmetric DNA segregation in clonal daughter cells. If a mutation is sustained during any stage of the cycle- from G1 to M- the cell can 'growth arrest' or exit the cell cycle to attend to DNA repair. Cyclin D and Cyclin E are two regulated proteins that progress the cell from 'G1' – the growth phase in which the cell prepares organelles for DNA duplication- to 'S' phase, the phase of the mitotic cell cycle in which DNA in one cell is actually doubled in preparation for symmetric segregation into two clonal daughter cells in M phase. (B) ERα activates transcription of the genes for cyclin D and cyclin E. ERα hyperactivity may be sufficient to progress mammary cells through G1 to S phase even when mutations have been sustained. Simply having fibrous breast tissue is also a risk factor for BC as a greater density of cells are susceptible to mutagenic assault over the course of a woman's lifetime (Santen and Mansel, 2005).

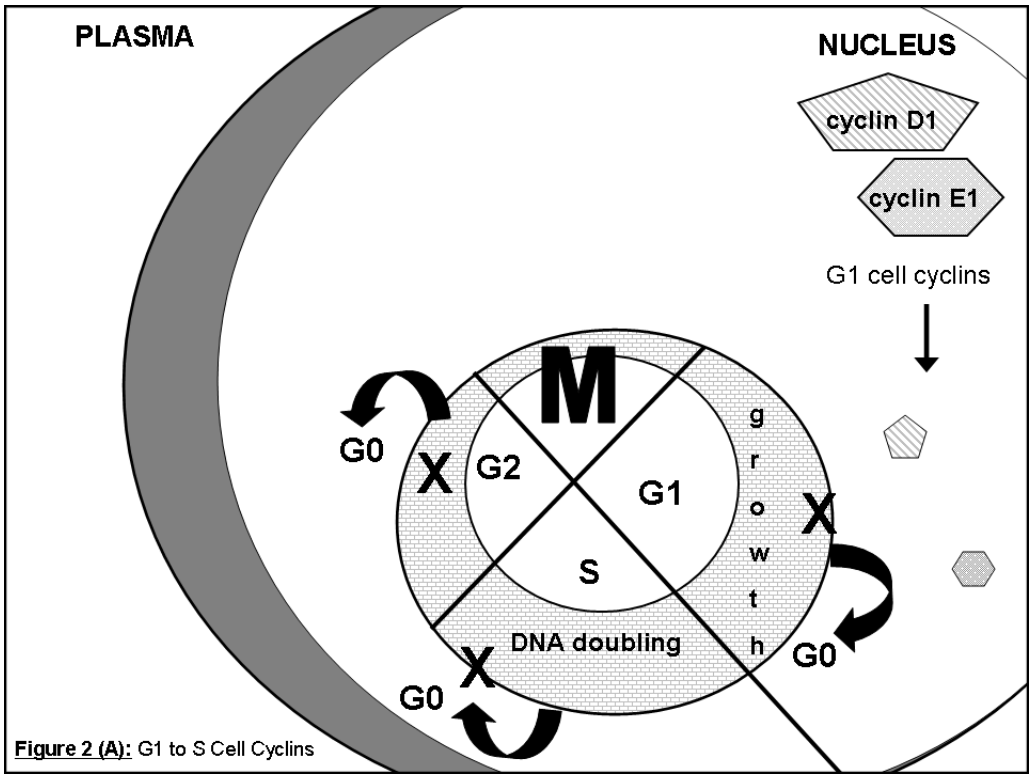


Figure 2 (A): G1 to S Cell Cyclins

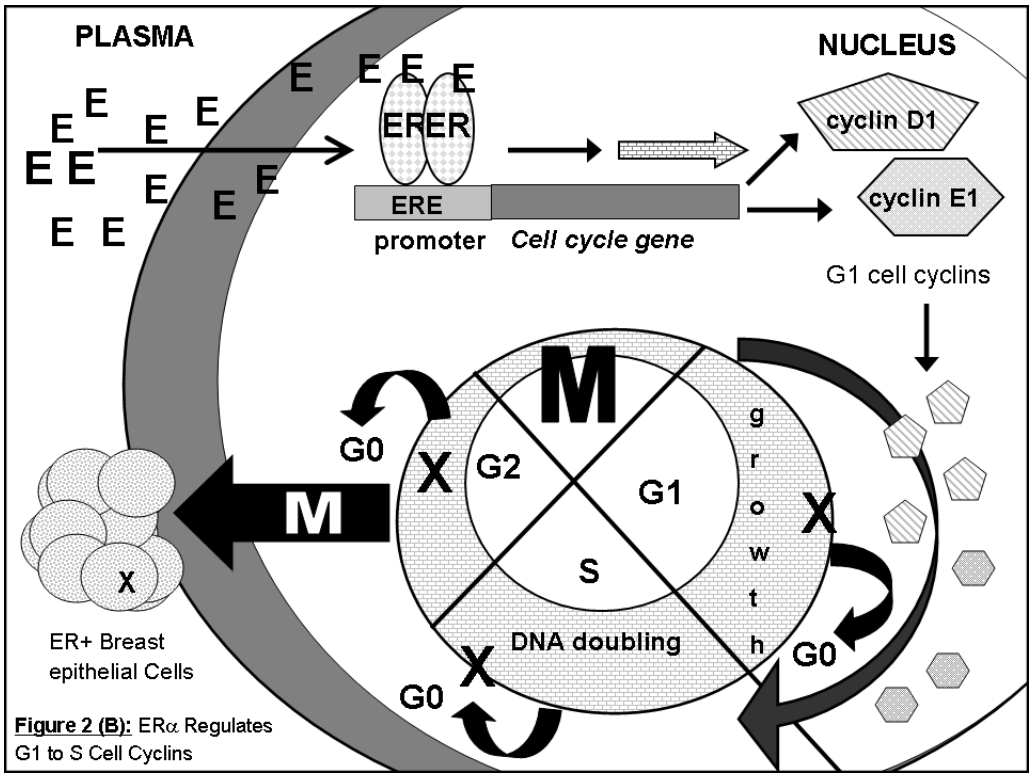


Figure 2 (B): ERα Regulates G1 to S Cell Cyclins

The National Academies of Science and Endocrine Society Designate Reducing Aggregate Environmental Estrogen Exposure an Imminent Public Health Priority

Estrogens are important promoters of ER+ (BCs) and elicit physiological actions in the picomolar range. Many emerging hormonal disorders are also influenced by estrogen, and in recent official position statements, The Endocrine Society (Diamanti-Kandarakis *et al*, 2009) and National Academies of Science have stated understanding the significance of exposure to environmental estrogens, or pollutants with the ability to impact estrogen signaling, is an imminent public health priority. Heightened research in the field of endocrine disruption reveals a troubling number of novel chemicals in our foods, home and work environments have estrogenic activity. Even at infinitesimally small doses individually, our aggregate exposure to such a number of 'xenoestrogens' is concerning, as unlike estrogens we produce endogenously- as a species we have not had sufficient time to evolve detoxification or degradative enzymes to contend with the varied estrogenic substances we've engineered, which may result in additive exposures to or body burdens of these chemicals that affect significant physiological and largely preventable consequences.

The Herbicide Atrazine is a Ubiquitous Contaminant that Elicits Anti-androgenic and Estrogenic Effects in every Vertebrate Class studied to date

Atrazine (ATR), a chlorotriazine herbicide and known endocrine disruptor used extensively on corn, sorghum, and sugarcane crops in North America, has remained one of the most heavily applied pesticides worldwide since its original registration by the Ciba-Geigy Corporation (Novartis subsequently; Syngenta currently) in 1958. ATR is the second-most frequently detected contaminant of ground, surface, and drinking waters in the U.S, and causes anti-androgenic and estrogenic reproductive effects at low, biologically relevant doses in every vertebrate class studied to date (Hayes *et al*, 2010).

Atrazine is relatively persistent to abiotic and biotic degradation, and is detectable in food, fish, and ecological and public water supplies far from application sites, being deposited remotely from agricultural run-off or atmospherically via rainfall- and has been detected at concentrations as high as 40 ppb in precipitation [Hornbuckle, K; 2000]. ATR is an especially potent endocrine disruptor in several amphibian species, and has been repeatedly shown to demasculinize and feminize dosed amphibians, sometimes resulting in hermaphroditism, at concentrations as low as 0.1 ppb (μg/L; Hayes *et al*, 2003). At the time of this writing, this level is thirty times less than the allowable ATR drinking water standard set by the U.S. Environmental Protection Agency (EPA), which is 3 ppb.

Owing to its high mobility, persistence, and pervasiveness as a groundwater contaminant, ATR was banned in European Union member states in 2003, one week before the US EPA announced approval for its continued use in Interim Reregistration Eligibility Decision (IRED) documents (Ivory, D, 2010; Sass and Colangelo, 2006). The EPA's decision was based on risk assessments and the final recommendations of two *ad hoc* science advisory panels (SAPs) the EPA itself composed of representatives from its own agency and Syngenta, the principle registrant and Switzerland-based manufacturer of ATR for the purpose of reviewing the significant ATR hazard and exposure data submitted to the EPA primarily by Syngenta and the Syngenta-funded contract and consulting lab, Exponent.

The EPA's 2003 evaluation of the human health risks of ATR, contrary to the EU's, may have reflected recent US declines in cumulative exposure to chlorotriazine pesticides used on other crops or registered with other manufacturers. The EPA restricted propazine application only to certain states and only on sorghum crops in authorized states in 1989. Further, in 2002, the EPA negotiated a phase-out of cyanazine with its principle registrant, Du Pont, over concerns surrounding cyanazine exposure and human reproductive cancer risk (Sass and Colangelo, 2006). While application of other potentially hazardous triazine herbicides in the U.S. is indeed gradually abating, application of ATR has generally increased apace with corn production over the past 50 years. Representative anti-androgenic and estrogenic effects of ATR in multiple species and human epidemiological studies are depicted in Table 2.

Table 2. Anti-androgenic and estrogenic reproductive effects of ATR documented in laboratory animal models, wildlife, human cell lines, and exposed humans

Author	Model	Study Type	Exposure	Outcome/Methods	Findings
Ingraham *et al*, 2008	Zebrafish *Danio rerio*	Laboratory	17 days post-fertilization immersed in 10^{-5} - 10^{-7}, 6 months duration for sex ratios; 3 days for gene expression	Altered sex ratios; RT-qPCR for *Cyp19*; histological sex determination	2.2 µg/L ATR induced gonadal *zCyp19a1*; 22 µg/l increased female to male ratios
Hayes *et al*., 2010	Frog *X. Laevis*	Laboratory	ZZ (males) larvae were reared in 2.5 ppb from hatching and post-metamorphosis	Analysis sexually dimorphic tissues; RIA; histology; competitive breeding assays; sex genotyping; RT-PCR	Demasculinized/ feminized larynges; reduced: T; spermatogenesis; fertility. Sex-reversal in 10% genetic males coincident w/gonadal CYP19 expression
Guilette *et al*., 1997	Alligator *T.scripta*	Laboratory	0.14, 1.4,14 ppm ATR dissolved in ethanol and applied to eggshells after stage 21 till hatching	Aromatase activity assayed via tritiated water release assay from gonadal-adrenal mesonephros (GAM)	ATR induced GAM CYP19 activity male hatchlings (testicular CYP19)
Fenton *et al*., 2004	Long-Evans Rat	Laboratory	Exposed transplacentally *in utero* from gavaged pregnant dams. Naïve and exposed offspring cross-fostered and exposed lactationally	Cross-fostering to delineate transplacental from lactational (dam-milk-hormone) effects on puberty and mammary gland development timing	Delayed mammary gland development all exposed litters; increased body weight and delayed pubertal timing in transplacental+lactationally exposed
Eldridge *et al*., 1994	SD and Fisher 344 rats	Laboratory	SD: 0, 70,400 (>MTD); F344: 0, 10, 70, 200, 400 ppm in 2 year feeding study. Ovariectomized and ovary-intact strains received chow *ad lib*	Estrous cyclicity; blood hormone levels; mammary and pituitary tumors; correlating tumors with ovarian estradiol produciton	≥70 ppm-dose-dependently increased: estrus days: estradiol: pituitary tumors: and fatal malignant mammary carcinomas in ovary-intact SD rats
Eldridge *et al*., 1998	F344 and SD rats	Laboratory	0, 10, 50, 70, 200, 400, 500 (>MTD), 1000 ppm in feeding study as above	Palpation for mammary tumors	Fibroadenoma, adeno- and malignant carcinoma at 70 ppm in intact SD rats
Eldridge *et al*., 1999	SD Rats	Laboratory	0, 10, 50, 70, 200 (>MTD), 400 ppm in feeding study as above	Estrous cyclicity; blood hormone levels; mammary and pituitary tumors; 70 ppm = ~ 2.3-3.5 mg/kg/day	≥70 ppm increased: estrus days: estradiol: fatal malignant mammary carcinomas in ovary-intact SD rats
Sanderson *et al*, 2001	Human Adrenal	Laboratory	H295R cells treated with 0- 30 µM ATR for 24 hours	CYP19 expression analyzed by RT-PCR; activity by ELISA and tritiated water release	CYP19 activity/ expression elevated by 30 µM ATR and cAMP
MacLennan *et al*. 2002	Men in Louisiana	Molecularly Epidemiologic	2045 employees of ATR manufacturing plant matched with general regional population	PSA screening performed by Ciba-Geigy; linkage with cancer registry, death certificates	Significant increase in prostate cancer in actively working over contract or inactive employees and non-employees
Donna *et al*, 1989	Women in Italy	Epidemiologic Case (65)– Referent (126)	Registered ovarian cancer cases compared to matched non-cases in highly agricultural corn-producing province	Retrospective questionnaire on occupational history/ triazine exposure issued to ovarian cancer cases and matched non-cases	Statistically significant increase in ovarian neoplasms with trazine exposure; Relative Risk = 2.7
Kettles *et al*, 1997	Women in Kentucky	Ecologic and Epidemiologic	Low, medium, or high triazine exposure estimated by water contamination, corn production and pesticide use in KY counties	Poisson regression analysis of KY breast cancer cases in registry with agency data on triazine water contamination and county corn acreage planted	Statistically significant increase in breast cancer incidence in medium OR= 1.14, and high OR = 1.2 exposure groups

Atrazine Inhibits Phosphodiesterases and Perturbs cAMP/PKA- and MAPK-signaling by Species-Conserved Mechanisms

Despite its demasculinizing and feminizing effects in a broad range of vertebrate models (Table 1), ATR does not bind androgen or estrogen receptors (Larsen *et al*, 2004). This finding suggests the hormonal effects of ATR are indirect and may result from alteration of signal transduction pathways. ATR potently inhibits phosphodiesterases (PDEs; IC_{50} 1.8 μM), which convert the ubiquitous and pleiotropic second messenger cAMP to physiologically inert 5'-AMP (ibid). Cyclic AMP is a master regulator of steroidogenesis, which it influences downstream of central releasing and tropic hormone signaling, via protein kinase A (PKA) and MAPK (mitogen activated kinase) activation and cAMP-response element binding protein (CREB) phosphorylation. Phosphorylation of CREB by these kinases results in *de novo* transcription of many steroidogenic genes and genes whose products regulate steroidogenic gene expression. The GATA family members GATA-3 and GATA-4- and the NR5A (nuclear receptor) family members SF-1 (steroidogenic-factor-1) and LRH-1 (liver receptor homolog-1)- are frequent transcriptional partners of CREB in regulating steroidogenic gene expression downstream of PKA and MAPK phosphorylation. Transcription of *lrh-1*, but not sf-1, is additionally regulated by cAMP/PKA signaling. A recent study published by Suzawa and Ingraham (2008) demonstrated biologically relevant concentrations of ATR increased: MAPK Phosphorylation; SF-1 and LRH-1 transcriptional activity in reporter studies; and steroidogenic gene expression in human placental carcinoma JEG-3 cells by cAMP/PKA-mediated mechanisms. Conserved mechanisms of steroidogenic gene expression as regulated by cAMP/PKA and MAPK signaling are depicted in Figure 3.

Figure 3:

Steroidogenic Gene Expression Proceeds via cAMP/PKA-, MAPK, GATA- and NR5A-Mediated Mechanisms. Steroidogenic gene expression in humans and rats proceeds by the conserved PKA- and MAPK- mediated mechanisms depicted. CREB, NR5A (SF-1 and LRH-1), and GATA (-3/4) are frequent transcriptional partners at cis-elements of steroidogenic genes. Contrary to SF-1- which is only post-translationally modified by PKA- *lrh-1* is also transcriptionally regulated by PKA, MAPK, GATA, and CREB via the indicated mechanisms.

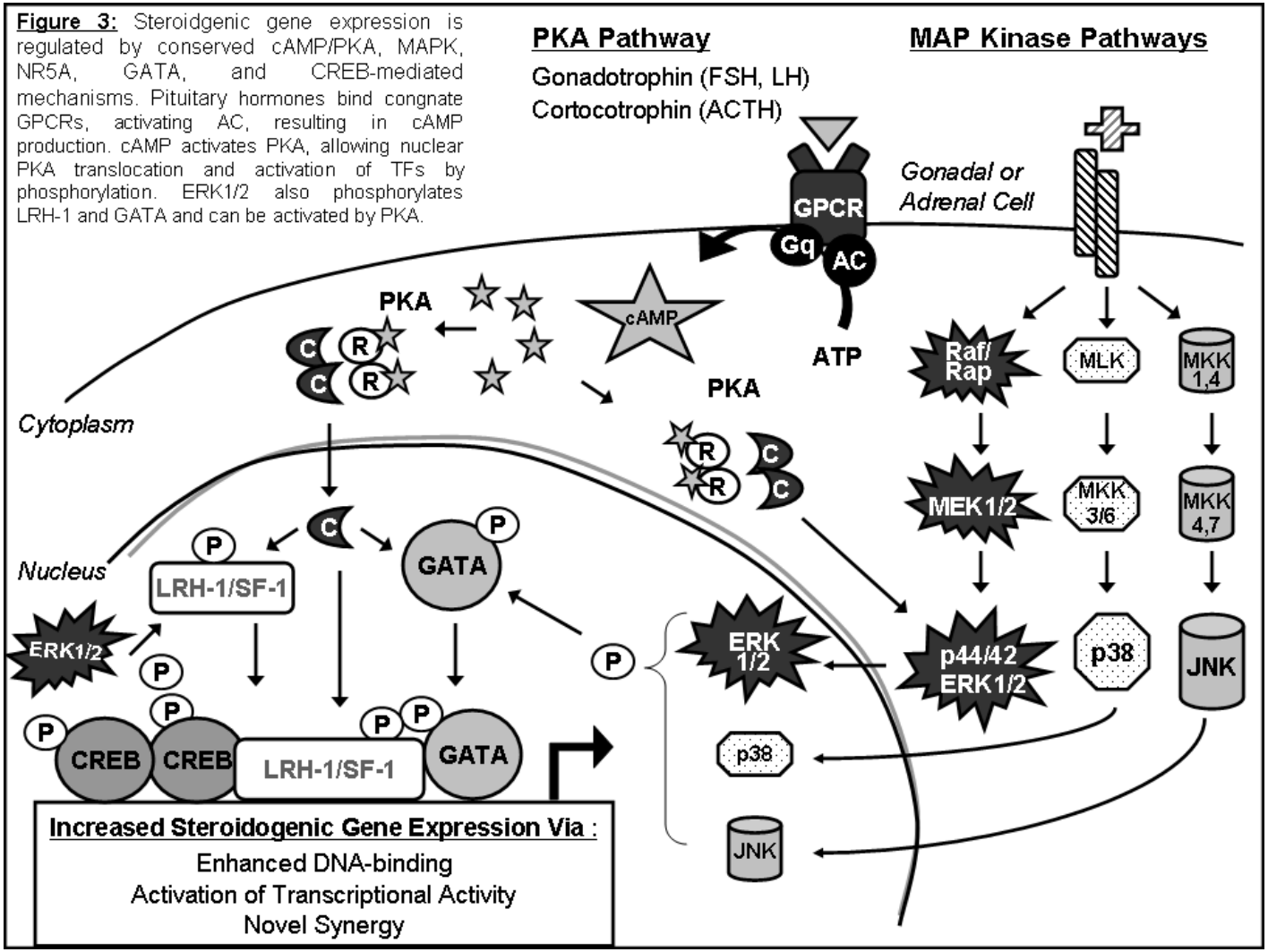


Figure 3: Steroidgenic gene expression is regulated by conserved cAMP/PKA, MAPK, NR5A, GATA, and CREB-mediated mechanisms. Pituitary hormones bind congnate GPCRs, activating AC, resulting in cAMP production. cAMP activates PKA, allowing nuclear PKA translocation and activation of TFs by phosphorylation. ERK1/2 also phosphorylates LRH-1 and GATA and can be activated by PKA.

Atrazine Induces Aromatase (CYP19), the Terminal Enzyme in the Estrogen Biosynthesis Pathway, by Conserved cAMP/PKA-, PI3K-, and NR5A-Mediated Mechanisms, in Human and Rat Steroidogenic Cells

In human adrenal carcinoma (H295R) cells, ATR increases cAMP levels and robustly induces the enzyme aromatase (CYP19; P450arom), which catalyzes the final and key step of estrogen biosynthesis through irreversible aromatization of androgen precursors (Sanderson *et al*, 2002] In gene-promoter reporter studies, Ingraham and Suzawa show ATR increases *Cyp19*-promoter activity by PKA-, PI3K-, and SF-1/LRH-1-mediated mechanisms in JEG3 cells. In addition to aromatase, ATR increases expression of CYP17- responsible for androgen conversion from progesterone, and StAR (steroidogenic acute regulatory protein)- responsible for cholesterol transport in the mitochondrial membrane, in rat leydig cells (Kovacevic *et al*, 2010). Thus, in addition to inducing CYP19- the terminal enzyme in the estrogen biosynthesis pathway- ATR may also increase: production of androgens, or aromatase substrate, through induction of CYP17; and steroidogenesis by upregulating the rate-determining step of steroid hormone production- mitochondrial cholesterol transport via StAR activity. The effect of ATR on CYP19 in particular may in part explain its potent anti-androgenic and estrogenic effects in several wildlife and laboratory models. Moreover, as 70% of human BC are growth-promoted by estrogen at the time of diagnosis (Bouchard *et al*, 2005), aromatase inhibitors, which globally suppress production of estrogens in the human body, are the current first-line therapy against this devastating disease. Thus, ATR induction of CYP19 in the described cell types may have implications for human BC risk. The steroidogenic pathway through androgen aromatization and estrogen biosynthesis is depicted in Figure 4. Proteins and enzymes ATR is known to affect are indicated.

Figure 4:

The Steroidogenic Pathway and Known Targets of ATR. Atrazine inhibits phosphodiesterases, leading to intracellular increases in the master regulator of steroidogenesis, cAMP. ATR is known to affect gene transcription and/or enzyme activity of the indicated targets along the pathway. ATR is also known to affect StAR mRNA levels, and StAR-mediated mitochondrial transport of cholesterol is the rate-determining step of steroidogenesis. In addition to transcriptional effects, the activity of some steroidogenic enzymes (e.g. CYP17) is post-translationally enhanced by PKA.

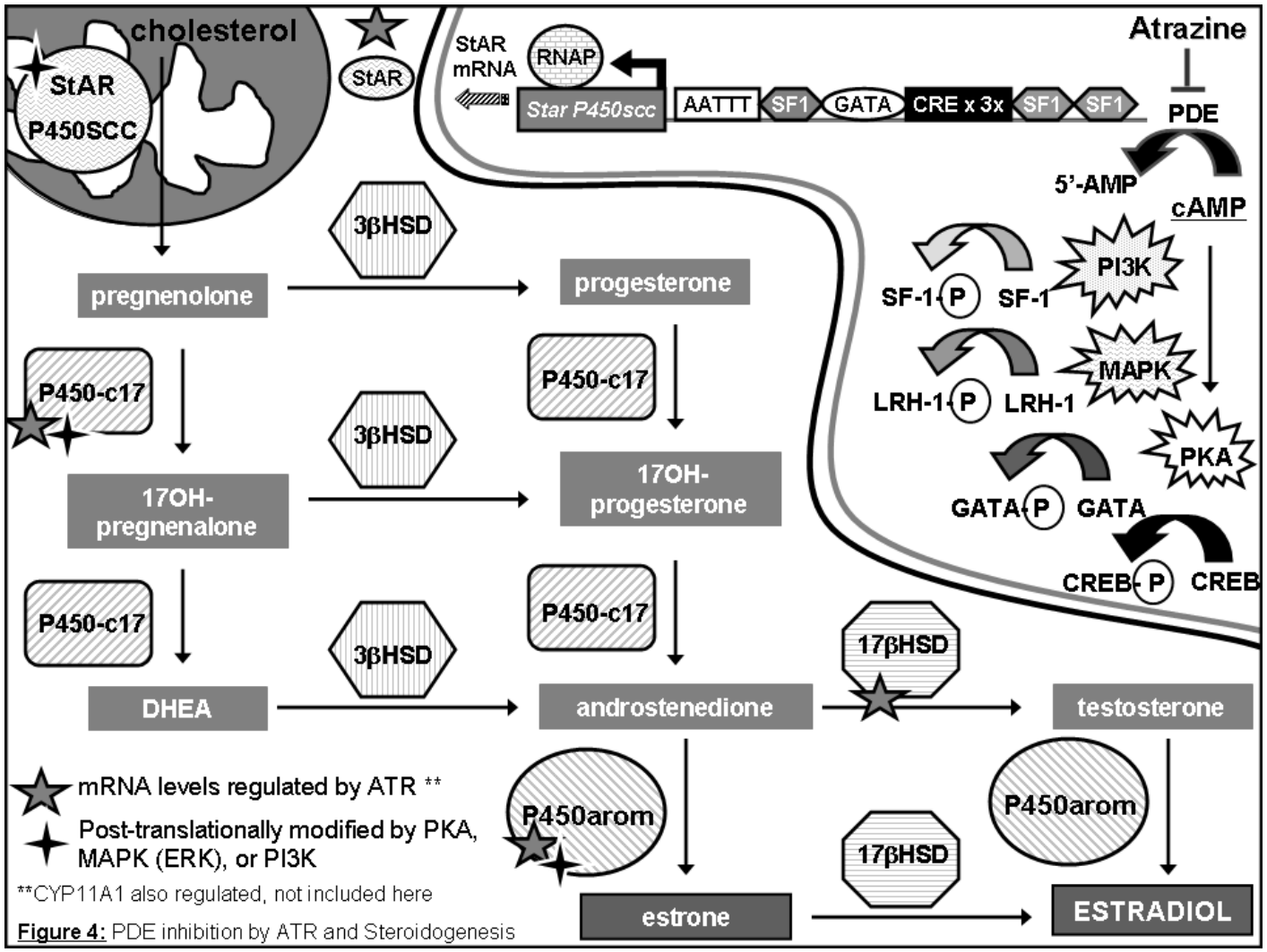


Figure 4: PDE inhibition by ATR and Steroidogenesis

Recent Findings Relevant to the Present Study

In EPA-reviewed, registrant-conducted lifetime feeding studies made publicly available more than fifteen years ago, ATR increased plasma estradiol levels and mammary tumor incidence in ovary intact but not ovariectomized (OVX) Sprague-Dawley (SD) rats, implicating ovarian estradiol as critical for ATR's action as a mammary carcinogen in this species. Although the EPA generally "presumes animal tumor studies to be indicative of human cancer potential" (Olin *et al*, 1996), the molecular mechanisms by which ATR elicits its endocrine-disrupting effects in female rodents, and the relevance of those mechanisms to women at risk for BC in particular, are still intensely debated. Atrazine, in addition to being broadly applied- since the seminal oncogenicitiy studies at least- is also one of the most widely studied herbicides. Though thousands of studies have been conducted in the fifteen years hence, ATR is presently classified under the EPA scheme as "not likely to be carcinogenic to humans" due to "lack of human and mechanistic data". Just this year, 2011, a group headed by Dr. Susan Laws at the EPA discovered ATR modestly increased CYP19 activity in ovarian granulosa cells derived from Wistar rats (Laws *et al*, 2011). In addition to bolstering the case for ovarian estradiol being critical for mammary tumor induction by ATR in the SD rat, Laws' findings have implications for human BC risk, as at the molecular level, aberrant *cyp19* regulation by near identical mechanisms in the rat ovary and human breast tissue leads to excessive estrogen biosynthesis and attendant mammary tumor promotion in both species.

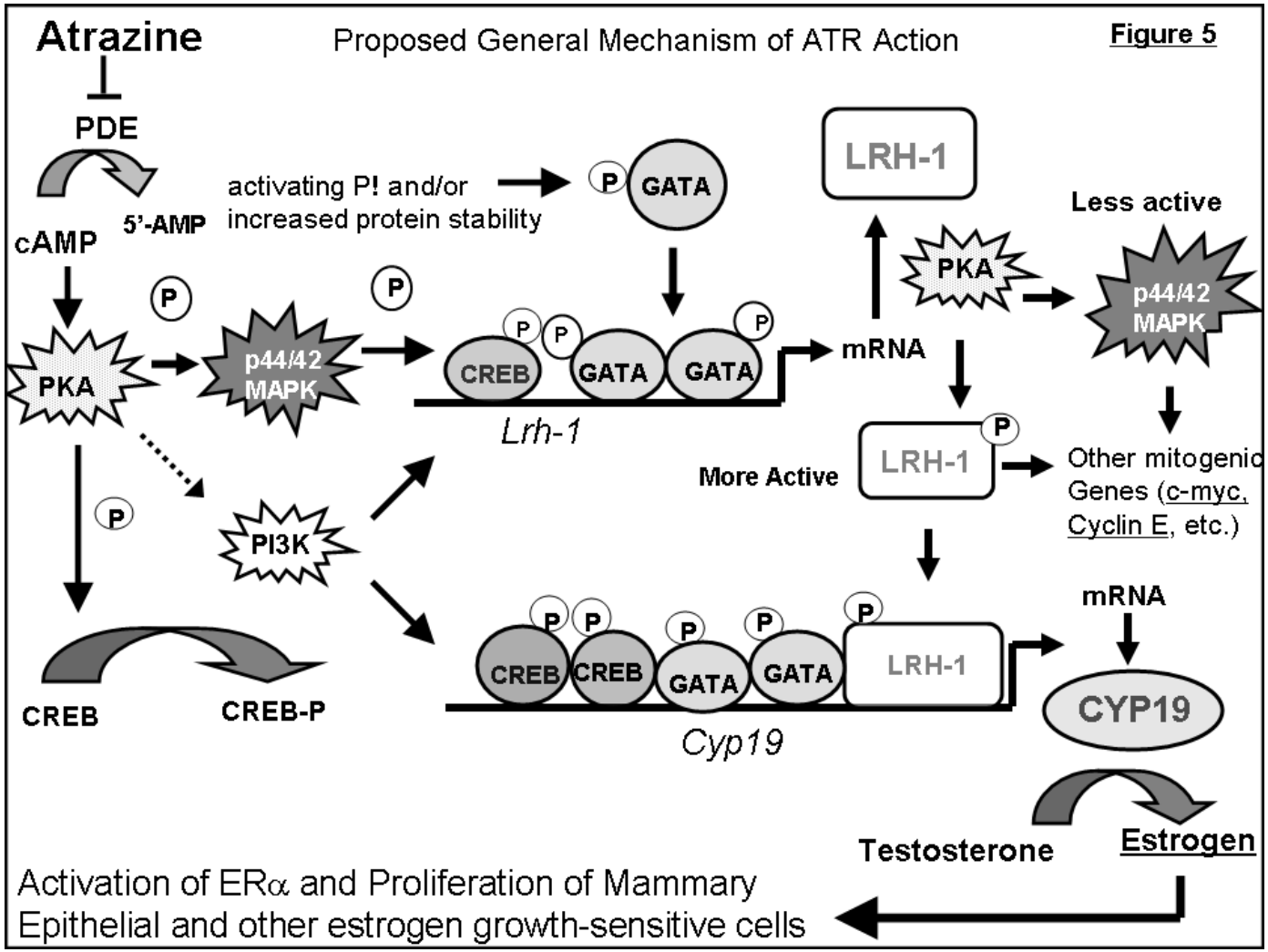
Significance and Rationale of the Present Study

The SD rat mammary tumor findings viewed in light of recent molecular discoveries may inform remaining questions as to the mechanisms by which ATR promotes mammary carcinogenesis in ovary-intact but not OVX SD rats and further, the relevance of those mechanisms to inter-individual human BC risk. Local estrogen concentrations breast tumor-containing tissue are often 10-50 times higher than patient plasma concentrations [Bouchard *et al*, 2005]. Quantitative mRNA analyses of tumor biopsies reveal these marked elevations in estrogen strongly correlate with increases in total aromatase transcript levels in cancerous breast tissue, and related to the premise of this study, this local induction of aromatase is critically linked to a switch in *cyp19* promoter preference. Significantly, aromatase transcripts in cancerous breast epithelial and supporting stromal tissue are chiefly derived, not from the distal and weak glucocorticoid-regulated promoter commonly activated in healthy breast tissue, but from proximal, strong, cAMP/SF-1-responsive gonadal promoters typically silenced in the breast (discussed in more detail in Chapter 1, Figure 7). ATR, a PDE inhibitor, was shown in promoter-reporter studies by Suzawa and Ingraham to activate *cyp19* transcription from these promoters in human placental and rat adrenal cells via LRH-1-mediated mechanisms. LRH-1 is overexpressed with GATA-3 and ERα in the major estrogen-sensitive BC subtype, and activates

gonadal cAMP-responsive c*yp19* promoters in human breast cancer, breast fibroblast, and rat ovarian cell lines. Aberrant derepression of these gonadal promoters in premalignant and cancerous breast tissue occurs when critical CpG dinucleotides in these are hypo- or demethylated, but the epigenetic mechanisms that precede this occurrence in breast tissue are poorly understood [25]. In a recent study profiling the methlyation status of gondal *cyp19* in adipose biopsies of four healthy female subjects however, aromatase in fibroblasts from one of the three subjects was atypically and robustly cAMP-inducible, and this finding was also correlated with promoter hypomethlyation in these fibroblasts relative to those from the three other subjects which were cAMP-refractory. Thus, as several biomonitoring studies of pesticide applicators (Fenske *et al*, 2000) and the general population (Barr et al , 2007) indicate ATR is absorbed dermally and in the gut, and it is possible that chronic exposure to this common drinking water contaminant may adversely affect estrogen-related health in high-risk individuals, especially women in which gonadal cAMP/SF-1-responsive *Cyp19* promoters are derepressed. The general mechanisms by which we propose ATR may affect rat ovarian granulosa cells, and human ER+ breast cancer and adipose fibroblast cells are provided in Figure 5.

Figure 5:

Conserved General Mechanisms of Cyp19 Induction by Atrazine in Susceptible Human Breast Cancer, Human Adipose Fibroblast, and Rat Ovarian Granulosa Cells. ATR is known to inhibit PDE (phosphodiesterase), which causes intracellular elevations in the master regulator of steroidogenesis, cAMP. We show ATR induces CYP19 by GATA, LRH-1, PKA- and PI3K-mediated mechanisms deregulated in malignant breast epithelial and tumor-proximal fibroblasts. Further, consistent with other reports conducted in JEG3 and H295R cells, we show ATR dose-dependently induces phospho-Akt (S473) and phospho-ERK-1/2 which, along with PKA, transcriptionally activate LRH-1 (Hu *et a*l, 2009) and GATA-4 at gonadal *cyp19* promoters downstream of FSH in rat ovarian granulosa cells (Stocco *et al*, 2007 and 2008).


Atrazine
Proposed General Mechanism of ATR Action
Figure 5
PDE
cAMP
5'-AMP
activating P! and/or
increased protein stability
P
GATA
LRH-1
Less active
PKA
p44/42
MAPK
CREB
GATA
GATA
mRNA
Lrh-1
More Active
LRH-1
Other mitogenic
Genes (c-myc,
Cyclin E, etc.)
PI3K
CREB
CREB-P
CREB
CREB
GATA
GATA
LRH-1
mRNA
CYP19
Cyp19
Testosterone
Estrogen
Activation of ERα and Proliferation of Mammary
Epithelial and other estrogen growth-sensitive cells

ACKNOWLEDGEMENTS

None of the present work nor my future in epigenetics research would be possible without the generosity, patience, and guidance of Professors: Chris Vulpe; Len Bjeldanes; Dale Leitman; and Gary Firestone of UC Berkeley. The laboratory, research communication, and science education training I received from each are invaluable to me, and I hope in my career to utilize their gifts in realization of significant advances in human and environmental health and disease prevention they can all be fiercely proud of impacting outside of the good work they do themselves. I additionally owe members of these professors' laboratories a great many thanks for their training and tolerance, specifically: Dr. Holly Nicastro, formerly of the Bjeldanes lab; Candice Herber of the Leitman lab; and Dr. Crystal Marconett, formerly of the Firestone Lab. Dr. Marconett is the Michael Jordon of epigenetics in my mind- and the keeper of my faith, inspiration, and sanity in so many phone calls. Mere words are inadequate for the gratitude I feel for Drs. Dale Leitman and Gary Firestone's contributions to my professional and personal resilience development; however I will work ceaselessly to let them and others here named know of it through my accomplishments.

I am further deeply indebted to Dr. Eileen Gregory and Dr. Erich Blossey of Rollins College for their mentorship and advice, especially for not letting on how difficult graduate school can be, and for disabusing me of notions class ceilings in education. Dr. Steven Kleimann of Rollins College- I miss our fights and hurricane parties.

I would have never obtained a bachelor's degree nor made it to Berkeley without the goading of Dr. Frances Frierson, Professor Susan Matthews, and my former lab manager, Ron Olmstead (departed)- of the biology department of Valencia Community College. I'm still not sure I shouldn't be driving ambulances, but Valencia will always be my home. I look forward to fetal pig and shark dissection prep when next I visit.

Mr. Fagan, your always impeccable timing steeled me with hope and patience through many an implausible scenario. I'd be waiting tables at Denny's without you.

Lastly, I have to thank my mother, who made me cheap-steak tough. After this post-doc thingy (it's not more school) we're going to Paris. Promise.

Curriculum Vitae

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EDUCATION

2005-present	Ph.D. Candidate in Molecular Toxicology; Principle Investigators (Co-chairs): Dale Leitman and Gary Firestone; University of California (UCB), Berkeley, CA, USA
2005	B.A. Emphasis in Biochemistry and Molecular Biology; Rollins College, Winter Park, Florida, USA. *Cum Laude*.
2003	A.A. Emphases in Biology, Human Anatomy/Physiology; Valencia Community College (VCC), Orlando, Florida. E.M.T. Emergency Medical Technician, Emergency Medical Services; VCC, Orlando, Florida.

RESEARCH EXPERIENCE/POSITIONS HELD

Fall 2010
Graduate Student Researcher for Translational Group (IARC, EPA, OEHHA, UCB) on California Breast Cancer Research Grant-sponsored work. Grant Title: *Toward the Development of a California Chemicals Policy that Considers Breast Cancer.* Contact: Megan Schwarzman, MD, MPH. Center for Occupational and Environmental Health School of Public Health, UCB.

Fall 2008- present
Editor, American Journal Experts. Edit English-language manuscripts of non-native-speaking researchers in several specialized areas of molecular biology for errors in word choice and grammar prior to journal submission.

2006-Present
Graduate Researcher in lab of Drs. Gary Firestone (MCB) and Dale Leitman (Integrative Biology, IB). Dissertation Research Project: *Common Molecular and Epigenetic Determinants of Atrazine Sensitivity In Three Different Models of Endocrine Disruption.*

2005-2006
Rotation Student in labs of Drs. Len Bjeldanes, Chris Vulpe, and Tyrone Hayes of UCB.

Summer 2004
Summer Research Intern under Dr. Erich Blossey in the Department of Chemistry at Rollins College in Winter Park, Florida. Research Project:

Immobilization of di-Rhodium Tetraprolinate Catalysts for Ritalin Synthesis on Solid Organic Polymer Supports.

Summer 2001
Summer Research Intern under Drs. Michael Barber and Veronica Pollock at the University of South Florida Medical School, Department of Biochemistry and Molecular Biology. Research Project: *Isolation, Purification, and Kinetics of Recombinant Spinach Nitrate Reductase.*

2000-2005
Laboratory Assistant in biology department of Valencia Community College, Orlando FL. Designed and prepared labs and lab practicums for courses in general biology, microbiology, anatomy, physiology, marine biology, and botany; 32 hours/week; Contact: Professor Susan Matthews (407) 299-5000 ext 1384 (former lab director, Ron Olmstead, is deceased).

2003-2004
Laboratory Assistant in biology/biochemistry and Rollins College, Winter Park, FL. Prepared labs and reagents for courses in biology, microbiology, anatomy, physiology, marine biology, biochemistry, and botany; (lab director: Ana Rodriguez).

TEACHING EXPERIENCE

Spring 2011
GSI (graduate student instructor) for NST 11 (Intro to Toxicology) with Drs. Len Bjeldanes, John Casida, Chris Vulpe; Introductory Course in Toxicology; principles for evaluation of natural and man-made hazards present in the environment, workplace, and consumables. Species selectivity, individual variations/resistance, mechanisms of toxin action, combined effects of toxic agents, and the impact of toxic agents on modern society are emphasized. UC Berkeley, Berkeley (UCB), CA.

Spring 2011
Reader/grader for NST 115 (Principles of Drug Action) with Dr. Dale Johnson. Basic principles and quantitative aspects of drug action and risk/benefit as applied to the discovery, design, and development of human therapeutics. Course emphasizes importance of integrating toxicology, pharmacokinetics, and pharmacogenomics (individual variation) in creation of treatments for human disease in an entrepreneurial model. Special emphasis on pharmacogenomics and variation in individual response. UCB.

Fall 2010
GSI for IB 137 (Human Endocrinology) with Dr. Tyrone Hayes. Responsibilities included: devising examinations; peer-review paper selection for discussion in

journal club; 3-5 hours committed to instruction in weekly discussions for lecture and related primary literature review in two sections of 30-40 students; grading ~200 short-essay midterms devised around problem solving in clinical endocrinology; tutoring/mentoring students and writing letters of recommendation for graduate and medical school. 40 hours/week. Gave an invited lecture on the influence of environment and lifestyle on breast and reproductive cancer risk. UCB.

Spring 2010
GSI for IB 140 (Human Reproduction) with Dr. Tom Carlson. Evaluation of human reproduction, infertility, and the influence of lifestyle and environment in different demographic groups. Provided instruction in three weekly discussion sections of 50 students each for lecture review; devised and disseminate weekly quizzes. UCB.

Fall 2009
GSI for IB 137 (Human Endocrinology) with Dr. Tyrone Hayes. Course description and duties described above (Fall 2011). Gave an invited lecture on the influence of environment and lifestyle on breast and reproductive cancer risk. UCB.

Spring 2008
GSI for NST 121 (Computational Toxicology) with Dr. Dale Johnson; Use of bioinformatics tools (e.g. QSAR) in linking chemical molecular features to mechanisms of toxicity elicited in biological systems. Instruction in using computer software to collect, organize, and relate chemistry and toxicology for the purpose of building models that predict chemical structure-toxicity relationships. Emphasis in application of these skills in drug development, food safety, and assessing hazards of environmental contaminants. Responsible for software tutorials/instruction (QSAR= Quantitative Structure-Activity Relationship; NCBI, PDB, GenBank, etc), project advising, lab report and midterm grading. UCB.

Fall 2007
GSI for NST 171 (Toxicology Lab) under Drs. Len Bjeldanes, Dale Leitman, and Ben Delumen. Toxicology laboratory course, emphasis in techniques used in human toxicology research, including harmonized GLP/EPA assays for genotoxicity and endocrine disruption. Helped students design, execute, and analyze experiments; responsible for lab reagent prep, lab report and quiz grading, mini-lectures in genotoxicity, writing for publication, and good laboratory practices (GLP) and technique. UCB.

Spring 2007
GSI for NST 11 (Intro to Toxicology) Drs. Len Bjeldanes, John Casida, Martyn Smith; course and duties described above. UCB.

2001-2005
Supplementary Instructor of Anatomy and Physiology under Drs. Frances Frierson and (Dean of Science) Ronald Keiper. Engineered lab practicums, graded examinations, gave weekly 1-2 hour lectures in human anatomy (organ systems) lab, coordinated and hosted after-hours and weekend open labs to serve working adult, non-traditional, and historically underserved and underrepresented nursing-track students [Contact: Dr. Frances Frierson, (407) 299-5000, ext 1324). Valencia Community College in Orlando, Florida.

PEER-REVIEWED PUBLICATIONS

Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). (2010) *Proceedings of the National Academy of Science (PNAS)* **107**(10): 4612–4617.

Indole-3-Carbinol Triggers AhR-dependent ER{alpha} Protein Degradation in Breast Cancer Cells Disrupting an ER{alpha}-GATA3 Transcriptional Cross-regulatory Loop. (2010) *Molecular Biology of the Cell.* **21**(7):1166-77.

