EFFECTS OF METHOXYCHLOR IN OVARIAN GRANULOSA CELL *IN VITRO*.

BY

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Written under the direction of Mehmet Uzumcu, D.V.M., Ph.D.

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ABSTRACT OF THE DISSERTATION

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Methoxychlor (MXC) has been shown to alter ovarian folliculogenesis through inhibition of granulosa cell steroidogenesis, which is required for normal reproductive physiology. The process of granulosa cell differentiation requires estrogen to augment follicle-stimulating hormone (FSH) activity and the ovulatory response to gonadotropins. This dissertation focuses on the effect of methoxychlor, which is suspected of acting through its metabolite, 2,2-bis-(p-hydroxyphenyl)-1, 1, 1-trichloroethane (HPTE), on granulosa cell function. HPTE acts as an estrogen receptor α agonist and an estrogen receptor β antagonist. The well-characterized immature rat granulosa cell model was used to generate samples for analysis of intracellular cAMP, protein, and gene expression. The transcriptional responses induced by MXC and HPTE were investigated using Affymetrix microarray Rat Genome 230 2.0. Alterations in guanine nucleotide binding protein-coupled receptors (GPCRs) signaling were suggested to be affected by MXC and HPTE.

G-protein coupled receptors (e.g. FSH and LHR) are among the most common and successful drug targets produced by pharmaceutical companies and are critical to granulosa cell differentiation.

Recent reports suggest that estrogen receptor β is required for FSH-induced granulosa cell differentiation, response to gonadotropins (i.e., LHR) in the pre-ovulatory follicle, and maximal cAMP production. MXC and HPTE reduced FSH-mediated LHR expression and cAMP levels. Our results using two estrogen receptor β antagonists, ICI 182,780 (fulvestrant or falsodex) and 4-[2-phenyl-5,7-bis (tri-fluoro-methyl) pyrazolo [1,5-a]pyrimidin-3-yl] phenol (PTHPP) support an association between estrogen receptor expression β and cAMP. However, co-treatment of granulosa cells with an estrogen receptor β agonist, diarylpropionitrile (DPN), did not reverse the effect of HPTE on cAMP. While there are clear differences in the morphology of FSH-stimulated granulosa cells treated with HPTE and MXC, as well as production of estradiol 17β (E_2) when compared to FSH-alone, the use of dibutyryl cAMP (dbcAMP) was protective against the effects of MXC and HPTE on gene expression and steroidogenesis. Furthermore, MXC and HPTE suppressed estrogen signaling through inhibition of estrogen receptor expression and E_2 production. We showed that cell viability was unaffected by MXC or HPTE at high doses, but MXC and HPTE did induce significant changes in gene expression and pathway activity. Gene ontology studies revealed changes in cell-cell interaction, signal transduction, transport, cell cycle, adhesion, differentiation, motility and growth, apoptosis, development, and metabolism. In summary, we found that MXC disrupts granulosa cell differentiation through inhibition of FSH-cAMP-mediated signaling and alters nuclear estrogen receptor signaling. This work suggests a novel

mechanism of action for the effect of MXC in granulosa cells and provides information about novel gene targets for endocrine-disrupting chemicals.

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DEDICATION

Dedicated to Dr. William MacArthur ("Mickey") Harvey Sr.

LIST OF ORIGINAL ARTICLES

Chapter 3: **Harvey, C.N**., Chen, J.C., Bagnell, C.A., **Uzumcu, M.** Methoxychlor and HPTE Inhibit cAMP Production and Estrogen Receptors α and β in the Rat Granulosa Cell In Vitro. In preparation

Chapter 4: **Harvey, C.N**., Esmail, M., Wang, Q., Brooks, A.I., Zachow, R., **Uzumcu, M.** (2009) Effect of the Methoxychlor Metabolite HPTE on the Rat Ovarian Granulosa Cell Transcriptome in Vitro. *Toxicological Science* 2009.

Chapter 5: **Harvey, C.N.**, Ovacik, M.A., Androulakis, I.P. **Uzumcu, M** Pathway Activity Analysis of Granulosa Cell Microarray: Gene Network changes induced in the Rat Granulosa Cell. In preparation

In addition to these works, some other unpublished data were included.

ABBREVIATIONS

FSH= follicle-stimulating hormone

cAMP= 3'-5'-cyclic adenosine monophosphate

dbcAMP= dibutyryl cAMP

LH= luteinizing hormone

MXC= methoxychlor, 1,1,1-Trichloro-2,2-bis(4-methoxyphenyl)ethane

HPTE= MXC metabolite 1,1,1-trichloro-2,2-bis(p-hydroxyphenyl) ethane

Mono-OH= MXC metabolite 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl) ethane

ERα= estrogen receptor alpha

ERβ= estrogen receptor beta

E_1= estrone

E_