Acknowledged in:
Pathways to Breast Cancer: A Case Study for Innovation in Chemical Safety Evaluation. *Report of the Breast Cancer and Chemicals Policy Project*, by the University of California, Berkeley and the Natural Resources Defense Council, with funding from the California Breast Cancer Research Program, University of California Office of the President. 2010.
http://coeh.berkeley.edu/greenchemistry/cbcrp.htm

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2000-2003 President's List, Valencia Community College (VCC); Orlando, Florida
2001-2003 Dean's List, VCC; Orlando, Florida
2001 Who's Who in America's Junior Colleges (VCC); Orlando, Florida
2003 Kathleen R. Johnson Scholarship, Rollins College (RC); Winter Park, FL
2003 Rollins Grant, RC; Winter Park, FL
2003 Pell Grant, RC; Winter Park, FL
2003 Archibald Bush Award for Scholastic Achievement in Mathematics and the Sciences, RC;
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2004 Kathleen R. Johnson Scholarship, RC; Winter Park, FL
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2005 Kathleen R. Johnson Scholarship. RC; Winter Park, FL
2005 Rollins Grant. RC; Winter Park, FL
2005 Archibald Bush Award for Scholastic Achievement in Mathematics and the Sciences. RC;
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2007 Ford Foundation Diversity Predoctoral Fellowship Honorable Mention, UCB; Berkeley, CA
2008 Award for Best Research Poster by a Graduate Student in Molecular Biology Research, UCB; Berkeley, CA

VOLUNTEER SERVICES / OUTREACH

Breast Cancer Action Network Volunteer (current)
(City of) Berkeley Symphony Volunteer (current)
Adopt-a-Block (Neighborhood Litter/Graffiti Removal) Volunteer; City of Oakland, Public Works (current)
Oakland East Bay Symphony Volunteer (current)
2000-2003 Second Harvest Food Bank Volunteer
2000-2003 Emergency Room and Dietary Services Volunteer
Health Central Hospital, Winter Garden, FL

PROFESSIONAL MEMBERSHIPS

American Association for the Advancement of Science (AAAS)
American Society for Biochemistry and Molecular Biology (ASBMB), Local Advocates Network (LAN)
American Chemical Society (ACS)
American Cancer Society (ACS), Cancer Action Network (CAN)
Breast Cancer Action Network
Federation of American Scientists, Students for International Security
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REFERENCES

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Chapter I

The Herbicide Atrazine Induces Molecular Hallmarks of the Major Estrogen Receptor Positive (ER+) Breast Cancer Subtype in a Model ER+ Human Breast Cancer Cell Line

ABSTRACT

Estrogens are produced by a single enzyme, aromatase, or 'CYP19', which catalyzes estrogen biosynthesis through androgen precursor aromatization. Two-thirds of BCs are growth-promoted by estrogen at the time of diagnosis, and CYP19 is typically upregulated 3-4 fold by unknown mechanisms in estrogen-sensitive tumor biopsies. Thus, ERα antagonists and CYP19 inhibitors are first-line therapies to treat the majority of early-stage BCs. The herbicide atrazine (ATR) causes anti-androgenic and estrogenic reproductive effects at biologically relevant doses in every vertebrate class studied to date. In lifetime feeding oncogenicity studies conducted more than 15 years ago, ATR also increased plasma estradiol levels and mammary tumor incidence in aging ovary-intact but not ovariectomized Sprague-Dawley rats (SD), implicating ovarian estrogen as critical for mammary tumor promotion by ATR. Despite being one of the most-studied environmental endocrine disruptors in history, the human health relevance of the incompletely defined molecular mechanisms by which ATR promotes mammary tumor development in ovary-intact SD rats and elicits primarily anti-androgenic and estrogenic endocrine-disrupting effects across vertebrate classes is still debated. In promoter-reporter studies conducted in both human H295R and JEG3 (Suzawa and Ingraham, 2008) cells, ATR induced gonadal tissue-specific *Cyp19* promoter, or 'pII' activity via cAMP/PKA and NR5A (SF-1) transcription factor-mediated mechanisms- which are conserved in rats and humans and robustly activated in ovarian granulosa cells of both species downstream of FSH. BC risk increases with age, and counterintuitively, ER+BC disproportionately afflicts post-menopausal (PM) women. CYP19 is overexpressed in malignant tissue of PM women through aberrant activation of cAMP-responsive gonadal promoters that are typically silenced in tumor-free breast tissue- the very same promoters activated in human and rat ovarian granulosa cells downstream of FSH, and which ATR, a phosphodiesterase (PDE) inhibitor, activates by PKA-mediated mechanisms in H295R AND JEG3 cells in *vitro*.

Using a model T-47D ER+BC cell line obtained from a stock routinely maintained by an LBNL laboratory, we show biologically relevant concentrations of ATR dose-dependently increase expression of: aromatase; GATA-4, known to regulate *Cyp19*; and LRH-1 and GATA-3- which, significantly, are known to regulate both *Cyp19* and *Esr1;* ERα; and the G1 to S cell cycle progressors, c-Myc, cyclins E, and D1. Further, via inhibition studies we show ATR elicits many of these expression changes through PKA-mediated mechanisms, which, especially as regards GATA-4 (Stocco *et al*, 2008) and LRH-1 activation (Hu *et al*, 2009), are highly conserved in the rodent ovary.

Preventing CYP19 expression from cAMP-responsive gonadal promoters and ablating ERα expression specifically in the breast are the lead foci of current chemical and gene therapies against ER+BC, and a growing body of literature

recommends targeting LRH-1 as a single strategy with potential for realizing both therapeutic measures. The human T-47D ER+BC cell line was heavily relied upon in all the studies here cited from which LRH-1 reduction emerged as a promising consensus strategy for both CYP19 and ERα breast-tissue specific ablation and ER+BC therapy; that biologically relevant concentrations of ATR significantly induce LRH-1 levels in conjunction with ERα, GATA-3, and CYP19 in this same cell line, suggest ATR is one of several contributory environmental endocrine disruptors for which exposure reduction should be carefully considered in BC prevention and BC hazard identification strategies.

INTRODUCTION

Aromatase is the Terminal Enzyme of Estrogen Biosynthesis, and is Transcriptionally Regulated in Many Human Tissues by Complex Alternative Splicing Mechanisms

Aromatase catalyzes the terminal and rate-determining step of estrogen biosynthesis through irreversible aromatization of androgens precursors. In humans, aromatase is expressed in a wide variety of tissues, including cells of bone-, placental-, central-, gonadal-, and adipose stromal origin, and the spatial and temporal molecular mechanisms of its regulation are commensurately complex. *Cyp19* is composed of a 30kb coding region spanning nine exons, corresponding to coding or translated exons 'II-X', and a 93kb regulatory region containing, to date, more than ten untranslated 'first exons I (I.1, I.2, I.3, I.4, I.5, I.7. I.f, and pII), each associated with its own unique tissue-specific promoter (Figure 6).

Cell-specific CYP19 expression in humans is achieved by alternative use of the more than 10 tissue-specific promoters associated with these unique 'first exons' I, which is driven by the distinct cohort of transcription factors in a cell that are recruited to regulatory sequences in DNA under the influence of various hormones, cytokines, and second messengers. Alternative activation of these promoters leads to splicing of an untranslated 'first exon' I to a highly promiscuous splice acceptor upstream of the translational start site of exon II (the first coding or translated exon), resulting in the production of cell and tissue-specific *P450arom* mRNA species containing distinct 5'-untranslated termini spliced to an identical coding region (exons II-X). The aromatase (*cyp19*) gene is shown in Figure 6.

Figure 6:

The human cAMP-responsive gonadal *cyp19* promoter 'pII' contains rat conserved cis elements. Species conserved cis- and trans-elements critical in normal gonadal and mammary-tumor promoting *cyp19* activation in rats and humans. Aromatase is differentially expressed in human tissues from alternative activation of more than ten tissue-specific promters, each variably activated by the unique constellation of transcription factors, cytokines, and second messengers defined by the cellular context in which transcription is driven. Although the rat aromatase gene is far less complex than human *cyp19*, the human gonadal cAMP-responsive aromatase promoter, 'pII', which is robustly activated in malignant breast tissue in the presence of PGE_2 is conserved in the rat *cyp19* promoter activated downstream of FSH in the estrus cycle. CpG dinuceotides in CRE of the gonadal promoters of both species are also conserved. Moreover, PKA motifs shown crucial in *Cyp19* pII activation by mutational analysis of critical serines in reporter studies are conserved in the primary sequences of GATA-3, GATA-4, and LRH-1 in rats and humans. ATR elicits estrogenic effects in every vertebrate class studied to date; indeed, though not shown depicted here in the interest of simplicity, the cis- and trans-factors critical in gonadal cAMP-mediated cyp19 regulation show in Figure 33 are perfectly conserved in: Xenopus; bovine; porcine; equine; chicken; and rabbit species. More detailed schematics of this remarkable degree of conservation in cis- and trans- gonadal *cyp19* regulation are provided in excellent detail in *Endocrinology* (2005) 146:4905-4916, by Bouchard *et al*.

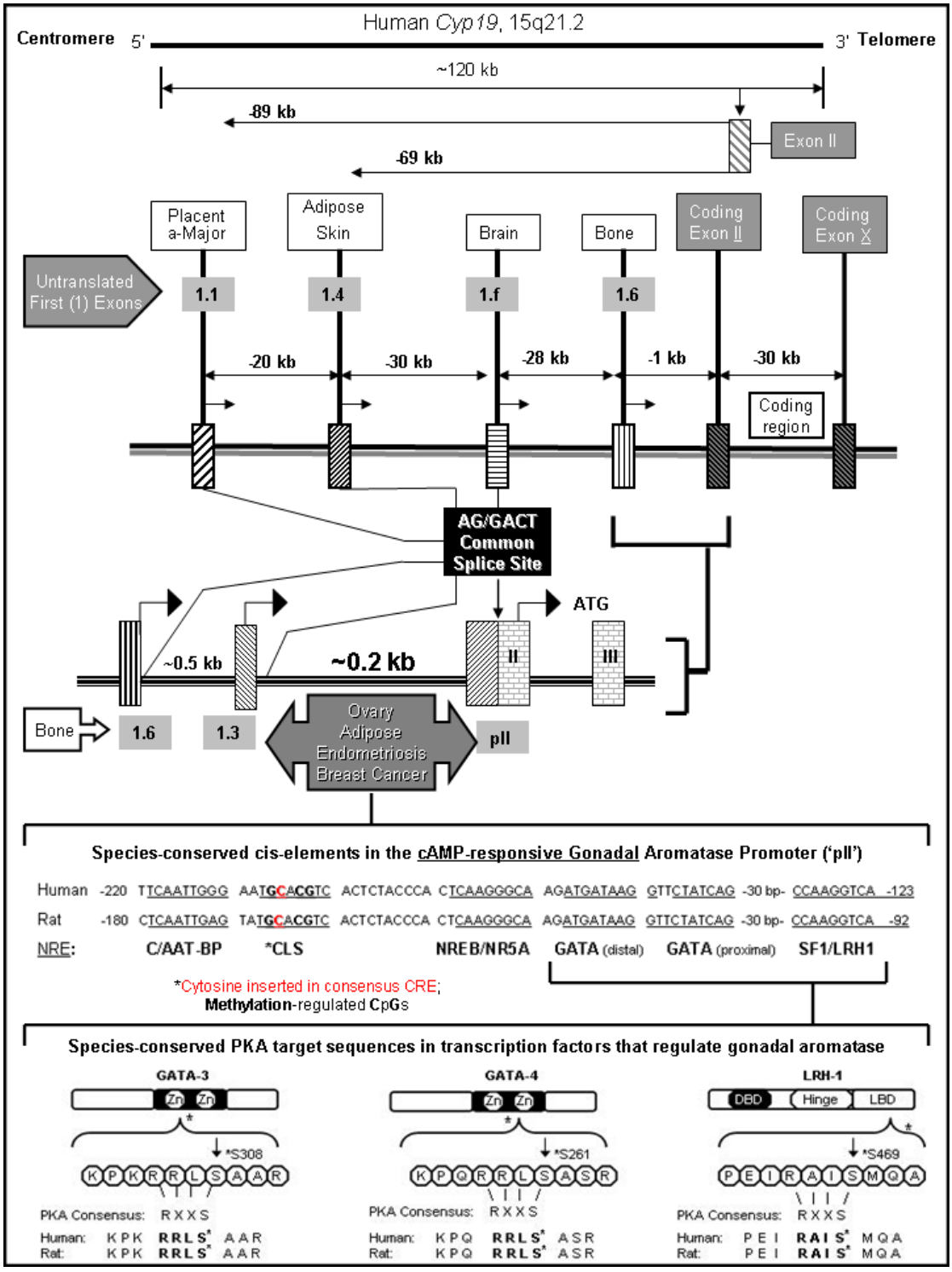

Human Cyp19, 15q21.2
Centromere
5'
3'
Telomere
~120 kb
-89 kb
-69 kb
Exon II
Placent a-Major
Adipose Skin
Brain
Bone
Coding Exon II
Coding Exon X
Untranslated First (1) Exons
1.1
1.4
1.f
1.6
-20 kb
-30 kb
-28 kb
-1 kb
-30 kb
Coding region
AG/GACT Common Splice Site
ATG
~0.5 kb
~0.2 kb
II
III
Bone
1.6
1.3
Ovary Adipose Endometriosis Breast Cancer
pII
Species-conserved cis-elements in the cAMP-responsive Gonadal Aromatase Promoter ('pII')
Human -220 TTCAATTGGG AATGCACGTC ACTCTACCCA CTCAAGGGCA AGATGATAAG GTTCTATCAG -30 bp- CCAAGGTCA -123
Rat -180 CTCAATTGAG TATGCACGTC ACTCTACCCA CTCAAGGGCA AGATGATAAG GTTCTATCAG -30 bp- CCAAGGTCA -92
NRE:
C/AAT-BP
*CLS
NREB/NR5A
GATA (distal)
GATA (proximal)
SF1/LRH1
*Cytosine inserted in consensus CRE;
Methylation-regulated CpGs
Species-conserved PKA target sequences in transcription factors that regulate gonadal aromatase
GATA-3
GATA-4
LRH-1
Zn
Zn
DBD
Hinge
LBD
*S308
*S261
*S469
K P K R R L S A A R
K P Q R R L S A S R
P E I R A I S M Q A
PKA Consensus: R X X S
Human: K P K RRLS* A A R
Rat: K P K RRLS* A A R
Human: K P Q RRLS* A S R
Rat: K P Q RRLS* A S R
Human: P E I RAIS* M Q A
Rat: P E I RAIS* M Q A

Figure 6

Tissue-Specific Aromatase Expression is Driven by Differential Tissue-Specific *Cyp19* Promoter Usage

Although the first exons of *Cyp19* are not translated, their presence in the mature transcript allows one to ascertain from which promoter transcription was driven, and similarly, which hormones and second messengers directed the process. For example, in placental tissue, the most distal promoter I.1 is constitutively activated under the influence of retinoids, resulting in elevated circulating estrogens in pregnant women 100-1000 times that typical of non-pregnant women (Bulun *et al* 2005). Conversely, the premenopausal ovary, transcription of *Cyp19* is induced in the follicular phase of the menstrual cycle downstream of FSH (follicle stimulating hormone), which binds its cognate GPCR (G-protein coupled receptor expressed in granulosa cells, stimulating associated adenylate cyclase, resulting in elevations in intracellular cAMP that drive aromatase transcription from a proximal promoter II (pII) in a CRE (cAMP-response element) and SF-1- dependent manner (Figure 7).

Figure 7:

Aromatase Tissue Distribution and Regulation. Aromatase is differentially expressed in many tissues via alternative activation of more than 10 tissue specific gene promoters- each responsive to different hormonal stimuli. In premenopausal women, the ovaries are the parenchymal tissue of estrogen biosynthesis, where Cyp19 is robustly induced via cAMP-responsive gonadal promoter 'pII/I.3' transactivation downstream of FSH release from the pituitary and subsequent LRH-1, CREB, and GATA-transcription factor activation by PKA. After menopause, adipose becomes the major tissue source of estrogen, where *Cyp19* is weakly activated from a distal glucocorticoid-regulated promoter termed '1.4', often in skin and adipogenic fibroblasts of the buttocks, thighs, and mammary adipose stroma. (Bottom panel). In healthy breast tissue, qPCR analyses reveal the majority of breast epithelial and adipose fibroblast Cyp19 transcripts derive from I.4. Conversely, in tumor- proximal containing tissue, total Cyp19 transcripts are elevated 3 to 4 fold generally, but the majority of transcripts derive from cAMP-responsive gonadal promoters 'pII/I.3' – which are typically silenced in healthy breast tissue via methylation of CpG dinucleotides, critically a CpG #5 contained in PII CRE.

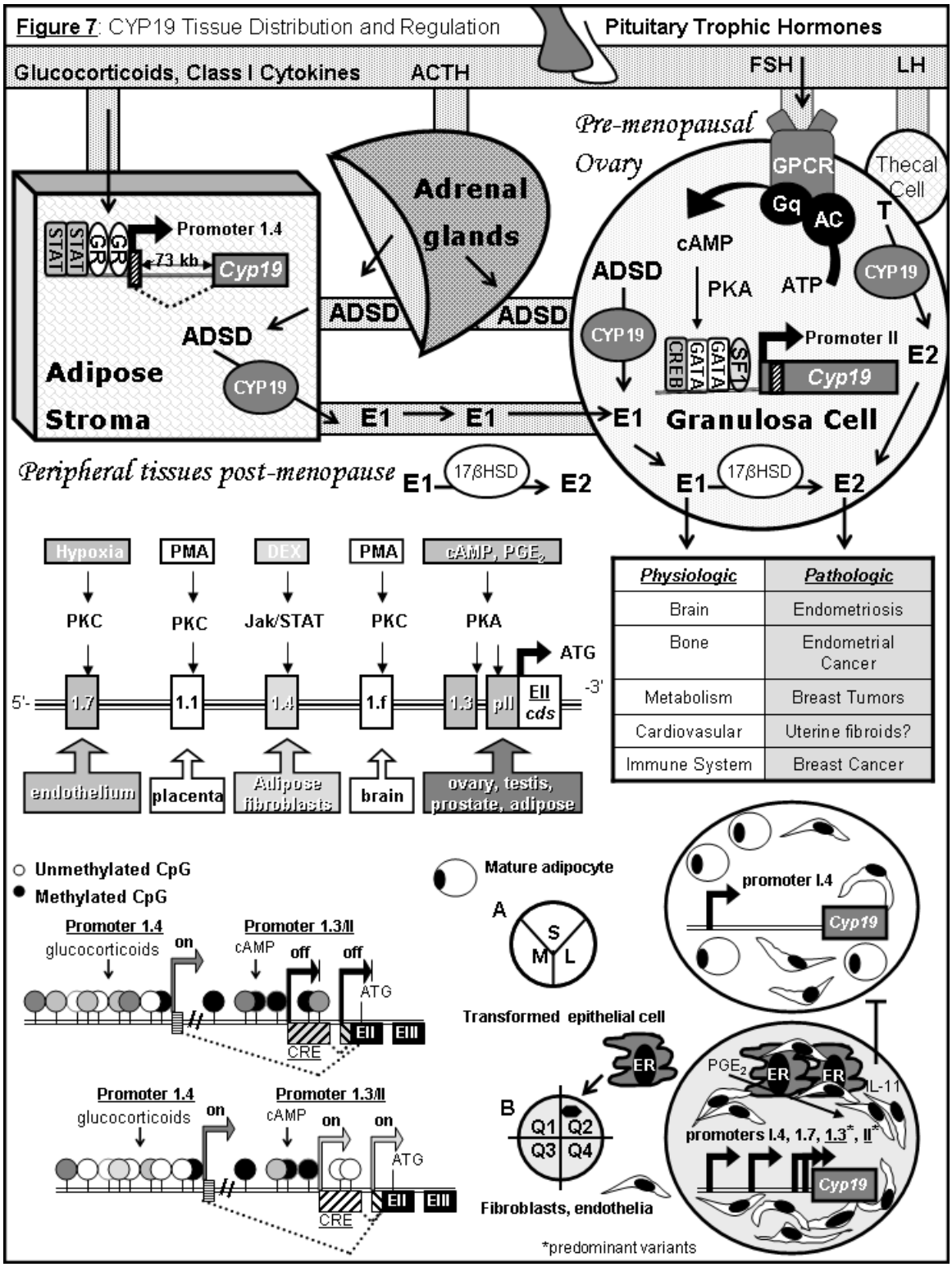


Figure 7: CYP19 Tissue Distribution and Regulation
Pituitary Trophic Hormones
Glucocorticoids, Class I Cytokines
ACTH
FSH
LH
Pre-menopausal Ovary
GPCR
Gq
AC
cAMP
ATP
PKA
T
CYP19
Thecal Cell
Adrenal glands
ADSD
ADSD
ADSD
CYP19
Promoter 1.4
73 kb
Cyp19
STAT
STAT
GR
GR
Adipose Stroma
E1
E1
E1
Granulosa Cell
Promoter II
CREB
GATA
GATA
SF1
Cyp19
E2
Peripheral tissues post-menopause
E1
17βHSD
E2
E1
17βHSD
E2
Hypoxia
PMA
DEX
PMA
cAMP, PGE2
PKC
PKC
Jak/STAT
PKC
PKA
ATG
5'-
1.7
1.1
1.4
1.f
1.3
pII
EII cds
-3'
endothelium
placenta
Adipose fibroblasts
brain
ovary, testis, prostate, adipose
Physiologic
Pathologic
Brain
Endometriosis
Bone
Endometrial Cancer
Metabolism
Breast Tumors
Cardiovasular
Uterine fibroids?
Immune System
Breast Cancer
Unmethylated CpG
Methylated CpG
Promoter 1.4
glucocorticoids
on
Promoter 1.3/II
cAMP
off
off
ATG
CRE
EII
EIII
Promoter 1.4
glucocorticoids
on
Promoter 1.3/II
cAMP
on
on
ATG
CRE
EII
EIII
Mature adipocyte
A
S
M
L
Transformed epithelial cell
B
Q1
Q2
Q3
Q4
Fibroblasts, endothelia
ER
PGE2
ER
ER
IL-11
promoter I.4
Cyp19
promoters I.4, 1.7, 1.3*, II*
Cyp19
*predominant variants

Aberrant Aromatase Promoter Usage is the Mechanism of Increased *In Situ* Estrogen Synthesis Necessary for Proliferation of ER+ Breast Cancer (BC) Cells

Quantitative RT-PCR analyses of healthy mammary adipose reveal CYP19 is typically expressed at low basal levels in this tissue from the distal and relatively weak promoter termed '1.4' under the influence of glucocorticoids and class I cytokines (Figures 3 and 8). In malignant estrogen growth-sensitive breast tissue of PM women, local estrogen concentrations are often 10 to 50 times that of circulating plasma levels (Bouchard *et al*, 2005), and these unusually high increases in estrogen are correlated with 3-4 -fold elevations in total Cyp19 transcript levels in the breast (Clyne *et al*, 2010). Importantly, this induction is the result of increased transcription from the breast-typical promoter I.4, but also results from aberrant activation of multiple other tissue-specific promoters (Bulun *et al*, 2005). In fact, in tumor epithelial and tumor-proximal fibroblasts, Cyp19 transcripts are chiefly derived, not from the distal breast-typical GC-regulated promoter I.4, but from strong, proximal, and SF-1 (steroidogenic-factor-1)-dependent gonadal-type pII and I.3 *Cyp19* promoters. These gonad-typical promoters 'pII' and 'I.3' are only separated by 215 bp (Figure 6, bottom panel) and together are robustly regulated by cAMP in the premenopausal ovary downstream of FSH (Figure 7)- but are typically quiescent in tumor-free breast tissue of both pre- and PM women.

Hypomethylation of CpG dinuleotides in Aromatase Promoters pI.4 and pI.3/pII May Contribute to Inter-individual Variation in Aromatase Overexpression, Estrogen Excess, and BC Risk

Because this switch in promoter preference, or 'promoter switching' as described in the literature is a pivotal phenomenon proceeding the local aromatase overexpression responsible for breast-tumor promoting estrogen production, the molecular mechanisms underlying both aberrant derepression and activation of pI.3/pII are presently intense areas of study. Activation of these promoters in the gonad occurs downstream of cAMP/PKA signaling, and sequence and mutational analyses have revealed a CRE (-292/285) and proximal CLS (cAMP-response element-like sequence, -211/-199) as crucial for their regulation. The proximal CLS, conserved in rats and humans, differs from consensus CRE by insertion of a single cytosine as the third nucleotide adjacent to guanine, which results in formation of an additional CpG dinucleotide in the CLS relative to the one CpG dinucleotide contained in consensus CRE sequence (Figure 6). In sum, six CpG dinucleotides, putative sites of DNA methylation, lie within pI.3/pII, and pI.4- the breast-typical *Cyp19* promoter also inappropriately activated in malignant breast tissue- contains 11 CpG dinucleotides (Figure 3) regulated by methylation (Knower *et al*, 2010).

In a recent study of 4 healthy women from which skin fibroblasts were isolated from buttock biopsies for other research purposes, Demura and Bulun at Northwestern University incidentally discovered CYP19 expression in fibroblasts

from one of the four subjects was cAMP-inducible and driven from the gonadal-typical cAMP-responsive pII/I.3- in contrast to fibroblasts from the other 3 subjects- in which, as expected, CYP19 was GC-inducible, driven from pI.4, and significantly, not cAMP-inducible (2008). This intriguing finding of fibroblast cAMP-sensitivity in one of the four subjects led Demura and Bulun to analyze the methylation status of the six CpG dinucleotides contained within pI.3/pII in fibroblasts derived from all four subjects; they discovered 3 of the 6 CpGs were unmethylated in the subject with cAMP-responsive fibroblasts, whereas all six CpGs were hypermethylated in subjects with cAMP-refractory skin fibroblasts (Figure 7). They next generated both methylated and unmethylated pI.3/pII promoter-reporter constructs, transfected these into skin fibroblasts from the cAMP-sensitive subject, and found pI.3/pII activity much higher in unmethylated versus methylated constructs. Finally, in EMSA assays, they found CREB (cAMP-response element binding protein) binding to methylated pII/I.3 CLS probes was significantly reduced relative to unmethylated CLS probes. In sum, they demonstrated hypomethylation of CpG #5 in the CLS of *Cyp19* pI.3/pII, via novel CREB promoter recruitment, conferred robust and atypical cAMP-responsiveness to fibroblasts in which the weaker GC-regulated *Cyp19* promoter pI.4 is typically utilized (Figures 3, 8). They and others suggest that the physiological significance for women with this skin or adiposal methylation profile is potential for systemic or local estrogen excess, and apprehending differences in the epigenetic landscape of both pI.4 and pII/I.3 between women could further our understanding of inter-individual variability in lifetime estrogen exposure and BC risk.

The mechanisms by which both gonadal and breast-typical *Cyp19* promoters are derepressed by hypomethylation in cells relevant to BC is still unknown, but a growing body of literature implicates aging (Kelsey *et al*, 2009), environmental hazard exposure (Chen and Xu, 2010), and low dietary folate- especially in combination with alcohol abuse (Wiencke *et al*, 2010), ask risk factors for low global methyl pools that may affect BC DNA methylation profiles. More recent developments in epigenetic BC risk factors in the context of *Cyp19* are discussed in greater detail in the next chapter.

The Herbicide Atrazine Activates Trans-Acting Factors in Steroidogenic Cells that Drive Aromatase Expression from cAMP-dependent Gonadal *Cyp19* Promoters 'I.3/pII'

In addition to epigenetic derepression, inappropriate utilization of gonadal cAMP-responsive *Cyp19* promoters in the breast is also critically influenced by aberrant expression and/or activation of several trans-acting factors with cis-elements in pI.3/pII. These trans-acting factors, not surprisingly, are frequent transcriptional partners of CREB in regulating steroidogenic gene expression; like CREB, they sustain transcriptionally activating phosphorylations by PKA and other kinases activated by upstream trophic hormones in steroidogenic tissues

(Figure 1), and are similarly phosphorylated downstream of cytokines from tumor epithelial or infiltrating immune cells in diseased breast tissue (Bulun *et al*, 2005). Relevant to the present study, at least one of these trans-acting factors, the NR5A family orphan nuclear receptor appropriately termed steroidogenic-factor-1 (SF-1), binds a NR5A NRHS (nuclear receptor half-site, -136/124) in pII (Figure 6), and was recently shown critical in pII induction in human adrenal (H295R; Fan *et al*, 2007) and human placental (JEG3; Suzawa and Ingraham) cells treated with the widely-applied herbicide atrazine (ATR). ATR is a well-established PDE inhibitor that increases intracellular cAMP levels in H295R cells (Sanderson *et al,* 2000, 2002), and in addition to aromatase, induces several other steroidogenic enzymes and genes (Figure 2) in multiple cell types (Hayes *et al*, 2010). In a startlingly comprehensive report published by Suzawa and Ingraham, ectopic expression and inhibitor studies indicate ATR activates *Cyp19* pII in JEG3 (human placental cells) via SF-1 activation through PKA, PI3K, and MAPK-(ERK1/2) mediated mechanisms (2008), which are conserved in human and rat ovarian cells.

Liver-Receptor Homolog-1 (LRH-1) is a cAMP-regulated Transcription Factor Expressed in ER+BCs and Proximal Stroma that Contributes to Aberrant *Cyp19* Promoter Use and Estrogen Production

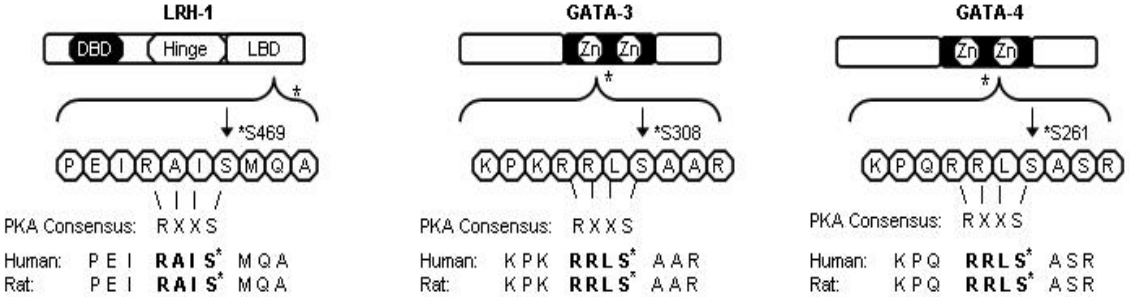
Previously, it was thought transcriptional activation of *Cyp19* from cAMP-dependent pII required binding of both CREB and SF-1. However, SF-1 expression is restricted mainly to steroidogenic tissues, and is not expressed in healthy breast or BC cells[6-9]. Several groups have since shown LRH-1, a NR5A family homolog of SF-1 that binds the same NRHS and is expressed in ER+ BC cell lines and mammary fibroblasts but not mature adipocytes, is in fact a potent activator of pII-specific *Cyp19* expression *in vitro (*Bouchard *et al,* 2005*)*. Furthermore, unlike SF-1, whose activation of pII is only regulated post-translationally via phosphorylation, both LRH-1's expression and activity are enhanced by cAMP/PKA (Figures 3 and 5). In the study by Suzawa and Ingraham cited above, LRH-1 promoter activity was also increased by ATR in JEG cells transfected with *Lrh-1* promoter-reporter constructs (2008). Thus, ATR-induced alterations in LRH-1 expression in steroidogenic or breast tissue cells in which pII/pI.3 are hypomethylated could promote systemic or local estrogen production and hence increase ER+BC and other estrogen-sensitive disease risk.

GATA-3 and GATA-4 Transcription Factors Activate LRH-1 and pII-driven *Cyp19* Expression in BC Cells by cAMP-mediated Mechanisms

Two consensus GATA binding sites, named so because their cognate proteins bind (A/T)GATA(A/G) motifs in genes via identical DNA-binding domains are located between the CLS and NR5A (SF-1/LRH-1) cis-elements of pII

(Figure 6). Occupation of these GATA sites by GATA members (typically GATA -3, -4, and -6) is increased in steroidogenic cells downstream of trophic pituitary hormones, which increases intracellular cAMP levels through their cognate GPCRs, and in PKA-, MAPK-, and PI3K-mediated phosphorylation of GATA members at conserved serine residues (Figures 3 and 6). This permits increased co-operation with SF-1, LRH-1, and CREB in activating genes involved in steroidogenesis and gonadal development (Tremblay and Viger, 2003; Stocco, C, 2008). Recently, GATA factors have also been implicated in the development of certain human cancers, with GATA-3 and -4 being upregulated in breast tumors (Bouchard *et al*, 2005). GATA factors transcriptionally regulate *Lrh-1* and also physically interact with mature LRH-1 protein to increase CYP19 expression from pII several BC cell lines, and these effects are markedly increased in the presence of cAMP or constitutively active PKA (ibid).

Conserved PKA-Target Residues in GATA-3, GATA-4, and LRH-1 Crucial in their Activation of *Cyp19* pII



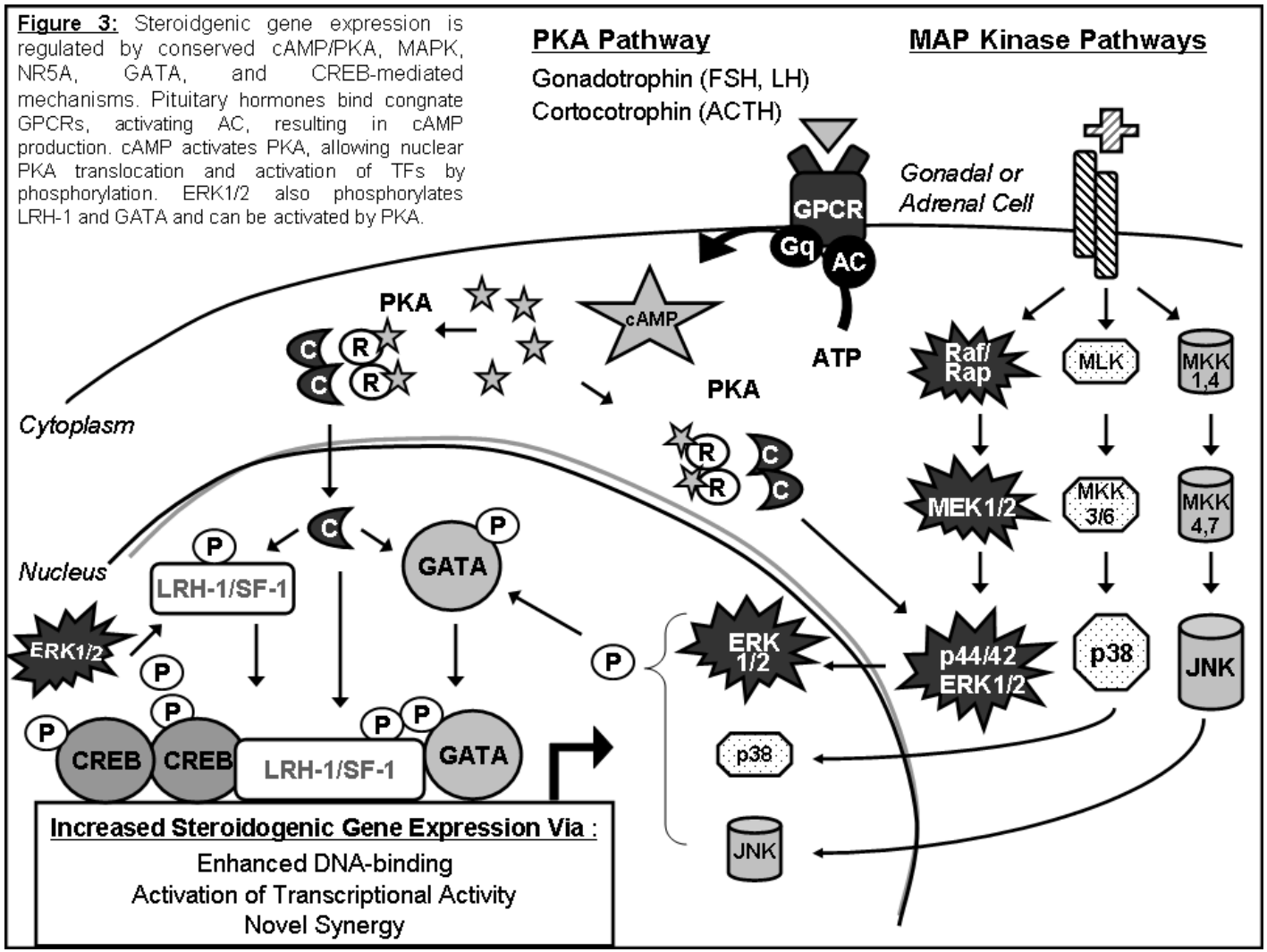


Figure 3: Steroidgenic gene expression is regulated by conserved cAMP/PKA, MAPK, NR5A, GATA, and CREB-mediated mechanisms. Pituitary hormones bind congnate GPCRs, activating AC, resulting in cAMP production. cAMP activates PKA, allowing nuclear PKA translocation and activation of TFs by phosphorylation. ERK1/2 also phosphorylates LRH-1 and GATA and can be activated by PKA.

GATA-3, ERα, and LRH-1 cross-regulate one another in a regulatory loop crucial for normal mammary epithelial cell differentiation and maintenance of the phenotype characteristic of the major ER+BC subtype

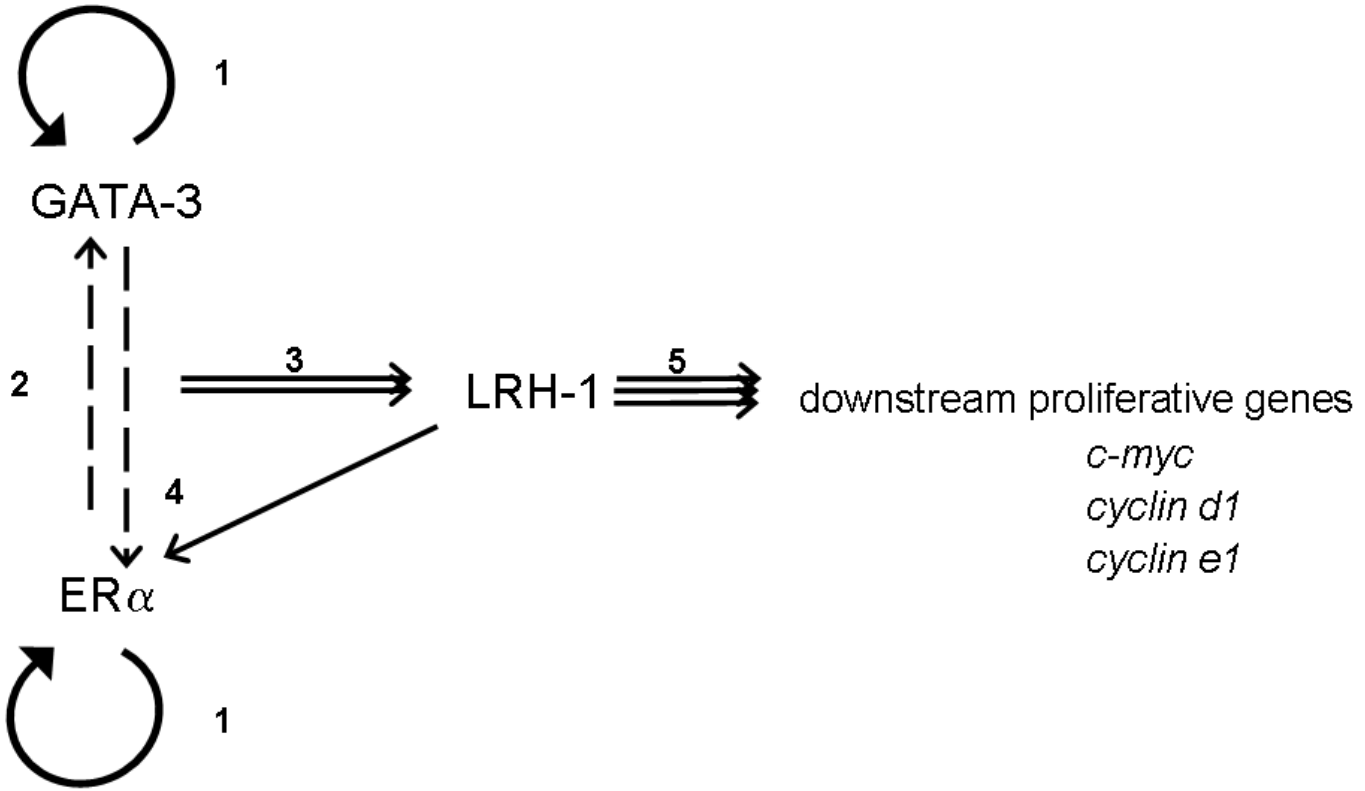
GATA-3 is highly correlated with ERα density in breast tumors, such that 97% of ER+ tumors are GATA-3+, and 76% of ER-negative tumors are GATA-3-negative (Hoch *et al*, 1999). GATA-3 has emerged as a critical transcriptional regulator of ERα expression in BC cells, and is reciprocally transcriptionally regulated by ERα in a positive cross-regulatory loop critical for both normal mammary epithelial cell differentiation in development (Werb *et al*, 2006; Visvader *et al*, 2007), and when deregulated, expression of molecular hallmarks characteristic of the ER+BC phenotype (Ali *et al*, 2011). Relevant to the present study, in addition to increasing LRH-1 and CYP19 expression in certain BC cell lines stimulated with cAMP- ERα, which 'cross-regulates' GATA-3 by the mechanism depicted in Figure 8, is reciprocally regulated by LRH-1 (Ali *et al*, 2011)- which may be induced by ATR in certain cell types. In sum, these findings suggest ATR-induced changes in cAMP/PKA could elicit increased GATA activity and/or expression and promote expression of molecular hallmarks characteristic of ER+BC risk.

Figure 8:

GATA-3-ERα–LRH-1 regulatory loop responsible for normal mammary epithelial differentiation in development and maintenance of the major phenotype of the ER+BC subtype. GATA-3 regulates ERα expression from two cognate cis sites located in the promoter of *esr1*. Reciprocally, ERα enhances Gata-3 expression from an ERE in the 3' *gata-3* untranslated region (UTR). GATA-3 and ERa regulate LRH-1 expression at the transcript level via cognate promoter and enhancer elements, respectively, and LRH-1 regulated ERα transcription via its cognate NRHS in the *esr*1 promoter. GATA-3, ERα, and LRH-1 all regulated G1 to S cell cycle progressors, including *cyclin d1* and *cyclin e*, in normal mammary epithelial cell differentiation and human BC cells.

Figure 8: GATA-3- ERα regulatory loop responsible for normal mammary epithelial differentiation in development and maintenance of the major phenotype of the ER+BC subtype.

1) Knock-down and RNAP II ChIP experiments reveal GATA-3 and ERα auto-regulate their own transcription (*solid black circles*).
2) Knock-down and RNAP II ChIP experiments reveal GATA-3 and ERα critically regulate one another (*dashed arrows*).
3) GATA-3 and ERα co-regulate many downstream genes involved in BC cell proliferation, including l*rh-l (double arrow*).
4) LRH-1 reciprocally partners with GATA-3 to regulate ERα transcription from cis-sites in the ESR1 promoter (*solid arrow*).
5) GATA-3, ERα, and LRH-1 are frequent transcriptional partners at promoters of mitogenic genes and BC hallmarks (*triple arrow*).



MATERIALS AND METHODS

Reagents

Atrazine (2-chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine) was purchased from Chemservice Inc. (Chester, PA). DMSO, H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride), dibutyryl cAMP (N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt), was purchased from Sigma Chemical Company (St. Louis, MO).

Cell Culture

T-47D cells were obtained from a stock routinely maintained by a LBNL BC laboratory. Cells were grown in Iscove's Modified Dulbecco's Media (IMDM) with 10 mM HEPES and 2mM L-glutamine from BioWhittaker (Walkersville, MD), supplemented with 10% fetal bovine serum from Mediatech (Manassas, VA), 50 U/ml penicillin, and 50 U/ml streptomycin from Sigma (St. Louis, MO). Cells were grown in a humidified chamber at 37^{o}C containing 5% CO_2. When cells reached 75-90% confluency, these were serum-starved for 24 hours prior to drug delivery. 0.01, 0.1, 1, and 10 mM (1000x) stocks of ATR were prepared in DMSO (stored at 4^{o}C) and then diluted 1:1000 in media which was added to cell culture plates for the exposures described. All exposures were conducted for 48 hours unless otherwise indicated, with cells being washed with PBS and treatments renewed 24 hours after the initial drug delivery.

Western Blotting

Following treatment, T47D cells were washed and harvested in PBS and pelleted by centrifugation at 4,000 rpm. Cell pellets were lysed in radio-immunoprecipitation buffer (RIPA; RIPA; 150 mM sodium chloride, 50mM tris, 0.5% deoxycholate, 0.1% NP-40, 0.1% sodium dodecyl sulfate (SDS) containing protease inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, and 10 μg/ml β-glycerophosphate). Protein contained within each sample was quantified by the Lowry method. Once normalized for protein content, samples were mixed with a pH 6.8 gel loading buffer containing 25% glycerol, 10% bromophenol blue, β-mercaptoethanol, 3.605% 0.5 M SDS, and electrophoretically fractionated on 8%-10% tris-glycine polyacrylamide/ 0.1% SDS resolving gels (Biorad). Fractionated proteins were then transferred to nitrocellulose membranes; following this, membranes were blocked for non-specific binding with 5% non-fat dry milk in TBST (10mM Tris-HCL, pH 8, 150 mM NaCl, and 0.05% Tween). After blocking, blots were rinsed briefly and incubated overnight at 4oC in antibodies diluted 1:500 to 1:2000 in TBST. GATA-3 (sc-269); GATA-4 (sc-25310); ERα (sc-8005); and HSP90 (sc-7947) were purchased from Santa Cruz Biotechnology. Cyclin D1 (sc-2926), c-Myc (sc-9402), and Cyclin E (sc-41295) antibodies were purchased from Cell Signaling Technology. LRH-1 antibody (ab 18293-200) was purchased from

Abcam Biosciences. CYP19 antibody (A7931) was purchased from Sigma. Immunoreactive proteins were detected by incubation with secondary horseradish peroxidase-conjugated antibodies diluted 3 x 10^{-4} in 1% NFDM in TBST. Blots were then treated with enhanced chemiluminescence reagents (Perkin Elmer, Wellesley, MA) and visualized on ECL film (Eastman Kodak, Rochester, NY).

RT-PCR

Total cell RNA was isolated from T-47D cells following the described treatments with TRI Reagent purchased from Sigma (St. Louis, MO) according to the manufacturer's protocol. Four μg of isolated RNA was used as a template for 1st-strand, cDNA synthesis performed with Moloney murine leukemia virus reverse-transcriptase (M-MLV RT), random hexamers as primers, and 10 mM dNTPs from Promega (Madison, WI). 2 μl cDNA was then subjected to PCR in a 50ul reaction containing Choice Taq polymerase from Denville Scientific, 2.5mM dNTPs (Promega), and primers specific to: LRH-1, ERα, cyclin D1, cyclin E, gata-3, gata-4, c-Myc, or GAPDH. All PCR primers were designed to span an exon-exon region to prevent genomic amplification. PCR products were resolved on a 1.2% agarose gel containing .0001% Gel Red from Biotium (Hayward, CA).

Nested PCR protocol

The following unique *Cyp19* sense (forward) primers were used together with a common antisense primer corresponding to exon III to amplify coding and promoter- specific Cyp19 transcripts:

Cyp19, coding region (corresponding exon II and total *Cyp19* transcript levels):
Forward: 5'- TTGGAAATGCTGAACCCGAT -'3

Antisense Cyp19 primer corresponding to exon III used with all above sense primers:
Reverse: 5'- CAGGAATCTGCCGTGGGGAT -3'

The forward and reverse primers for the remaining targets were as follows:

LRH-1:
Forward: 5'- ATTGAGGATTTTGGCCAGGT -'3
Reverse: 5'- TCACTGCAGCTTCTGTCTCC-'3

ERα:
Forward: 5'-AGCACCCAGTGAAGCTACT -'3
Reverse: 5'- TGAGGCACACAAACTCCT-'3

GATA-3
Forward: 5'-CTCATTAAGCCCAAGCGAAG -'3

Reverse: 5'- TTTTTCGGTTTCTGGTCTGG-'3

Cyclin D1:
Forward: 5'- CATGGAACACCAGCTCCTGTG -'3
Reverse: 5'- TGCACCACCAACTGCTTAG -'3

GATA-4:
Forward: 5'- ACGGGTCACTATCTG TGC A -'3
Reverse: 5'- GACATCGCACTGACTGAGAAC- '3

Cyclin E:
Forward: 5'- TTTCAGGGTATCAGTGGTG -'3
Reverse: 5'- CTGCAACAGACAGAAGAGAA -'3

GAPDH:
Forward: 5'-TGAAGGTCGGAGTCAACGGATTTG -'3
Reverse: 5'- CATGTGGGCCATGAGGTCCACCAC-'3

Chromatin Immunoprecipitation (ChIP) Assays

T-47D cells were grown to near confluency and treated for 48 hours with vehicle (DMSO) control or 10 μM ATR. Formaldehyde was added to living cells (1% final concentration) and incubated for 15 minutes in shaking plates to cross-link protein to DNA. Cross-linking reaction was quenched by addition of 2.5 M glycine. Cells were then lysed with ChIP lysis buffer (50 mM HEPES pH 7.5; 140 mM sodium chloride; 1% Triton X-100; 0.1% sodium deoxycholate) containing protease inhibitors in concentrations described for western blotting. Samples were then sonicated and supernatants were assayed for protein content by the Lowry method (Biorad, Hercules, CA). Each sample used for immunoprecipitation was normalized to contain 1 mg protein, and 1% of the final aliquot from each sample was reserved and stored at -80 for 'Input' controls until further analysis. Normalized samples were pre-cleared by incubation with 100 μl Sepharose-G beads (GE Healthcare) for one hour, after which, two μg of the ChIP-grade LRH-1 (abcam) antibody were added for immunoprecipitation of DNA-protein complexes. Samples were nutated at 4^{o}C with antibody overnight, and then precipitated with Sepharose-G beads. Beads were washed twice with ChIP lysis buffer, twice with 2X ChIP wash buffer (10 mM pH 8 tris; 250 mM lithium chloride; 0.5% NP-40; 0.5% sodium deoxycholate; 1mM EDTA), and 2x TE. DNA in all samples, including input samples, was eluted at 65% overnight in elution buffer (50 mM pH 8 tris, 1% SDS, 10 mM EDTA). DNA was in these samples was then cleaned and concentrated with the Qiagen PCR purification kit (Germantown, MD). A 150 bp fragment of the cAMP-responsive gonadal region of the *Cyp19* promoter containing LRH-1, GATA, and CREB binding sites was PCR amplified with pII-*Cyp19*-specific primers. DNA "No IP" and "input" samples were PCR amplified for 31 cycles. DNA in samples precipitated with

LRH-1 antibody was PCR amplified for 42 cycles (30 seconds/94°C, 30 sec/56°C, 30 seconds/72°C after a 94°C 1-min hot start) with the following ChIP primers specific to *Cyp19* pII (containing CREB, GATA, and LRH-1 binding sites):

Forward: 5'- AGGCAAGACTAATTTATGGTTACAAGTC -3'
Reverse: 5'- TCCACCTCTGGAATGAGCTT -3'

Products were visualized on a 1.2% agarose gel stained with ethidium bromide and buffered with TBE.

RESULTS

ATR dose-dependently increases CYP19 and LRH-1 protein levels in a T-47D human breast cancer cell line derived from stocks routinely maintained by a LBNL breast cancer laboratory

To preliminarily assess whether ATR affects overall CYP19 expression in cell lines typically used to screen xenobiotics for human reproductive hazard potential: Ishikawa (human endometrial cancer); 3T3-L1 (mouse fibroblast); MCF-7 (human ER+ BC); and passage 14 (p14) T-47D (human ER+ BC) cells obtained from stocks routinely maintained by an LBNL laboratory- here after described 'LBNL T-47D'- were seeded and grown to 70% confluence and exposed to increasing concentrations of ATR (0.01-10 μM) in serum-free media for 12, 24, or 48 hours following a 24 hour serum-starvation period. These concentrations of ATR were chosen as a review of the literature indicated chronic exposure to 0.01 μM ATR was sufficient to feminize genetic male *X. laevis* (Hayes *et al*, 2010), and 10 μM was effective in doubling Cyp19 transcript levels in the H295R human adrenal carcinoma cell line *in vitro*. Serum-free media following a 24 hour period of serum starvation was chosen as the diluent medium as at the time of the study pioneering undefined protein components in sera were widely reported to significantly inhibit transcription of *Cyp19* from the cAMP-regulated gonadal promoter, pII (Lephardt and Simpson, 1997)- the same promoter shown in mutagenesis studies to be critical for ATR induction of overall CYP19 expression in H295R cells (Fan *et al*, 2007). Following the 48 hour exposure period, levels of the indicated proteins (Figure 10) were assessed by western blot analysis of fractionated cell extracts as described in the Materials and Methods.

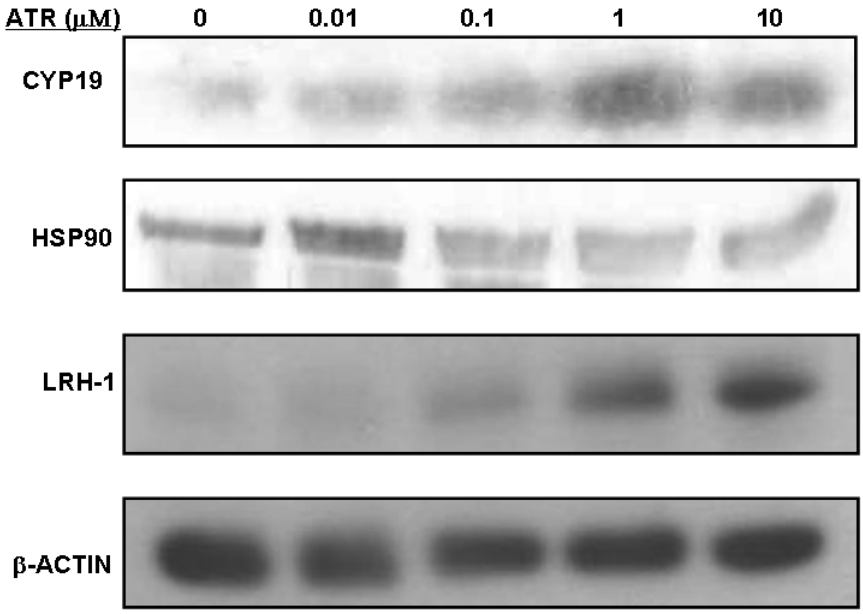
Under the culture conditions employed in this preliminary screen, ATR did not significantly affect CYP19 protein (nor transcript) levels in Ishikawa, 3T3-L1, nor MCF-7 cells at any dose or time-point tested (data contained in two 3'' binders not shown). However, in contrast to MCF-7 cells, ATR did dose-dependently induce CYP19 protein levels after 48 hours in a T-47D BC line (Figure 1) obtained from a stock maintained by the Lawrence Berkeley National Laboratory (LBNL). Moreover, under the same culture conditions and in the same LBNL T-47D cell line, ATR robustly induced protein levels of the NR5A nuclear receptor LRH-1 (Figure 1), which, in combination with GATA-4, GATA-6, and GATA-3, regulates *Cyp19* transcription from pII downstream of cAMP signaling in human and rat gonadal cells (Stocco, Carlos, 2008) and cells of the human breast tumor microenvironment (Bulun *et al*, 2005). In contrast to SF-1, the other nuclear receptor in the NR5A family that regulates *Cyp19* from pII, LRH-1 is itself transcriptionally regulated by cAMP- and the robust induction of LRH-1 protein by ATR would not be anticipated for SF-1- which is post-translationally modified (PTmod) but not regulated at the gene level by cAMP/PKA. These preliminary findings are consistent with a more recent report published by Suzawa and Ingraham (2008), in which ATR potentiated *Cyp19* pII activity in SF-1-transfected

cells but did not affect SF-1 expression. While both SF-1 and LRH-1 bind the same nuclear receptor half-site (NRHS) in pII after PKA-mediated PTmod, SF-1 is not expressed in healthy or malignant breast epithelial cells or fibroblasts, and LRH-1 is frequently enriched in breast tumor epithelial cells and tumor-proximal tissue (Chand *et al*, 2011), where it is coordinately overexpressed with GATA factors, ERα, and G1 cell cycle progressors described in the next experiment- and is an integral hallmark of the early ER+BC phenotype.

Figures 9 and 10:

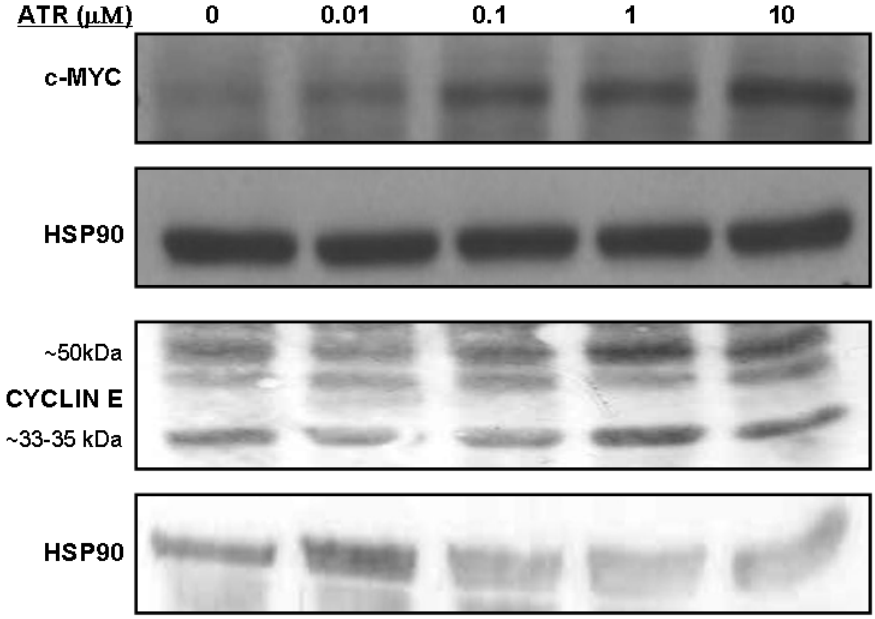
ATR increases CYP19, LRH-1, and c-MYC protein levels in LBNL T-47D Cells. (Figure 9) ATR dose-dependently increases CYP19 and LRH-1 and (Figure 10) c-Myc protein levels in a T-47D cell line. Human T-47D BC cells obtained from a stock maintained by an LBNL BC lab were serum-starved for 24 hours prior to exposure to the indicated doses of ATR in serum–free media for an additional 48 hours. Protein expression at the end of the exposure period was assessed by western blot with antibodies against the indicated proteins as described in the Materials and Methods. Samples were normalized for protein content prior to fractionation by electrophoresis using HSP90 and β-actin as loading control as they were not affected by vehicle or any concentration of ATR at any time point tested in this cell line. Modest induction of LRH-1 was also observed after 24 hours of ATR exposure (Figure 15), however CYP19 was not significantly induced in preliminary time course experiments until 48 hours, which further implicates an upstream role for LRH-1 in CYP19 induction by ATR in BC cells.

Figure 9



48 hour exposure

Figure 10



48 hour exposure

Aromatase overexpression, through locally enhanced estradiol biosynthesis, is a critical promoter of endometriosis, endometrial cancer, and ER+BCs and its induction by ATR in a T-47D cell line is a significant finding. However, LRH-1 induction by ATR observed in the previous experiment, apart from CYP19, may be as significant a finding, as: LRH-1 regulates several steroidogenic enzymes involved in cortisol and androgen biosynthesis (which ATR also affects), and its aberrant regulation in this context may contribute to immune and/or other endocrine related-disease; LRH-1 is aberrantly regulated in several cancer cell types and overgrowth developmental phenotypes which CYP19 does not promote; and LRH-1 is often coincidently overexpressed with mitogen-regulated G1 cell cycle progessors dysregulated 60 to 80% of early-stage breast adenocarcinomas (Chand *et al*, 2011). For these reasons, the robust induction of LRH-1 by ATR observed in T-47D cells may have significant implications for breast tumor promotion beyond CYP19, and if operative in steroidogenic or endodermal tissues, may adversely affect disease outcomes in which LRH overexpression plays a role.

LRH-1 exerts positive pressure on cell proliferation through transcriptional regulation of the G1 cell cycle promoters c-Myc, and the D- and E- type G1 cell cyclins. LRH-1 dysregulation contributes to the pathogenesis of colorectal cancer, and ectopic expression of LRH-1 in gut endoderm is sufficient to drive previously serum-starved Go/G1-arrested cells into S-phase. Further, ERα and LRH-1 reciprocally regulate one another in BC cells as later described in more detail (Figure 8), and knock-down of LRH-1 in MCF-7 and T-47D BC cells inhibits estradiol-stimulated proliferation (Ali *et al*, 2011).

Because LRH-1 was robustly and dose-dependently induced by ATR in T-47D cells, protein levels of its downstream G1 cell cycle effectors c-Myc, Cyclin E1 and Cyclin D1, were also assessed in similarly dosed T-47D cells by western blot analysis. .Figure 10 B shows that ATR dose-dependently and robustly increased c-Myc protein levels, and only moderately (but equally) affected the three isoforms of Cyclin E1 (~33 to 50 kD). The available antibody for cyclin D1 proved inadequate for detection by western blot at the time of this experiment; however, attempts with other aliquots depicted later (Figure 15) and RT-PCR analysis of cyclin D1 mRNA levels described in the next experiment indicate that in addition to the established breast tumor promoter, c-Myc, ATR dose-dependently induced cyclin D1 expression in the LBNL T-47D cell line. Thus, in addition to CYP19 and LRH-1 these studies for the first time demonstrate ATR induces other hallmarks and therapeutic targets of the ER+BC phenotype in ER+ human BC cells.

The robust induction of LRH-1 by ATR coincident with increased c-MYC and CYP19 protein levels raises the possibility that ATR enhances LRH-1 transcriptional activity, or *Lrh-1* transcription- which is also regulated by cAMP (Bouchard *et al*, 2005), or both. In enterohepatic cells, LRH-1 transcriptionally regulates *cyclin E* expression from a perfect consensus NR5A nuclear

consensus half-site (NRHS) in the *cyclin E* promoter- and is co-activated there by β–catenin (Auwerx *et a*l, 2004), which is robustly expressed in T-47D cells (data not shown). Reciprocally, LRH-1 acts as a potent co-activator of β-catenin on the *cyclin D1* promoter in gut cells, but whether it directly regulates cyclin D1 transcription from the five imperfect LRH-1 REs contained within this promoter is unclear (ibid).

While cAMP stimulation alone is not widely reported to induce transcription of c-Myc or the D- and E-type G1 cell cyclins in BC cells, cAMP from PGs and other common tumor-derived factors does enhances transcription of *Lrh-1* (Bouchard *et al*, 2005). Ectopic expression of LRH-1 in gut and colorectal cancer cells is sufficient to progress G0/G1- arrested cells through G1 to S phase (Auwertx *et al*, 2004), and knock-down of LRH-1 reduces ERα-mediated proliferation in BC cells (Fajas *et al*, 2005). Hence, ATR induction of LRH-1 expression could subsequently drive transcription of G1 cyclins from LRH-1-regulated promoters as described above and contribute to breast tissue expression profiles typical of the estrogen-sensitive BC subtype and elevated BC risk.

In addition to driving LRH-1 expression, PKA post-translationally phosphorylates LRH-1, which enhances its transcriptional activity in BC cells by increasing its interaction with other transcription factors at regulated gene promoters (Bouchard et al, 2005). Indeed, as shown by mutagenesis studies elsewhere (ibid) and ectopic expression and inhibitor studies described here (Chapter 2), PKA-phosphorylation of LRH-1 results in striking synergism with GATA-3 at pII of *Cyp19* (Figure 3), another effector of BC cell proliferation downstream of LRH-1 induction. A scheme of the possible mechanisms by which ATR regulates G1 progressors through LRH-1 in BC cells is included in Figure 6.

To interrogate the hypothesis that ATR upregulates *Cyp19*, *Lrh-1*, or LRH-1-regulated cell cycle progressors by affecting their expression at the transcript level, LBNL T-47D cells were exposed to increasing concentrations of ATR as described for the previous experiments, and relative mRNA levels of the named targets- with the exception of Cyp19- were determined by RT-PCR. As is widely reported, low expression levels and multiple splice variants significantly complicated traditional RT-PCR amplification of Cyp19 transcripts; therefore, nested PCR was employed in assessment of Cyp19 levels as described in the Materials and Methods.

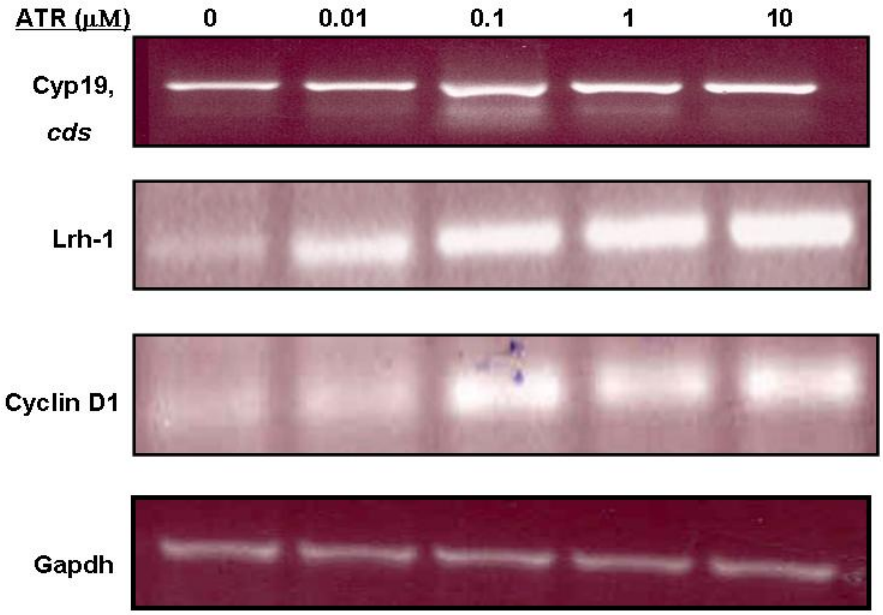
As shown in Figure 11, ATR robustly induced Lrh-1 mRNA levels in T-47D BC cells at concentrations as low as 0.01 μM- which equates to 2 ppb (parts per billion)- or 1 ppb less than the current U.S. ATR drinking water standard. Multiple attempts to amplify c-myc and cyclin E transcripts were unsuccessful; nevertheless, in addition to Lrh-1, ATR also modestly induced both cyclin D1 and Cyp19 mRNA levels beginning at 0.1 μM, which equates to 20 ppb, or half the

concentration of ATR detected in rainfall near application sites. While induction of Cyp19 mRNA levels was indeed slight, estrogens elicit physiological activity in the picomolar range, and 4-fold elevations in CYP19 expression in PM breast tissue (Knower *et al*, 2010) are reported to affect local estrogen concentrations 10 -50 times higher than circulating levels [Bouchard et al, 2005].

Figure 11:

ATR dose-dependently increases Cyp19, Lrh-1, and Cyclin D1 mRNA levels in LBNL T-47D breast cancer cells. Nested PCR results for Cyp19 mRNA and RT-PCR results for LRH-1 and Cyclin d1 levels in LBNL T-47D cells exposed to the indicated concentrations of ATR in serum-free media for 48 hours. Nested and RT-PCR conditions and primer pairs are described in the Materials and Methods. Samples were normalized for RNA content prior to RT. GAPDH was included as a loading control as levels were not affected by vehicle or ATR. Attempts to amplify cyclin E and c-myc transcripts with available primer pairs were unsuccessful.

Figure 11



48 hour exposure

Cyp19 and *Lrh-1* are robustly induced in the breast tumor microenvironment downstream of PGE_2 and other pathological secretory products of BC cells, and cAMP-regulated cis-acting elements, including CRE and GATA binding sites, are common to both the LRH-1 promoter and the gonadal *Cyp19* promoters 1.3 and pII- which are only separated by 215 bp- and are coordinately regulated by cAMP (Figures 3, 12A). Interestingly, the c*yclin d1 gene*, in addition to being regulated by LRH-1, contains a GATA motif in its promoter that is occupied by GATA-3 in neuroblastoma cells (Molenaar *et al*, 2010).

GATA factors are implicated in the development of certain human cancers, with GATA-3 and -4 being upregulated in breast tumors (Bouchard *et al*, 2005). PKA phosphorylation of GATA -3 (S308) and -4 (S261) is known to increase their stability and (Figure 9) markedly enhance their transcriptional activity at *Cyp19* and *Lrh-1* promoters in BC cells (ibid), and in the case of GATA-4, in ovarian granulosa cells downstream of FSH (Stocco C, 2008). GATA-3 and LRH-1 have also emerged as a positive transcriptional regulators of ERα. in human BC cells, and are reciprocally transcriptionally regulated by ER in a 'positive cross regulatory loop' critical for normal mammary gland differentiation (Ali *et al* 2011). In addition to cross-regulating one another in BC cells, GATA-3 and ERα 'autoregulate' their own transcription as evidenced by knock-down and RNAP II ChIP experiments, and co-regulate shared downstream genes from cis-sites, including *Lrh-1* and *cyclin d1* (Eeckhoute *et al*, 2007). Cross-, auto-, and co-regulation by ERα and GATA-3 is critical for normal differentiation of mammary epithelial cells in development but is also likely responsible for the well-differentiated phenotype of early-stage ER+ breast tumors in which ERα, GATA-3, LRH-1, CYP19, c-MYC, and CYCLIN D1 overexpression define the major subtype (Ali *et al* 2011). A scheme of the 'ERα - GATA-3 regulatory loop' is provided in Figure 6.

GATA-3 was recently shown to act synergistically with LRH-1 at the *Esr1* promoter in MCF-7 BC cells (Ali *et al*, 2011). ChIP analysis revealed PKA did not affect their recruitment to *Esr1* in MCF-7 cells *per se*, however whether PKA modulated their physical interaction at *Esr1* was not examined (ibid). Combinations of *Cyp19* promoter-reporter, ChIP assays, and immunoprecipitation and GST pull-down experiments in another study in MCF-7 cells indicate PKA-enhanced physical interaction of GATA-3 and LRH-1 may be more significant than their individual promoter recruitment in evoking the synergy these two transcription factors elicit at pII in MCF-7 cells treated with FSK (Bouchard *et al*, 2005). Taken together, these findings indicate in addition to decreasing their degradation, PKA may modulate physical interactions of GATA-3 and LRH-1 at *Esr1*, and/or ERα and GATA-3 interaction at *Lrh-1*, if not promoter recruitment *per se.* PKA modulation of GATA-3, LRH-1, and ERα interaction and activation of gene promoters relevant to BC is likely highly nuanced, minimally at the level of co-activators, and especially with estradiol

bioavailability- *in vitro* within and between cell types- and *in vivo* also depending on subtype and stage.

PKA phosphorylation of ERα at serine 305: increases its ligand-independent activity; decreases its degradation by proteosomes; modulates its transcriptional response to estrogen; and is associated with tamoxifen-resistance (Al-Dhaheri and Rowan, 2007) . ERα expression is also activated by cAMP in rat pituitary lactotrophs (Tsai *et al*, 2004). In addition to sharing GATA and CRE elements with pII of *Cyp19*, the *Lrh-1* gene contains an ERE located at - 2338 to -2323 bp (relative to +1), and transcription of LRH-1 is enhanced by ERα recruitment to this cis-element in MCF-7 cells following estradiol stimulation. Whether PKA modulation of ER affects its recruitment to *Lrh-1* is unknown; however, ERα synergizes with GATA-3 at other gene promoters *(cyclin d1*), and as described above, PKA phosphorylation of GATA-3 at S308 increases it stability and potentiates transcriptional pressure it exerts from two cognate cis sites in the *Lrh-1* promoter in MCF-7 cells (Bouchard *et al*, 2005). Furthermore, PKA phosphorylation of ERα increases its recruitment to the *c-myc* promoter in MCF-7 cells, and ATR up-regulation of c-MYC levels in combination with cyclin d1 mRNA levels indicate ATR could affect phosphorylation of ERα to modulate its stability, promoter recruitment, or interaction with LRH-1 and GATA-3 at DNA regulatory elements of ATR-targets.

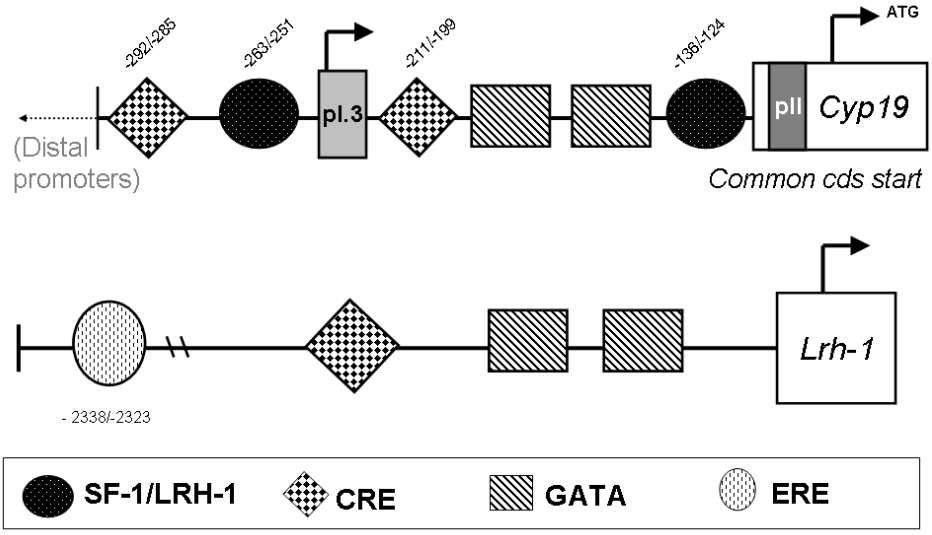
To examine whether GATA-3 and/or ERα might be regulated by ATR upstream of Cyp19, Lrh-1, and Cyclin d1 mRNA induction in T-47D cells, LBNL T47Ds were exposed to increasing concentrations of ATR in serum-free media as previously described and GATA-3 and ERα protein levels were evaluated by western blot as described in the Materials and Methods.

Remarkably, as shown in Figure 12 B, ATR robustly and dose-dependently increased ERα levels in LBNL T-47D cells. ATR also regulated GATA-3 at the lowest dose (0.01 μM) tested, with a maximal response occurring between 0.1 and 1 μM ATR. These observations suggest this effect may be due to ATR increases in PT phosphorylation and concomitant stabilization of GATA-3, and not *de novo gata-3* transcription per se, which preliminary RT-PCR data of gata-3 mRNA levels support (data not shown). The robust induction of ERα protein by ATR in LBNL T-47D cells, while highly intriguing, was incompletely explored mechanistically. Preliminary RT-PCR analyses, analogous to the case of GATA-3, suggest ATR may affect ERα protein stability and not *de novo* transcription *per se*. Nevertheless, LRH-1, GATA-3, and ERα are all modulated by PKA in BC cells, and in LNBL-T-47D cells, by ATR. These findings have significant implications for the role of ATR in contributing to hallmarks of the ER+BC subtype, estrogen-sensitive disease risk, and indicate PKA may be significant in mediating these effects- a hypothesis explored in the next experiment.

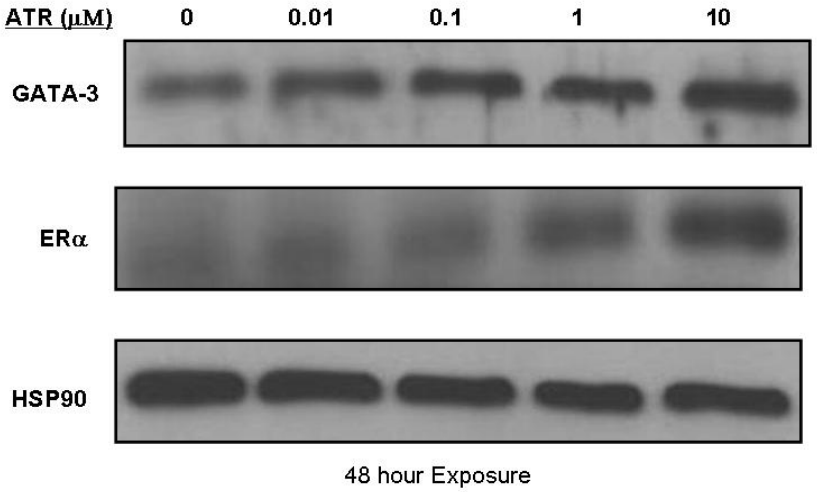
Figure 12:

ATR dose-dependently increases ERα and GATA-3 protein levels in LBNL T47D cells. (A) *Lrh-1* and pI.3/pII of *Cyp19* share common CRE and GATA cis-elements, and *Lrh-1* contains a distal ERE that is positively regulated by ERα in BC cells treated with E_2.. (B) ERα and GATA-3 protein levels detected by western blot in LBNL T-47D BC cells treated with the indicated concentrations of ATR in serum-free media for 48 hours as described in the Materials and Methods. HSP90 is included as a loading control. Auto-, cross-, and co-regulation of ERα and GATA-3 is depicted in Figure 8.

Figure 12 (A): The Human Gonadal cAMP-responsive *Cyp19* Promoters 'I.3/pII' and LRH-1 Promoter Contain GATA and CREB Elements



12 B



The combination of RT-PCR and western blot data for GATA-3 and ERα suggest ATR may phosphorylate these to increase their stability, and possibly their interaction or recruitment to downstream ATR targets of the ER+BC phenotype, including *c-myc*, *cyclin d1*, and *lrh-1*. Combinations of site-directed mutagenesis and *Cyp19 pII*-reporter studies conducted in MCF-7 cells revealed mutation of the consensus PKA phosphorylation sites in GATA3 at S308, GATA4 at S261, and LRH1 at S469, reduced their individual activation of *cyp19* pII by 60, 37, and 64% respectively, and mutation of LRH-1 at S469 reduced its synergism with both transcriptional partners at pII by half (Bouchard *et al*, 2005). A schematic of these PKA-target motifs in each transcription factor, conserved in human and rats, is provided in Figure 6. While PKA-dependence for synergism of these transcription factors at *cyp19* is fairly well-characterized, as described previously, the effect of PKA on ERα, GATA-3, and LRH-1 recruitment to, interaction at, or activation of other genes regulated by ATR is less understood. Moreover, PKA phosphorylation is known to stabilize levels and activate the activity of P450c17, the enzyme responsible for androgen production and hence just upstream of CYP19 in the estrogen biosynthesis pathway; whether CYP19 is similarly regulated by PKA is not well described currently, but a finding in the affirmative might reconcile observations of low Cyp19 transcript levels with robust and sustained protein levels in granulosa cells downstream of FSH in the follicular or estrogen-producing phase of the menstrual cycle.

Thus, to determine if PKA is involved in ATR up-regulation of target proteins in BC cells, LBNL T-47D cells were treated with vehicle or 10 μM ATR for 48 hours 30 minutes after being treated with vehicle, 10 or 20 μM of the PKA inhibitor, H89. Protein levels at the end of the treatment period were determined by western blot as described in the Materials and Methods.

As shown in Figure 13 B, ATR induced protein levels of CYP19, GATA-4, ERα, and c-Myc, and these affects were attenuated or ablated by H89. PKA inhibition also attenuated GATA-4, ERα, c-Myc, and GATA-3 levels in vehicle-treated cells, which reaffirms other reports suggesting protein levels of these targets are modulated by PKA phosphorylation. LRH-1 and GATA-3 induction by ATR appears modest, and the effect of PKA induction less clear. These results likely reflect the poor quality of our on-hand LRH-1 antibody, and the replacement of my α-GATA-3 aliquot with a shoddy substitute by another otherwise generous and kind researcher in the lab.

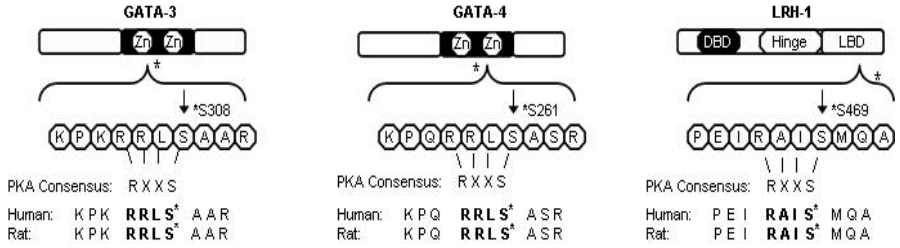
Nevertheless, taken together with findings detailed above, these H89 studies demonstrate PKA is critically involved in ATR upregulation of CYP19, GATA-4, ERα, and c-Myc protein levels, and likely LRH-1 and GATA-3 protein levels in LBNL T-47D cells. Furthermore, effects of H89 in vehicle-treated cells suggest PKA may impact rates of normal turnover of GATA-4, ERα, and GATA-3 in BC cells. Considered with what is incompletely understood about the ERα - GATA-3 regulatory loop critical for both normal differentiation of mammary epithelial cells and maintenance of the ER+BC phenotype, these findings

suggest ATR could adversely impact both at the levels of LRH-1, GATA-3, and ERα- by multiple conserved mechanisms detailed in a schematic provided in Figure 6. The rat and human mammary gland develop shared morphological features and hormone responsiveness at a similar biological pace, and a high degree of conservation in regulators of the ERα-GATA-3 loop may explain recent findings that exposure to ATR transplacentally *in utero* and post-natally in cross-fostering studies alters both developmental timing and structural features of the rodent mammary gland (Fenton *et al*, 2004).

Figure 13:

Involvement of PKA in ATR induction of CYP19, ERα, GATA-4, c-Myc, and other Hallmarks of the ER+BC phenotype. (A) Species-conserved PKA-target motifs in GATA-3, GATA-4, and LRH-1. (B) Effect of PKA inhibition on normal turnover and ATR induction of the indicated proteins. LBNL T-47Ds were treated with vehicle or 10 μM ATR 30 minutes after being treated with vehicle or the indicated concentrations of H89 in serum-free media. Cells were harvested after 48 hours and protein levels were assessed by western blot as described in the Materials and Methods. Samples were normalized for protein content, and H89 treatment did not appear to affect cell survival/death or HSP90 expression- which is included as a loading control.

Figure 13
(A)


GATA-3
Zn Zn
*S308
K P K R R L S A A R
PKA Consensus: R X X S
Human: K P K RRLS* A A R
Rat: K P K RRLS* A A R
GATA-4
Zn Zn
*S261
K P Q R R L S A S R
PKA Consensus: R X X S
Human: K P Q RRLS* A S R
Rat: K P Q RRLS* A S R
LRH-1
DBD
Hinge
LBD
*S469
P E I R A I S M Q A
PKA Consensus: R X X S
Human: P E I RAIS* M Q A
Rat: P E I RAIS* M Q A

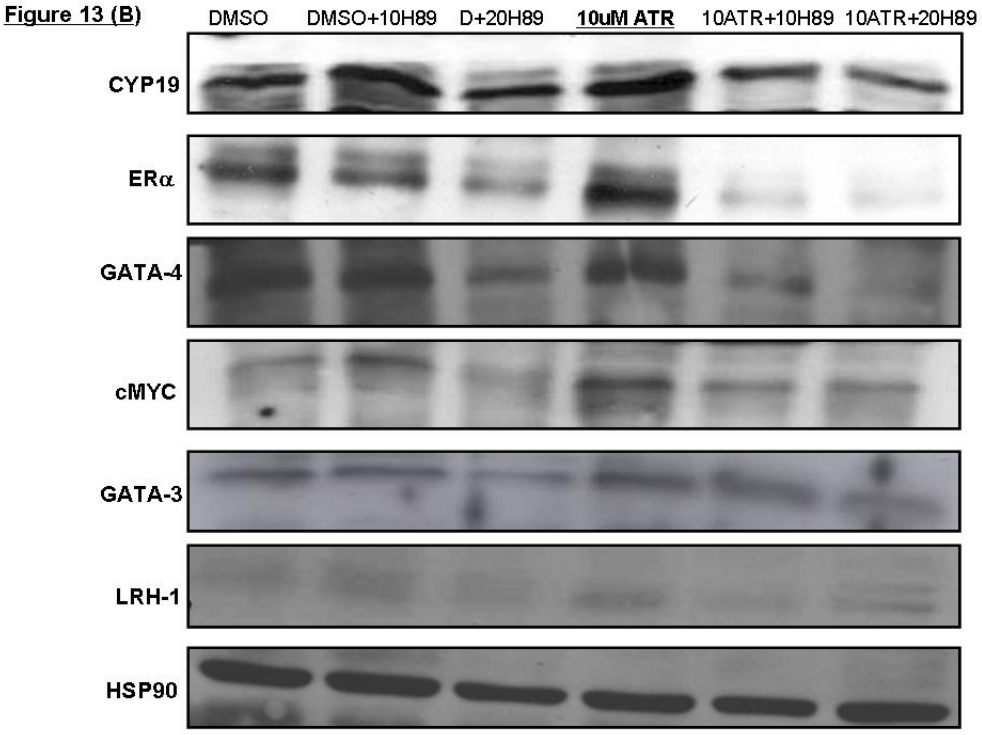


Figure 13 (B)
DMSO
DMSO+10H89
D+20H89
10uM ATR
10ATR+10H89
10ATR+20H89
CYP19
ERα
GATA-4
cMYC
GATA-3
LRH-1
HSP90

Figure 14:

Hypothetical mechanisms by which ATR and PKA may affect the ERα-GATA-3-LRH-1 regulatory loop critical for normal mammary epithelial development and phenotypic maintenance of the major ER+BC subtype. Stars indicate serine phosphorylation by PKA. Solid lines indicate mechanisms for which strong evidence is provided here or elsewhere; dashed lines indicate putative mechanisms and vast opportunities for further research.

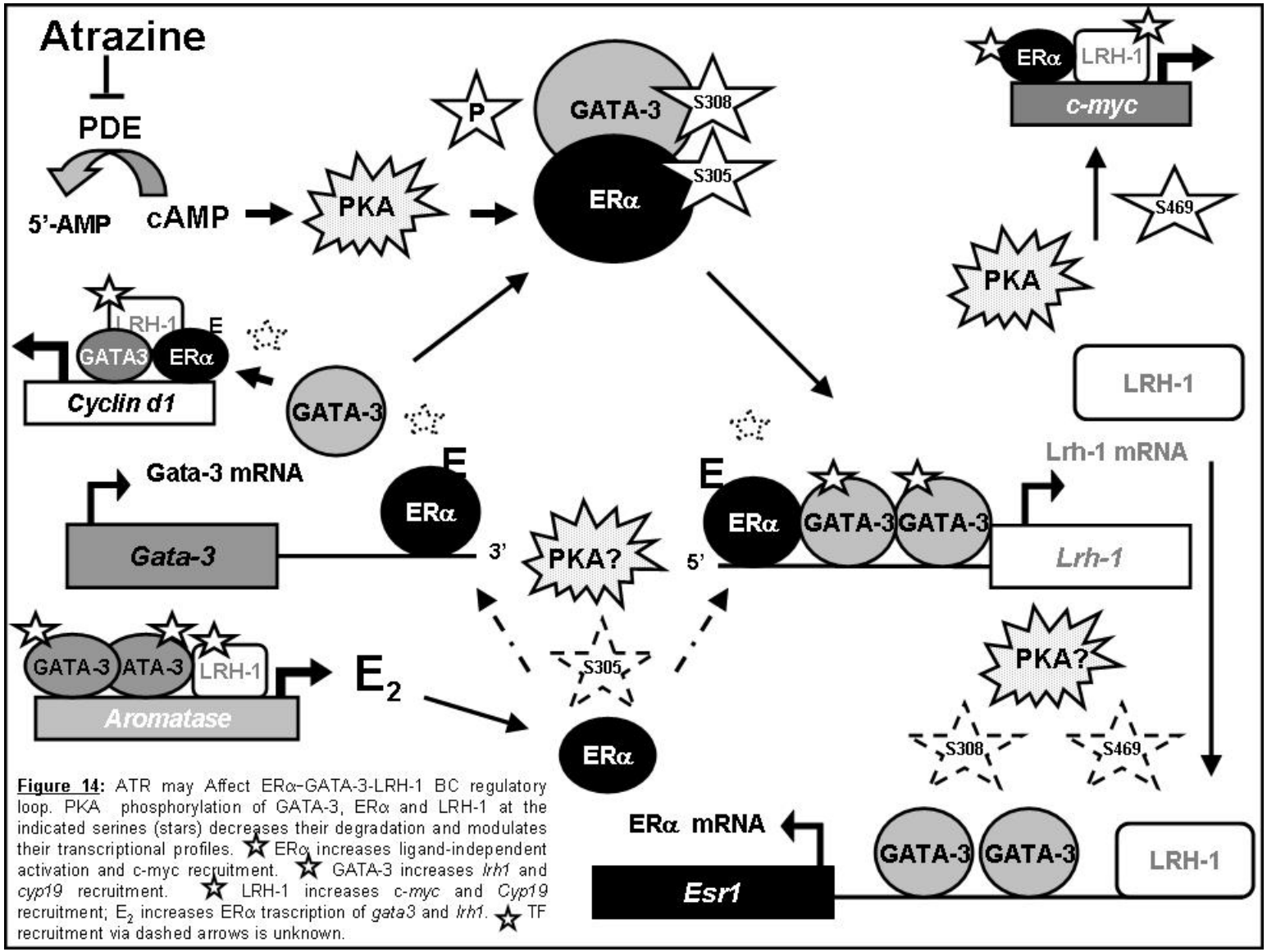


Figure 14: ATR may Affect ERα-GATA-3-LRH-1 BC regulatory loop. PKA phosphorylation of GATA-3, ERα and LRH-1 at the indicated serines (stars) decreases their degradation and modulates their transcriptional profiles. ☆ ERα increases ligand-independent activation and c-myc recruitment. ☆ GATA-3 increases *lrh1* and *cyp19* recruitment. ☆ LRH-1 increases *c-myc* and *Cyp19* recruitment; E_2 increases ERα trascription of *gata3* and *lrh1*. ☆ TF recruitment via dashed arrows is unknown.

Because GATA-3 and LRH-1 together regulate ERα, ERα regulates GATA-3, and GATA3 and ERα together regulate LRH-1, establishing the kinetics of ATR induction of these targets may inform future strategies of elucidating transcriptional- and protein- level mechanisms of ATR induction of these targets in BC and other cell types.

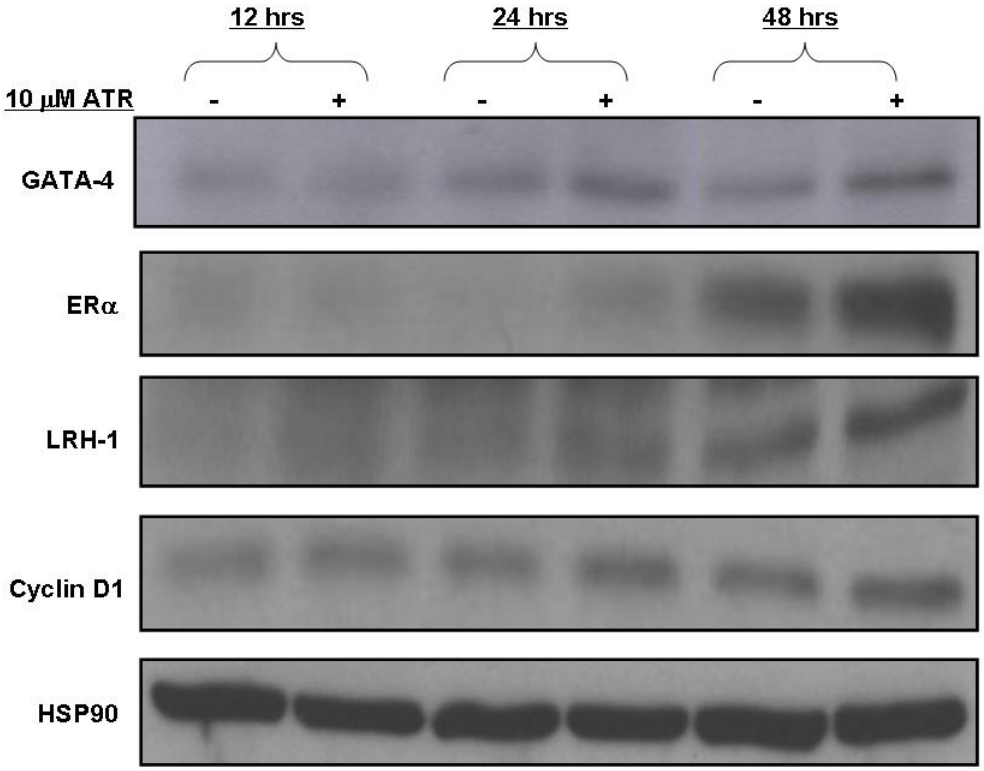
To this end a timecourse of protein induction of these and related targets by ATR was attempted. As shown in Figure 15, none of the targets were regulated at 12 hours; GATA-4 was induced at 24 hours; ERα, LRH-1, and CYCLIN D1 were induced at 48 hours.

GATA-4: is dysregulated in BC, is rapidly phosphorylated at S261 by PKA in BC cells (Bouchard et al, 2005) and in human and rat ovarian cells downstream of FSH (Stocco C, 2008); regulates transcription of Lrh-1 in BC and ovarian cells- an effect dramatically enhanced by PKA (80-fold) in *Lrh-1* promoter-reporter studies in *MCF-7* cells; and synergizes with LRH to activate transcription of *Cyp19* from pII in MCF-7 and HeLa cells.

Figure 15:

Timecourse of induction of molecular breast cancer risk factors by ATR in LBNL T47D breast cancer cells. LBNL T-47D cells were treated with vehicle or 10 μM ATR for 12, 24, or 48 hours in serum-free media and harvested at the end of the exposure period for detection of the indicated proteins by western blot as described in the Materials and Methods. Samples were normalized for protein content prior to fractionation and HSP90 is included as a loading control as levels did not change with time or treatment in T-47D cells.

Figure 15



Unlike SF-1, which sustains post-translational activating phosphorylations but is not itself regulated at the transcript level by PKA, the activity and expression of LRH-1 are robustly induced by cAMP/PKA signaling; this difference in expression inducibility by PKA between the two NR5A members is well-established (Clyne *et al*., 2004), and is consistent with findings presented here (Figure 16). Because, unlike SF-1, overall LRH-1 protein levels change with PKA activation, it is possible that LRH-1 promoter recruitment to PKA-regulated genes can be in part driven by mass action, or simply an increase in intracellular LRH-1 protein levels, which could subsequently translate into a larger number of LRH-1 proteins being available for transcriptionally activating and possibly promoter-recruiting phosphorylations by PKA. Therefore changes in downstream LRH-1 promoter recruitment that in part result from upstream cAMP/PKA induction of overall LRH protein levels, may be detectable by chromatin immunoprecipitation-absent phospho-antibodies against-LRH-1 (which unfortunately were not commercially available at the time this experiment was first undertaken). This is in contrast to SF-1, whose activation of promoters downstream of cAMP is likely only induced by PKA phosphorylation of transactivation domains, phosphorylation-induced conformational changes and co-activator recruitment, but not by changes in SF-1 protein levels or expression-related differences in downstream promoter occupancy (however in cases where LRH-1 and SF-1 are co-expressed changes in LRH-1 expression can affect one TF displacing the other at shared NR5A NRHSs, as happens in granulosa cells in different phases of the rat estrus cycle (Weck and Mayo, 2006). Promoter occupancy would also not be expected to change in cells poorly inducible or refractory to LRH-1 induction by cAMP stimulation, which has reported for breast adipose fibroblasts (Chand *et al*, 2011) and other BC cell lines (Ali *et a*l, 2011). However, promoter-reporter studies reveal ectopic expression of LRH-1 is sufficient to increase *cyp19*-pII activity absent cAMP-stimulation in breast fibroblasts and BC cells (Bulun *et al*, 2007), which may indicate post-translational modification by PKA is dispensable in mediating differences in LRH-1 *Cyp19* promoter occupancy when LRH-1 is robustly induced in one group but not the other to which it is compared. Because:

1) both LRH-1 protein levels were robustly induced by ATR in LBNL T-47D cells
2) LRH-1 is transcriptionally activated by PKA at *pII* of *Cyp19*
3) both Cyp19 transcript and protein levels were modestly induced by ATR in LBNL T-47D cells (Figure 16 B)
4) ATR induction of LRH-1 protein was highest after 48 hours exposure in the previous timecourse experiment (Figure 16 C)
5) Phospho-LRH-1 antibodies were not commercially available at the time this experiment was undertaken

LRH-1 'pII' recruitment was ascertained by chromatin immunoprecipitation (ChIP) of LRH-1 on the endogenous cAMP-responsive *Cyp19* gonadal promoter 'pII' in LBNL T-47D cells treated with vehicle or 10 μM ATR for 48 hours as

described in the Materials and Methods. Briefly, following the 48 hour exposure period: protein associated with endogenous DNA in vehicle- and ATR-treated cells was cross-linked with formaldehyde; total chromatin was sheared by sonication; total LRH-1 was immunoprecipitated from whole-cell extracts with an α-LRH antibody bound to sepharose beads; isolated LRH-1-antibody complexes were released from beads in a series of washes; DNA-protein cross-links were reversed; and primers specific to a 150 bp region of the cAMP-responsive c*yp19* pII spanning the CRE, GATA, and NR5A response elements (shown in panel 16 D) were used to PCR amplify this specific region of pII with endogenous DNA only pulled-down with α-LRH-1 antibodies as template. ~ 1% of the total whole-cell extracts was reserved apart prior to incubation with α-LRH-1 and carried through the procedure as described as a loading control representative of total DNA for the respective ATR-treated and control groups.

As shown in Figure 16 D, 48 hour ATR exposure did appear to modestly increase LRH-1 recruitment to its cognate NRHS in the cAMP-responsive gonadal *Cyp19* promoter 'pII'. This finding is in contrast to other reports conducted in BC cell lines and breast adipose fibroblasts (BAF), where FSK treatment did not affect LRH-1 NRHS binding (EMSA, BC lines) or pII occupancy (ChIP, BAF), respectively. However the cell lines employed in the aforementioned promoter recruitment studies were also refractory to LRH-1 induction by FSK, which is in sharp contrast to LBNL T-47D cells, where LRH-1 protein was robustly induced by ATR- which may account for the difference. In accordance with the large magnitude of LRH-1 inducibility in ATR-treated LBNL T-47D cells observed in previous experiments (panels B and C), LRH-1 appeared to be virtually absent from pII in vehicle-treated cells, which may also explain widely reported and here verified low basal Cyp19 mRNA expression levels in T-47D and other BC cell lines. LRH-1 pII recruitment at this 48 timepoint was modest, and exposure of shorter duration may have yielded different results. This preliminary endeavor was undertaken after a 48 hour exposure period as previous timecourse studies indicated LRH protein induction was highest at this timepoint (Figure 16), and the kinetics of cAMP/PKA-dependent steroidogenic promoter recruitment is reported to proceed at a similar pace in other cell types. Antibodies against phospho-LRH-1 were not commercially available at the time this experiment was undertaken, which would have been highly preferable to antibodies against total LRH-1, especially as more recent reports document pII occupancy by phospho-LRH-1 is changed by FSK even in cells refractory to LRH-1 expression induction via cAMP stimulation. Nevertheless, these preliminary findings indicate LRH-1 recruitment to the highly conserved pII of *Cyp19* was enhanced by ATR in LBNL human T-47D BC cells.

The results presented here should be interpreted with caution, as in these experiments all the appropriate controls, including non-specific primers, and a 'no IP' control with an irrelevant anti-body, were not included, and the results presented here in LBNL-T47D cells were not repeated as soon after this experiment the doubling time, morphology, and ATR-responsiveness of this stock

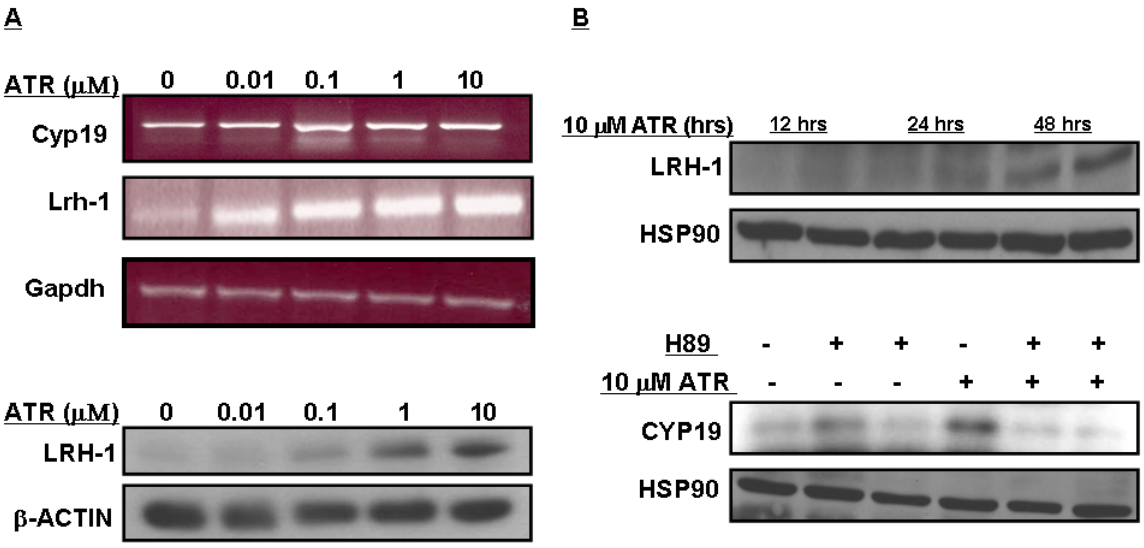
fundamentally changed as described and explored in great detail in the next chapter.

As our current lead gene therapies against ER+BC seek to suppress LRH-1 recruitment to pII, that physiologically relevant concentrations of ATR enhance this interaction in LBNL T-47D cells has significant implications for its role in affecting lifetime estrogen exposure and BC risk.

Figure 16:

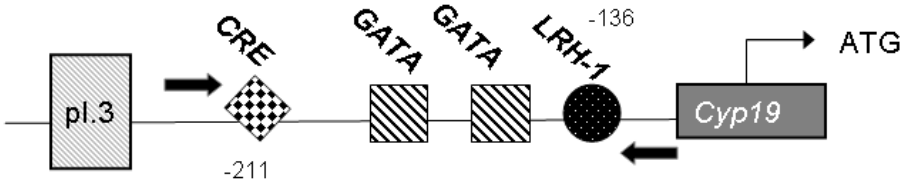
LRH-1 is recruited to the cAMP-responsive gonadal *Cyp19* promoter 'pII' by ATR in LBNL T-47D ER+ human breast cancer cells. (A) Cyp19 and Lrh-1 mRNA are dose-dependently induced by ATR in LBNL T47D cells treated for 48 hours. (B) LRH-1 protein levels are highly induced by ATR at 48 hours. (C) Chromatin Immunoprecipitation (ChIP) of LRH-1 on the endogenous *Cyp19 pII* reveals ATR induces LRH-1 recruitment to its cognate RE in LBNL T-47D cells exposed for 48 hours in serum-free media. *Cyp19* pII-specific primers were designed to flank a 150 bp of pII containing the CRE, GATA, and NR5A2 elements, and were employed to PCR-amplify (30 cycles) endogenous DNA isolated from chromatin cross-linked to LRH-1 and immunoprecipitated from vehicle and treated cells with α-LRH-1 following the exposure period. 1% of the whole cell extracts from each treatment group was reserved prior incubation with α-LRH-1 and represents total DNA input and was used as the loading control. Products were visualized on a 1.2% agarose gel with ethidium bromide staining.

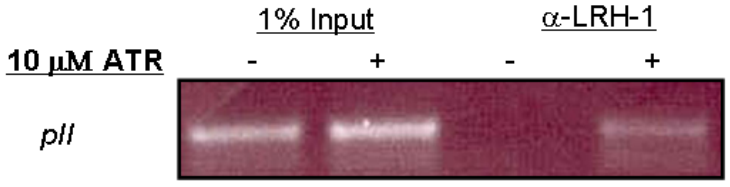
Figure16



C

Predicted ATR-regulated Transcription Factor Binding Sites within the *cAMP-responsive gonadal Cyp19 promoter* 'pII'





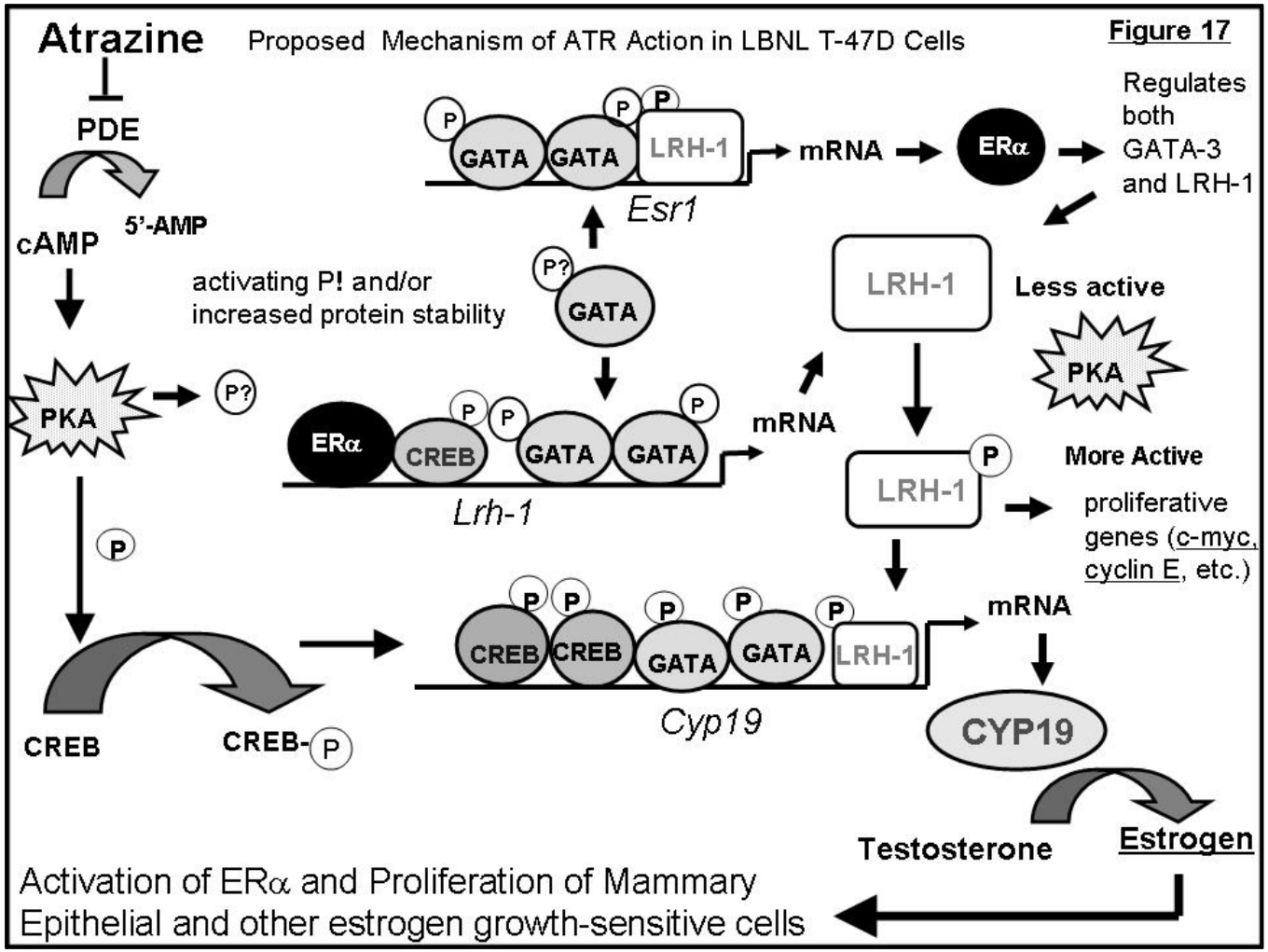
48 hour Exposure

DISCUSSION

These studies establish, consistent with other reports conducted in steroidogenic cancer cell lines, that physiologically relevant concentrations of the widely applied herbicide ATR induce CYP19 and LRH-1 by highly species-conserved and largely PKA-mediated mechanisms in a human ER+ T-47D BC line. As summarized in Figures 6 and 9, these studies also for the first time show ATR upregulates expression of ERα, GATA-3, GATA-4, c-Myc, and Cyclin D1- all highly relevant actors in the gene regulatory network characteristically deregulated in the major ER+BC subtype. Each of these targets critically participate in normal mammary epithelial development, and an increasing body of animal studies suggests dysregulation of any of these factors prenatally or in other developmental windows of susceptibility may result in genetic or epigenetic markers that predispose women to higher risk of BC or other estrogen-sensitive disease. A summary scheme of the mechanisms by which we propose ATR affects ER+BC hallmarks in LBNL T-47D cells is provided in Figure 17.

Figure 17:

Proposed Mechanisms of *cyp19* Induction by ATR in LBNL T-47D Cells. Our findings presented here implicate highly conserved: PKA-, GATA-3-; GATA-4, and LRH-1-mediated mechanisms as molecular determinants of *cyp19*-inducibility by ATR in ER+ LBNL T-47D BC cells. ATR inhibits PDE, elevating intracellular cAMP levels and enhancing PKA activity. Enhanced PKA activity may increase the transcriptional activity of LRH-1 and/or its interaction with GATA factors and CREB at pII of *Cyp19* in cells in which one or both CpG dinucleotides located in CRE of pII are hypomethylated.


Atrazine
Proposed Mechanism of ATR Action in LBNL T-47D Cells
Figure 17
PDE
5'-AMP
cAMP
PKA
P?
activating P! and/or
increased protein stability
GATA
LRH-1
mRNA
ERα
Esr1
Regulates
both
GATA-3
and LRH-1
Less active
PKA
More Active
proliferative
genes (c-myc,
cyclin E, etc.)
CREB
CREB-P
Lrh-1
Cyp19
CYP19
Testosterone
Estrogen
Activation of ERα and Proliferation of Mammary
Epithelial and other estrogen growth-sensitive cells

Significance of Aromatase Induction by ATR in Light of Current BC Prevention and Treatment Strategies

Significantly, our current first-line defense against the majority of BCs cases- 2/3 of which are growth-promoted by estrogen at the initial time of diagnosis (Eeckhoute *et al*, 2007)- is treatment with aromatase and estrogen receptor antagonists. Fadrozole, one of the first therapeutic aromatase inhibitors described in 1987, was initially developed at Ciba-Giecy- which later merged with AstraZeneca to form Novartis- formerly the principle manufacturer and registrant of ATR as Novartis Crop Protection. Following public notification of ATR-induced rat mammary oncogenicity findings in 1986 (described in Chapter 3), a Novartis demerger ensued, resulting in formation of two pharma companies and an agribusiness. Astrazeneca and Novartis constitute the former- and these currently hold the patents on the two most widely prescribed aromatase inhibitors for BC- anastrazole and letrozole, respectively (Brodie *et al*, 2009). The 'spin off' of the latter in the demerger resulted in formation of agrichemical giant Syngenta, the current registrant and manufacturer of ATR- ATR being formerly registered with and manufactured by the Novartis Crop Protection branch of Novartis (Hayes, T, 2004).

Aromatase inhibitors and ERα antagonists, while highly effective against early stage ER+BC, globally suppress estradiol production and action respectively, and BC patients minimally suffer severe cardiovascular, mental, gynecological, metabolic, and bone-loss side effects associated with frank bodily estrogen deprivation as a result of these treatments (Chen *et al*, 2009). Current chemical and gene therapies against ER+BC BC, the major subtype diagnosed that also disproportionately affects PM women via localized aromatase and ERα deregulation, are therefore aimed at breast tissue-specific CYP19 and ERα-action ablation while preserving these in other tissues. As aberrant *cyp19* 'promoter switching', or derepression and activation of cAMP-responsive gonadal *Cyp19* promoters 'pI.3' and 'pII' in breast tumor epithelial cells and tumor-proximal fibroblasts is paramount in driving tumor-promoting estradiol production in PM cases, understanding the cascade of events that underlie and exacerbate the phenomenon of 'promoter switching' will provide us with a greater number of molecular tissue-restricted targets we can exploit therapeutically to prevent and treat BC, ideally on idiosyncratic basis that accounts for each patient's unique internal and external environmental risk factors. While presently our understanding of *Cyp19* promoter switching in ER+BCs is far from complete, a growing body of literature has advanced the orphan nuclear receptor LRH-1 as the most promising molecular target for breast tissue-specific aromatase inhibition; knockdown of LRH-1 in BC cell lines results in significantly attenuated aromatase expression resulting from near ablation of pII utilization- the ideal *in vivo* consequence being suppression of breast tissue estrogen synthesis and corollary tumor regression.

Significance of ATR Induction of Hallmarks of the Major ER+BC Subtype

LRH-1 was immunohistochemically detected in 43% of breast tumor biopsies in a recently studied Japanese cohort diagnosed with invasive breast adenocarinoma (Chand *et al*, 2011). In addition to driving aromatase expression from cancer-associated promoters critical for estrogen-sensitive breast tumor-promotion, LRH-1 knockdown in MCF-7 cells reduces ERα transcript levels by half (Ali, *et al* 2011), and because ERα reciprocally regulates LRH-1, and the two regulate mitogenic genes as transcriptional partners, LRH-1 knockdown also significantly blunts estradiol-stimulated (ER-mediated) BC cell proliferation (Figure 8). LRH-1 regulation of the G1 progressors *c-myc*, *cyclin d1*, and *cycin e1* independent of ERα is also significant; ectopic LRH-1 expression promotes cell proliferation, invasion, and migration in gastric, colonic, and also ER (-) BC models (Chand *et al* 2011)- the latter indicating LRH-1, contrary to ERα- may retain its value as significant therapeutic target when ER+BC cases progress to estrogen-insensitive stages.

Preventing CYP19 expression from cAMP-responsive gonadal promoters and ablating ERα expression specifically in the breast are the lead foci of current chemical and gene therapies against ER+BC, and a growing body of literature recommends targeting LRH-1 as a single strategy with potential for realizing both therapeutic measures (Ali *et al*, 2010). The human T-47D ER+BC cell line was heavily relied upon in all the studies here cited from which LRH-1 reduction emerged as a promising consensus strategy for both CYP19 and ERα breast-tissue specific ablation and ER+BC therapy; that biologically relevant concentrations of ATR, the second-most widely applied herbicide in the U.S., significantly induce LRH-1 levels in conjunction with ERα and CYP19 in this same cell line, suggest ATR is one of several emerging environmental endocrine disruptors for which exposure reduction should be considered in ER+BC prevention strategies.

GATA-3 is another critical effector of maintenance of the major ER+BC subtype, and is expressed in 97% of ER+ breast tumors. As knock-down experiments reveal GATA-3 is critical for estradiol-dependent BC proliferation (Eeckhoute *et al*, 2007), and pII-drivien CYP19 expression (Bouchard *et al*, 2005), chemical and biological strategies to ablate GATA-3 expression specifically in the breast, like those against LRH-1, are being intensely perused as novel BC therapies. In addition to maintaining the ER+BC phenotype in the cross-regulatory loop depicted in Figures 8 and 14, in the same way, GATA-3 and LRH-1 are crucial for normal breast epithelial differentiation early in development. LRH-1 and/or GATA-3 deregulation at one or several levels of the loop, prenatally or later in life, may predispose women to ERα, GATA-3, LRH-1, CYP19, c-MYC, and/or CYCLIN D1 overexpression in the breast, promoting further deregulation of the 'loop', and phenotypic acquisitions characteristic of the ER+BC phenotype, which represent 2/3 of cases at the initial time of diagnosis. Thus, that ATR affects expression of hallmarks and therapeutic targets at every

level of the 'loop' responsible for normal mammary development and maintenance of the major ER+BC subtype has implications for risk of BC predisposition, promotion, and progression.

Aromatase pI.3/II CpG Dinucleotide Methylation Status as a Critical Epigenetic Determinant of ATR Susceptibility Relevant to Inter-Individual BC Risk

Counterintuitively, PM women are disproportionately affected by BC. After menopause, adipose becomes the major organ of estrogen biosynthesis, where *Cyp19* expression in adipogenic fibroblasts of adiposal compartments of the thighs, buttocks, and mammary stroma in healthy women is chiefly driven from the distal, weak, glucocorticoid and class I cytokine- fat/breast-typical *Cyp19* promoter termed I.4, resulting in both low systemic and local estrogen levels produced from weak adrenal androgen conversion (Figure 7). Conversely, estrogen levels in breast tumor-containing tissue are frequently elevated upwards of ten-fold relative to plasma concentrations in PM patients (Bouchard *et al*, 2005) owing to increases in total CYP19 expression in the breast, which results primarily from aberrant activation of cAMP-responsive gonadal *Cyp19* promoters termed 'pII' and 'pI.3' typically silenced in healthy breast tissue (Knower *et al,* 2010). This phenomenon of derepression and activation of gonadal pI.3/II by tumor epithelial and proximal fibroblasts is nominally described in the literature as 'promoter switching', and in conjunction with aberrant activation of several of the more than ten alternative *Cyp19* promoters results in 4-5 fold elevations of total CYP19 expression in cancerous breast tissue and ultimately hyperestrogenicity conducive to tumor growth promotion (Figure 7). Thus, in addition to characterizing the transacting factors that exacerbate the phenomenon of 'promoter switching', i.e. LRH-1 and GATA-3, elucidating the epigenetic mechanisms underlying pI.3/II derepression, which are permissive for subsequent transactivation, is fundamentally crucial for development of BC prevention and treatment strategies.

Differential methylation of CpG dinucleotides is one of the best understood epigenetic modifications of DNA that partition the genome into active and inactive regions in different developmental stages or environments (Liu and Ward, 2010), and disruption of the epigenome is increasingly understood to play a significant role in cancer risk and malignant transformation (Chen and Xu, 2010). In addition to varying between tissues, aromatase expression also varies greatly between women, and recent evidence suggests the epigenetic landscape of *Cyp19* may in large part account for inter-individual variability in *Cyp19* promoter usage and attendant disparities in estrogen production (Demura and Bulun, 2008). Recent sequence and DNA methylation analyses reveal 6 CpG dinucleotides lie within a -571 bp fragment of *Cyp19* I.3/pII, and two of these lie within pII CRE (ibid). Demura and Bulun at Northwestern University recently discovered Cyp19 expression in skin fibroblasts derived from one of four healthy subjects was robustly cAMP-inducible, but cAMP-refractory- and as expected, glucocorticoid-

regulated cyp19 I.4-dominant- in fibroblasts derived from the other three volunteers. Subsequent DNA methylation analyses revealed CpGs in pII/I.3 were hypomethlyated in cAMP-inducible fibroblasts, critically CpG #5 in pII CRE. Moreover, Demura and Bulun demonstrated *in vitro* methylation of pII CRE disallowed CREB recruitment in subsequent gel shift assays, indicating hypomethylation of CpGs in pII is a requisite epigenetic determinant of robust aromatase induction by cAMP, and further that some women may be more or less susceptible to CYP19 induction by ATR. Revelations in the role alternate methylation patterns have in inter-individual disease susceptibility and promotion are only very recent; while much remains to be discovered in this exciting field, preliminary epidemiological and molecular studies indicate aging, environmental exposures, and dietary folate and other B vitamin intake may modulate the epigenome at the level of methylation (Chen and Xu, 2010).

Tonic Inter-cell Line Variability in ATR responsiveness as a Function of I.3/pII CpG Dinucleotide Methylation

Determining to what degree epigenetic changes owing to lifestyle factors are reversible is of great public health interest. Demura and Bulun's incidental 2008 discovery of inter-individual sensitivity to Cyp19 induction by cAMP in subject's buttock fibroblasts prompted Knower *et al* in 2010 to characterize the methylation status of *Cyp19* pI.3/II in the breast epithelial cell line MCF10, and most widely- studied model of ER+BC model- the MCF-7 cell line. Consistent with Bulun's report, Knower *et al* found the degree of CpG #5 methylation in pII CRE was lower in MCF-7 relative to MCF10A cells, and that pII-specific expression was similarly inversely correlated with CpG # 5 methylation, or far higher in MCF-7 versus MCF10A cells. Most relevant to the present study, the authors also demonstrated treatment with 5'-aza-dC resulted in a ~40-fold induction in of CYP19 expression, strongly indicating, indeed, pII-specific CYP19 expression in BC cells is tonically inhibited by methylation of certain CpG dinuceotides, and this inhibition is reversible in culture.

Regretfully or serendipitously, in the next chapter we provide further evidence for the reversibility of CpG dinucleotide methylation in pII CRE in BC cells in culture. Following ChIP of LRH-1 on *Cyp19* pII illustrated here in Figure 16, we attempted ChIPs of multiple other trans- and cis-factors in LBNL T-47D cells, but subsequent to marked changes in the morphology and dividing time of this subline, were unable to reproduce our original finings of robust CYP19 induction by ATR. This initially devastating occurrence of decreasing ATR responsiveness and possibly genomic instability with population doublings of the LBNL T-47D variant in culture prompted us to obtain T-47D cells direct from ATCC, henceforward termed 'ATCC T-47D cells'. Contrary to the LBNL T-47D variant characterized in the present chapter, in considerable detail, we illustrate in the next chapter ATCC T-47D cells are inherently poorly sensitive to ATR- but this finding also provided us a unique opportunity to demonstrate LRH-1 and

GATA-3 are indeed molecular determinants of CYP19 induction by ATR- in agreement with all the findings ofthe present chapter.

In summary, in light of current BC treatment strategies and the growing body of literature that suggests a high degree of inter-individual and inter-cell line variability exists for *cyp19* pII methylation, attendant cAMP-inducibility, and related lifetime estrogen exposure, our finding that brief exposure durations of physiologically relevant concentrations of ATR enhance expression of ER+BC hallmarks and LRH-1 pII recruitment has significant implications for risk of BC and other estrogen-related disorders associated with chronic low-dose drinking water, home, and occupational exposure.

Chapter II, Part A

LRH-1, GATA Factors, and Aromatase promoter hypomethylation sensitize human ER+ T-47D BC cells to aromatase induction by the herbicide atrazine: conserved molecular and epigenetic determinants of susceptibility with predictive and interpretive value in risk assessment

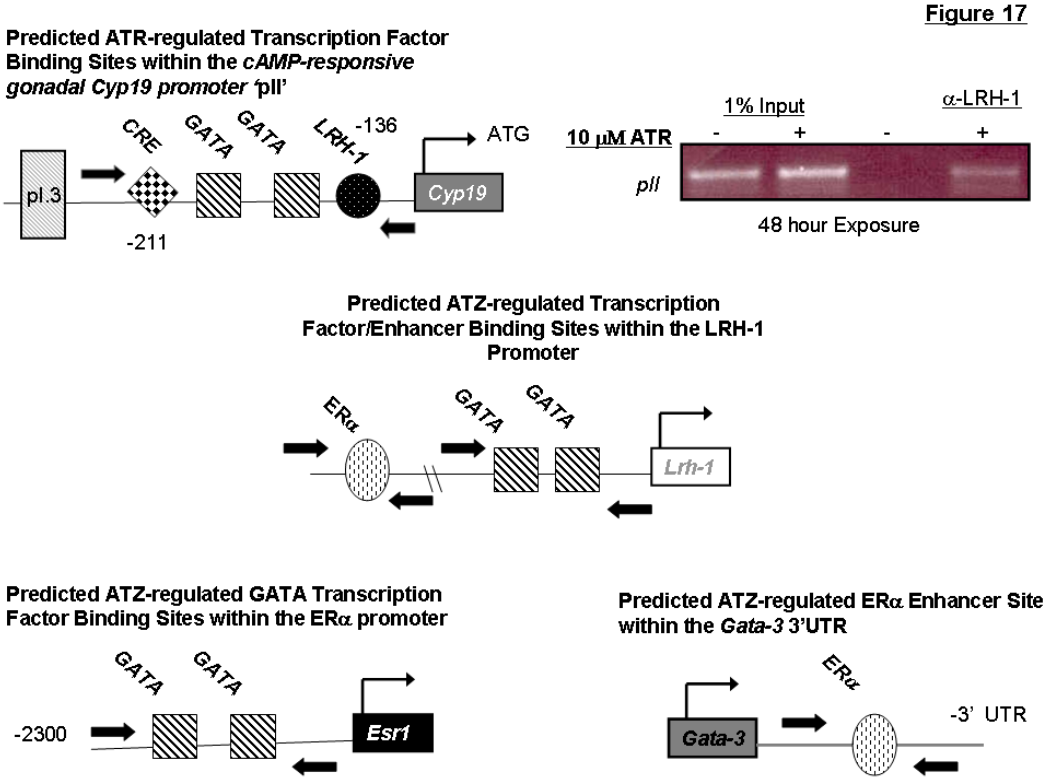
ABSTACT

The herbicide atrazine (ATR) is a widespread water contaminant with estrogenic effects in every vertebrate class studied to date. Atrazine induces aromatase expression through cAMP-mediated mechanisms in several human cell lines, and decreases the latency of mammary tumor incidence in ovary-intact Sprague Dawley but not Fisher 344 rats. In this study, we show sublines of the human T-47D ER+ BC cell line maintained by different laboratories have varied sensitivity to aromatase induction by ATR, possibly owing to epigenetic determinants, specifically varied methlyation status of CpG dinucleotides in the cAMP-responsive gonadal-type *Cyp19* promoter termed 'pII' . The magnitude of aromatase induction in each subline was correlated positively with expression of highly conserved GATA and LRH-1 transcription factors that coordinately regulate cAMP-responsive gonadal aromatase promoters termed promoters 'I.3' and 'pII' in addition to several other molecular hallmarks of ER+BC phenotype described in the previous chapter. Further, the magnitude of subline-specific responses may be inversely correlated with methylation status of CpG dinucleotides located in CREs of the aforementioned promoters, as aromatase mRNA levels were induced 5-fold by atrazine in a previously non-responsive T-47D subline following treatment with a DNA methylation inhibitor. Characterization of epigenetic and molecular determinants of ATR responsiveness may further our understanding of inter-strain, inter-species, and inter-laboratory variability and allow us better integrate the existing rat mammary gland tumor and molecular data into risk assessment models that predict inter-individual susceptibility and human risk.

INTRODUCTION

Following chromatin immunoprecipitation (ChIP) of LRH-1 on the gonadal cAMP-responsive *Cyp19* promoter 'pII' in LBNL T-47D cells described in the last chapter (Chapter 1, pictured here below), multiple other ChIPs were attempted to determine the effect of ATR on: GATA recruitment to *Cyp19*; GA recruitment to *Lrh-1*; and GATA and ERα recruitment to each oth own cognate regulatory cis-elements in LBNL T-47D cells.

Figure 17



ChIP-grade antibodies were pursued, gene-element-specific primers were designed, protocols and reagent lists were formatted for specificity and efficiency. Because the prior experiment with LRH-1 in LBNL T-47D cells indicated additional ChIP endeavors might provide mechanistic insight into the effects of ATR observed in a wide range of vertebrates and could further reveal novel implications for its influence on human estrogen-sensitive cancer risk, the researcher elected to apply for two related grants while ChIP reagents were being amassed and final exams were ongoing, and froze-down her stocks of LBNL T-47D cells (-80) for a period of two weeks to attend to grant writing and grading. Upon return to experimentation, a large number of cell vials were seeded in anticipation of the amount of starting chromatin required to perform ChIPs of multiple transcription factors on several genes. During this time the laboratory experienced several cycles of cell incubator failure and systemic contamination issues likely precipitated by transfer of seeded culture plates to distant working incubators, and as the researcher seeded many vials of cells in

anticipation of several ChIP experiments, she lost much of her LBNL T-47D stock this way. These unfortunate events at the time did not give the inexperienced researcher much pause however as many other T-47D subline stocks close in passage number were maintained and frozen by other members of the laboratory and were available for her use. Once the incubator was repaired and related contamination issues subsided, the remaining LBNL T-47D stocks in possession of the researcher were seeded for the purposes of amplifying and storing LBNL stock by freezing prior to further experimentation. However, upon seeding and passage, several differences in the behavior and morphology of the remaining stocks relative to that observed previously for LBNL T-47D cells became evident; these are described in the following section.

Firstly, after seeding, the remaining stocks LBNL T-47D stocks in possession of the researcher divided at a much faster rate than did T-47D cell stocks previously worked with or concurrently in use by other researchers. Secondly, the morphology of the more rapidly dividing LBNL stocks was more spindle-like and sparsely-oriented than the typically spherical and clustered arrangement of T-47D cells worked with prior to this point. For these reasons, the large number of visibly changed LBNL-stocks were again frozen down, and T-47D subline or variant stocks maintained by other researchers were seeded for the purposes of stock-building and undertaking the ChIP experiments described (Figure 17). The researcher obtained passage 13 and passage 15 (p13 and p15) T-47D cell stocks, respectively, from two other members of the laboratory, and with these (p13 and p15) sublines preliminary ChIP time-course experiments for ATR-induced target recruitment to the *Cyp19* and *Lrh-1* promoters were undertaken. In performing 12, 24, and 48 hour timecourse ChIP experiments in both new sublines however, CYP19 was not induced after 48 hours in the exposure experiments included in parallel as a positive technical control for ATR action. A time-course for CYP19 protein induction in these sublines was performed several times with newly made ATR derived from different commercial batches (lot numbers) from the manufacturer (Chem Service), however the robust induction of CYP19 protein levels previously observed in the LBNL T-47D variant was repeatedly found lacking. Upon recommendation of other researchers, T-47D sublines maintained by other laboratories were then obtained, amplified, and tested for sensitivity to CYP19 protein and upstream target induction after 48 hours ATR exposure.

<u>T-47D human breast cancer stocks routinely maintained by different laboratories have varied sensitivity to aromatase induction by the herbicide Atrazine (ATR)</u>

T-47D sublines obtained from five different campus laboratories, ranging from p12 to p18, in addition to T-47D cells ordered and shipped direct from the American Tissue Type Culture Collection (hereafter referred to as 'ATCC cells'; received as passage '1' but cultured through 10 doublings prior to experimentation), shared an indistinguishable morphology and doubling time at the same density consistent with that reported for T-47D cells in the ATCC

literature. However, the same ATCC subline (p10) and five laboratory T-47D variants, while close in passage number and grown to similar confluence, exhibited remarkably varied sensitivity to ATR after 48 hours exposure, and surprisingly, basal expression levels of ER+ breast cancer hallmarks, with GATA-3 and ERα overexpression tending to segregate together (Figure 18).

At the time of diagnosis approximately 50 to 70% of breast tumors are ERα positive (ER+) without amplification of the ERB2/HER2 gene (Shioda *et al*, 2009); relative to T-47D cells, the more extensively studied *in vitro* model of ERB2/HER2- breast cancer, the polyclonal MCF-7 cell line, is known to consist of highly heterogeneous cells that especially between laboratories differ remarkably in estrogen-responsiveness and other phenotypic and cytogenetic characteristics (Shioda *et al*, 2009; Jones *et al*, 2000). While gradient studies have revealed the MCF-7 line contains a fraction of stem cells capable of generating as many as six different subpopulations of clonal variants (Theillet *et al* 2003), the highly heterogenous nature of MCF-7 cells is rarely cited as a significant factor in estrogen-responsiveness or -resistance studies, and at the time of this writing, has not been thoroughly described for T-47D cells. Nevertheless, differences in sensitivity to ATR between ATCC and laboratory T-47D variants was repeatedly observed, and thus elucidating the molecular determinants of ATR sensitivity in variably-responsive T-47D sublines was set as a new objective for the purpose of gaining predictive insight into inter-laboratory model and inter-individual ATR susceptibility and risk, and is the focus of this chapter.

MATERIALS AND METHODS

Reagents

Atrazine (2-chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine) was purchased from Chemservice Inc. (Chester, PA). DMSO; H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride); forskolin; PDB *(*phorbol 12,13-dibutyrate*)*; 17β-estradiol (1,3,5-estratrien-3,17-β-diol), testosterone (17β-Hydroxy-3-oxo-4-androstene); 5'-aza-deoxycytosine were purchased from Sigma Chemical Company (St. Louis, MO). All chemicals purchased were of the highest quality available.

Plasmids

pc3-DNA-GATA3 was the kind gift of Dr. John Colligan (NIH/NIAID); pc3-CMV-LRH-1 was the kind gift of Drs. Donald McDonnell And Rachid Safi, Duke University Medical Center. Pgl2-*cyp19*-pII-luc was the generous gift of Dr. Sedar Bulun at Northwestern University. e*-GFP was kindly provided by Dr. Jen-Chywan Wang at UC Berkeley.

Cell Culture

'ATCC' T-47D cells were obtained from the American Type Culture Collection (Manassas, VA). T-47D cells were grown in Iscove's Modified Dulbecco's Media (IMDM) with 10 mM HEPES and 2 mM L-glutamine from BioWhittaker (Walkersville, MD), supplemented with 10% fetal bovine serum from Mediatech (Manassas, VA), 50 U/ml penicillin, and 50 U/ml streptomycin from Sigma (St. Louis, MO). 'Lab 1' and other sublines of T-47D cells were obtained from T-47D cell stocks maintained by different labs on campus.

SGBS human fibroblasts were the kind gift of Drs. Martin Wabitsch and Alexandra Killian, University of Ulm (Germany). SGBS cells were grown in Dulbecco's Modified Eagle Media/Nutrient Mixture F-12 (DMEM/F-12) supplemented with biotin (1.6×10^{-5} g/ml; Sigma #B-4639), pantothenate (8×10^{-6} g/ml; Sigma, #P-5155)), 10% calf serum (Mediatech, Manassas, VA), 50 U/ml penicillin, and 50 U/ml streptomycin (Sigma; St. Louis, MO).

ATCC T-47D subline transfections

'ATCC' T-47D cells grown to 60-70% confluence in 6-well plates were transfected with 3-6 mg/well of the indicated expression construct (CMV –lrh1, -gata3, or –pc3 (control) using Fugene 6 transfection reagent in a 12 μl Fugene6:2 μg DNA ratio according to the manufacturer's instructions. 'CMV-empty', 'CMV-lrh1', and 'CMV-gata-3' transgenics were all generated by transfection with 6 μg/well of the corresponding plasmid in full media lacking antibiotics; co-transfection of 'CMV–lrh1' and 'CMV–gata3' was performed using 3 μg/well of each of the corresponding plasmid as previous transfection efficiency

experiments with CMV-GFP indicated a 12 ul fugene:2 μg DNA ratio optimal for T-47D cell transfection and DNA in greater amounts would have warranted toxic doses of transfection reagent to maintain this efficiency/ratio. Twenty-four hours post-transfection cells were selected in full media containing 750 μg/ml G418 sulfate (Gibco, Carslbad) for several weeks for generation of stable pools.

Prior to luciferase assays, ATCC cells were stably transfected with empty-vector (pc3, 2 μg) or LRH-1 (2 μg); following a selection period, the resulting stable pools (CMV-pc3 or CMV-LRH-1) were transiently transfected with *cyp19*-I.3/pII-luc (1 μg) or Cyp19-I.3/pII-luc and GATA-3 (2 μg; the CMV-gata-3 plasmid is a constitutive expression construct lacking a eukaryotic selection/resistance gene so only transient transfections could be performed).

Exposure Procedures and Protocols.

Cells were grown to sub-confluence in a humidified chamber at 37^{o}C containing 5% CO_2. One, 10, and 30 μM stocks of ATR were prepared in DMSO and then diluted 1:1000 for delivery to serum-free cell culture media containing 0.1% BSA that was added to PBS-rinsed cells following a 24-hour serum starvation for the exposures described. Cyp19 transcription was found to be significantly affected by cell density (data not shown) and thus all SGBS cells were grown maximally to 60-70% confluence prior to the 24 hour serum-starvation preceding treatments described.

DNA methylation inhibition experiments with 5'aza-dC

To asses the combined effects of DNA methylation inhibition and ATR exposure in ATCC T-47D cells-prior to vehicle or ATR treatment, ATCC T-47D cells were grown to ~50% confluence in full media, which was replaced for 5 days there after with media containing vehicle or 100 μM 5-aza-dC. For the initial 3 days of exposure, cells were exposed to full media containing 5'-aza-dc or vehicle. On the fourth day, cells were exposed to serum-free media containing 5'-aza-dc or vehicle (24 hour serum-starvation). On the fifth day, cells were exposed to serum-free media containing 5'aza-dC or vehicle and ATR or the equivalent volume of vehicle.

Western Blotting

Following the indicated treatments, cells were washed and harvested in PBS and pelleted by centrifugation at 4,000 rpm. Cell pellets were lysed in radio-immunoprecipitation buffer (RIPA; purchased pre-made from Santa Cruz). Protein contained within each sample was quantified by the Lowry method. Once normalized for protein content, samples were mixed with a pH 6.8 gel loading

buffer containing 25% glycerol, 10% bromophenol blue, β-mercaptoethanol, 3.605% 0.5 M SDS, and electrophoretically fractionated on pre-cast 10% tris-glycine resolving gels (Biorad). Fractionated proteins were then transferred to PVDF membranes; following this, membranes were blocked for non-specific binding with 5% non-fat dry milk in TBST (10 mM Tris-HCL, pH 8, 150 mM NaCl, and 0.05% Tween). After blocking, blots were rinsed briefly and incubated overnight at 4^{o}C in antibodies diluted 1:500 to 1:2000 in TBST. GATA-3 (sc-268), and HSP90 (sc-7947) were purchased from Santa Cruz Biotechnology. Phospho-'p' p-44/42/ERK1/2 (cs-4377), p-Akt-S473 (cs-3787), antibodies were purchased from cs-9402), and Cyclin E (cs-2925) antibodies were purchased from Cell Signaling Technology. LRH-1 antibody was purchased from Abcam. CYP19 (A7981) and GAPDH antibody were purchased from Sigma. Immunoreactive proteins were detected by incubation with secondary horseradish peroxidase-conjugated antibodies diluted 3 x 10^{-4} in 1% NFDM in TBST. Blots were then treated with enhanced chemiluminescence reagents (Perkin Elmer, Wellesley, MA) and visualized on film (Eastman Kodak, Rochester, NY).

qRT-PCR (quantitative real-time PCR)

Total RNA from cultured cells was extracted with an Aurum RNA minikit (Biorad) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA with the iScript cDNA synthesis kit (Biorad, #170-8891) according to the manufacturer's instructions. qRT-PCR was performed using 2 ul of the cDNA product and the primer pairs described below with the iQ SYBR Green Supermix real-time PCR kit (Biorad, #170-8880) according to the manufacturer's instructions. All qPCR primer pairs were designed to span an exon-exon boundary and were subjected to melt-curve analysis (Applied Biosystems) to ensure single-product amplification prior to use in experiments. Data obtained from PCR reactions was analyzed using the comparative CT method. cDNA was amplified as detailed on an ABI with specific primers of the following sequences:

Lrh-1: Forward: 5'-GGCCCAAACTTATTCCTTCC -'3
Reverse: 5'-TGTCCCGTGTGTGGAGATAA- '3

Gata-3:
Forward: 5'- CTCATTAAGCCCAAGCGAAG -'3
Reverse: 5'-TTTTTCGGTTTCTGGTCTGG-'3

Gapdh:
Forward: 5'- TGAAGGTCGGAGTCAACGGATTTG -'3
Reverse: 5'- CATGTGGGCCATGAGGTCCACCAC -'3

Melting curve analyses revealed different primer pairs for Cyp19 performed differently in SGBS and T-47D cells for unknown reasons; therefore the following sets were used in related experiments for the indicated cell type,

Cyp19 primer pairs used for SGBS cell experiments:
Forward: 5' -ACCCTTCTGCGTCGTGTCA -'3
Reverse: 5'- TCTGTGGAAATCCTGCGTCTT -'3

Cyp19 primer pairs used for T-47D cell experiments:
Forward: 5' - CCCTTCTGCGTCGTGTCA -'3
Reverse: 5'- CTTTCGTCCAAAGGGATCCT -'3

Luciferase Assays

Cells grown to 60-70% confluence in 6-well plates were transfected with 2 μg/well of the indicated plasmid construct. Transfections were performed in serum-free media with Fugene6 from Roche (Nutley, NJ) according to the manufacturer's instructions. Cells were treated post-transfection for the indicated times in phenol red- and serum- free media in the presence or absence of vehicle (DMSO) or 30 μM ATR. Cells were then lysed in passive lysis buffer provided with the Promega Luciferase Assay Kit (Madison, WI) and relative luciferase activity was determined on a luminometer according to the manufacturer's protocol. Relative luciferase activities in each sample were normalized for protein content in each sample and reported as background- subtracted and the mean of triplicate treatments with standard error. The reproducibility of these findings was verified by three independent determinations of values for three replicates for each treatment and construct.

RESULTS

T-47D human breast cancer stocks routinely maintained by different laboratories have varied sensitivity to aromatase induction by the herbicide Atrazine (ATR)

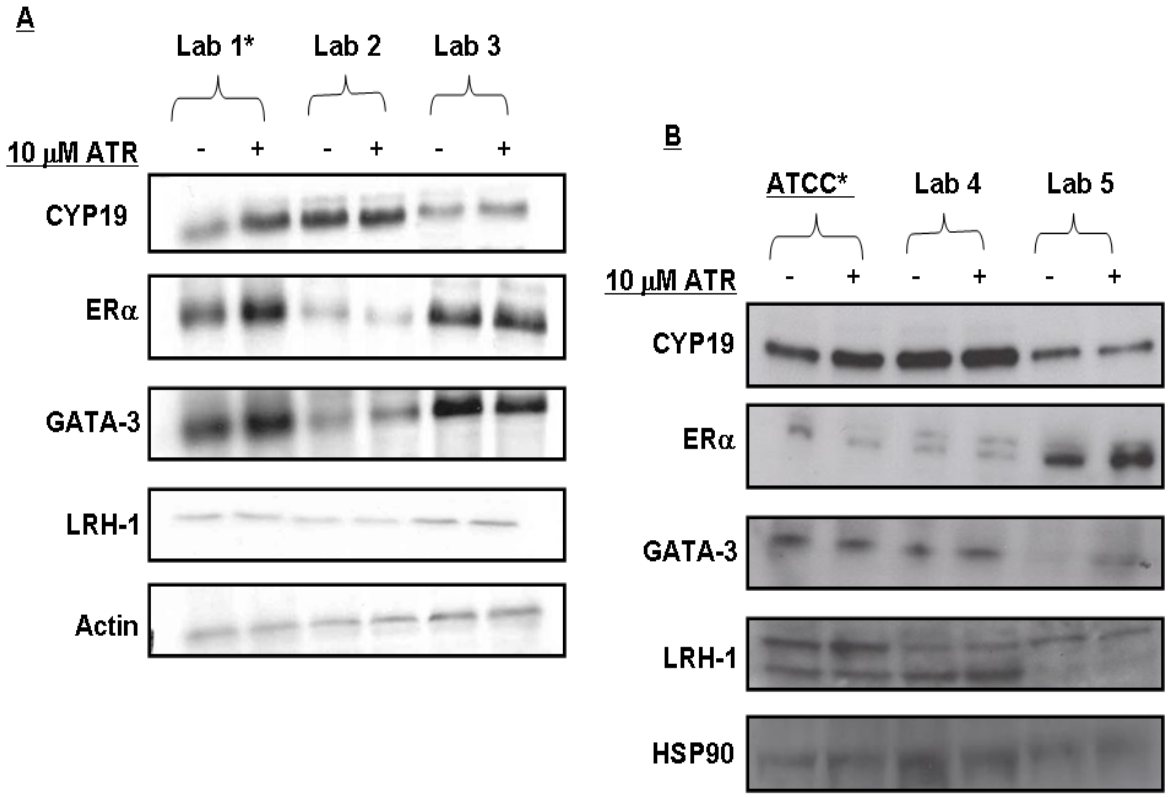
To initially determine whether the wealth of results produced in the last chapter from experimentation with the LBNL T-47D variant were reproducible in other T-47D sublines, T47D stocks from five different nearby laboratories were obtained and exposed to ATR as previously described, and levels of the indicated proteins were detected as before by western blot. When it became clear reportedly 'clonal' laboratory T-47D sublines were variably responsive to ATR, p1 T-47Ds were purchased and shipped direct from ATCC, and underwent ~ 10 passages only in the hands of the researcher prior to being compared against the five indicated laboratory T-47D sublines- which, according to laboratories from which they were obtained from- underwent between 12-18 passages before being handled by the author. As shown in Figure 18, when ATCC and laboratory sublines were compared for ATR-responsiveness against one another, the magnitude of CYP19 induction by ATR was greatest in 'Lab 1' T-47D cells, in which, as expected, ERα and GATA-3 were also expressed at relatively high levels and inducible by ATR. In all the newly obtained T-47D sublines, consistent with previous observations in the LBNL variant, basal LRH-1 expression was low. Inconsistent with the LBNL variant however, LRH-1 was not inducible in any subline tested; further, two bands, having molecular weights between 55 and 72 kD, were detected with the LRH-1 antibody and differentially expressed in ATCC cells and two of the five T-47D lab variants. Five isoforms of LRH-1 are described in the literature, and the two most prevalent in breast and colorectal cancer have molecular weights of 56 and 61 kD; it is also possible that the two bands observed in Lab 4, 5, and ATCC sublines correspond to phosphorylated and unphosphorlated versions of the same LRH-1 isoform. Regardless, unlike LBNL T-47D cells, absolute or isoform levels of LRH-1 were not correlated with GATA-3 or ERα expression levels in any subline tested- even in 'Lab 1' and ATCC cells- which, in the context of CYP19 induction- were respectively highly and poorly ATR-responsive (Figure 18).

Figure 18:

T-47D human breast cancer stocks routinely maintained by different laboratories have varied sensitivity to aromatase induction by the herbicide Atrazine (ATR). (A and B) T-47D cells purchased and shipped direct from ATCC and sublines maintained by and obtained from different laboratories have varied sensitivity to ATR after 24 hours exposure. (A) Basal and ATR-induced expression of the depicted targets varies in sublines maintained by three different laboratories, with ERα and GATA-3 protein expression levels segregating together, and CYP19 protein levels only robustly inducible in Lab 1 cells (p17), in which GATA-3 and ERα were also induced. (B) T-47D cells obtained direct from ATCC (p10) and from two other laboratories (p12 Lab 4; p18 Lab 5) have varied sensitivity to ATR. CYP19, but not LRH-1, GATA-3, or ERα, was modestly induced in ATCC T-47D breast cancer cells. All lab variants depicted were reportedly within five passages of one another, and while the ATCC subline underwent fewer passages (p10) than the highest (p18, Lab 5) lab variant at the time of experimentation, no trends in inducibility were correlated with the reported passage number. All exposures were performed for 24 hours in subconfluent cells grown to a similar density. Samples were normalized for protein content prior to fractionation by electrophoresis as described in the Materials and Methods. HSP90 and β-actin are included as loading controls. Similar findings were observed at 48 hours exposure (data not shown).

Figure 18

T-47D Breast Cancer Cell Stocks Maintained by Different Laboratories Have Varied Sensitivity to ATR

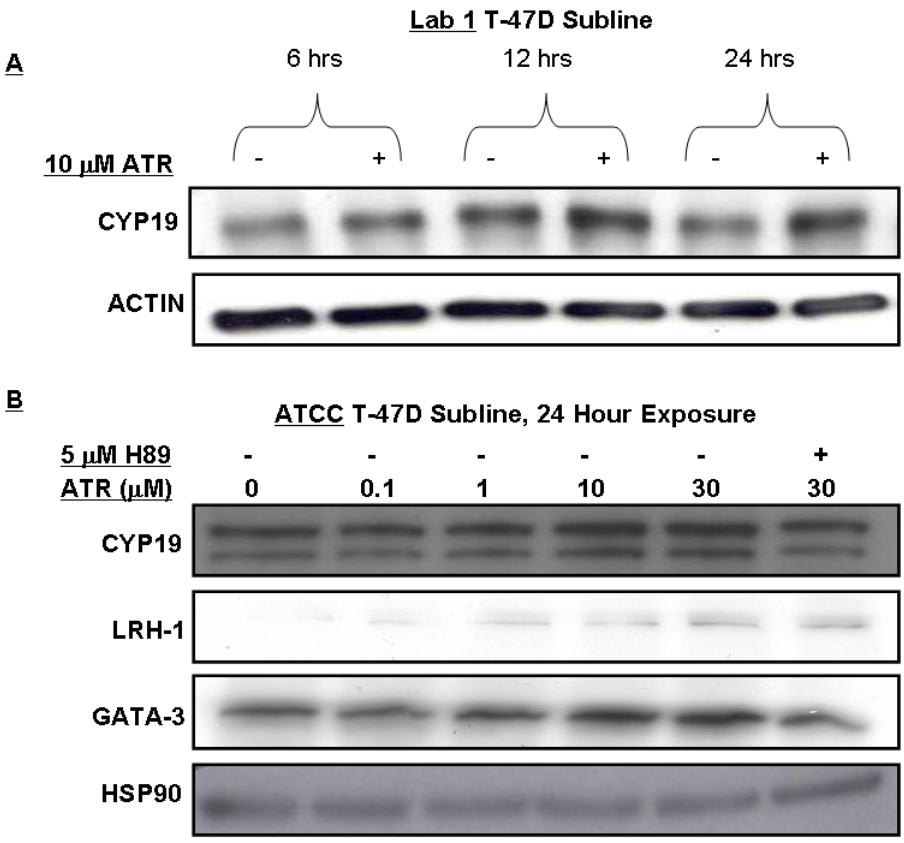


Because CYP19 protein levels appeared modestly inducible in ATCC T-47D cells exposed to 10 μM ATR for 24 hours (Figure 18)- absent concurrent GATA-3 and LRH-1 induction and 24 hours earlier than that previously observed for significant CYP19 induction in LBNL cells (Figure 10A, significant induction at 48 hours) , a time-course for CYP19 and expanded dose-response for CYP19 and upstream targets in the ATCC subline were performed. Additionally, because preliminary experiments in the ATCC subline indicated CYP19 induction with 10 μM ATR exposure was more modest than in LBNL and Lab 1 T-47D cells, the effects of a higher dose of ATR (30 μM ATR) alone or in combination with H89 (chemical PKA inhibitor) were also tested. As shown in Figure 19, after 24 hours exposure, CYP19 protein levels in ATCC T-47D cells were indeed dose-dependently but modestly inducible by ATR, and this effect was attenuated in cells pre-treated with 5 μM of the PKA inhibitor, H89. Consistent with LBNL cells, basal LRH-1 expression was low; in agreement with findings reported in sublines described in the previous experiment, LRH-1and GATA-3 were also not as robustly induced by ATR as they were in LBNL and Lab 1 T-47D cells, in which the magnitude of CYP19 induction by ATR was more pronounced.

Figure 19:

CYP19 is modestly but dose-dependently induced in ATCC T-47D cells by PKA-mediated mechanisms. To assess whether the modest CYP19 induction observed in ATCC cells at 24 hours absent concurrent LRH-1 AND GATA-3 induction was reproducible, a time-course and expanded 24 hour ATR dose-response were performed in ATCC T-47D cells, where levels of the indicated proteins were assessed by western blot. (A) CYP19 is induced in ATCC cells after 24 hours exposure to 10 μM ATR. (B) CYP19 is modestly but dose-dependently induced after 24 hours ATR exposure by PKA-mediated mechanisms in ATCC cells, though effects on GATA-3 and LRH-1 are less significant.

Figure 19


A
Lab 1 T-47D Subline
6 hrs
12 hrs
24 hrs
10 μM ATR
- + - + - +
CYP19
ACTIN
B
ATCC T-47D Subline, 24 Hour Exposure
5 μM H89 - - - - - +
ATR (μM) 0 0.1 1 10 30 30
CYP19
LRH-1
GATA-3
HSP90

Because CYP19 was only modestly induced in ATCC T-47D cells relative to the (Chapter 1) LBNL T-47D variant in which GATA-3 and LRH-1 were strongly expressed and inducible by ATR, the contributions of LRH-1 and GATA-3 in sensitizing ER+ T-47D cells to CYP19 induction by ATR were preliminarily explored in ectopic expression experiments in which constitutive LRH-1 and GATA-3 expression vectors were co-transfected with a gonadal *Cyp19* 'I.3/pII' promoter-reporter (*Cyp19*-I.3/pII-luc) construct in ATCC T-47D cells. The promoter region of the *Cyp19*-I.3/pII-luciferase construct spans -517 bp of *Cyp19*, and encompasses the cAMP-responsive 'pII' and I.3 gonadal *Cyp19* promoters. As described in the general introduction (Figure 6), the cAMP-responsive gonadal *Cyp19* promoters termed pII and I.3 are: only separated by 217 bp; coordinately regulated *in vitro* with cAMP stimulation; typically activated downstream of FSH in ovarian granulosa cells in the follicular phase of the menstrual cycle; and aberrantly and robustly activated in the majority of CYP19+ breast tumors and fibroblasts of tumor-proximal adipose stroma. *Cyp19*-I.3/pII-luc was the kind gift of Dr. Sedar Bulun at Northwestern University.

Additionally, because GATA-3 was highly induced by ATR in conjunction with CYP19 protein levels in the Lab 1 T-47D variant (Figure 18), *Cyp19* pII activity in 'wild-type' Lab 1 cells was assessed in parallel with *Cyp19* I.3/pII activity in transgenic ATCC cells co-transfected with *Cyp19*-I.3/pII-luc as described above.

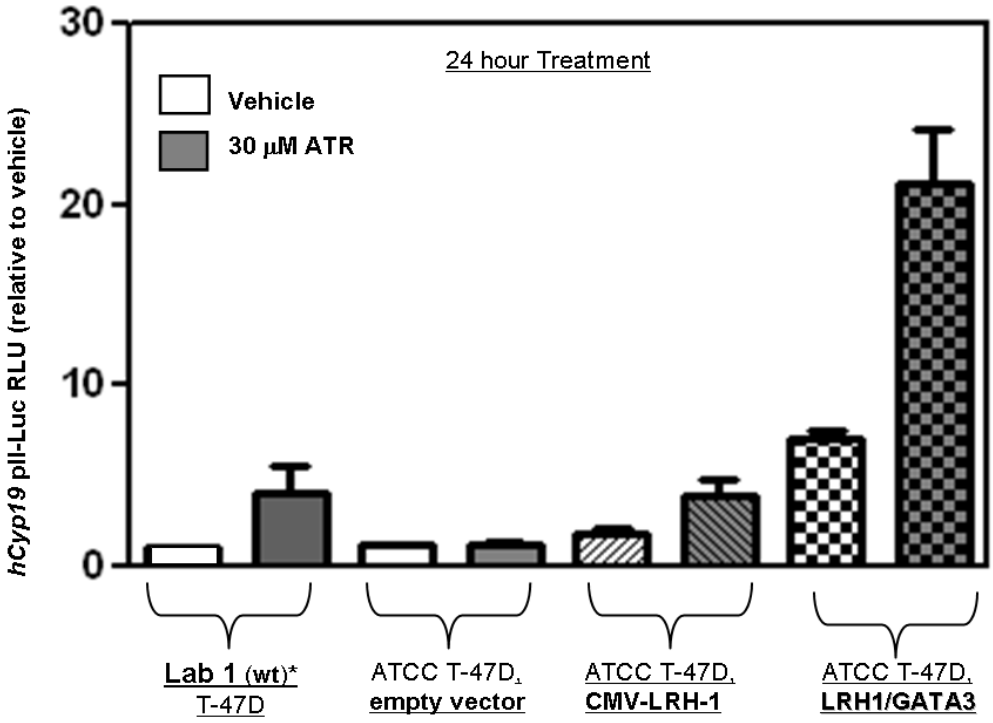
As shown in Figure 20, *Cyp19* I.3/pII promoter activity was increased > 5 fold by 30 μM ATR in 'wild-type' (wt) Lab 1 cells- 'wild-type' here indicating Lab 1 cells transfected with *Cyp19*-I.3/pII-luc but not LRH-1 or GATA-3. In contrast, in ATCC cells transfected only with a control (empty) vector, or transgenetically analogous to 'wild-type' Lab 1 cells, *Cyp19* I.3/pII promoter activity was not increased by ATR- indicating there exist innate differences *Cyp19* transfactor expression and ATR-sensitivity between the Lab 1 and ATCC T-47D variants. Further, transfection of ATCC cells with constitutive LRH-1 increased sensitivity to *Cyp19* I.3/pII promoter induction by ATR (fold relative to vehicle-treated empty-vector transfected), and transfection of ATCC cells with both GATA-3 and LRH-1 resulted in increased basal () and ATR-stimulated *Cyp19*-I.3/pII-luc activity (), with a magnitude far exceeding that in wt Lab 1 cells and consistent with the literature (Bouchard *et al*, 2005). These findings indicate GATA-3 and LRH-1 are indeed molecular determinants of *Cyp19* promoter I.3/pII-inducibility by ATR in human ER+ T-47D BC cells.

Figure 20:

GATA-3 and LRH-1 are molecular determinants of ATR *Cyp19* promoter I.3/pII-inducibility in human ER+ T-47D breast cancer cells. Lab 1 cells, in which GATA-3 is inducible by ATR and basally expressed at higher levels than in ATCC T-47D cells, are susceptible to *Cyp19* promoter I.3/pII induction by ATR as demonstrated in 'wild-type' (wt) Lab 1 cells transfected with *Cyp19*-I.3/pII-luc and treated with vehicle or 30 μM ATR for 24 hours (> 5-fold induction w/ATR). In contrast, transgenetically analogous ATCC cells transfected only with a control ('empty', pc3) vector and the *Cyp19*-I.3/pII-luc reporter construct are not susceptible to *Cyp19* promoter I.3/pII induction by ATR. LRH-1 transfection modestly sensitizes ATCC cells to *Cyp19* I.3/pII induction by ATR. Consistent with the literature, co-transfection of GATA-3 and LRH-1 increases basal *Cyp19* promoter I.3/pII activity in ATCC T-47D cells, and as we show for the first time, greatly enhances *Cyp19* I.3/pII induction by ATR in ATCC T-47D cells. Prior to this experiment, transgenic ATCC sublines were generated with the indicated expression constructs as described in the Materials and Methods. In preparation for luciferase studies, all sublines depicted were transfected with 2 μg h*Cyp19*-I.3/pII-luc for 24 hours prior to exposure to vehicle (DMSO) or 30 μM ATR for 24 hours. Following the treatment period, relative luciferase activity was assessed in cell lysates with a luciferase assay kit (Promega) according to the manufacturer's instructions and as detailed in the Materials and Methods. Transfection efficiency and background fluorescence were respectively validated and corrected for via transfections with CMV-luc (constitutive) and pgL2 constructs (data not shown). RLU (relative light units) are normalized to protein content and expressed relative to vehicle and are the mean of three replicates with standard error. Similar results were obtained in two independent experiments.

Figure 20

Effect of Transgene Expression *Cyp19*-I.3/pII Activity in T-47D Cells from ATCC



ATR is a known PDE inhibitor; we previously demonstrated CYP19 protein induction by ATR is influenced by PKA in T-47D cells (Figures 9). Further, forskolin or constitutive PKA greatly enhance pII-driven *Cyp19* transcription in MCF-7 cells co-transfected with LRH-1 and GATA-3/4, and a recent study published by Ingraham and Suzawa (2008) demonstrated ATR upregulates *Cyp19* pII-reporter activity in SF-1-transfected human placental carcinoma (JEG3) cells by PI3K-mediated mechanisms. In the present study, to preliminarily assess whether PI3K and PKA are additional determinants of *Cyp19* inducibility by ATR in T-47D cells, ATCC cells co-transfected with GATA-3, LRH-1, and h*Cyp19*-I.3/pII-luc as described in the previous experiment were exposed to vehicle or 30 μM ATR for 24 hours 30 minutes after being pre-treated with vehicle, the PKA inhibitor H89, or the PI3K inhibitor LY294002 (hereafter indicated as 'LY' in figure legends). As shown in Figure 21 (follows with Figure 22), our inhibitor studies indicate PKA and PI3K are indeed key participants in GATA-3 and LRH-1 sensitization of T-47D breast cancer cells to *Cyp19* promoter I.3/pII induction by the herbicide ATR.

As the previous two experiments indicated ectopic expression of GATA-3 and LRH-1 greatly enhanced ATR-induction of *Cyp19* promoter I.3/pII activity (> 15-fold) in ATCC T-47D cells, logically, we next tested whether ectopic expression of these transcription factors also enhanced ATR induction of endogenous Cyp19 mRNA levels in transfected ATCC T-47D cells. Surprisingly, as shown in Figure 22, multiple quantitative PCR experiments revealed ectopic GATA-3 and LRH-1 expression did not sensitive ATCC T-47D cells to endogenous Cyp19 mRNA induction by ATR- which was initially difficult to reconcile with the previous finding of marked exogenous *Cyp19*-I.3/pII promoter induction seen in similarly transfected and treated ATCC cells (Figure 20, 21), and further with the modest increase in CYP19 protein by ATR in wild-type (no transgene expression) ATCC T-47D cells (Figure 19). Similar ATR exposures in highly responsive H295R cells (Figure 24) indicated lack of Cyp19 mRNA induction in ATCC cells was not attributable to compromised efficacy of our on-hand ATR lot or stocks, and transfection efficiency studies with green fluorescent protein, western blots for GATA-3, and qPCR of LRH-1expression in transfected ATCC cells similarly indicated that poor transfection efficiency was also not responsible for ATR-unresponsiveness in transfected ATCC T-47D cells (Figure 24).

Figure 21:

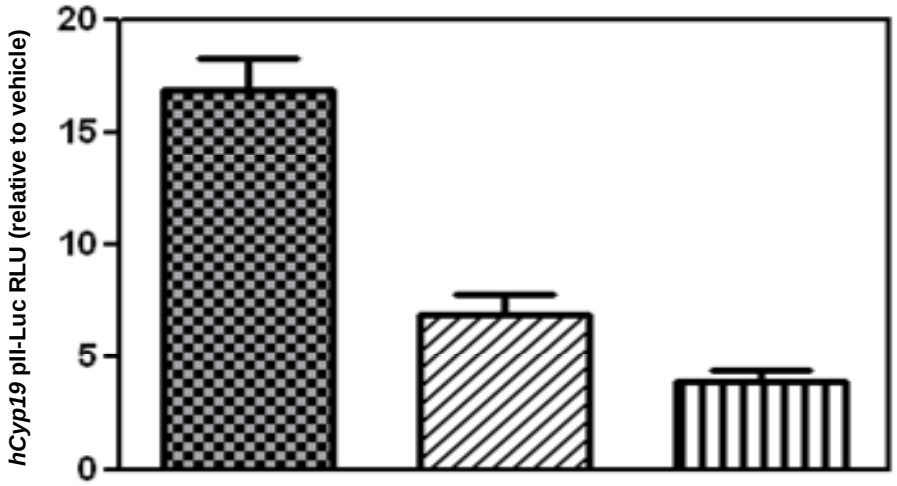
ATR robustly upregulates *Cyp19* I.3/pII promoter activity in GATA-3 and LRH-1- transfected ATCC T-47D cells by PI3K and PKA-mediated mechanisms. ATCC T-47D cells were transfected with LRH-1, GATA-3, and h*Cyp19*-I.3/pII-luc as described in the Materials and Methods. Following transfection, cells were treated with vehicle (DMSO) or ATR for 24 hours 30 minutes after being pre-treated with vehicle or the indicated amounts of PKA or PI3K (LY294002, indicated as 'LY') inhibitors. RLU (relative light units) are normalized to protein content and expressed relative to vehicle and are the mean of three replicates with standard error. Similar results were obtained in two independent experiments.

Figure 22:

Endogenous Cyp19 mRNA are not inducible by ATR in LRH-1 and GATA-3-transfected ATCC T-47D cells. ATCC T-47D cells were transfected with constitutive LRH-1 and GATA-3 expression vectors, and following transfection, cells were treated with vehicle (DMSO), 1 or 10 μM ATR for 24 hours as described in the previous Cyp19 pII-reporter experiment (Figure 21). Following treatment endogenous Cyp19 mRNA levels were quantified by qPCR with primers for total Cyp19 as described in the Materials and Methods. Surprisingly, contrary to our observations in the prior ectopic pII-reporter study (Figure 21), transfection of GATA-3 and LRH-1 did not confer endogenous Cyp19 mRNA inducibility to ATCC T-47D cells. Similar results were obtained in more independent experiments than the researcher can bear to admit.

Figure 21

h*Cyp19*-pII Luciferase Activity in CMV-LRH1/GATA3 ATCC T-47D Cells

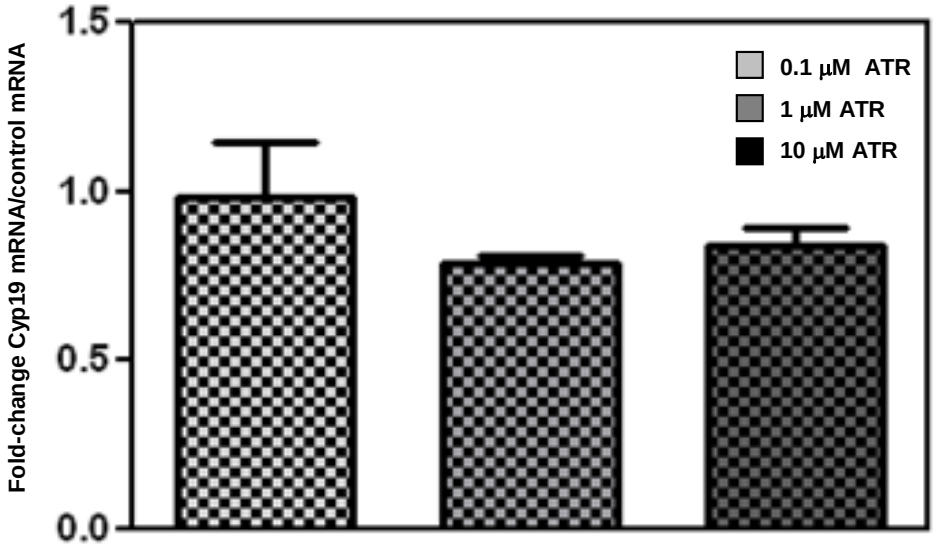


24 hour Treatment:

ATR (30 μM)	+	-	+
H89 (20 μM)	-	+	-
LY (5 μM)	-	-	+

Figure 22

Endogenous Cyp19 Transcript Levels in CMV-LRH1/GATA3 ATCC T-47D Cells



24 hour Treatment

Figure 23:

Poor transfection efficiency is not responsible for lack of Cyp19 mRNA induction in ATR-treated GATA-3 and LRH-1-transfected cells. Transfection efficiency in ATCC T-47D cells was tested with the indicated ratios of transfection reagent (Fugene 6 and Fugene HD) to DNA (GFP) as detailed in the Materials and Methods. A constitutive green fluorescent protein expression vector was used to determine optimal transfection reagent (μL): expression vector (μg DNA) ratios. Based on our observations represented in this figure, a ratio of 12:2 (reagent:DNA) with Fugene 6 was used for all transfection studies, including exogenous *Cyp19* pII-reporter studies (Figure 21) and endogenous Cyp19 mRNA studies (Figure 22). Because cells are transfected for 24 hours prior to 24 hour ATR treatment, we estimate transfection efficiency at 48 hours with the 12:2 reagent:DNA ratio to be between 15-20% (bottom panel).

Figure 24:

Neither poor transfection efficiency (panels A and B), nor ATR stock or Cyp19 qPCR protocol efficacy (panel C) are responsible for an absence of Cyp19 mRNA induction in constitutive GATA-3 and LRH-1- transfected ATCC cells. To verify lack of Cyp19 mRNA induction in transfected ATCC T-47D cells was not merely attributable to poor transfection efficiency or activity loss of on-hand ATR stocks, GATA-3 (A) and LRH-1 (B) expression in transfected ATCC-T47D cells were assessed relative to empty-vector-transfected cells by western blot (A) and qPCR (B) respectively, and endogenous Cyp19 mRNA inducibility by ATR in H295R human adrenal cells (C) was tested to verify both efficacy of our Cyp19 primers/qPCR protocol and prepared ATR stocks.

Figure 23

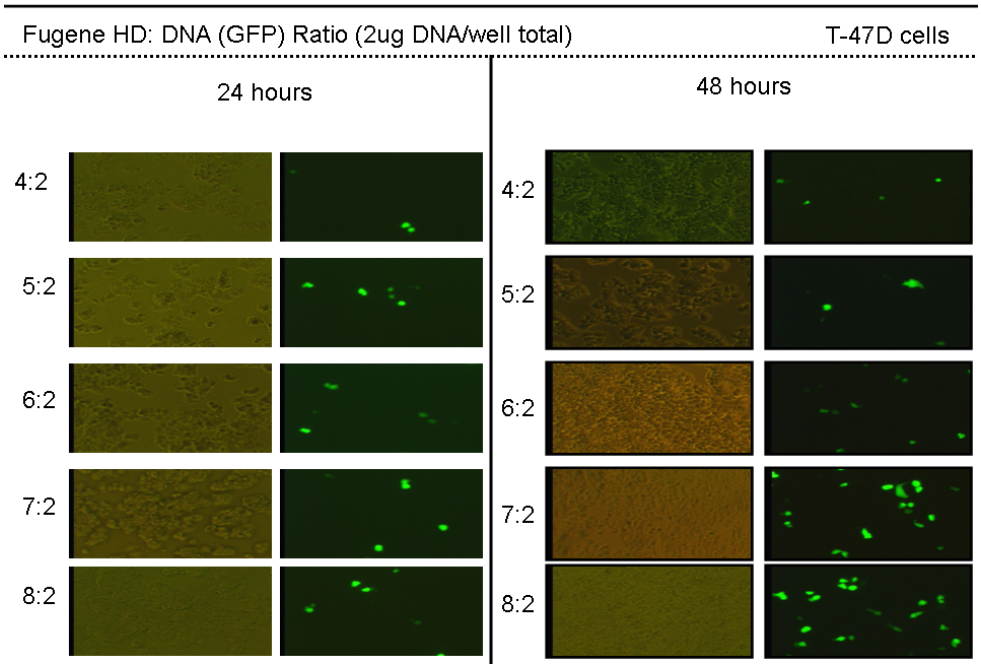
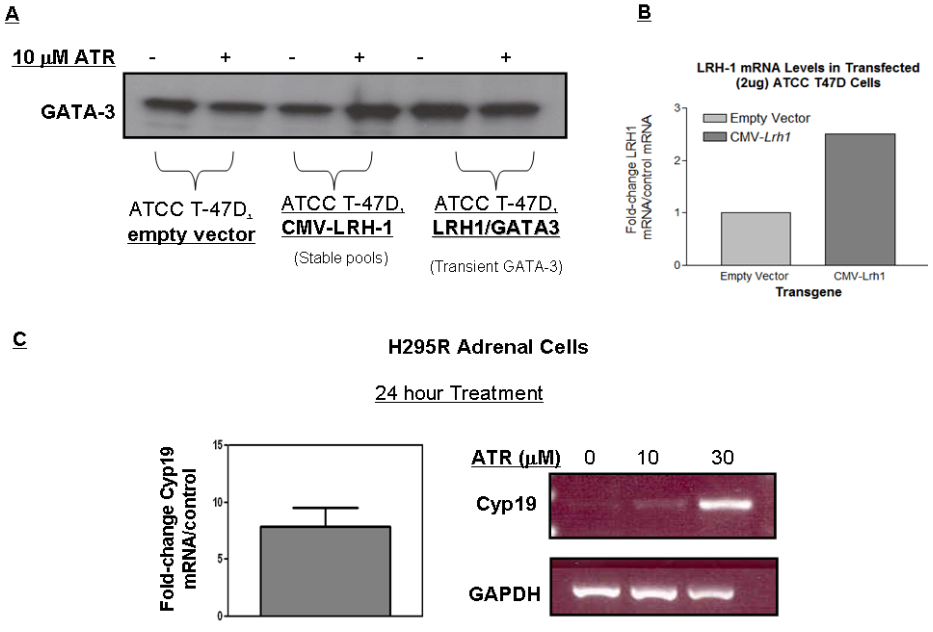


Figure 24

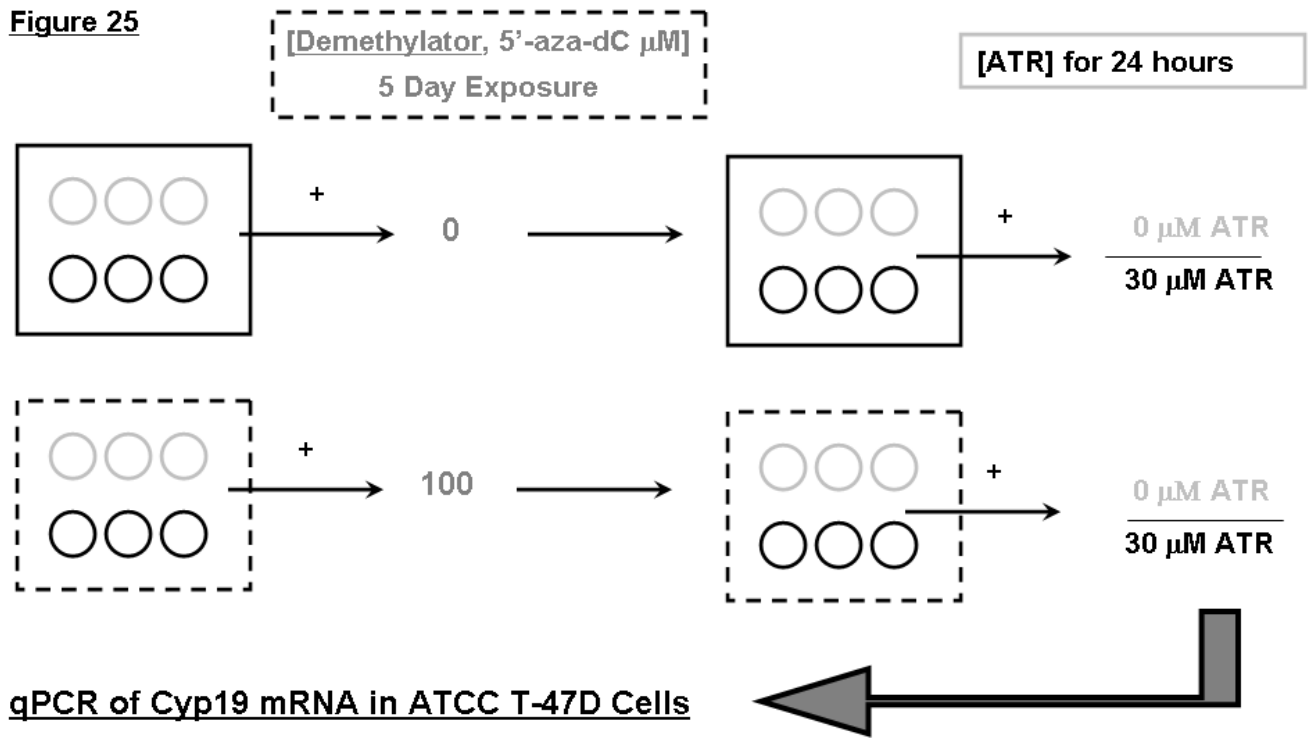


Attempts to reconcile strong *Cyp19* pII-reporter inducibility with an absence of endogenous Cyp19 mRNA induction in ATCC T-47D cells, in light of our previous experience with the LBNL T-47D variant- which changed from highly responsive to irresponsive in culture coincident with ostensible loss of typical mitotic controls- led us to a recent report of variability in cAMP-responsiveness owing to inter-individual epigenetic modifications of *Cyp19* in skin fibroblasts between four healthy female volunteers (Demara and Bulun, 2007). Specifically, Demura and Bulun showed four of six CpG dinucleotides in *Cyp19* pII, crucially including two located in pII CRE, were unmethylated in fibroblasts derived from one subject in which *Cyp19* transcription was cAMP-inducible. Conversely, in fibroblasts derived from the other three subjects in which Cyp19 expression was cAMP-refractory, all six CpG dinucleotides in pII of *Cyp19* were methylated, which additionally disallowed CREB recruitment to pII following cAMP stimulation in further *in vitro* EMSA studies in non-responsive fibroblasts (CpG dinucleotides in Cyp19 pII depicted in Figure 3). More recently, Demura and Bulun's 2007 finding of inter-individual variability in fibroblast *Cyp19* cAMP-transactivation owing to CpG dinucleotide methylation led *Knower et al* in 2010 to the discovery that inhibition of DNA methylation with the non-specific 5'-aza-2'-deoxycytosine (5'-aza-dC) conferred robust *Cyp19* cAMP-responsiveness to formerly poorly-responsive breast adipose fibroblasts (BAFs) and MCF-7 breast cancer cells. Taken together, these reports led us to the hypothesis that 5'-aza-dC treatment might confer ATR-responsiveness to treated ATCC T-47D cells. Thus, to preliminary interrogate this hypothesis, ATCC-T47D cells were treated with vehicle or 100 μM 5'-aza-dC for a total of 5 days as described in Bulun's report (2007) and detailed in the Materials and Methods, and for the last 24 hours of the exposure were treated with vehicle or 30 μM ATR for 24 hours in serum-free media following a 24 hour serum-starvation period (during these last 48 hours cells were also exposed to vehicle or 5'-aza-dC in serum-free media). Cells were harvested at the end of the treatment period and Lrh-1, Gata-3, and (total) Cyp19 mRNA levels were assessed by qPCR as described in the Materials and Methods; a schematic of this exposure protocol is provided in Figure 25.

Figure 25:

Schematic of Exposure protocol to Inhibit DNA methylation with non-specific inhibitor 5'-aza-dC in ATCC T-47D Cells. In experiments to determine whether Cyp19 promoter methylation is an epigenetic determinant of CYP19 inducibility by ATR in human breast cancer cells, DNA methylation was inhibited via 5-day 5'aza-dC exposure in ATCC T-47D cells as depicted. Two six-well plates were seeded with T-47D cells; one was treated with vehicle for a total of 5 days; the other was treated with the methylation inhibitor 5'-aza-Dc. During the last 48 hours of the exposure, full media in both plates was replaced with serum-free media. During the last 24 hours of the treatment, three wells (replicates) of the 5 day vehicle- and 5'aza-dC plates were treated with either vehicle or ATR in serum free media. *Cyp19* is anticipated only to be induced by ATR in T-47D cells in which DNA (promoter) methylation was previously inhibited with 5'aza-dC.

Figure 25



Anticipated Results if CpG dinucleotides in pII CRE are methylated in T-47D cells obtained from ATCC:

ATCC T47D cells + 5-day Vehicle Exposure		vs.	ATCC T47D cells + 5-day 5'-aza-Dc Exposure	
DMSO	**ATR**		**DMSO**	**ATR**
-	-		-	+

As shown in panel A of Figure 26, Gata-3 and Lrh-1 inducibility were not affected by pre-treatment with the methylation inhibitor 5'-aza-dC. At the same time, while Cyp19 mRNA levels were not induced by ATR in ATCC T-47D cells exposed to vehicle for five days, 5'-aza-dC pre-treatment resulted in a 4.4-fold increase in Cyp19 mRNA levels in ATR-treated cells relative to vehicle (Figure 26, B). These findings were only reproduced once before our qPCR thermocycler failed permanently and was replaced months later with a new instrument requiring new software and chemistry both temporally and financially prohibitive for replicate experiments. Howvever preliminary, taken together with previous reports indicating CpG dinucleotide methylation within pII of *Cyp19*, especially within the two CpGs located within pII CRE, is highly variable between cell lines and female subjects and further inversely correlated with cAMP transcription-level inducibility, our findings implicate *Cyp19* pII methylation status as an epigenetic determinant of ATR sensitivity in human breast cancer cells. Further, in light of recent revelations indicating gene expression regulation by methylation in cancer cells is highly dynamic and often progressively misregulated in the process of transformation, our findings in ATCC cells may provide an epigenetic explanation for the change in sensitivity of LBNL T-47D cells in culture (Chapter I) and differences in *Cyp19*-induciblity by ATR we detected in T-47D sublines maintained by different laboratories (Figure 18).

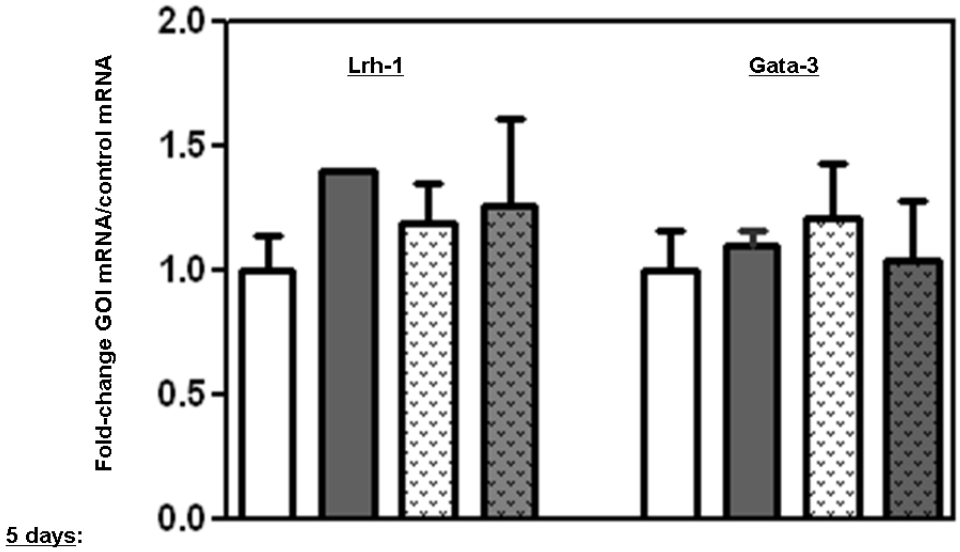
Figure 26:

Pre-treatment of ATCC T-47D cells with the non-specific methylation inhibitor 5'-aza-dC does not affect Gata-3 nor Lrh-1 mRNA-level inducibility (A) but sensitizes cells to Cyp19 mRNA induction by ATR (B). ATCC T-47D cells were exposed to either vehicle or 5'-aza-dC for a total of five days and vehicle or 30 μM ATR in serum-free media for last 24 hours of this period following 24-hour serum starvation as detailed in the Materials and Methods and depicted in Figure 25. (A) 5-aza-dC pre-treatment does not affect Lrh-1 or Gata-3 mRNA levels in vehicle nor ATR-treated cells as assessed by qPCR. (B) Pre-treatment of ATCC T-47D cells with the non-specific methylation inhibitor 5'-aza-dC results in a 4.4-fold increase in Cyp19 mRNA levels in ATR-exposed relative to vehicle-exposed (and methylation-inhibited cells). Results are representative of findings reproduced in two independent experiments conducted before our thermocycler broke for several months, are normalized to Gus mRNA levels (housekeeping gene), and are the mean of three replicates with standard error.

Figure 26

A

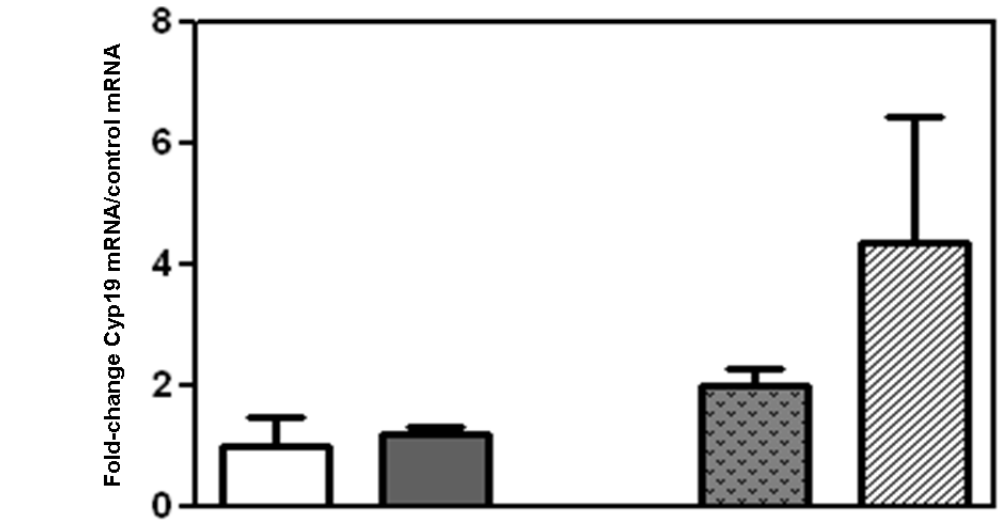
Lrh-1 and Gata-3 Transcript Levels in ATCC T-47D Cells -/+ Demethylating Agent



5 days:								
5'aza-dC (100 μM)	-	-	+	+	-	-	+	+
Last 24 hours:								
30 ATR (30 μM)	-	-	+	+	-	-	+	+

B

Cyp19 Transcript Levels in ATCC T-47D Cells -/+ Demethylating Agent



5 days:				
5'aza-dC (100 μM)	-	-	+	+
Last 24 hours:				
30 ATR (30 μM)	-	+	-	+

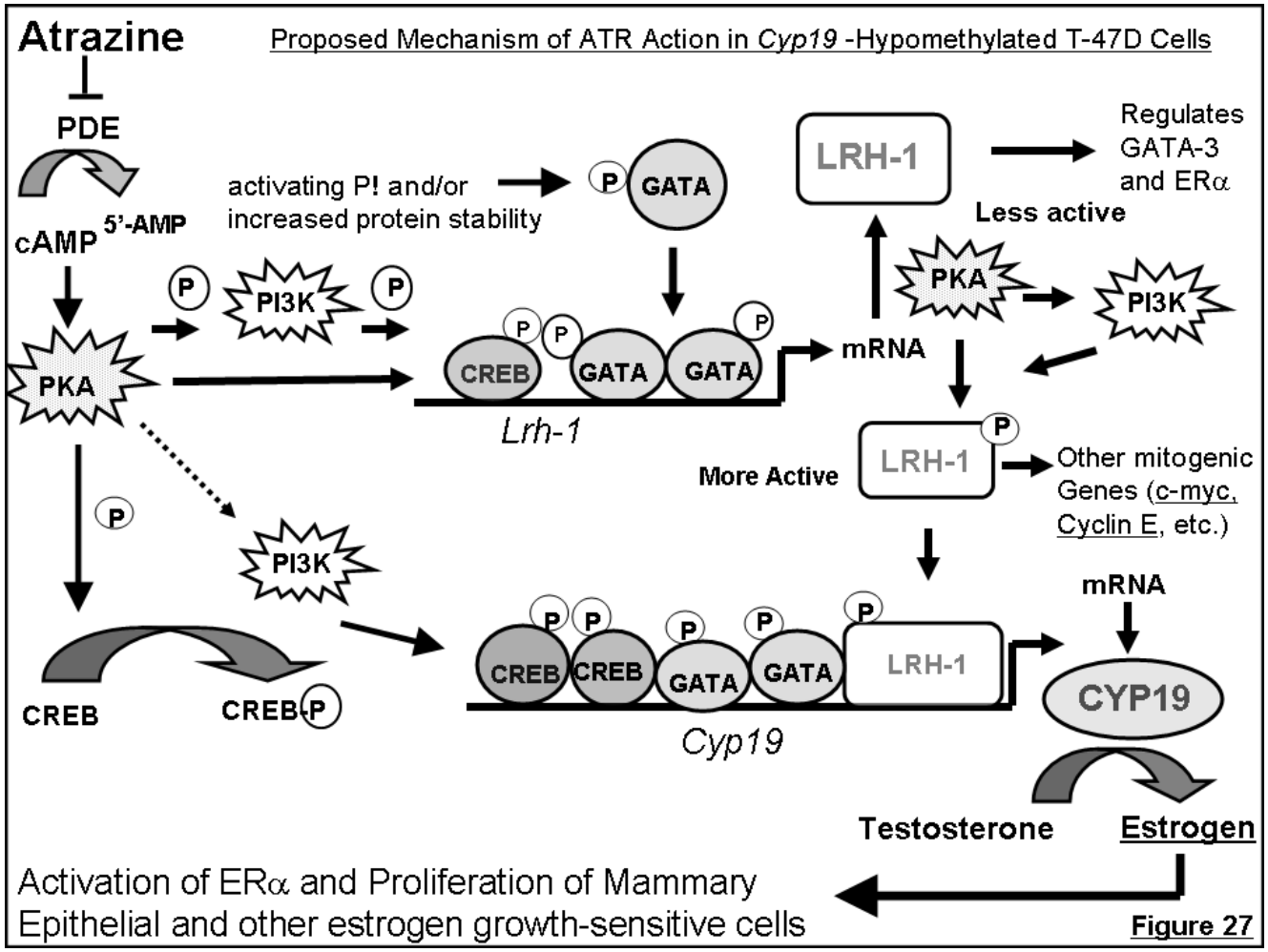
DISCUSSION

Our findings in the LBNL T-47D variant (Chapter 1) taken together with those in laboratory-specific sublines and ATCC T-47D cells presented here strongly implicate *cyp19* pII hypomethylation and GATA-3 and LRH-1 expression as epigenetic and molecular determinants, respectively, of aromatase inducibility by ATR. In addition to GATA-3, GATA-4 was robustly induced in the LBNL variant described in the previous chapter, and although we did not have an expression vector for GATA-4 on hand, this transcription factor binds the same cis-elements as GATA-3 in BC cells, is similarly modulated by upstream kinases, is upregulated in endometriosis and ovarian disorders (Xu *et al*, 2003; Stocco *et al*, 2007; Bouchard *et al,* 2005), and may be important in predicting ATR susceptibility in tissues in which GATA-3 is absent or less abundant. Contrary to LBNL T47D cells, we were not able to reproducibly detect GATA-4 in ATCC T-47Ds here or in rat granulosa cells described in the next chapter- which is unfortunately consistent with numerous reports indicating GATA-4 expression is rapidly lost in human breast and ovarian cancer cells in culture (Xu *et al*, 2003). Yet, as we here for the first time report in addition to NR5A members, ATR affects GATA -3/4 activity and protein levels in certain T-47D sublines, constitutive expression vectors for each transcription factor may be useful in further mechanistic studies our findings should prompt.

Our results suggest ATR regulates aromatase through LRH-1, GATA-4, and GATA-3 in BC cells, and additional *Cyp19* pII-reporter studies performed with the kinase inhibitors H89 and LY492002 ('LY', Figure 21) indicate ATR activates *cyp19* pII-driven transcription through PKA- and PI3K-mediated mechanisms in T-47D cells, consistent with promoter studies in JEG3 cells published by Suzawa and Ingraham (2008). Beyond aromatase, PKA and PI3K are involved in regulation of many steroidogenic enzymes, which they in part affect at the transcriptional level by phosporylating LRH-1 and/or its homolog, SF-1- permitting enhanced interaction with GATA (3 or 4) and CREB at conserved regulatory motifs in many steroidogenic genes. Although the present study focused solely on CYP19, our finding that brief durations of low doses of ATR affect these highly conserved upstream effectors of steroidogenesis in human BC cells has strong implications for ATR's role in human and wildlife developmental and hormone-related disorders- supported by the fact that ATR elicits potent endocrine-disrupting effects in every vertebrate class studied to date (Laws *et al*, 2003). A schematic of the mechanisms by which we propose ATR affects CYP19 in cells of the human breast is provided in Figure 27.

Figure 27:

Proposed mechanisms by which ATR may induce CYP19 in pII-hypomethylated and GATA-3- and LRH-1- expressing cells of the human breast. In addition to *Cyp19* pII hypomethlation as an epigenetic risk factor of ATR sensitivity, our findings presented here and in the previous chapter implicate the highly conserved steroidogenic factors, PKA, PI3K, GATA-3, and LRH-1 as molecular determinants of CYP19-inducibility by ATR in ER+ T-47D BC cells. ATR inhibits PDE, elevating intracellular cAMP levels and enhancing PKA activity. ATR upregulates PI3K activity by incompletely described mechanisms, however PKA is known to activate PI3K downstream of FSH in granulosa cells. Enhanced PKA and PI3K activity may increase the transcriptional activity of LRH-1 and/or its interaction with GATA factors and CREB at pII of *Cyp19* in cells in which one or both CpG dinucleotides located in CRE of pII are hypomethylated.


Atrazine
Proposed Mechanism of ATR Action in Cyp19 -Hypomethylated T-47D Cells
PDE
cAMP
5'-AMP
activating P! and/or
increased protein stability
P
GATA
LRH-1
Regulates
GATA-3
and ERα
Less active
PI3K
PKA
CREB
GATA
mRNA
Lrh-1
More Active
Other mitogenic
Genes (c-myc,
Cyclin E, etc.)
CREB
CREB-P
Cyp19
CYP19
Testosterone
Estrogen
Activation of ERα and Proliferation of Mammary
Epithelial and other estrogen growth-sensitive cells
Figure 27

We here show for the first time T-47D stocks maintained by different laboratories are variably sensitive to ATR (Figure 18). As expected in light of our discoveries in the LBNL T-47D variant characterized in the last chapter, 'Lab 1' T-47D cells, which were inherently highly responsive to CYP19 induction by ATR, were also innately GATA-3- and LRH-1 ATR-inducible. Similarly, ectopic GATA-3 and LRH-1 expression conferred ATR-responsiveness to formerly insensitive *Cyp19*-pII-luc-transfected ATCC T-47D cells, further underscoring the importance of elucidating GATA-3 and LRH-1 expression and activity in predicting mode; susceptibility to *Cyp19* induction by ATR.

Our incidental and initially surprising discovery of inter-laboratory variability in ATR-responsiveness in 'clonal' T-47D cells is in actuality quite consistent with comprehensive but infrequently cited literature in which a high degree of phenotypic and cytogenetic heterogeneity in inter- and intra-tumoral cells and MCF-7 BC cells derived both from a single stem cell progenitor (Shioda *et al*, 2009) and different laboratory stocks (Theillet *et al*, 2003; Jones *et al*, 2000) is characterized. Moreover, our finding that DNA methylation inhibition with 5'-aza-dC sensitized non-responsive ATCC T-47D cells to CYP19 induction by ATR is similarly bolstered by recent studies which uncovered inter-individual- (Demura and Bulun, 2008), breast adipose fibroblast-, and inter-breast cancer cell line (MCF-7, MCF10A)- variability (Knower *et al*, 2010) in the methylation status of c*yp19* CpG dinucelotides in pII- especially two CpGs in pII CRE critical for cAMP-mediated transactivation. Thus, in addition to the epithelially and stromally expressed molecular determinants GATA and LRH-1, our findings strongly implicate *Cyp19* pII hypomethylation- an increasingly frequent finding in epithelial and adipose stromal cells of malignant breast tissue- as an epigenetic determinant of ATR susceptibility.

Our discovery that *cyp19* pII methylation is a critical epigenetic determinant of ATR susceptibility may also explain why we were the first group to demonstrate ATR affects both Cyp19 mRNA and protein levels in the LBNL T-47D variant characterized in the previous chapter. Numerous groups have repeatedly shown ATR does not affect CYP19 expression in MCF-7 or Ishikawa cells (Suzawa and Ingraham, 2008)- the most widely studied breast and endometrial cancer cell lines, respectively. However as NR5A and GATA factors are abundantly expressed in both cell lines, it is reasonable to hypothesize that *Cyp19* pII hypomethylation- induced experimentally or as a sporadic occurrence in subpopulation doublings- would similarly sensitize these cell lines to *Cyp19* induction by ATR. In the same way, because *Cyp19* pII methylation and cAMP-responsiveness was recently discovered to vary widely in fibroblasts derived from ostensibly healthy female subjects (Demura and Bulun, 2008), it is likely that exposed individuals may be alternately susceptible to aromatase induction and estrogen-related disorder promotion by chronic low-dose occupational or drinking water ATR exposure. Examples of variable susceptibility have been demonstrated in rat strains and amphibian species, where ATR-dosed SD but not Fisher rats (Eldridge *et al*, 1999) develop mammary tumors, and specific lab *X.*

laevis colonies and wild northern leopard frogs obtained by different groups exhibit different sensitivity to ATR. Because the two CpG dicucleotides located in CRE of the cAMP-responsive gonadal *Cyp19* promoter ('pII' in humans) are conserved across vertebrate classes (rodent, amphibian, ovine, bovine, and avian genes), varied methylation status of pII could account conflicting reports of model susceptibility in the literature.

In summary, consistent with other reports, we show ATR upregulates aromatase expression by LRH-1-, PKA-, and PI3K-mediated mechanisms- but here for the first time show ATR affects *Cyp19* in human ER+ BC cells- and establish GATA factors and *cyp19* pII hypomethylation as key determinants of susceptibility with high predictive value for other models and future studies.

Chapter II, Part B

Atrazine dose-dependently increases endogenous aromatase mRNA and protein levels in a human non-malignant adipogenic fibroblast cell line: implications for breast cancer and estrogen-sensitive disorder risk

ABSTRACT

Obesity is well-established risk factor for breast cancer (BC) as undifferentiated adipogenic fibroblasts of adipose stromal compartments of the thighs, buttocks, and mammary tissue express aromatase from the glucocorticoid and class I cytokine-regulated *Cyp19* promoter '1.4', and aromatase expression, and hence estrogen production, increases in adipose with advancing weight and age. Adipose is the primary organ of estrogen biosynthesis in PM women- those at highest risk for BC. Aromatase in mammary tumor-proximal fibroblasts is enriched via increased utilization of multiple *cyp19* promoters, but primarily from depressed cAMP-regulated gonadal c*yp19* pI.3/II also depressed in malignant epithelial cells. DNA methylation profiling of four healthy subjects by Demura and Bulun (2008) recently revealed CYP19 expression in skin fibroblasts derived from one woman was cAMP-inducible owing to hypomethylation of CpG dinucleotides in CRE of pII, suggesting healthy individuals may be variably susceptible to environmental hazards that exert transcriptional pressure on this promoter as a mechanism of estrogenicity. We here we demonstrate ATR induces cAMP-mediated transcription, and dose-dependently increases endogenous aromatase mRNA and protein levels in the novel clonal human adipogenic fibroblast cell line, SGBS. Our preliminary findings further validate conserved mechanisms of ATR action we proposed previously in T-47D human BC cells, and with childhood and PM obesity on the rise, have implications for ATR's role in affecting precocious puberty, estrogen-related disorders, and risk of BC development and promotion.

INTRODUCTION

In addition to *Cyp19* pII hypomethylation (Knower *et al*, 2010; Bulun *et al* 2009), GATA-3 and LRH-1 overexpression are hallmarks of the estrogen-sensitive BC phenotype and may be deregulated in a positive feedback loop in epithelial and stromal cells of the breast in the process of transformation leading to establishment of the major ER+BC subtype (Ali *et al* 2010; Chand *et al*, 2011; summarized in Figures 8 and 14). In the previous sections we shown low, biologically relevant concentrations of ATR enhance expression, activity, or the effect of all the described ER+BC hallmarks in the well-characterized model human BC cell line, T-47D (Figures 10-13), and our findings have implications for BC promotion and progression. However if the mechanisms by which we propose ATR affects CYP19 in BC cell lines are also operative in non-malignant mammary epithelial or stromal cells *in vivo*, such findings may additionally have implications for ATR's role in risk of BC development, as cumulative estrogen exposure (through aromatase or ERα activity) and deregulation of the GATA-3/LRH-1/ERα regulatory loop are highly correlated with BC risk *in vivo* and well-established transformative factors *in vitro* (Ali *et al* 2011).

The Importance Of Paracrine Epithethial And Adipose Fibroblast Interactions In The Estrogen-Sensitive Breast Tumor Microenvironment

In premenopausal women, the ovaries are the primary site of estrogen biosynthesis, where aromatase transcription is driven from pII downstream of FSH in granulosa cells (Figure 1). In post-menopausal (PM) women however, adipose becomes the major organ of estrogen biosynthesis, and aromatase is expressed primarily from skin/adipose-typical promoter I.4 in adipogenic fibroblasts of the thighs, buttocks, and mammary adipose stroma under the influence of glucocorticoids and class I cytokines. Postmenopausal women are disproportionately affected by BC; obesity has lately been understood to increase risk particularly in this group as increased adiposity over time or with advancing age may result in increased bodily CYP19 expressed from adiposal pI.4 in a greater number of adipogenic fibroblasts, leading to systemic or local estrogen excess (McInnes *et al* 2008; Demura and Bulun, 2008). Interestingly, in the malignant breast, where local estrogen concentrations may be 10 times greater than circulating plasma levels in PM women, biopsies reveal *Cyp19* is elevated 3 to 4 fold (McInnes *et al* 2008) in tumor-proximal fibroblasts of the mammary stroma but not fully differentiated adipocytes. These marked elevations in Cyp19 result in part from pI.4-driven transcription, but the majority of transcripts in tumor-proximal fibroblasts derive predominantly from cAMP-responsive gonadal promoters pII and 1.3- which are derepressed by unknown mechanisms only recently discovered to involve hypomethylation of two crucial CpG dinucleotides in pII CRE (ibid) discussed in the previous section of this chapter; being

derepressed in tumor-proximal fibroblasts, these cAMP-gonadal promoters are further transactivated by cytokines, such as prostaglandin e2 (PGE2), secreted by adjacent transformed epithelial cells. Thus, *cyp19* 'promoter switching', from the breast-typical glucocorticoid and class I cytokine-regulated promoter 1.4, to cAMP-responsive gonadal promoters, is a critical phenomenon in tumor-proximal fibroblasts as well as preneoplastic and malignant cells of the estrogen-sensitive phenotype (Bulun *et al*, 2005 and 2009). A scheme of major sources tissue sources of estrogen and CYP19 regulation in premenopausal, PM, and tumor-containing breast tissue is provided in Figure 28 (same as in General Introduction).

Figure 28:

Major sites and regulation of estrogen biosynthesis in premenopausal and PM women and malignant breast tissue. In the premenopausal ovary, Cyp19 expression is driven from cAMP-responsive gonadal promoters pII/I.3 downstream of FSH in the follicular phase of the menstrual cycle; FSH binds its cognate GPCR in granulosa cells- elevating cAMP levels- which results in PKA and p44/42-mediated activating phosphorylations of GATA-4 and SF-1, trasactivation of Cyp19 pII/I.3, and increased estrogen production associated with thickening of the endometrial lining in the follicular phase. In PM women, adipose is the primary organ of estrogen biosynthesis, where aromatase expression is driven from the skin/adipose-typical glucocorticoid and class I cytokine -regulated promoter 1.4 via glucocorticoid receptor (GR) and JAK/STAT activation. *Cyp19* expression from this promoter is enriched in skin fibroblasts and adipogenic fibroblasts of disease-free adiposal compartments in thigh-, buttock-, and the mammary adipose stroma.

Lower Panel:

In disease-free breast tissue, Cyp19 transcript pools derive primarily from the GC-regulated adipose/breast-typical promoter 1.4. Quantitative PCR analyses of tumor-bearing quadrants reveal an overall increase in CYP19 expression relative to tumor-adjacent quadrants, primarily from increased utilization of cAMP-responsive gonadal pI.3/pII, or 'promoter switching', in both tumor-proximal fibroblasts and malignant epithelial cells of the tumor mass. Malignant breast epithelial cells secrete pathological levels of cytokines (ILL-11, TNF-a) that inhibit adipogenesis and expand undifferentiated fibroblasts in their periphery. Secretion of PGE_2 by tumor cells also enhances 'promoter switching' the dense layer of fibroblasts surrounding the tumor, resulting in marked elevations in local estrogen conducive to tumor growth. This expansion and recruitment of aromatase-expressing undifferentiated fibroblasts by malignant epithelial cell secretory products is termed the 'desmoplastic reaction'.

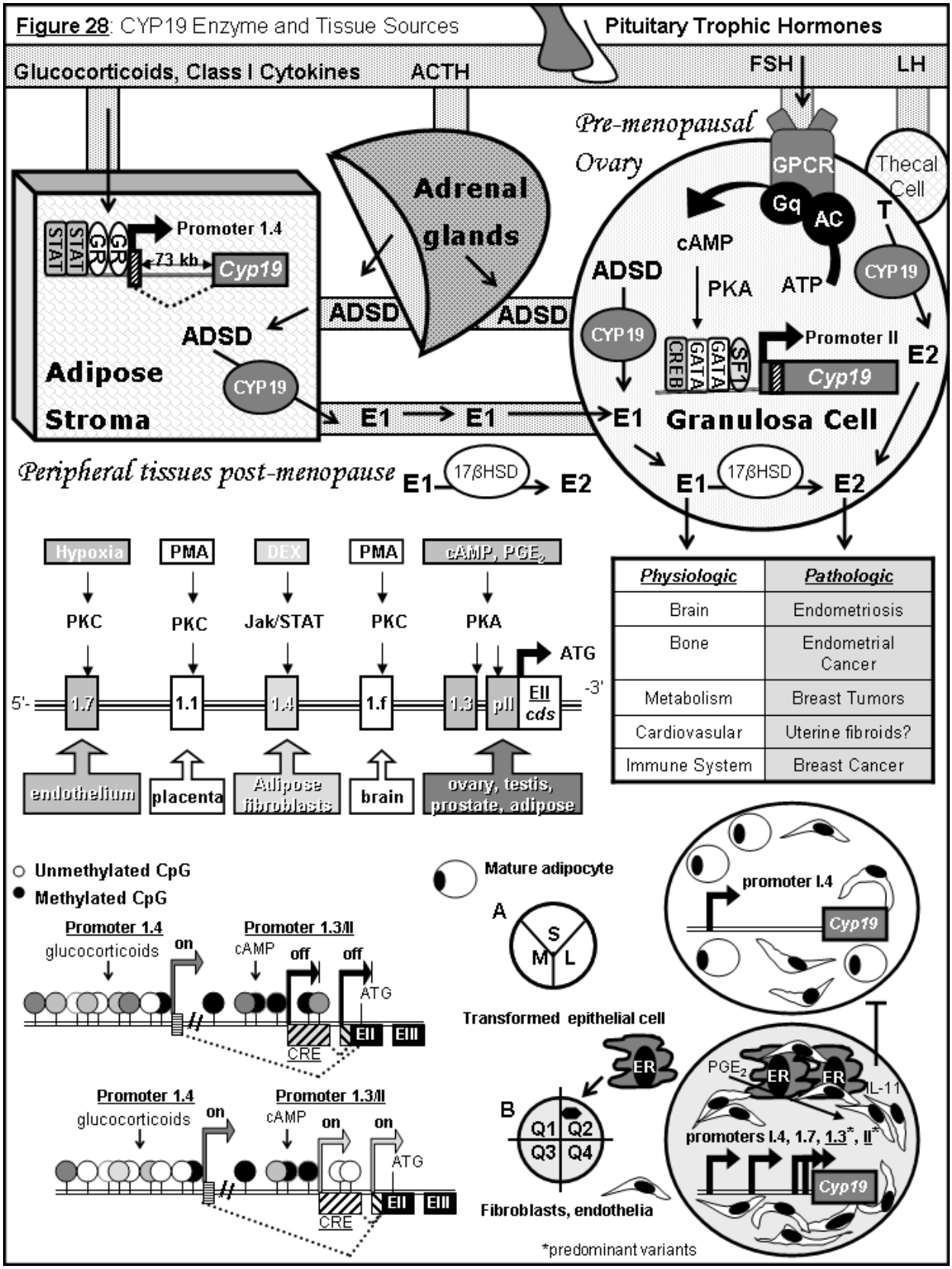


Figure 28: CYP19 Enzyme and Tissue Sources
Pituitary Trophic Hormones
Glucocorticoids, Class I Cytokines
ACTH
FSH
LH
Pre-menopausal Ovary
GPCR
Gq
AC
cAMP
ATP
PKA
T
CYP19
Thecal Cell
Adrenal glands
ADSD
ADSD
ADSD
CYP19
Adipose Stroma
E1
E1
E1
Granulosa Cell
Promoter 1.4
73 kb
Cyp19
STAT
STAT
GR
GR
Promoter II
CREB
GATA
GATA
SF1
Cyp19
E2
Peripheral tissues post-menopause
E1
17βHSD
E2
E1
17βHSD
E2
Hypoxia
PMA
DEX
PMA
cAMP, PGE2
PKC
PKC
Jak/STAT
PKC
PKA
ATG
5'-
1.7
1.1
1.4
1.f
1.3
pII
EII cds
-3'
endothelium
placenta
Adipose fibroblasts
brain
ovary, testis, prostate, adipose
Physiologic
Pathologic
Brain
Endometriosis
Bone
Endometrial Cancer
Metabolism
Breast Tumors
Cardiovasular
Uterine fibroids?
Immune System
Breast Cancer
Unmethylated CpG
Methylated CpG
Promoter 1.4
glucocorticoids
on
Promoter 1.3/II
cAMP
off
off
ATG
CRE
EII
EIII
Promoter 1.4
glucocorticoids
on
Promoter 1.3/II
cAMP
on
on
ATG
CRE
EII
EIII
Mature adipocyte
A
S
M
L
Transformed epithelial cell
B
Q1
Q2
Q3
Q4
ER
Fibroblasts, endothelia
promoter I.4
Cyp19
PGE2
ER
ER
IL-11
promoters I.4, 1.7, 1.3*, II*
Cyp19
*predominant variants

The Desmoplastic Reaction

Up to 70% of breast tumors diagnosed by pathologists are described as the 'schirrhous' type- which denotes a rock-like consistency of these tumors resulting from a dense layer of undifferentiated adipogenic fibroblasts being accumulated around a transformed epithelial core (Bulun *et al*, 2005). This common phenomenon of proliferation and sequestration of CYP19+ adipose fibroblasts by mammary tumor epithelial cells is termed the 'demsoplastic reaction', which is thought to occur via secretion of pathological levels of certain cytokines by malignant epithelial cells, such as IL-11 (interleukin 11), TNF-α (tumor necrosis factor), and PGE_2 (prostaglandin E_2); these paracrine secretory products inhibit nearby adipocyte differentiation, induce fibroblast expansion and migration, and upregulate aromatase expression in tumor-proximal preadipocytes - resulting in structural support and increased local estrogen levels conducive to tumor growth (Chen *et al*, 2009).

In stromal mammary adipose, ninety percent of aromatase activity derives from undifferentiated fibroblasts rather than mature lipid-laden adipocytes (Bulun *et al*, 2009). Paracrine secretion of TNF-α and IL-11 by malignant epithelial cells blocks differentiation of proximal fibroblasts by enhancing GATA-3 and LRH-1 expression in these cells- which inhibits the adipogenic transcription factors C/EBPα and PPARγ in fibroblasts, creating a dense fibroblast layer around the transformed epithelial core. Further, paracrine PGE_2 secretion by malignant epithelial cells results in, sequentially: increased intracellular cAMP/other second messenger levels in proximal fibroblasts; PKA/other kinase-mediated activating phosphorylations of GATA-3 and LRH-1; robust transactivation (promoter-switching) of gonadal *Cyp19* I.3/II in fibroblasts; and finally increased tumor-promoting estrogen produced by fibroblasts in response to pathological cytokines secreted by neighbor epithelial cells. Thus, as we previously demonstrated ATR enhances GATA-3, LRH-1, and CYP19 activity and/or expression in T-47D breast cancer cells by PKA-mediated mechanisms, it is conceivable that ATR may also exacerbate both the 'desmoplastic reaction' and 'promoter switching' in fibroblasts in which pII/pI.3 are derepressed. A scheme of the 'desmoplastic reaction' and promoter switching in tumor-proximal fibroblasts in response to pathological secretory products of malignant epithelial cells is provided in Figure 28, bottom panel.

Potential Effects of ATR In Adipogenic Fibroblasts And Risk Of BC Development

With obesity prevalence increasing in western societies, the need to elucidate mechanisms and consequences of *Cyp19* pII/I.3 derepression in adipogenic fibroblasts is great. However, *in vitro* and mechanistic studies of primary human preadipocytes have to date been significantly limited by the difficulty of obtaining sufficient numbers of primary adipogenic fibroblasts and preadipocytes that are challenging to isolate and have a limited lifespan in culture. Mouse fibroblast models, particularly the commonly used 3T3-L1 cell line, are also proving less relevant in the study of human breast phenomena as CYP19 is temporally and molecularly regulated far differently in mouse fibroblasts and differentiated adipocytes. For instance, contrary to human adipose fibroblasts, *Cyp19* is not induced in 3T3-L1 cells by cAMP or glucocorticoids, and where CYP19 expression decreases with adipogenesis in the human scenario, CYP19 expression actually increases in adipogenic mouse fibroblasts as they differentiate and persists in fully differentiated adipocytes (McInnes *et al*, 2008). The sum of these challenges were recently met by McInnes *et al*, who characterized an CYP19+ human adipogenic fibroblast cell line, SGBS, derived from an infant with the rare Simpson-Golabi-Behmel overgrowth phenotype. In their characterization, McInnes *et al* demonstrated these clonal adipogenic fibroblasts maintain their capacity to differentiate in adipogenic media after 30 population doublings, express CYP19 from pII/I.3 when stimulated with cAMP, and express LRH-1 (reported but not shown). These findings suggest CpG dinucleotides in critical CREs of Cyp19 II/I.3 are hypomethylated in SGBS cells, and recommend the SGBS model as a good candidate to study the potential of ATR to affect non-maliganant stromal cells that may contribute to BC development or transformation of mammary epithelial through estrogen excess.

Further, as discussed in far greater detail in Chapter 1, in a recent study of four healthy women from which skin fibroblasts were isolated from buttock biopsies, Demura and Bulun at Northwestern University incidentally discovered CYP19 expression in fibroblasts from one of the four subjects was cAMP-inducible and driven from the gonadal-typical cAMP-responsive pII/I.3- in contrast to fibroblasts from the other three subjects- in which, as expected, CYP19 was GC-inducible, driven from pI.4, and significantly, not cAMP-inducible (2008). This intriguing finding of fibroblast cAMP-sensitivity in one of the four subjects led Demura and Bulun to analyze the methylation status of the six CpG dinucleotides contained within pI.3/pII in fibroblasts derived from all four women. They discovered 3 of the 6 CpGs were unmethylated in the subject with cAMP-responsive fibroblasts- in particular CpG #5 located in CRE of pII- whereas all six CpGs were hypermethylated in subjects with cAMP-refractory skin fibroblasts (Figure 2). Additional gel shift assys also demonstrated that the degree of CpG dinucleotide methylation was inversely correlated with CREB recruitment. Thus, as transient or permanent *Cyp19* pII CpG dinucleotide hypomethylation is a common finding even in fibroblasts of healthy women, it is reasonable to suggest

that some individuals may be more susceptible to adverse CYP19 expression and estrogen-related disorders as a consequence of lifetime low-dose ATR exposure from contaminated drinking water.

Thus, to preliminarily assess whether ATR could affect CYP19 expression in non-malignant cells highly relevant to BC risk, SGBS fibroblasts were obtained as a gift from the Watisbchi lab in Germany, and this brief subsection describes our preliminary findings in this novel and incompletely characterized cell line.

RESULTS

ATR stimulates cAMP-mediated transcription and increases endogenous aromatase mRNA and protein levels in the human adipogenic fibroblast cell line, SGBS

To preliminary assess the potential of ATR to affect SGBS cells, these were grown to 50% confluence and transfected with a CRE-luciferase (cAMP-reporter) construct as described in previous experiments and detailed in the Materials and Methods, and were treated with vehicle or 30 μM ATR for 24 hours in full media. As shown in panel A of Figure 30, ATR exposure resulted in robust CRE-promoter reporter activation (~ 15-fold) relative to vehicle. This finding indicated ATR may affect transcription of genes susceptible to cAMP-transactivation in human adipogenic fibroblasts.

Having established SBGS cells are highly responsive to CRE-mediated transcription by ATR, we next sought to determine whether ATR could affect endogenous Cyp19 mRNA levels in this cell type. To this end, we exposed SGBS cells to vehicle, increasing concentrations of ATR, or FSK in serum-free media for 25 hours after a 24 hour serum-starvation period as frequently done in experiments with the LBNL T-47D variant described in Chapter 1, and assessed endogenous Cyp19 mRNA levels by qPCR with specific primers described in the Materials and Methods. As shown in Panel B of Figure 30, also dose-dependently increases endogenous Cyp19 mRNA levels in SGBS cells (~ 2.5-fold induction).

Next, to determine whether protein levels of CYP19 and upstream activators induced in the LBNL T-47D variant were affected by ATR in SGBS cells, the latter were treated with vehicle, increasing concentrations of ATR, or FSK and phorbol dibutyrate (PDB) as described in the previous experiment, and CYP19, LRH-1, and GATA-3 levels were assessed by western blot as described in the Materials and Methods. As shown in panel C of Figure 30, ATR but not FSK/PDB enhances GATA-3 levels in SGBS cells, and ATR dose-dependently increases CYP19 levels in SGBS cells. Phorbal acetate (PMA), an potent activator of PKC (<10 nanomolar range), is reported to enhance PKA activity and CYP19 expression in human fibroblasts. As PMA was not available at the time of experimentation, PDB (8 nM), another activator of PKC, was assumed to have a similar activity and was included with FSK as a putative positive control. No induction of CYP19 relative to vehicle was observed in this treatment group however, which may be the result of weaker PKC activation by PDB relative to PMA (reported) or ineffectively low FSK (25 μM) or PDB concentrations (8 nM).

LRH-1 was also not detectable with any titer of the LRH-1 antibody we employed to detect expression in T-47D cells. In preliminary qPCR studies, contrary to T-47D cells, we discovered SGBS Cyp19 mRNA levels significantly reduced with increasing confluence (30% reduction from 50 to 75% confluence;

data not shown). As such, SGBS cells were maximally 70% confluent at the time of harvest following the indicated treatments and protein levels may not have been high enough for LRH-1 detection. These limitations standing, our preliminary findings indicate ATR enhances both GATA-3 and CYP19 expression in SGBS cells.

Figure 30:

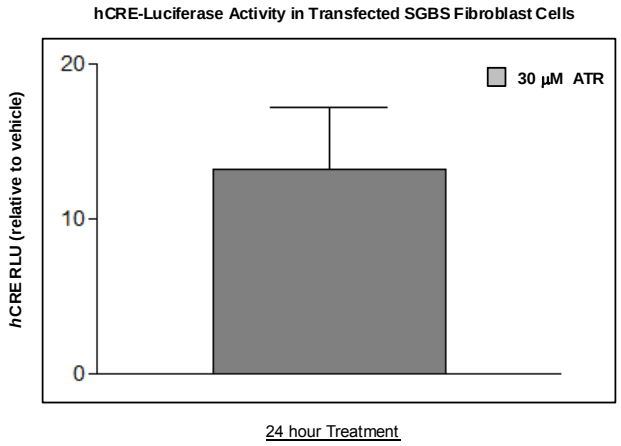
(A) ATR robustly induces CRE-mediated transcription in human SGBS fibroblasts. SGBS cells were grown to 50% confluence and transfected for 24 hours with the hCRE-luc construct as described in the Materials and Methods. Following transfection, cells were exposed to vehicle or 30 μM ATR in full media for 24 hours and harvested for assessment of luciferase activity with the Promega Luciferase Assay kit according to the manufacturer's instructions. Relative luciferase activity (RLU) is expressed relative to vehicle and is the mean of three replicates with standard error normalized for protein content.

(B) ATR dose-dependently increases Cyp19 mRNA levels in SGBS cells. SGBS cells were grown to ~50-60% confluence, serum-starved for 24 hours, and exposed to the indicated treatments for 24 hours. Following the exposure period, cells were harvested for qPCR and Cyp19 RNA levels were detected with specific primers as described in the Materials and Methods. Fold-change is expressed relative to vehicle, is normalized to Gus expression (housekeeping gene), and is reported as the mean of three replicates with standard error. Similar results were obtained in two independent experiments.

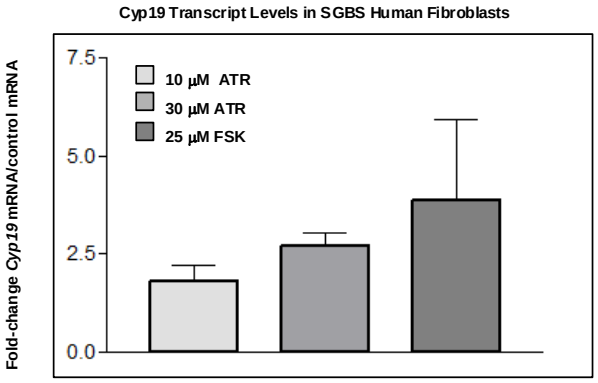
(C) ATR enhances GATA-3 protein levels and dose-dependently increases CYP19 expression. SGBS cells were treated with the indicated treatments as described in the panel above, and following this were harvested for detection of the indicated proteins by western blot. Samples were normalized for protein content prior to fractionation by electrophoresis, and GAPDH is included as a loading control. Similar results were obtained in several independent experiments.

Figure 30

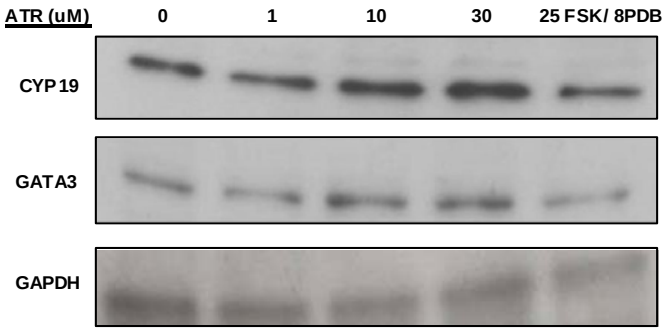
A



B



(C)



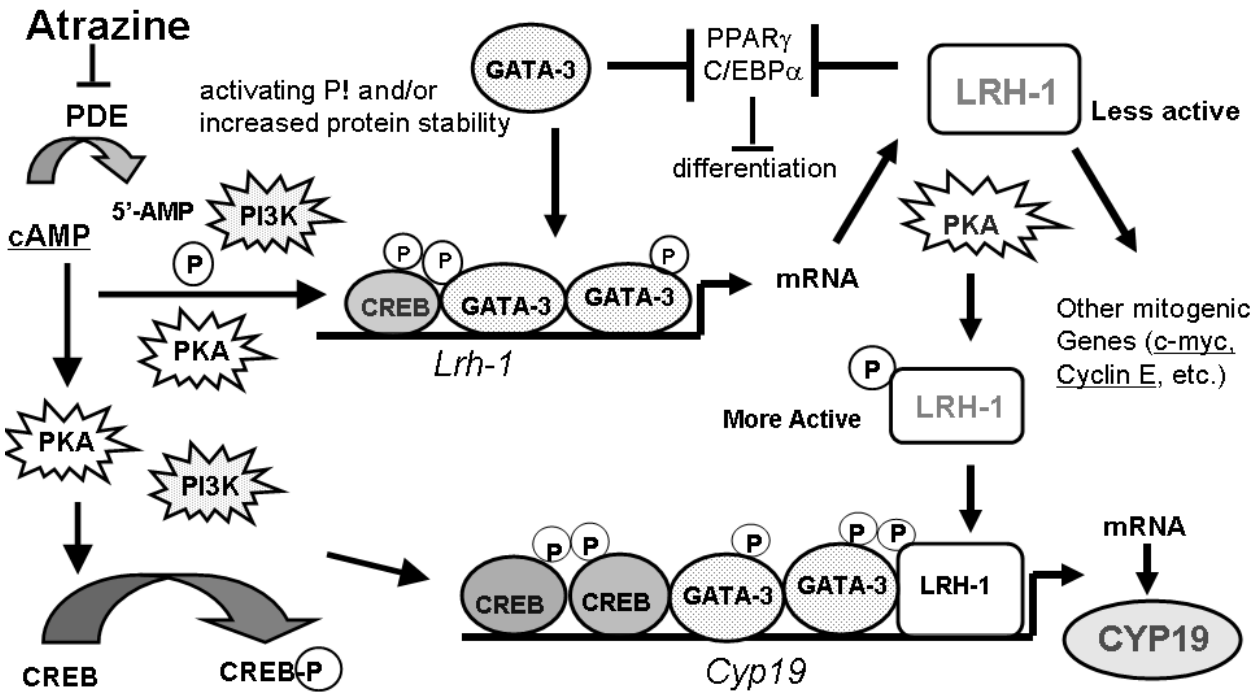
DISCUSSION

With obesity and estrogen-related disorder prevalence increasing in western societies across demographic groups, the importance of investigating the effects of environmental hazards on CYP19 expression in adipose fibroblasts is paramount. Here for the first time we show low dose and short duration ATR exposure induces cAMP-mediated transcription, and dose-dependently increases endogenous Cyp19 mRNA and protein levels in a human adipogenic fibroblast line. Taken together with previous reports and our own findings in ATCC T-47D cells, our results suggest critical CpG dinucleotides in pII/I.3 are hypomethylated in the SBGS cell line, recommending it as a good candidate to study inter-individual variability in susceptibility to ATR and other environmental endocrine disruptors that affect aromatase induction through gonadal promoters. A summary scheme of the mechanisms by which we propose ATR affects CYP19 and risk factors of estrogen-related disease if provided in Figure 31.

Figure 31:

Clinical Consequences and Proposed Mechanisms of ATR Action in Human Adipogenic Fibroblasts. Our findings in SGBS human adipose fibroblasts indicate ATR upregulates cAMP-mediated transcription and induces GATA-3 expression. In human adipose fibroblasts, GATA-3 upregulates LRH-1 expression by PKA-mediated mechanisms; GATA-3 and LRH-1 together may subsequently partner to inhibit differentiation of fibroblasts by opposing the depicted adipogenic transcription factors. Moreover, PGE_2 secretion by malignant epithelial cells permits *Cyp19* pI.3/II transactivation by GATA-3 and LRH-1 in tumor-proximal fibroblasts via cAMP/PKA-mediated mechanisms. In addition to effects on cAMP-mediated transcription and GATA-3, we also show ATR dose-dependently induces endogenous aromatase mRNA and protein levels in SGBS fibroblasts at low concentrations. Taken together with our previous findings, these results indicate ATR may inhibit adipogenesis and further induce CYP19 expression in human adipose fibroblasts in which critical CpGs in pII CRE are transiently or permanently derepressed. These molecular and epigenetic phenomena are thought to predispose susceptible individuals to precocious puberty and/or menarche, endometriosis, breast cancer, and other estrogen-related disorders.

Proposed General Mechanism of ATR Action in Human Adipose Fibroblasts **Figure 31**



Activation of ERα: Precocious Puberty; Proliferation of Mammary Epithelial Cells; Endometriosis

Future Studies in the SGBS Human Fibroblast Line

Obesity and lifetime estrogen exposure are known risk factors for BC, as higher growth-promoting local or systemic estrogen levels decrease the time dividing epithelial cells have for DNA repair, and increase mammary tissue density or the number of breast epithelial cells susceptible to mutagen exposure over a woman's lifetime. Moreover, precocious puberty, also on the rise in western societies, is another established risk factor for BC, and environmental estrogens, like ATR, are increasing implicated as exerting a significant role in altering pubertal timing following *in utero* and/or critical developmental window exposure (Fenton *et al*, 2004 and 2007). Because ATR affects CYP19 expression in human adipogenic fibroblasts, it is reasonable to conceive that similar effects *in vivo* might render overweight and obese girls particularly susceptible to altered pubertal timing and estrogen-related disorder or BC risk via repeated exposure. While our novel findings provoke more questions than answers for ATR's role in affecting non-cancer, obesity- and estrogen-related developmental and fertility disorders, they also recommend the human SGBS adipose fibroblasts cell line as a valuable tool in future timely studies.

Chapter 3

The Herbicide Atrazine Induces Aromatase in Rat Ovarian Cells via Highly Conserved Mechanisms: Implications for Rat Mammary Tumorigenesis and Human Breast and Reproductive Cancer Risk

ABSTRACT

Atrazine (ATR) elicits anti-androgenic and estrogenic reproductive effects at biologically relevant doses in every vertebrate class studied to date (Hayes *et al*, 2010). In lifetime feeding oncogenicity studies conducted by the registrants more than 15 years ago, ATR also increased mammary tumor incidence in aging ovary-intact but not ovariectomized (OVX) Sprague-Dawley (SD) rats, implicating ovarian estrogen as critical for mammary tumor promotion by ATR in this strain (Cooper *et al*, 1996; Eldridge *et al*, 1999). While seminal oncogenicity findings in the ovary-intact SD rat ignited concern in many in the independent scientific community regarding the sustainability of continued widespread ATR use (Gammon *et al*, 2005), their relevance to human health have in fact been repeatedly discounted by the registrants themselves as similarly dosed mice and Fischer 344 (F344) rats suffered no increase in mammary carcinoma incidence over controls (ibid). Further, in 'mechanistic studies' performed by the registrants in OVX and tumor-insusceptible rat strains (F344 and Long Evans), ATR causes suppression of LH secretion at the level of the hypothalamus, which the registrants extrapolate both as: (1) a direct mechanism of ATR critical for only secondarily increased estradiol synthesis in unovulated follicles in mammary tumor-susceptible ovary-intact SD rats; and (2) one irrelevant to human risk as BC afflicts primarily post-menopausal (PM) women whose ovaries are irresponsive to the upstream central gondotropins LH and FSH (Eldridge *et al*, 1994 and 1999; Cooper *et al*, 1996 and 2007). In the present work we interrogate the more parsimonious but less considered putative mechanism of enhanced rat ovarian estradiol production by ATR- that ATR directly induces CYP19 expression in rat ovarian granulosa cells independent of upstream neuroendocrine influence (Gammon *et al*, 2005). Indeed, we show ATR directly and dose-dependently induces cAMP-mediated transcription, endogenous rat aromatase mRNA and protein, and upstream MAPK and Akt kinases. Most compellingly, we show cis- and trans- ATR-regulated factors between rats and humans are conserved to the degree that ATR induces human gonadal *Cyp19* pII-luciferase reporter activity in transfected rat cells by PKA-mediated mechanisms, as we did in the previous chapter in human T-47D BC cells. Our findings therefore have novel implications for conserved mechanisms of rat mammary tumor promotion by ATR and their significance to human breast and reproductive cancer risk.

INTRODUCTION

In addition to being one of the most widely applied herbicides the world over, atrazine (ATR) is also one of the most frequently studied herbicides. Concerns regarding the sustainability of its continued use have grown in prevalence among academic and policy advisory scientists since 1994, when ATR was first publicly disclosed in registrant-conducted chronic feeding studies to significantly and dose-dependently increase estradiol levels and mammary tumor incidence in ovary-intact but not ovariectomized Sprague-Dawley (SD) rats (Eldridge *et al*, 1994). These mammary tumors were apparently fatal, as afflicted rats given the highest doses of ATR (500 and 1000 ppm) died at nearly twice the rate (57% and 63%) of controls (38%) after two years (Gammon *et al*, 2005). Though the majority of tumors induced in ovary-intact SD rats were malignant and fatal, ATR did not induce mammary tumors in similarly-dosed ovariectomized (OVX) SD rats, male SD rats, nor in mice, or Fisher-344 (F344) rat strains of either sex (ibid). In the remarkably few studies in which estradiol levels were measured in conjunction with tumor assessment, blood estradiol levels were similarly unaffected by ATR in the tumor-insusceptible Fisher rat strain (Wetzel *et al*; 1999). Industry-funded researchers have since discounted findings in the ovary-intact SD strain as not relevant to humans; they claim incidence of spontaneous mammary tumors with advancing age, or time in reproductive senescence in the SD strain is relatively high, and rather than promote mammary tumorigenesis, “atrazine could be promoting a premature senescence of reproductive and mammary tissues” and as it

> merely enhanced tumor incidence from approximately one-fourth to one-half of the animals might not be a direct carcinogen as much as a modulator of endogenous hormone secretions, serving to stimulate an earlier appearance in a greater number of subjects.
>
> (Eldridge *et al*, 1994)

Importantly, in contrast to mice, the majority of human and rat mammary (Russo and Russo, 1996; Cooper *et al*, 2007) tumors are strongly dependent on estrogen for growth- and in early stages are effectively treated with estrogen-receptor antagonists or inhibitors the enzyme aromatase- which is the key enzyme of estrogen biosynthesis in every steroidogenic tissue in both humans and rats. As risk of hormone-dependent mammary carcinoma increases with lifetime estrogen exposure in both species, incidence, counterintuitively, increases with years in reproductive senescence (proxy for advancing age and cumulative ovarian estradiol production) in both humans and rats (Eldridge *et al*, 1994, and 1999). Although our common risk is strongly correlated with advancing age and estrogen exposure, the parencyhmal tissues of aromatase dysregulation and attendant mammary-tumor promoting estrogen in each differs. In the PM female, ovarian granulosa cells cease responding to the pituitary gonadatrophins FSH and luteinizing hormone (LH)- which may be secreted in high amounts in the general circulation- and primary tissues of breast tumor-promoting estrogen

are extraovarian. Conversely, in the Long-Evans (LE) and SD senescent rat strains, the gonadal axis is fails at the central level, where secretion of LH diminishes with age, and immature and anovulatory ovarian 'follicular' or granulosa cells (same cell type), which express aromatase, are the parencymal source of mammary tumor-promoting estrogen- which may be secreted in high amounts in the plasma (Cooper *et al*, 1996 and 2007). Thus, although the majority of mammary tumors in both species are estrogen growth-sensitive and the result of aromatase misregulation, our plasma profiles of estrogen and upstream gonadaotropins are reversed in advancing age. A table comparing ATR tumor- susceptible and -insusceptible rat strains (Panel A) and differences in hypothalamic-pituitary-gonadal axis failure in senescing rats and humans (Panel B) is provided in Figure 32. While LE (Long Evans) rats were never tested for mammary tumor promotion by ATR, they have been used as a model in several later described 'mechanistic studies' the registrants and those they advise have conducted since the seminal oncogenicity findings the SD rat strain were made available (Cooper *et al*, 2007); the effect of ATR on LE rat strain hormones are therefore included in Figure 32 in the absence of tumor data for this strain.

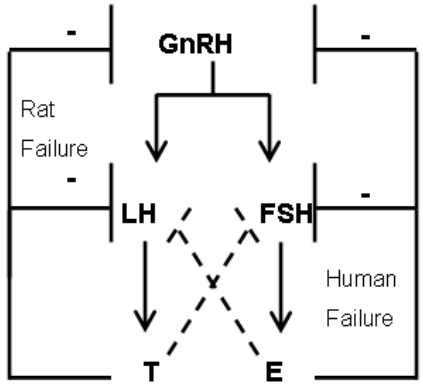
Figure 32:

Comparison of susceptible and insusceptible rat strains (A) and differences in HPG failure in senescing rats and humans. (A) In registrant-conducted lifetime feeding studies, only SD rats with their ovaries intact were susceptible to mammary tumor induction by ATR. As blood estradiol levels were elevated by ATR in ovary-intact tumor-susceptible SD rats, and ovariectomy protected SD rats from mammary tumor induction by ATR, aberrant ovarian CYP19 induction is a highly plausible mechanism of oncogenecity by ATR in the SD strain. Moreover, ATR-dosed ovary-intact F344 rats did not suffer significant increases in mammary tumors over controls, but this strain was also insusceptible to estradiol elevations by ATR. Registrant studies indicate LH levels/surges are lowered by ATR in LE rats; however estradiol levels are not affected in this strain (so LH attenuation has no relevant consequence), and the effect of ATR on mammary oncogenicity has not been reported for this strain. (B). In the PM female, ovarian granulosa cells cease responding to the central gonadatropins, FSH and LH- possibly through cognate receptor insensitivity- which results in elevated FSH and LH (reduced feedback) secretion and far diminished estrogen in the circulation; still, in PM women, aromatase and estrogen may be elevated locally in the malignant breast. In the senescing SD rat, LH levels diminish with age. Consequently, no regular LH surges occur, and immature, unovulated ovarian follicles are retained; these continue to secrete estradiol in the general circulation resulting in promotion of ER+ mammary tumor growth. Abbreviations: LH= luteinizing hormone; FSH= follicle stimulating hormone; T= testosterone; E = estrogen.

Figure 32 (A): Effect of Strain and Ovary Status on Estradiol Induction and Mammary Tumor Incidence in Rats Fed ATR in Lifetime Feeding Studies

Rat Strain**	Ovary Status	Plasma Estradiol Levels	Mammary Tumor Incidence
Sprague-Dawley (SD)	Intact	Increased	Increased
Sprague-Dawley (SD)	Ovariectomized	Unaffected	Unaffected
Fisher-344 (F344)	Intact	Unaffected	Unaffected
Fisher-344 (F344)	Ovariectomized	Unaffected	Unaffected
Long-Evans (LE)	Intact	Unaffected	Untested
Long-Evans (LE)	Ovariectomized	Unaffected	Untested

(B) HPG (Hypothalamus-Pituitary-Gonadal) Axis in Rats and Humans in Senescence



Hormonal Profile in Senescence

Species	Arm of Axis Failure	LH/ FSH	Ovarian Estradiol
Rat	GnRH ↓ LH/FSH	low	high
Human	FSH ↓ E	high	low

*Registrant (CIBA-GIECY formerly, Syngenta/Novartis currently)- conduced studies

**Males of any rat strain and mice of either sex are insusceptible to mammary tumor formation by ATR

While tissue sources of estrogen are different between senescing rats and humans, as discussed in detail in the previous chapter, at the molecular level, aromatase is misregulated in rats and humans by near-identical mechanisms in the context of mammary tumor promotion. To wit: in malignant human breast tissue, aromatase is overexpressed primarily from aberrant activation of highly conserved gonadal- not breast-typical- cAMP-responsive *Cyp19* pII/I.3, which share conserved cis-elements in the rat gonadal c*yp19* promoter (Figure 33). Further, in malignant human breast tissue, *Cyp19* pII/I.3 promoters are transctivated by LRH-1 and GATA-3/4- which similarly have homologs (SF-1 and GATA-4, respectively) responsible for normal robust activation of *cyp19* pII downstream of FSH signaling in both the rat (estrus) and human (follicular phase) ovary (Figure 2 and 28; Stocco, C, 2008). Further, PKA motifs, targets for transcriptionally-activating phosphorylations downstream of FSH/cAMP stimulation, are conserved in the primary sequences of both rat and human GATA-3, GATA-4, and LRH-1 proteins (Figure 33). Moreover, recent work indicates these transcription factors gain access to highly-conserved gonadal cAMP-responsive *Cyp19* pII/I.3 promoters typically silenced in the human breast when these are derepressed in malignant tissue through hypomethlyation of critical CpGs- at least two of which are located in human pII CRE and are in fact perfectly conserved in the rat gonadal *cyp19* promoter (Figure 33). Thus: as we previously demonstrated ATR induces *Cyp19*-transription via cAMP/PKA-, GATA (3/4)-, NR5A (LRH-1)- mediated mechanisms in human breast cancer cells; these mechanisms of induction are conserved in the rat ovary (Stocco *et al*, 2007 and 2008; Hu *et al*, 2009); and ovariectomy protects ATR-susceptible SD rats from mammary tumor induction (Eldridge *et al*, 1999), it is highly plausible to suggest ATR, at least in part, promotes mammary tumorigenesis in the SD rat by upregulating ovarian aromatase via mechanisms nearly identical to those critical for malignant transformation in the human scenario, and findings of rat mammary tumor induction by ATR should therefore be considered highly relevant to human breast cancer risk. The conserved cis- and trans- elements critical in normal gonadal and mammary-tumor promoting *Cyp19* activation in rats and humans cited above are depicted in Figure 33.

Figure 33:

Species conserved cis- and trans-elements critical in normal gonadal and mammary-tumor promoting *cyp19* activation in rats and humans. Although the rat aromatase gene is far less complex than human *cyp19*, the human gonadal cAMP-responsive aromatase promoter, 'pII', which is robustly activated in malignant breast tissue in the presence of PGE_2, is conserved in the rat *cyp19* promoter activated downstream of FSH in the estrus cycle. CpG dinuceotides in CRE of the gonadal promoters of both species are also conserved. Moreover, PKA motifs, shown crucial in *Cyp19* pII activation by mutational analysis of critical serines in reporter studies, are conserved in the primary sequences of GATA-3, GATA-4, and LRH-1 in rats and humans. ATR elicits estrogenic effects in every vertebrate class studied to date; indeed, though not shown depicted here in the interest of simplicity, the cis- and trans-factors critical in gonadal cAMP-mediated cyp19 regulation show in Figure 33 are perfectly conserved in: Xenopus; bovine; porcine; equine; chicken; and rabbit species. More detailed schematics of this remarkable degree of conservation in cis- and trans- gonadal *cyp19* regulation are provided in excellent detail in *Endocrinology* (2005) **146**:4905-4916, by Bouchard *et al*.

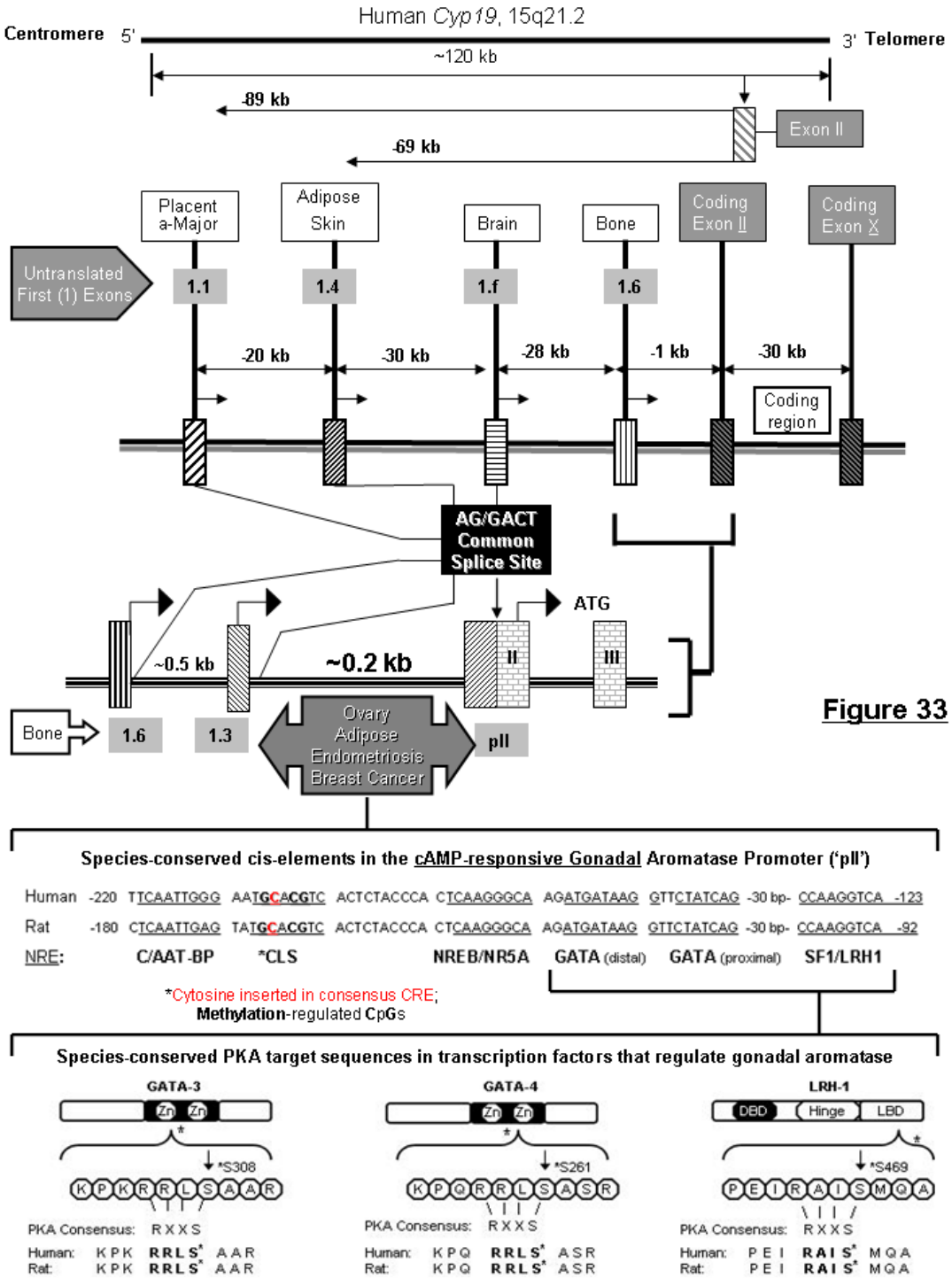

Human Cyp19, 15q21.2
Centromere
5'
3'
Telomere
~120 kb
-89 kb
-69 kb
Exon II
Placent a-Major
Adipose Skin
Brain
Bone
Coding Exon II
Coding Exon X
Untranslated First (1) Exons
1.1
1.4
1.f
1.6
-20 kb
-30 kb
-28 kb
-1 kb
-30 kb
Coding region
AG/GACT Common Splice Site
ATG
~0.5 kb
~0.2 kb
II
III
Bone
1.6
1.3
Ovary
Adipose
Endometriosis
Breast Cancer
pII
Species-conserved cis-elements in the cAMP-responsive Gonadal Aromatase Promoter ('pII')
Human -220 TTCAATTGGG AATGCACGTC ACTCTACCCA CTCAAGGGCA AGATGATAAG GTTCTATCAG -30 bp- CCAAGGTCA -123
Rat -180 CTCAATTGAG TATGCACGTC ACTCTACCCA CTCAAGGGCA AGATGATAAG GTTCTATCAG -30 bp- CCAAGGTCA -92
NRE:
C/AAT-BP
*CLS
NREB/NR5A
GATA (distal)
GATA (proximal)
SF1/LRH1
*Cytosine inserted in consensus CRE;
Methylation-regulated CpGs
Species-conserved PKA target sequences in transcription factors that regulate gonadal aromatase
GATA-3
Zn Zn
*S308
K P K R R L S A A R
PKA Consensus: R X X S
Human: K P K R R L S* A A R
Rat: K P K R R L S* A A R
GATA-4
Zn Zn
*S261
K P Q R R L S A S R
PKA Consensus: R X X S
Human: K P Q R R L S* A S R
Rat: K P Q R R L S* A S R
LRH-1
DBD
Hinge
LBD
*S469
P E I R A I S M Q A
PKA Consensus: R X X S
Human: P E I R A I S* M Q A
Rat: P E I R A I S* M Q A

Figure 33

The findings of mammary tumor promotion by ATR in the SD rat strain are frequently undermined in registrant-funded and EPA-weighted regulatory literature on the basis that similarly-dosed mice and F-344 rats suffered no increase in malignant mammary carcinoma over controls (Cooper *et al*, 1996; Eldridge *et al* 1999; FIFRA 2010). Though the SD strain, owing to its particular commonalities with humans in terms of hormonal sensitivity and temporality in tumor sporadicity is historically and currently preferred as a model for mammary hazard identification by the EPA (Spearow J, 2004), in literature cited in the specific case of regulatory ATR hazard assessments, effects of ATR on the SD strain are discounted by SAPs (scientific advisory panels) composed of registrants and EPA members (FIFRA 2000-2005) as insufficient or irrelevant to human health as the SD strain has a high incidence of spontaneous mammary tumor formation in old age (like humans) and is possibly uniquely susceptible to ATR (as are some human cell lines).

Differential *cyp19* pII CpG methylation may account for Variation in Ovarian Cell Susceptibility Over Time and Between Laboratory Models

In the previous chapter we demonstrated sublines of the same 'clonal' human BC line are variably sensitive to CYP19 induction by ATR, and in the present chapter, we aim to bolster the above registrant and EPA claims of rat-strain specific susceptibility- for the purpose of understanding whether additional insight could inform inter-individual human susceptibility and risk. As others have shown methylation of perfectly conserved pII CRE CpG dinucleotides (depicted above in Figure 33) is inversely proportional to cAMP-inducibility and varies widely between even healthy subjects (Demura and Bulun, 2008) and human breast cell lines (Knower *et al*, 2010), we here suggest it may also be likely that gonadal *cyp19* methylation, or at least the manner in which it is regulated in the estrus cycle and senescence, varies between alternately ATR-susceptible rat strains. Regarding reproductive or life cycle time of exposure and ATR-susceptibility, others have shown gonadal *cyp19* methylation, and hence cAMP-insensitivity, increases post-ovulation or progesterone/thecal cell-dominant 'diestrus' (Figure 34) in rat and bovine species (Vanselow and Fürbass, 2010) and suggest gonadal c*yp19* promoter methylation may be more dynamically regulated throughout the far shorter reproductive cycles of non-human mammals (ibid; the rat estrus cycle repeats on average every four days; see Figure 34). Thus, it is also conceivable that timing of dosing regimens and use of post-ovulatory or luteinized granulosa cells may significantly factor into predictiona of susceptibility.

Natural Variation in Estrogen, Progesterone, and LH Hormone Profiles between Senescent F344, LE, and SD Rat Strains

While 'ATR-induced' and 'natural' reproductive senescence in the ATR-mammary tumor susceptible/ovary-intact SD rat strain is characterized by low circulating LH and high estrogen produced from immature follicles typical if the of

the rat ‘estrus’ phase (analogous to follicular phase in human menstrual cycle; Figure 34)- ATR treatment results in increased days in progesterone-dominant ‘diestrus’ or ‘pseudopregnancy’ in the tumor-untested but blood estradiol-unaffected LE strain (Figure 34). Further, the hormonal milieu of the ATR tumor- and blood estradiol-insusceptible aging F344 rat strain differs fundamentally from the respectively tumor-susceptible and tumor-untested SD and LE rats strains, where instead of being estrogen-dominant, F344 senescence is characterized by high progesterone levels, or increased days in diestrus/cycles of ‘pseudopregnancy’ (Cooper *et al*, 1996). Notably, where estrogen promotes sporadic mammary tumor formation in both aging SD rats and humans, in addition to being ATR-insensitive, spontaneous tumor incidence in the progesterone-dominant aging F344 is also far lower than in the in estrogen-dominant senescing SD rat strain (Cooper *et al*, 2007).

Considering the above observations in sum, it is conceivable that gonadal c*yp19* promoter methylation is a critical epigenetic determinant of ATR-susceptibility in rats as well as humans. If so, rat gonadal *cyp19* promoter methylation, and hence ATR-susceptibility, may vary significantly between rat strains- and even within the same rat strain in different life cycle phases or phases of the typical four-day rat estrus cycle- and could thus account for variations in strain-specific and time/duration-dependent ATR-susceptibility reported by different groups in the literature (Cooper *et al*, 1996 and 2007; Eldridge *et al*, 1999; Gammon *et al*, 2005). A scheme detailing hormones of the rat HPG (hypothalamic-pituitary-gonadal) axis, and changes in central hormone secretion, steroid biosynthesis, and gonadal Cyp19 promoter methlylation in different phases of the estrus cycle is provided in Figure 34.

Figure 34:

Hormones of the rat HPG axis and estrus cycle. The 4-day rat estrus cycle and mechanisms of cyp19 regulation throughout. Post-ovulation, in 'diestrus', LH secreted by the pituitary stimulates conversion of cholesterol to progesterone and androgens by thecal or luteinized granulosa cells by the depicted mechanisms, and c*yp19* transactivation is repressed by gonadal promoter methylation. In proestrus/estrus, pituitary FSH stimulates follicular maturation and Cyp19 expression in granulosa cells in which the gonadal cAMP-responsive *cyp19* promoter Is hypomethylated, resulting in conversion of androgens to estrogens by aromatase. Ovarian estradiol produced in rats can promote mammary tumor growth and suppress hypothalamic GnRH (gonadotropin releasing hormone) and pituitary gonadotropin (LH and FSH) secretion via ERα-mediated mechanisms.

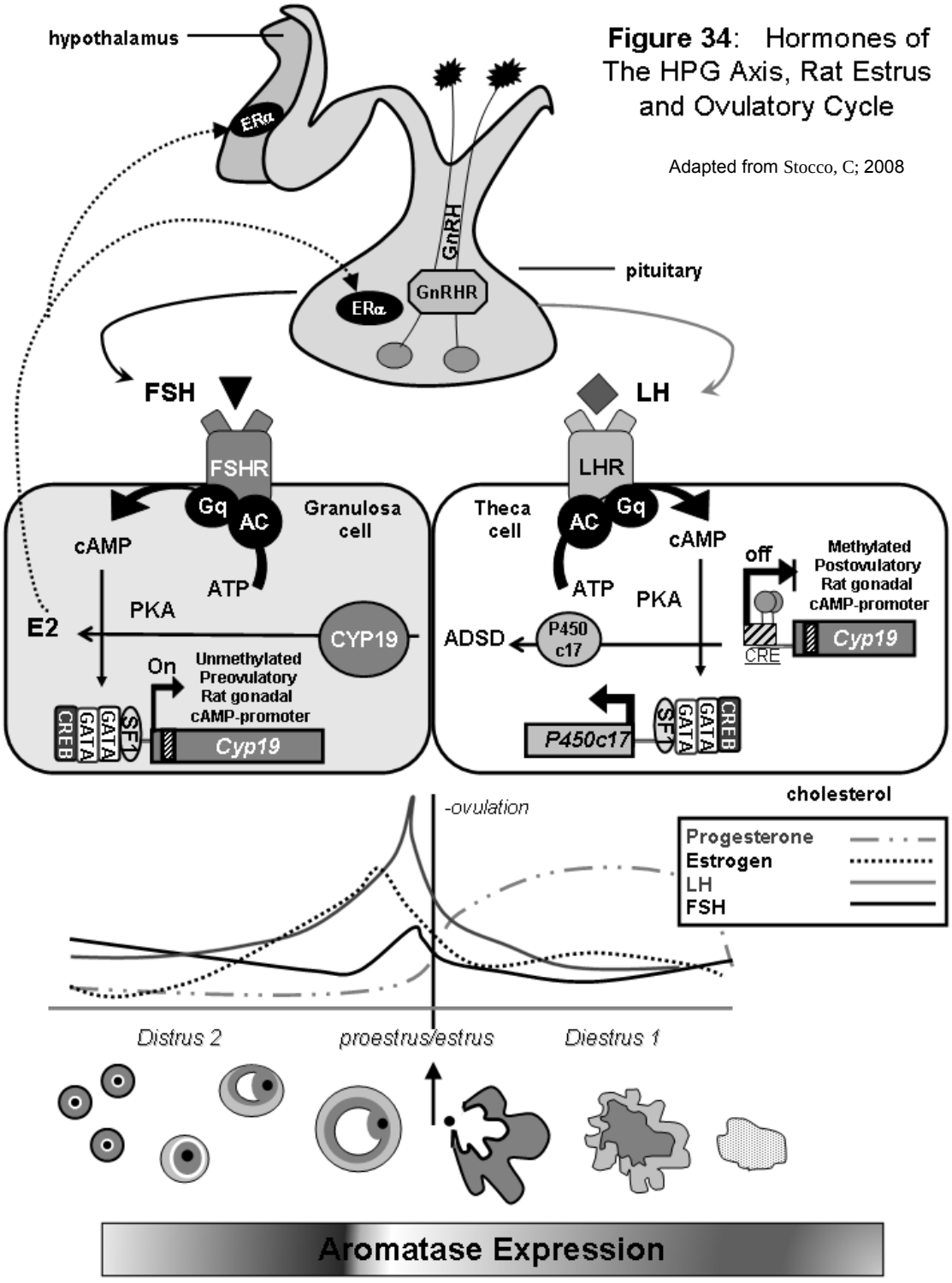


Figure 34: Hormones of The HPG Axis, Rat Estrus and Ovulatory Cycle

Adapted from Stocco, C; 2008

Although ATR is known to induce CYP19 in both human and rat adrenal cells by PKA-mediated mechanisms conserved in both human malignant breast and rat ovarian tissue (Suzawa and Ingraham, 2008), in the more than 15 years since the original oncogenicity studies, industry-funded researchers have repeatedly concluded mechanisms of mammary tumor promotion in the ovary-intact SD rat are not relevant to human health (Eldridge *et a*l, 1999; Cooper *et al,* 2007). To put forward this hypothesis, registrant-funded researchers have generated a preponderance of confounding data related to the effect of ATR on the brain of OVX F344 and LE rats, and claim ATR's effect of central LH suppression in these OVX, respectively tumor-insusceptible and tumor-untested strains, can be extrapolated as the mechanism of mammary tumor promotion in the ATR-susceptible ovary-intact SD rat strain (Figure 32 summarizes mammary tumor susceptibility by ATR as a function of strain and ovary status). They argue, because ATR causes LH suppression in OVX tumor-insusceptible/untested (respective) F344 and LE strains, this 'neuroendocrine' mechanism observed in irrelevant models- if operative in the tumor-susceptible, ovary-intact- and untested SD strain- could suppress ovulation and indirectly lead to increased 'days in estrus' or ovarian estradiol production by CYP19+ immature anovulatory follicles (see Figure 34 for rat estrus cycle). Further, as this purely theoretical 'neuroendocrine' or hypothalamic/pituitary-level mechanism of LH suppression tested most often OVX LE and F344 strains is analogous to the natural SD strain-unique phenomenon of senescence, where indeed, contrary to humans and the tumor-insusceptible F344 (Cooper *et al* 1996) rat, LH levels decrease and estrogen levels as a result increase with age- industry-funded researchers finally conclude ATR only 'hastens the onset' of mammary tumors in the SD rat by inducing 'premature reproductive senescence in the brain' of the SD strain (LH suppression)- which only secondarily results high plasma estradiol levels and so as a mammary tumor-promoting mechanism is not relevant to human health- as menopause (senescence) in humans is characterized by quiescent follicles and low plasma estradiol levels (Eldridge *et al*, 1999; Cooper *et al* 2007). These 'mechanistic' studies being conducted only in tumor-insusceptible and tumor-untested and so irrelevant rat models not withstanding, the proposed mechanism of ATR oncogenecity only accelerating 'naturally occurring senescence' in the rats is also difficult to take seriously given that all the studies this supposition is based on are conducted in models researchers themselves have surgically senesced or castrated before administering ATR (Cooper *et al*, 2007).

Even if the brain site-of-action proposal tested in OVX tumor-insusceptible F344 and tumor-untested LE strains is actually valid in tumor-susceptible SD rats with their ovaries intact, it cannot be counted as the sole mechanism of tumor promotion by ATR, as no vigorous investigations into the direct effects of ATR on CYP19 expression or estradiol production in the SD rat ovary or pre-ovulatory granulosa cells have been undertaken [granulosa cells derived from the Wistar rat strain (Laws *et al*, 2011) and luteinized human cells (Holloway *et al*, 2008) have been tested], contrary to what one would expect for a *bona fide* rat

mammary carcinogen whose action has been known for more than 15 years to critically depend on ovarian estradiol production for tumor production.

The more parsimonious but far less interrogated hypothesis of tumor promotion by ATR in ovary-intact SD rats is that ATR- rather than (or in addition to) directly/firstly suppressing LH at the central level permitting only secondarily increases ovarian estradiol production- directly upregulates CYP19 expression and attendant mammary tumor-promoting estrogen biosynthesis in ovarian granulosa cells of intact SD rats. In addition to mammary tumor promotion, in intact rats, increased ovarian estradiol biosynthesis by this mechanism could further result in estrogen receptor-mediated negative feedback on GnRH and LH secretion (see Figures 32 and 34)- which as described, has been repeatedly observed in registrant studies conducted in other strains.

As of the last Interim Re-registration Eligibility Decision (IRED) documents on ATR made available in 2010, despite rat mammary tumor findings and a wealth of studies characterizing ATR's effect of *Cyp19* induction by conserved and human breast cancer-relevant cAMP-mediated mechanisms in several other human and rat steroidogenic cells *in vitro*, ATR is currently classified by the EPA as 'not likely carcinogenic to humans' due to 'lack of human and mechanistic data' (FIFRA, 2010; Gammon *et al*, 2005). For these reasons, and because the direct effects of ATR on rat ovarian granulosa cells have not been materially tested *in vitro* lo these many years, in the present work we characterize the effects of ATR on spontaneously immortalized pre-ovulatory rat granulosa cells (SIRGCs) derived from Berlin Duckey rats; lines derived from SD rats that met the study criteria were unavailable at the time of experimentation. Nevertheless, in the present chapter we show ATR induces Cyp19 mRNA and protein levels in this rat ovarian line by conserved PKA and PI3K-mediated mechanisms independent of neuroendorcrine influence, and so here disprove the registrant-advanced and EPA- touted hypothesis that central LH attenuation is principally critical for secondary ovarian aromatase dysregulation, hyperestrogenicity, and ultimately mammary tumor promotion by ATR in rats. Most compellingly, we show cis- and trans- ATR-regulated factors between rats and humans are conserved to the degree that ATR induces human gonadal *Cyp19* pII-luciferase reporter activity in transfected rat (SIRGC) cells by PKA- mediated mechanisms. These findings may provide mechanistic insight critically needed to inform remaining questions as to differences in ATR susceptibility between rat strains and meaningful strain choice in ATR risk assessment, and have novel implications for conserved mechanisms of rat mammary tumor promotion by ATR and their significance to human breast and reproductive cancer risk.

MATERIALS AND METHODS

Reagents

Atrazine (2-chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine) was purchased from Chemservice Inc. (Chester, PA). DMSO, H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride), dibutyryl cAMP (N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt), 17β-estradiol (1,3,5-estratrien-3,17-β-diol), and testosterone (17β-Hydroxy-3-oxo-4-androstene) were purchased from Sigma Chemical Company (St. Louis, MO).

Plasmids

Pgl2-*cyp19*-pI.3/II-luc was the generous gift of Dr. Sedar Bulun at Northwestern University. eGFP was kindly provided by Dr. Jen-Chywan Wang at UC Berkeley.

Cell Culture

Preovulatory SIRGCs were the kind gift of Dr. B Burghardt at Texas A&M. These cells were derived from preovulatory follicles of 45-day-old Berlin Duckrey (BD IV) rats (HarÃan Breeding Laboratories, Indianapolis, IN) in proestrus as described by Stein *et al*. SIGCs were grown in Dulbecco's Modified Eagle Media/ Nutrient Mixture F-12 (DMEM/F-12) supplemented with 5% fetal bovine serum from Mediatech (Manassas, VA), 50 U/ml penicillin, and 50 U/ml streptomycin from Sigma (St. Louis, MO). Cells were maintained in a humidified chamber at 37^{o}C containing 5% CO_2.

Treatment Procedures

Sub-confluent cells were washed in phospho-buffered saline (PBS) and serum-starved for 3 or 24 hours prior to treatment as indicated in the Results section For exposure experiments, 0.01, 0.1, 1, 10, and 30 mM stocks of ATR and 20 mM H89 were prepared in DMSO and then diluted 1:1000 in serum-free media for delivery to sub-confluent cell culture plates as indicated.

Transient Transfection of the human Aromatase gonadal promoter 'pI.3/II''-Luciferase (h*Cyp19*pII-*Luc*) construct

SIGC cells grown to 50-70% confluency in 6-well tissue culture plates from Nunc (Fisher Scientific, Rochester, NY) in full-media were washed with PBS and switched to full media lacking antibiotics two hours prior to the transfection procedure. Transfection of 2 μg h*Cyp19*pII-*Luc*/well was performed using Fugene 6 transfection reagent from Roche in a Fugene (μl):DNA (μg) ratio of 6:1 ratio

diluted in Opti-MEM (Invitrogen) prior to addition to cells according to the Fugene 6 protocol. All transfections were performed 24 hours prior to the indicated drug treatments, at which time transfection media was replaced with phenol-red and serum-free media as described for luciferase assays below. Preliminary transfection efficiency studies with a constitutive GFP (green fluorescent protein) construct indicated the transfection efficiency using this procedure was 20-30%.

Luciferase Assays

Cells grown to 60-70% confluence in 6-well plates were transfected with 2 μg/well of the indicated plasmid construct. Transfections were performed in serum-free media with Fugene6 from Roche (Nutley, NJ) according to the manufacturer's instructions. Cells were treated post-transfection for the indicated times in phenol red- and serum- free media in the presence or absence of vehicle (DMSO) or 30 μM ATR. Cells were then lysed in passive lysis buffer provided with the Promega Luciferase Assay Kit (Madison, WI) and relative luciferase activity was determined on a luminometer according to the manufacturer's protocol. Relative luciferase activities in each sample were normalized for protein content in each sample and reported as background- subtracted and the mean of triplicate treatments with standard error. The reproducibility of these findings was verified by three independent determinations of values for three replicates for each treatment and construct.

Western Blotting

Following the indicated treatments, SIGCs were washed and harvested in PBS and pelleted by centrifugation at 4,000 rpm. Cell pellets were lysed in radio-immunoprecipitation buffer (RIPA) from Santa Cruz Biotechnology (Santa Cruz, CA) containing 1% PMSF, sodium orthovanadate, and protease inhibitor cocktailed supplied by the manufacturer. Protein contained within each sample was quantified by the Lowry method. Once normalized for protein content, samples were mixed with a pH 6.8 gel loading buffer containing 25% glycerol, 10% bromophenol blue, β-mercaptoethanol, 3.605% 0.5 M SDS, and electrophoretically fractionated on 8%-10% polyacrylamide/ 0.1% SDS resolving gels from Bio-Rad (Hercules, CA). Fractionated proteins were then transferred to nitrocellulose or PVDF membranes; following this, membranes were blocked for non-specific binding with 5% non-fat dry milk in 1x TBS purchased from Bio-Rad (Tris-buffered saline) containing 0.05% Tween (TBST). Blots were then rinsed briefly and incubated overnight at 4^{o}C in antibodies diluted 1:500 to 1:2000 in TBST. HSP90 (sc-7947), phospho-p44/42 ERK1/2MAP kinase (sc-101760), and LRH-1 (sc-25389) antibodies were purchased from Santa Cruz Biotechnology. The phospho-(S473) Akt (cs-4058) antibody was purchased from Cell Signaling Technology. The CYP19 and antibody was purchased from Sigma. GAPDH and GATA-4 (ab61767) antibodies were purchased from Abcam Biosciences (Cambridge, MA). Immunoreactive proteins were detected by incubation with secondary horseradish peroxidase-conjugated antibodies (Bio-Rad) diluted 3 x

10^{-4} in 1% NFDM in TBST. Blots were then treated with enhanced chemiluminescence (ECL) reagents (Perkin Elmer, Wellesley, MA) and visualized on ECL film (Eastman Kodak, Rochester, NY).

qRT-PCR (quantitative real-time PCR)

Total RNA from cultured cells was extracted with an Aurum RNA minikit (Biorad) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA with the iScript cDNA synthesis kit (Biorad, #170-8891) according to the manufacturer's instructions. qRT-PCR was performed using 2 μl of the cDNA product and the primer pairs described below with the iQ SYBR Green Supermix real-time PCR kit (Biorad, #170-8880) according to the manufacturer's instructions. All qPCR primer pairs were designed to span an exon-exon boundary and were subjected to melt-curve analysis (Applied Biosystems) to ensure single-product amplification prior to use in experiments. Data obtained from PCR reactions was analyzed using the comparative CT method. cDNA was amplified as detailed on an ABI with specific primers of the following sequences:

Rat rCyp19:

Forward: 5'- CTGCTGATCATGGGCCTCC-'3
Reverse: 5'- CTCCACAGGCTCGGGTTGTT- '3

Rat L-19 (housekeeping/control mRNA):

Forward: 5'- CTGAAGGTCAAAGGGAATGTG -'3
Reverse: 5'- GGA CAG AGT CTT GAT GA CTC - '3

RESULTS

Atrazine (ATR) increases human cAMP-responsive gonadal *Cyp19* 'promoter II' (pII) activity in reporter-construct transfected rat ovarian granulosa cells

To assess:

1) whether ATR directly affects *Cyp19* expression in rat ovarian granulosa cells (independent of upstream CNS influence)

2) whether molecular mechanisms and transcription factors involved in *Cyp19* induction in by ATR in the rat ovary are conserved in humans and relevant to human breast cancer- in which aromatase is overexpressed in breast tissue primarily owing to derepression and activation of the conserved cAMP-responsive gonadal *Cyp19* promoter termed 'promoter II' (pII),

spontaneously immortalized rat ovarian granulosa cells (SIRGCs) were transfected with a (cAMP-responsive) h*Cyp19*-pI.3/II luciferase (h*Cyp19*-pII-luc) reporter construct (-571 bp fragment, human and rat cis element alignment depicted Figure 35 A) and treated with: vehicle (DMSO); 30 μM ATR; ATR + 20 μM H89; or 50 μM forskolin (FSK) for 24 hours in serum-free media. These concentrations of ATR and the PKA-inhibitor, H89, were effective in respectively inducing and attenuating h*Cyp19*-pI.3/II-luc activity in other cell types described here and elsewhere, and serum-free media was chosen for the exposure period as sera components are reported to inhibit transcription specifically from *Cyp19* pI.3/II *in vitro* The -571 bp human *Cyp19*-pI.3/II construct contains conserved C/AAT, CRE, GATA, and SF1/LRH1 cis-elements depicted in Figure 33 and the simplified vector map in Figure 35, panel A.

As shown in Figure 35 B, both ATR (2.66-fold) and FSK (2.83-fold) significantly increased h*Cyp19*-pI.3/II-luc activity in rat SIRGC cells relative to vehicle treatment by 24 hours, and induction by ATR was modestly attenuated in cells pre-treated with the PKA inhibitor H89 (1.75-fold).

Consistent with findings in other steroidogenic cell lines, these data demonstrate, in addition to induction secondary to central LH attenuation reported elsewhere in OVX rat models, ATR directly induces CYP19 expression in rat ovarian granulosa cells, in part by PKA-mediated mechanisms, independent of CNS influence.

Further, ATR induction of human gonadal *Cyp19*-pII promoter-reporter activity in transfected rat ovarian granulosa cells indicates ATR regulates transcription factors expressed in the rat ovary with a high degree of functional

homology to those transcription factors misregulated in estrogen-sensitive breast tumors, which are responsible for driving *Cyp19* overexpression from conserved cAMP-responsive promoters pII/I.3 in mammary tumor and supporting stromal cells.

ATR increases human cAMP-response element (CRE) promoter activity in reporter-construct transfected rat ovarian granulosa cells

Aromatase catalyzes the final step of estrogen biosynthesis in humans and rats through irreversible aromatization of androgen precursors. Cyclic AMP response element (CRE)-mediated transcriptional mechanisms are critical in gonadal aromatase expression, generation of androgen precursors specifically, and steroidogenesis generally, as

1) CREs are contained in: the gonadal *Cyp19* promoter, 'pII' (Figure 33); the StAR promoter (Figure 3); and regulatory regions of genes encoding most of the enzymes of steroidogenesis

2) CREs are also regulated and contained in the genes of some transcription factors, such as LRH-1, that exert pressure on CRE elements of steroidogenic genes described above, such that overall expression levels of these transcription factors (upstream, e.g. LRH-1) is also increased through CRE-mediated transcription mechanisms which ultimately increase CRE-regulated steroidogenic enzyme expression (downstream, e.g. p450-c17, CYP19; see Figure 3)

the CREs described above are regulated in all cases by both cAMP-dependent and -independent mechanisms, dependending on the cell type and contextual gene architecture, and both cAMP-dependent and -independent regulation of CRE occupancy have been desribed for regulation of human gonadal *cyp19* pI.3/II. Because CYP19 generates estrogens from androgen precursors, and the ovary is the parenchymal tissue of mammary tumor-promoting estrogen in ATR-susceptible SD rats, it is important to understand if ATR, in addition to inducing CYP19, can directly affect steroidogenesis in the rat ovary generally. Further, if ATR can indeed impact rat ovarian steroidogenesis, it would also be informative to elucidate the degree to which involved mechanisms are conserved in humans.

Thus, as in the previous experiment, the ability of ATR to directly regulate CRE-mediated transcription in the rat ovary, as a proxy of steroidogenesis-and the degree to which regulatory mechanisms are conserved in rats and humans-was assessed in rat SIGC cells transfected with a human CRE-luc construct (Invitrogen) for 24 hours. Following transfection, rat SIGC cells were treated for a 24 hour period with: vehicle; 25 μM FSK (positive control); 10, or 30 μM ATR in full media, after which a luciferase assay was performed as described above. As

shown in Figure, both ATR (1.66 and 3.82-fold for 10 and 30 μM doses) and FSK (12.56-fold) significantly increased human hCRE-promoter activity in rat SIRGC cells relative to vehicle. These findings indicate that ATR, in addition to directly activating *Cyp19* transcription, may affect cAMP-mediated transcriptional programs involved in rat ovarian steroidogenesis (estrogen precursor) generally, as has been reported for *Star* and *P450-Cyp17* (Kovacevic *et al*, 2010) in rat Leydig (testicular) cells, and *P450-Cyp11A1* (Suzawa and Ingraham, 2008) in human placental JEG-3 cells, treated with ATR *in vitro*.

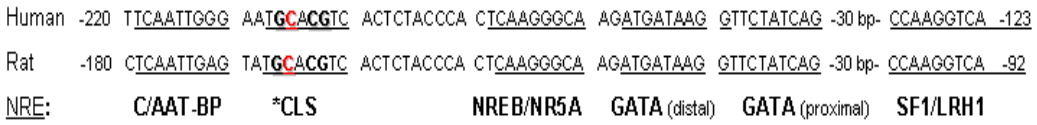
Figure 35:

Effect of ATR on human gonadal h*Cyp19*-pI.3/II-luc reporter and human CRE-reporter activity in transfected rat SIGCs. (A) Alignment of conserved cis-elements in humans and rats contained within the (-571 bp fragment) h*Cyp19*-I.3/pIIluc construct. (B) Rat ovarian granulosa cells (rat SIRGC cells) were transfected with 2 μg h*Cyp19*-I.3/pII-luc for 24 hours prior to exposure to vehicle (DMSO) or the indicated treatments for 24 hours. (C) Rat SIRGCS were transfected with 2 μg hCRE-luc for 24 hours prior to exposure to vehicle (DMSO) or the indicated treatments for an additional 24 hours. Following the treatment period, relative luciferase activity (RLU) was assessed in cell lysates with a luciferase assay kit (Promega) according to the manufacturer's instructions and as detailed in the Materials and Methods. Transfection efficiency and background fluorescence were respectively validated and corrected for via transfections with eGFP, CMV-luc (constitutive), and pgL2 constructs (data not shown). RLU (relative light units) are normalized to protein content and expressed relative to vehicle and are the mean of three replicates with standard error. Similar results were obtained in two independent experiments.

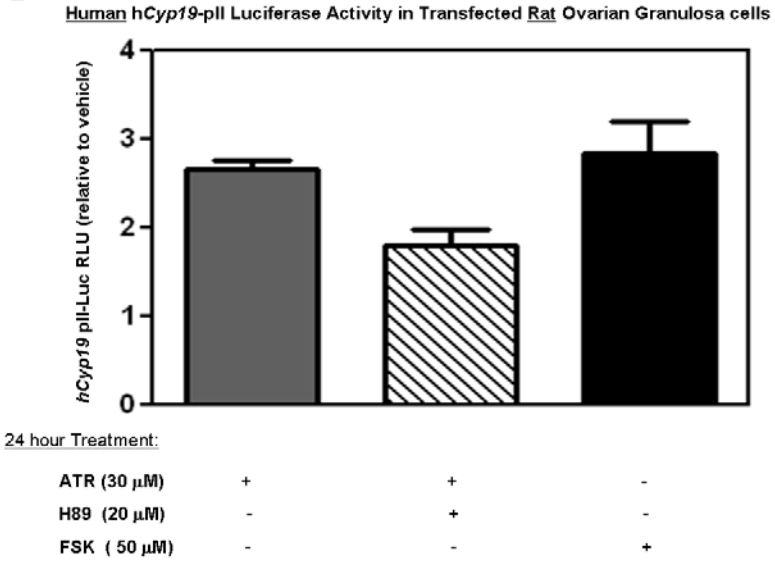
Figure 35

A Species-conserved cis-elements in the cAMP-responsive Gonadal Aromatase Promoter

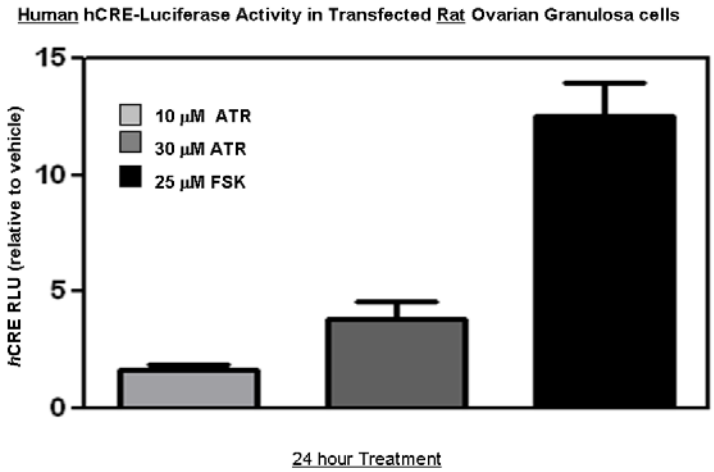
('pII', contained within the human -571 bp h*Cyp19*-pI.3/II-luc construct)



B



C



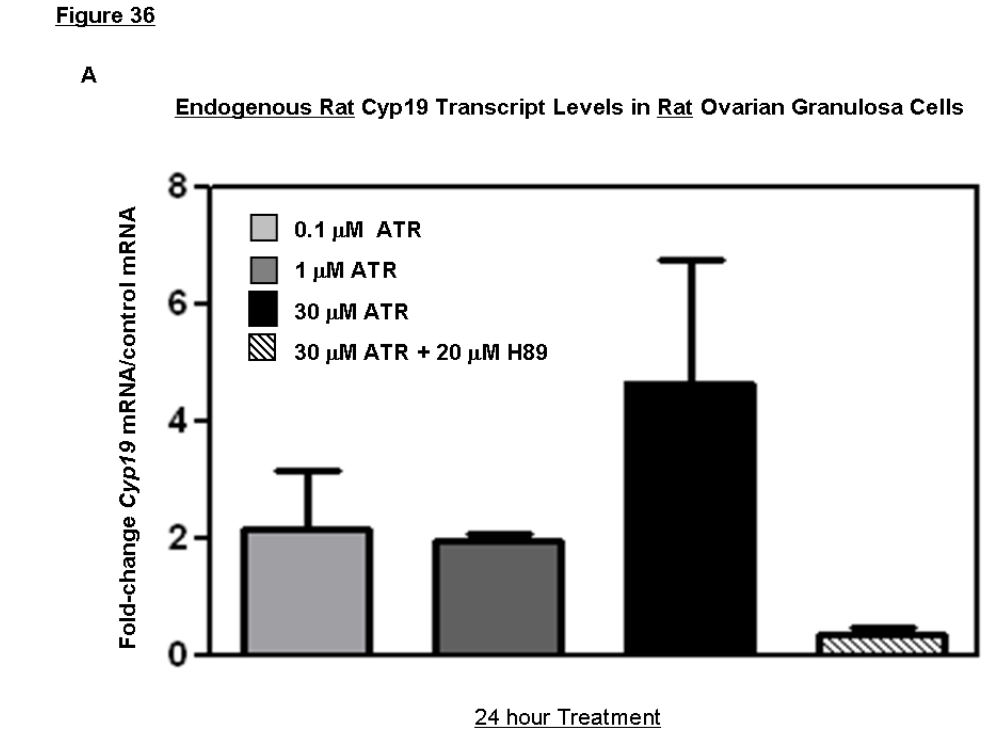
Effect of ATR on endogenous Cyp19 mRNA and protein levels in ovarian rat granulosa cells

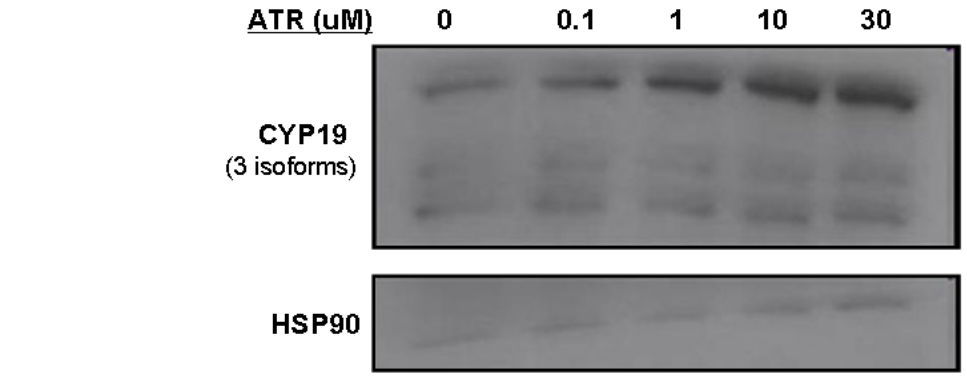
To assess the effects of ATR on endogenous Cyp19 expression at the mRNA level in rat ovarian granulosa cells, rat Cyp19 transcripts were analyzed by quantitative RT-PCR (qPCR) analysis of isolated total RNA collected from rat SIGC cells treated for 24 hours with vehicle or 30 μM ATR in the presence or absence of 20 μM of the PKA inhibitor H89. As shown in Figure 36 A, ATR induced Cyp19 transcript levels by PKA-mediated mechanisms 4-fold over vehicle, however our standard error in quantification was high. At least four Cyp19 mRNA splice variants in the rat ovary are reported the literature; translation of three of these result in non-functional CYP19 protein lacking the heme-containing region of the P450 oxidase domain. The presence of splice variants in cells of the rat ovary are widely reported to complicate quantification of Cyp19 transcripts; the primer pairs used in the depicted experiment were designed to anneal to the transcript region encoding the heme domain of CYP19 in the functional protein, and preliminary gradient curves with this primer pair indicated amplification of a single (specific) gene product.

To assess the effects of ATR on endogenous CYP19 expression at the protein level, SIRGCs were treated as described in the experiment above, and CYP19 levels were detected by western blot as described previously. As shown in Figure 36 B, indeed, biologically relevant doses of ATR dose-dependently induced CYP19 in SIRGCs. Surprisingly in light of numerous exposures performed in human cells, ATR also appeared to modestly induce HSP90 in SIRGCs, which we previous employed as a reliable loading control in T-47D cells. While the biological consequence of the incidental HSP90 induction by ATR we observed in SIRGCs is not known at the time of this writing, in subsequent experiments, we use GAPDH as a loading control in SIRGCs.

Figure 36:

Effect if ATR on endogenous (A) Cyp19 mRNA and (B) protein levels in rat SIRGCs Cells. (A) Q-PCR (B) WESTERN.





ATR increases CYP19, LRH-1, phospho-ERK ½, and phospho-AKT (S473) protein levels in rat SIRGC cells

Both CYP19 and LRH-1 expression are regulated at the transcript level by PKA, PI3K, and MAPK-signaling in ovarian granulosa cells, breast cancer cells, and breast adipose fibroblasts. Additionally, ATR was recently reported to regulate *Cyp19* pII promoter activity in human placental JEG3 cells by MAPK and PI3K-mediated mechanisms (Suzawa and Ingraham, 2008), and cooperation of LRH-1 with GATA factors at *Cyp19* pII is enhanced by phosphorylation of LRH-1 and GATA factors at conserved amino acid residues by the same kinases (Bouchard *et al*, 2005). Moreover, protein levels of of LRH-1 (and GATA factors) may be stabilized by phosphoylation when PKA, PI3K, and MAPK are activated by upstream regulators of steroidogenesis (Stocco, Carlos 2008; Figure 2).

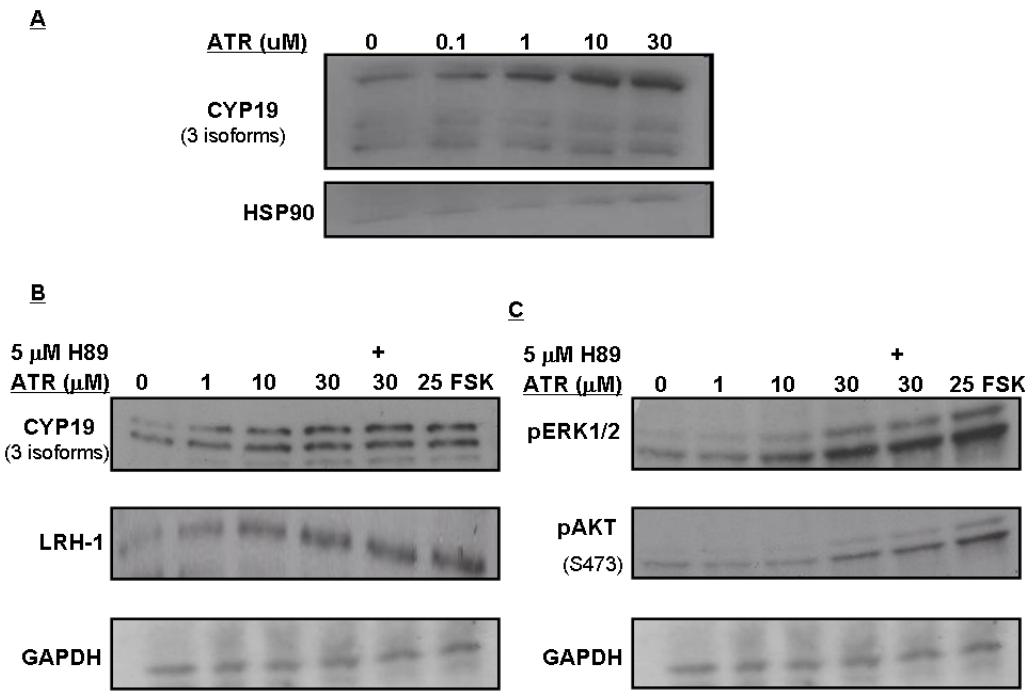
While post-translational modification (PTmod) of CYP19 is not widely reported at the time of this writing, P450-c17, critical for androgen production and just upstream of CYP19 in the estrogen biosynthesis pathway (Figure 3), is stabilized by PKA PT phosphorylation, and PT mods to CYP19 by any of the named kinases could account for sustained levels of aromatase expression in the human follicular, and rat proestrus/estrus phases that are difficult to reconcile with low Cyp19 mRNA abundance in the same tissues.

To assess the effects of ATR on endogenous CYP19 protein levels in the rat ovary and putative upstream kinases reported to regulate ovarian Cyp19 expression at the transcript level, rat SIGC cells were treated with vehicle or increasing concentrations of ATR in the presence or absence of 5 μM H89, and levels of the indicated proteins were assessed by western blot analysis of fractionated cell extracts as described in the Materials and Methods. Exposures were performed in serum-free media for 24 hours in cells serum-starved 24 hours prior to treatment. As shown in Figure, ATR dose-dependently induces: all three of the CYP19 isoforms observed in rat SIGC cells (up to four reported in ovarian cells from other rat strains); LRH-1; and consistent with other tested rat cell lines but not human T-47D breast cancer cells, HSP90. ATR modestly increased phospho-Akt (S473) levels and robustly increased phospho-ERK ½ levels at 24 hours, which is further consistent with a recent study in which ATR activation of *Cyp19* pII was attenuated in JEG3 cells pre-treated with inhibitors of these kinases prior to ATR exposure [Suzawa and Ingraham, 2008]. Similar kinase inhibitor studies could not be replicated here as rat ovarian granilosa cells, unlike JEG3 cells, are strongly dependent on cAMP and PI3K signaling for survival, and preliminary experiments indicated that concentrations of H89 and LY294002 higher than 5 μM resulted in cellular death that could confound interpretation of results. Nevertheless, these studies demonstrate ATR increases CYP19, LRH-1, phospho-AKT and phospho-ERK levels- molecular mediators of ovarian estradiol biosynthesis in the parenchymal tissue of rat mammary tumor promotion, indepedent of the primary central LH attenuation postulated solely responsible for mammary tumor induction by ATR in intact SD rats.

Figure 37 :

ATR dose-depedently increases CYP19, LRH-1, HSP90, phospho-ERK1/2 and phospho-Akt levels in rat ovarian granulosa cells. (A) Dose-response for CYP19 and HSP90. (B) Dose respose for CYP19, LRH-1, and (C) upstream kinases that regulate these. Rat SIGCs were serum-starved for 24 hours prior to exposure to the indicated treatments in serum –free media for an additional 24 hours. A western blot analysis with antibodies against the indicated proteins was performed following the treatment period as described in the Materials and Methods. Samples were normalized for protein content prior to electrophoresis and GAPDH was selected as a loading control as levels were not affected by vehicle or any concentraion of ATR tested. These results were verified in two or more independent experiments.

Figure 37


A
ATR (uM) 0 0.1 1 10 30
CYP19
(3 isoforms)
HSP90
B
5 μM H89 +
ATR (μM) 0 1 10 30 30 25 FSK
CYP19
(3 isoforms)
LRH-1
GAPDH
C
5 μM H89 +
ATR (μM) 0 1 10 30 30 25 FSK
pERK1/2
pAKT
(S473)
GAPDH

DISCUSSION

Like the majority of early human cases, mammary tumor growth in rats is estrogen-dependent, in that ovariectomy or chemically blocking estrogen receptor (ER) action effectively attenuates mammary tumor growth (Eldridge *et al*, 1999; Russo and Russo, 1996). Similarly and perhaps counter-intuitively, in both species, it is actually females in reproductive senescence that are disproportionately afflicted with mammary carcinomas, as women in menopause and anovulatory rats (Cooper *et al*, 2007) constitute the majority of estrogen-dependent cases. Although aberrant aromatase regulation and consequent elevated estrogen promote mammary tumor growth in both species, at the tissue level, the principal site of estrogen synthesis in each differs.

In PM women, estrogen dysregulation occurs primarily in several different cell types that comprise the mammary gland and stroma; derepression and robust aberrant activation of multiple *cyp19* promoters typically quiescent in healthy breast tissue leads to marked local estrogen elevations in malignant tissue without affecting plasma concentrations (Bouchard *et al*, 2005). Conversely, in the senescent Sprague Dawley (SD) and LE rat strains, anovulatory aromatase-positive ovarian granulosa cells are the principle source of estrogen hypersecretion (Cooper *et al,* 1996 and 2007; Eldridge *et al*, 1999), and estrogen produced from these follicles travels in the blood to mammary tissue where it promotes the growth of ERα^+ neoplasms.

Atrazine was shown to increase mammary tumor incidence and elevate plasma estradiol levels in intact but not ovariectomized (OVX) SD rats, implicating a critical role for ovarian estradiol in ATR-induced rat mammary tumorigenesis, in registrant-conducted lifetime feeding studies publicly disclosed at least as early as 1996 (Cooper *et al*, 1996). Plasma estradiol levels were unaffected in Fisher 344 rats subjected to an analogous dosing schedule by the registrants, and tellingly, regardless of ovary status, F344 rats are also insuceptible to mammary carcinogenesis by ATR. In the many years since the seminal SD mammary oncogenicity findings were made publicly available, a large number of industry-funded 'mechanistic' studies have been conducted in OVX and respectively tumor-insusceptible and tumor-untested F344 and Long Evans (LE) rat strains. In these strains irrelevant to the question of ATR-induced oncogenicity in the ovary-intact SD rat, registrant-funded scientists repeatedly demonstrate ATR suppresses luteinizing hormone (LH) secretion at the central level; they extrapolate observed LH suppression in OVX F344 and LE rats as a theoretical mechanism of mammary tumor promotion by ATR in intact SD rats.

Briefly, in untested ovary-intact SD rats, registrants speculate direct LH surge insufficiency or suppression by ATR at the hypothalamic/pituitary level observed in other strains theoretically:

(1) is critical for secondary/indirect ovulation failure, retention of immature CYP19+ follicles, and attendant secretion of mammary tumor-promoting levels of estrogen into the plasma of SD rats;

(2) is analogous to the 'natural' reproductive aging process in the SD rat, in which senescence is characterized by low LH and consequent high plasma E_2;

(3) means ATR does not induce mammary tumors in the ovary-intact SD rat *per se*, but rather "atrazine could be promoting a premature senescence of reproductive and mammary tissues" (Eldridge *et al* 1994); or "accelerates the rate of developing pathology" (Eldridge *et al*, 1999); or "promotes development of mammary gland tumors by inducing a premature reproductive senescence and thus creating a hormonal milieu conducive to tumor growth" (Eldridge *et al*, 1994).

In so many words, the registrants hypothesize ATR '*accelerates the natural reproductive aging process*' (Eldridge and Cooper), so that dosed SD rats merely present with mammary tumors in the prime of their reproductive life rather than only in senescence. Because the registrants take as a premise theoretical direct and central LH-suppression by ATR is critical for secondary anovulation, retention of CYP19+ ovarian follicles, and attendant tumor-promoting estradiol secretion typical of SD senescence, they further argue this unvetted mechanism of rat mammary tumor promotion is irrelevant to human BC risk as menopause in women is characterized by high LH, quiescent or LH-irresponsive ovaries, and low plasma E_2 (Eldridge *et al* 1999; Cooper *et al*, 2007).

As discussed at length in the previous chapter and in the present's introduction, at the molecular level, aromatase is overexpressed in malignant breast tissue primarily from deprepession of conserved gondal cAMP-responsive *cyp19* promoters 'pI.3/II' typically silenced in healthy breast tissue, and the GATA and NR5A family members that transactivate these gonadal promoters in malignant epithelial and tumor-proximal cells are conserved in the rat ovary and are in fact robustly regulated downstream of FSH/cAMP in estrus. Thus, as we previously demonstrated ATR induces CYP19 and affects expression or activity of conserved upstream regulators by PKA-mediated mechanisms in human T-47D cells, we herein sought to determine whether ATR could affect expression of these same factors in rat ovarian granulosa cells independent of neuroendocrine or LH influence.

Indeed, for the first time, we show physiologically relevant concentrations of ATR dose-dependently induce endogenous aromatase expression at the

mRNA (Figure 36 A) and protein levels (36 B) by PKA-mediated mechanisms in pre-ovulatory spontaneously immortalized rat ovarian granulosa cells (SIRGCs) derived from BD (Berlin Duckey) rats. Consistent with previous reports on rat ovarian cells, we detected three CYP19 isoforms in SIRGCs by western blot (36 B). Also consistent with other reports, multiple Cyp19 mRNA splice variants may have complicated mRNA-level assessments by qPCR in the present work (Figure 36), as our standard error in quantification was high; however, after a survey of the literature before experiments were begun, we designed specific qPCR primters to span an exon-exon boundary contained with the fully-functional (heme-domain containing) rat Cyp19 mRNA splice variant, and preliminary gradient curves with this primer pair indicated amplification of a single (specific) gene product. Of the three rat CYP19 isoforms we detected by western blot (Figure 36), we found that of the highest molecular weight (52 to 60 kD) ATR-inducible. Though the ATR-inducible band we observed is in the range of the molecular weight reported for the active CYP19 isoform in the literature (contains P450 heme domain that is spliced-out exonally in non-functional/truncated lower molecular weight variants), aromatase activity assays or other measures of estradiol production are warranted to determine if the CYP19 isoform induced by ATR in SIRGCs is indeed active (examples of future studies are provided in the next chapter). Surprisingly in light of countless other experiments we performed in human T-47D cells, we found HSP90, used commonly as a loading control by others and in the present work, was modestly inducible by ATR in SIRGCs (Figure 36 and 37). HSP90 is a molecular chaperone of several steroid receptors; though estrogen is synthesized via CYP19 activity in rat granulosa cells for the purpose of proximal endometrial expansion in estrus, its receptor, ERα, is not reported as having functional significance in granulosa cells themselves, and the biological consequences of HSP90 induction by ATR in SIGCs, while intriguing, are not known to us at the time of this writing. GAPDH was used as a reliable loading control in all western blots performed subsequent to our incidental discovery of HSP90 induction in SIRGCs by ATR.

Also for the first time in rat ovarian cells, consistent with previous reports in other cell types (Suzawa and Ingraham, 2008), we show ATR dose-dependently induced upstream activators of *Cyp19* known to be regulated by PKA-mediated mechanisms in human breast cancer, breast adipose fibroblast, and rat ovarian granulosa cells, including LRH-1, phospho-ERK1/2 (MAPK), and (serine 473) phospho-Akt (Figure 37). We demonstrated ATR activates *cyp19* transcription by PKA- and PI3K mediated mechanisms in human T-47D cells in the previous chapter; our finding of phospho-Akt induction in rat granulosa cells further supports novel PI3K-mechanisms for ATR, and findings in *Cyp19*-pII reporter (Figure 35) and mRNA studies (Figure 36) in SIRGCs here validate PKA-mediated mechanisms of ATR. Rat ovarian granulosa cells, *in vivo* being strongly dependent on FSH for survival, *in vitro* are highly sensitive to PKA-inhibition, and unfortunately the degree to which PKA mediates the effects of ATR on many of the described upstream factors could not be robustly interrogated as treatment of SIGCs with effective concentrations of the PKA

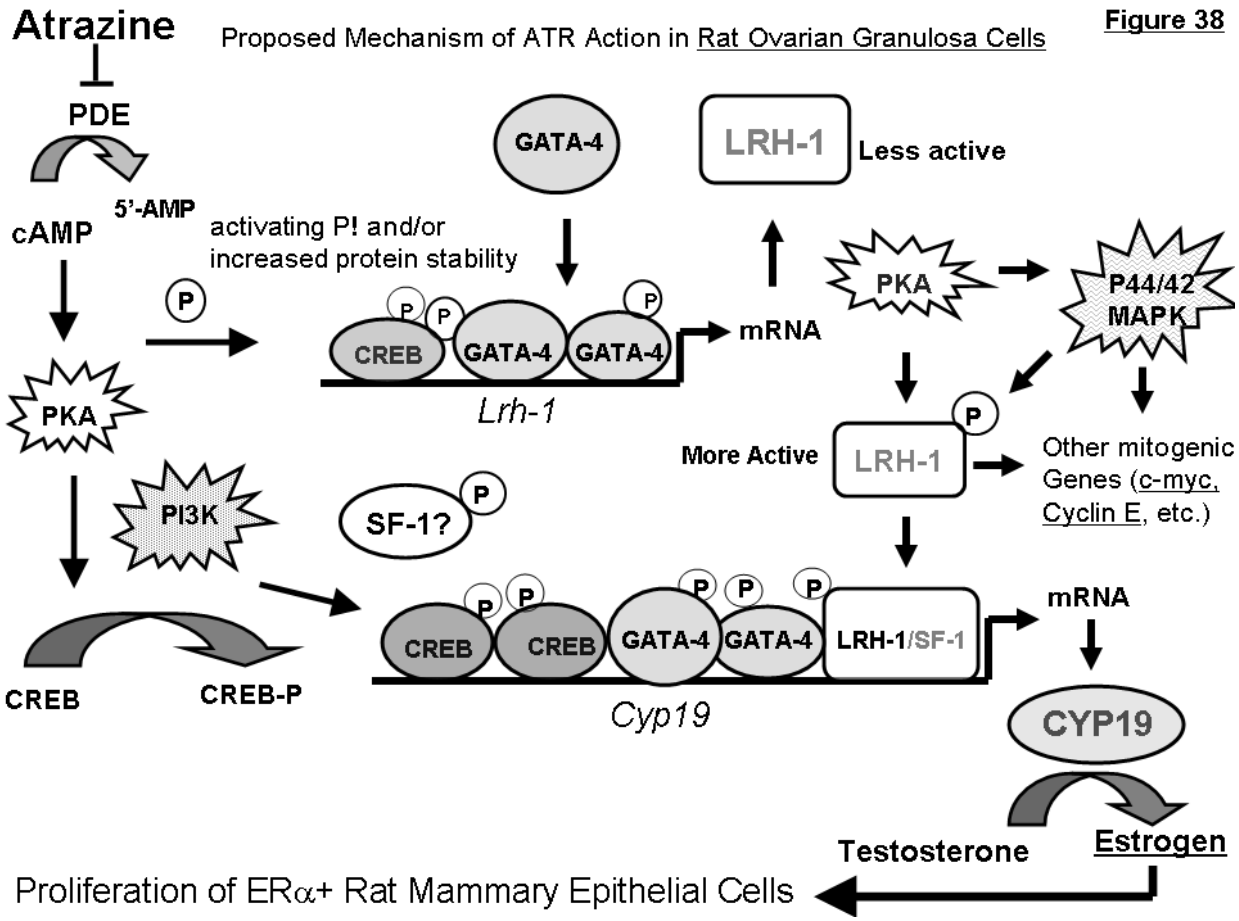
inhibitor H89 (> 5 μM; 20 μM used in our previous studies in T-47D cells) frequently resulted in cell death in culture. The strong dependence of these cells on cAMP for survival also complicated upward titrations of the positive control for cAMP, FSK (forskolin, activator of adenylate cyclase; we only used 25 μM), as SIRGCs in 5% FBS-supplemented media divide in less than 24 hours and confluence is a concern in studies of CYP19 expression. Nevertheless, although phospho-CREB antibodies were not available at the time of experimentation, further indication that ATR may affect many of the described factors by conserved PKA-mediated mechanisms is provided in Figure 35 C, where we show ATR dose-dependently increases human CRE- (cAMP-response element) reporter activity (~4-fold) in transfected rat promoter cells. We show phospho-ERK1/2 is induced by ATR in SIGCs; certain MAPKs are known to be activated downstream of PKA; we also show LRH-1 is induced in SIRGCs- which, along with GATA-4 (lost in the majority of established ovarian cell lines in culture as it was here; Xu *et al*, 2003), is further known to sustain protein-stabilizing and transcriptionally activating phosphorylations by PKA, MAPK, and PI3K/Akt (Stocco, 2008 and 2007; Tremblay and Viger, 2003) in rat granulosa cells.

Despite SD rat mammary tumor findings and a wealth of studies characterizing ATR's effect of *Cyp19* induction by conserved and human BC-relevant cAMP-mediated mechanisms in several other human and rat steroidogenic cells *in vitro*, ATR is currently classified by the EPA as 'not likely carcinogenic to humans' due to 'lack of human and mechanistic data' (FIFRA; Gammon *et al*, 2005). As depicted in Figure 33, aromatase is critically overexpressed from cAMP-responsive gonadal promoters via highly conserved epigenetic and molecular mechanisms in the parenchymal cell types of hyperestrogenicty and mammary tumor promotion, of ovarian- and breast tissue-origin, respectively, in rats and humans. We illustrate this high degree of conservation in the present work most compellingly in Figure 35, where ATR induces human gonadal *cyp19*-pII/I.3-reporter (-571 bp) activity (transcription of human aromatase driven from cAMP-responsive gonadal promoters) in transfected rat ovarian granulosa cells (ATR-regulated rat trans-acting factors share homology sufficient to transactivate human *cyp19* transcription from pII/I.3). Taken together with dose-dependent inductions of endogenous CYP19, LRH-1, phospho-Akt and ERK1/2, our findings in SIRGCs demonstrate ATR can directly induce CYP19 in rat ovarian cells by highly conserved cAMP/PKA-mediated mechanisms independent of central neuroendorcrine influence, which undermines the hypothesis that hypothalamic/pituitary-level LH suppression/attenuation is critical for secondary: anovulation; ovarian aromatase dysregulation; hyperestrogenicity as a result of persistent estrus; and ultimately mammary tumor promotion by ATR in rats. Further, as the SIRGC cells we employed in these experiments are derived from Berlin-Duckey (BD) rats, our findings suggest ATR may induce ovarian aromatase and estradiol biosynthesis in other strains in addition to the SD rat. The mechanisms by which we propose ATR directly induces CYP19 in rat ovarian granulosa cells are provided in Figure 38.

Figure 38:

Putative Conserved Mechanisms of Aromatase Induction by ATR in Spontaneously Immortalized Rat Ovarian Granulosa Cells (SIRGCs). ATR is inhibits PDE (phosphodiesterase), leading to intracellular elevations in the master regulator of steroidogenesis, cAMP. We show ATR induces CYP19 by PKA-mediated mechanisms, and dose dependently induces LRH-1, a known transcriptional activator of rat Cyp19 in ovarian cells with congnate cis-elements in both human and rat gonadal *cyp19* promoters. Although, as here, GATA-4 is frequently lost in the majority of established ovarian cell lines in culture (Xu *et al*, 2003), we previously found it induced by ATR in T-47D cells (Chapter 1), and propose on this basis and recombinant studies performed in rat granulosa cells GATA-4 may also regulate *cyp19* and *lrh-1 i*n ATR-stimulated SIRGCs. Further, consistent with other reports conducted in JEG3 and H295R cells, we show ATR dose-dependently induces phospho-Akt (S473) and phospho-ERK-1/2, which, along with PKA, transcriptionally activate LRH-1 (Hu *et a*l, 2009) and GATA-4 at gonadal *cyp19* promoters downstream of FSH in rat ovarian granulosa cells (Stocco *et al*, 2007 and 2008). We posit direct induction of ovarian CYP19 by the indicated conserved pathways as plausible molecular mechanisms of observed elevations in plasma estradiol levels and attendant mammary tumor growth induced by ATR in intact SD rats, which is underscored both by countless molecular studies of ATR action in other cell types, and by the fact that ovariectomy protects susceptible SD rats from elevated plasma estradiol and mammary tumor induction by ATR. These depicted mechanisms are conserved in human BC cells, breast tumor-proximal fibroblasts, and endometrial cells. Thus, addition to providing key molecular insight into ATR-induced rat mammary tumorigenesis, our findings have significant implications for human breast and reproductive cancer risk.

Figure 38


Atrazine
Proposed Mechanism of ATR Action in Rat Ovarian Granulosa Cells
PDE
5'-AMP
cAMP
activating P! and/or
increased protein stability
GATA-4
LRH-1
Less active
PKA
P44/42
MAPK
CREB
GATA-4
GATA-4
mRNA
Lrh-1
PKA
More Active
LRH-1
Other mitogenic
Genes (c-myc,
Cyclin E, etc.)
PI3K
SF-1?
CREB
CREB-P
CREB
CREB
GATA-4
GATA-4
LRH-1/SF-1
mRNA
Cyp19
CYP19
Testosterone
Estrogen
Proliferation of ERα+ Rat Mammary Epithelial Cells

Chapter IV

Conclusions and Future Directions

Relevance of the Present Work to Previous Studies and Gaps in Mechanistic Understanding of Inter-Individual Risk

We for the first time demonstrate ATR induces aromatase and hallmarks of ER+BC in human ER+ T-47D BC cells, human adipose fibroblasts, and rat ovarian granulosa cells by conserved GATA-, LRH-1-PKA-, and PI3K- mediated mechanisms relevant to rat mammary tumor promotion by ATR and highly relevant to human breast and reproductive cancer risk. Though the cell lines interrogated in our studies are novel, the mechanisms by which we propose ATR affects these cells- apart from those mediated by GATA- are well-established.

The aggregate of data collected in the fifteen years since the original rat mammary carcinogenecity studies demonstrate ATR is a potent endocrine disruptor which elicits anti-androgenic and estrogenic reproductive effects by conserved mechanisms at biologically relevant doses, often far below the current US EPA drinking water standard of 3 ppb, in laboratory and wildlife models of every vertebrate class studied to date. The estrogenic effects of ATR via CYP19 induction in particular have also been verified in human and rat cancer cell lines derived from placental, adrenal, testicular and ovarian tissues (Suzawa and Ingraham, 2008). Collectively these findings suggest chronic ATR exposure may be hazardous to human health, as risk for several emerging estrogen-related disorders ranging from infertility to obesity are influenced by cumulative lifetime estrogen exposure- as is risk of carcinogenesis of both the human breast and rat mammary gland (Laws *et al*; 2003; Rudel *et al*, 2007). Although the EPA generally "presumes animal tumor studies to be indicative of human cancer potential" (Olin *et al*, 1996), and the SD rat strain is historically and currently the preferred model of the EPA for screening environmental endocrine disruptors and mammary cancer hazards (see *Bibliography of EDCs*; and Spearow J, 2004), in their IRED documents for ATR published in 2003, the EPA states LH surge insufficiency observed in experiments conducted in OVX and tumor-insusceptible (LE and F344) models can be extrapolated as a theoretical mechanism underlying confirmed elevations in blood estradiol and mammary tumor incidence in the intact SD rat. On the basis of this theoretical neuroendocrine hypothesis never tested in intact SD rats, the EPA further states ATR promotes "mammary tumors by a species-, strain-, and sex- specific mode of action that is the result of prolonged exposure to endogenous estrogen", and further affirms "there is no established or even likely neuroendocrine path through which atrazine could induce cancer in humans by a mode similar to that occurring in female SD rats relevant to humans" (FIFRA, 2000-2005).

The IRED (Interim Registration Eligibility Decision) documents here cited were made publicly available by the EPA one week after EU member states announced a ban on ATR use in 2003, the same year the EPA held approximately 50 private meetings with Syngenta for the purposes of soliciting *ad hoc* committee advisors from Syngenta and the Syngenta-funded contract lab,

Exponent, to inform its IRED documents on ATR (Ivory, D 2010; Sass and Colangelo, 2006). Exponent contract scientists funded by General Motors, Mobil Corporation, Proctor and Gamble, and numerous other conglomerates have published extensive literature contrary to consensus scientific opinion on the health risks of asbestos, tobacco, chromium, and cell phone use regulatory agencies have cited in decisions setting acceptable exposure levels for these hazards (see *NRDC Comments on Asbestos Panel*, 2007). Exponent is generally poorly regarded in the independent community as a 'product defense firm' stakeholders pay for the purpose of influencing policy (ibid).

Although neuroendocrine-independent, species-conserved, direct, and cAMP-mediated mechanisms of CYP19 inducibility by ATR have been robustly characterized in animal and cell line models in many labs outside the stakeholder research community, these are largely not included as relevant literature cited in SAP (scientific advisory panel solicited by the EPA from the stakeholder community) risk assessments of ATR, as Syngenta-funded (registrant) studies are presumed by the EPA to 'supercede' other studies, being, as senior EPA policy analyst William Jordan stated in 2006

> scientifically more robust than are the studies generated by people in academia, generally because companies spend more money on their studies and can attend to details that are potentially important that people in academia just can't afford to do. I know that people might not agree with this proposition, but I believe that the scientists at EPA constitute a peer-review. Our scientists go over the studies with a fine tooth comb.
>
> Jordan quoted in interview cited in 2010 *Scientific American* Article by Danielle Ivory and Huffington Post Investigative Fund

<u>Relevance of the Present Work to Previous Mammary Oncogenicity Findings in the SD Rat Model</u>

While financial backing and statistical power are irrefutably more easily attained in stakeholder-affiliated labs, many independent scientists are skeptical about the rigor and efficiency the EPA assesses these tremendous resources are utilized in the case of ATR in EPA-exclusive peer-reviews (Ivory, D, 2010). Critics of the process argue legitimate attempts to uncover mechanisms of mammary tumorigenesis in the ovary-intact SD rat at the molecular level and their relevance to human health are lacking, as in the more than 6,000 largely unpublished stakeholder studies submitted to the EPA for ostensibly this purpose- contrary to less cited studies undertaken in independent labs- none by stakeholders have actually been conducted in intact SD rats or human or rat pre-ovulatory ovarian cell lines. Dr. Wagner, who studies ATR at the University of Texas, commented in an NRDC interview, "what worries me is the possibility that there isn't time or energy within EPA to give a lot of oversight to this unpublished,

industry-funded research, especially when the number of unpublished studies for a chemical like atrazine are in the thousands." (quoted in article by Ivory, D, 2010)

Though the SD rat model is not perfect, it remains the long-standing preferred strain of the EPA and NTP (National Toxicology Program) in screens of environmental endocrine disruptors and human BC hazards, except for where ATR is concerned (Olin *et al*, 1996; Fenton *et al* 2007; see also *Bibliography of EDCs*). Despite tissue-source differences in estrogen production in senescence, ERα activation promotes the growth of the majority of early stage mammary tumors in both humans and SD rats, and carcinomas in both species develop similar molecular and morphological hallmarks at an analogous pace with a similar sporadicity (Fenton *et al*, 2007). Though the SD rat exhibits a high incidence of spontaneous estrogen-dependent mammary tumors in senescence relative to the ATR-insensitive and progesterone-dominant F344 rat strain- this further strengthens the case for it as a useful model of human BC risk (Rudel, R, 2006)- as one in eight women will be diagnosed with BC in their lifetimes, and the majority of cases are estrogen-dependent and afflict PM women.

CONCLUSIONS

Aromatase inhibitors, which were initially vetted for clinical efficacy in the intact SD rat, are currently our first-line therapy against human BC (Brodie *et al*, 2009). In the present work we show ATR directly induces aromatase expression in (BD-derived) rat ovarian granulosa cells independent of upstream neuroendocrine influence, by conserved mechanisms we also showed operative in human ER+ T-47D BC cells and adipose fibroblasts. These findings may provide mechanistic insight critically needed to inform remaining questions as to differences in ATR susceptibility between rat strains and meaningful strain choice in ATR risk assessment. Moreover, taken together with findings presented in the previous two chapters, the simple human *cyp19*-pII/I.3 reporter studies conducted in BD rat granulosa cells here have novel implications for conserved mechanisms of rat mammary tumor promotion by ATR and may inform remaining questions surrounding the relevance of SD rat mammary tumor induction by ATR to human inter-individual BC risk.

FUTURE STUDIES AND DIRECTIONS

Though ATR is known mammary carcinogen in SD rats, and the conserved mechanisms by which it induces CYP19 in many human and rat steroidogenic cells relevant to human BC are well-characterized elsewhere, ATR is presently classified by the EPA as ‘not likely carcinogenic to humans’ due to ‘lack of human and mechanistic data’. Thus, in the present work, we sought to address gaps in our understanding of mechanisms of ATR action enumerated in

the regulatory literature, yet much remains to be explored in terms of the functional consequences of the mechanisms we uncovered in human adipose fibroblasts and BC cells, and additionally rat ovarian granulosa cells.

Potential Assay Designs to Determine Functional Consequence of CYP19 Induction by ATR

The most imminent question is whether CYP19 induction by ATR in human BC or adipose fibroblasts promotes the proliferation of ER+ BC cells. CYP19 converts androgens to estrogens, and the most obvious experimental set-up to determine mitogenic potential by ATR would simply involve co-incubation of androgen substrate in ATR-treated cells of interest that are pathologically growth-promoted by estrogens. Expression of functional androgen receptor (AR), which may have independent effects on the cell-cycle at least in the in the case of AR+ T-47D cells, complicates this set-up- theoretically and in our hands. Potentially, chemical or biological knock-down of AR in T-47Ds, or substitution of an ATR-susceptible, ERα+, AR- BC of relevant cell line in place of T-47D may obviate the problem of androgen-mediated effects on the cell cycle in experiments to determine whether CYP19 induction results in mitogenic estrogen production. Additionally, as depicted in Figure 39, co-culture or 'conditioned media' experiments could be performed- where human fibroblasts or rat ovarian granulosa cells are incubated with testosterone and vehicle or ATR for a 24 hour period after which, CYP19+-cell 'conditioned media' is transferred to (ER+ or ERE-reporter-gene transfected) human breast or rat mammary cancer cells, respectively.

Figure 39:

Possible Experimental Strategies To Elucidate Functional Consequences Of CYP19 Induction By ATR In The Cell Types Described. Androgens are critically aromatized by CYP19 in estradiol biosynthesis, however AR-mediated effects on cell cycle progression may confound results in experiments to determine estrogen-mediated proliferation. Co-culture experiments may in part obviate this problem, as may ERE-reporter genes and/or chemical or biological steroid receptor knock-down. While not as functionally significant, a simple CYP19 assay could similarly be performed, where E2 IS measured by ELISA or RIA, possibly in combination with tritiated water release (as testosterone is aromatized), in ATR and testosterone (tritated testosterone depending on assay design) co-treated cells (tritiated water release for CYP19 activity described in Lephart and Simpson, 1991; performed with ELISA described in Sanderson *et al*, 2004).

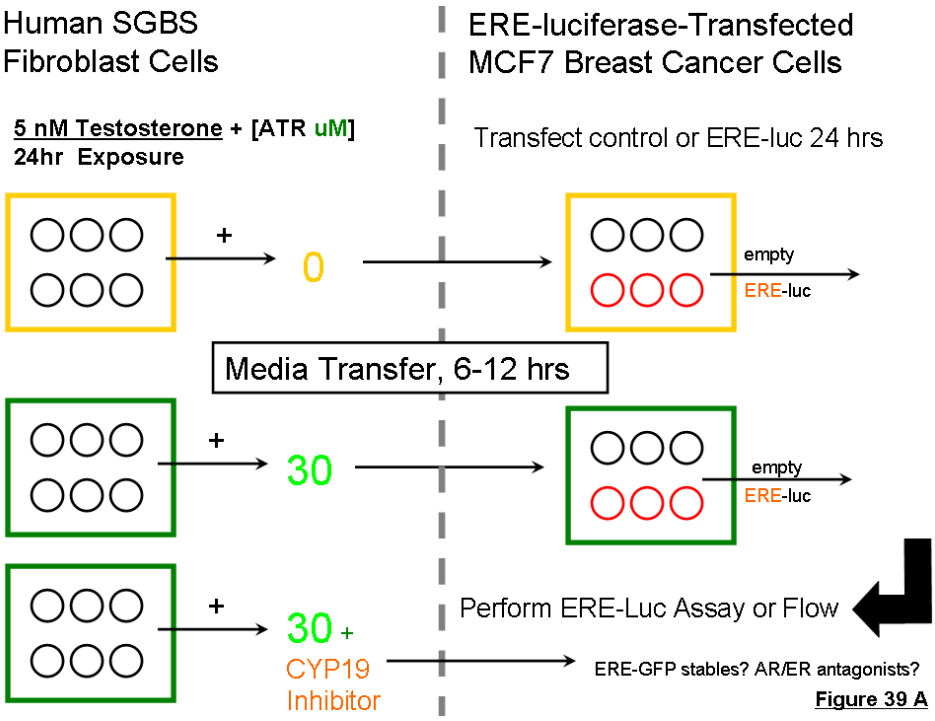


Figure 39 A

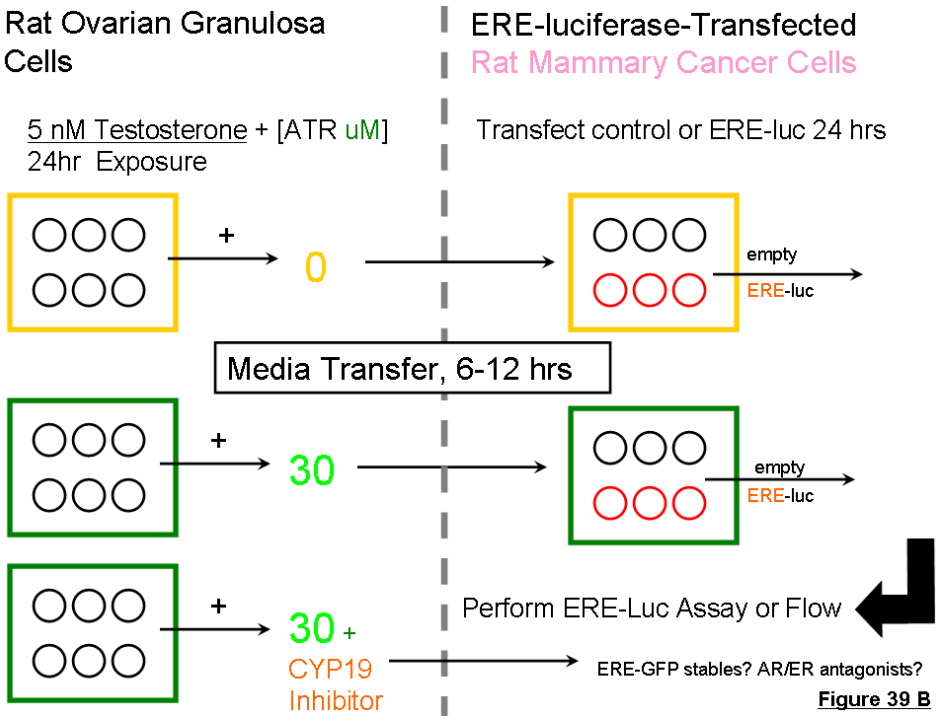


Figure 39 B

Future ChIP Assay Designs

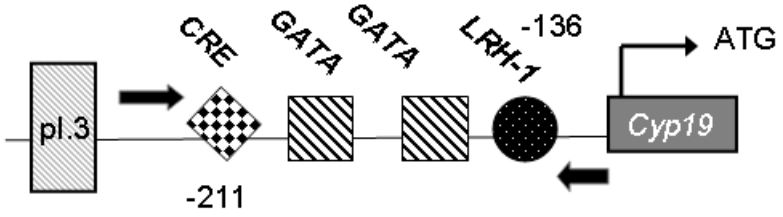
Because: we demonstrated LRH-1 is recruited to *cyp19* in the (putatively) hypomethlyated LBNL variant which thereafter became ATR-irresponsive after undergoing drastic phenotypic changes, and others have shown CpG dinucleotide hypomethylation is critical for pII-CRE recruitment in gel shift assays (Demura and Bulun, 2008); CREB recruitment may precede LRH-1 and GATA-recruitment to ATR-regulated genes of interest; in addition to CYP19, ATR induced LRH-1, ERα, and GATA expression at least at the protein level in the LBNL T47D variant, ChIP assays depicted in Figure 40 may provide additional mechanistic insight in ATR-responsive cell line of interest.

Figure 40:

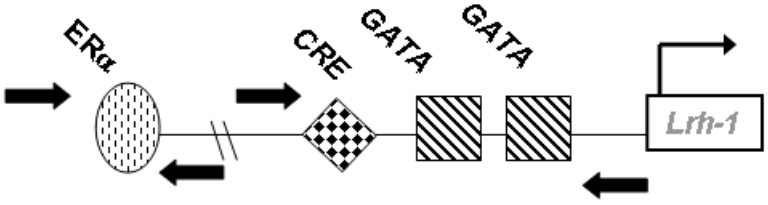
Potential ChIP Assay Designs in the study of ATR. Cis- and trans- ChIP targets that could inform mechanisms of action in ATR-responsive cell lines of interest are depicted. An ERE in the 5'- enhancer region exists > -1000 bp from +1 in the *lrh-1* gene; an ERE also exists in the 3'- enhancer of the *gata-3* gene.

ChIP Experiments That Could Inform Mechanisms of ATR Action in pII-hypomethylated breast cancer or adipose fiboblast cells

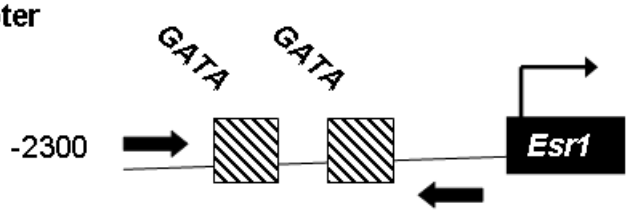
Predicted ATR-regulated Transcription Factor Binding Sites within the *cAMP-responsive gonadal cyp19 promoter* 'pII'



Predicted ATZ-regulated Transcription Factor/Enhancer Binding Sites within the *lrh*-1 Promoter



Predicted ATZ-regulated GATA Transcription Factor Binding Sites within the *esr1* (ERα) promoter



Predicted ATZ-regulated ERα Enhancer Site within the *Gata-3* 3'UTR

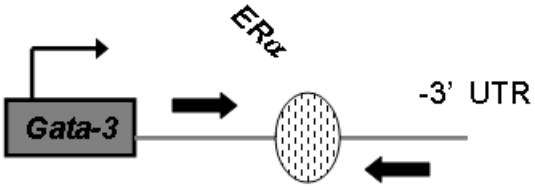


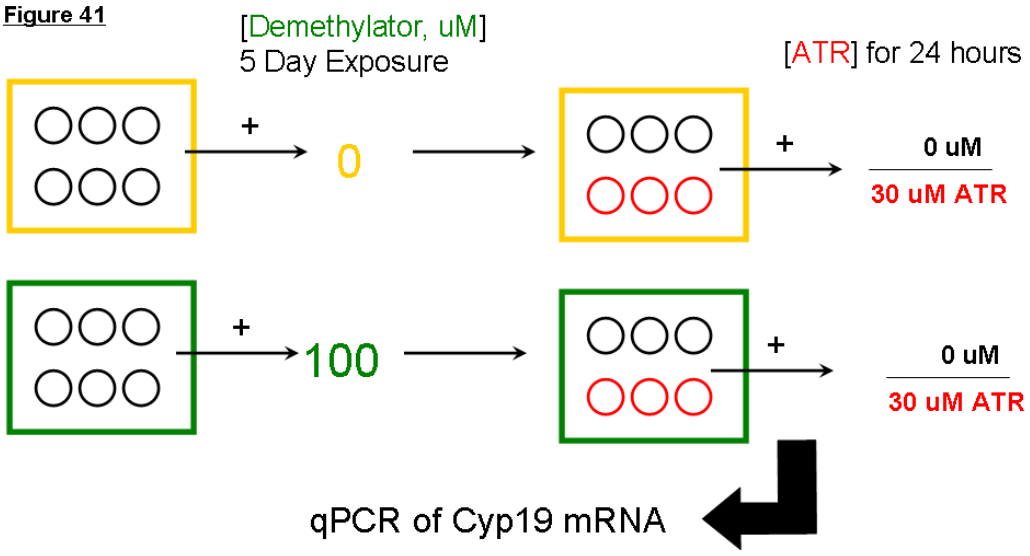
Figure 40

Lastly, of particular interest to the author here writing, is whether DNA methylation was actually responsible for the loss of ATR-responsiveness in the LBNL T-47D variant characterized in Chapter 1. Because we demonstrated DNA methylation conferred ATR-responsiveness to formerly poorly responsive 'ATCC' T-47D cells, if the LBNL variant sustained methylation changes in culture that affected its response to ATR, treating non-responsive LBNL variants with 5'-aza-dC may re-establish the robust ATR-responsiveness seen in this T-47D subline before it underwent drastic phenotypic changes after freezing and subsequent population doublings in culture. Treatment protocols of interest only to the author are depicted in Figure 41; in combination with these experiments, methylation-specific PCR of *cyp19* pII in alternately responsive T-47D cell lines described in Chapter 2 would also be interesting to the author.

Figure 41:

DNA methylation Inhibition Assay Designs that Could Inform Questions Surrounding Event that Spontaneously Rendered LBNL T-47D Cells Irresponsive to ATR in Culture. If, as possible in ATCC T-47D cells, *cyp19* II methylation is an epigenetic determinant of ATR responsiveness in the post-freezing-spontaneously-changed LBNL T-47D variant, than treatment of this subline with the DNA methylation inhibitor 5'-aza-dC may reestablish its robust ATR-responsiveness characterized before the phenotype-altering even occurred as detailed in Chapter 1.

Figure 41



ATCC T47D cells	v.	ATCC T47Ds+ LRH/GATA-3	v.	Original (LBNL)T47D cells that responded to ATR but mutated in culture	v.	Original T47D cells w/LRH and GATA-3 *knockdown*
+		++		++		-

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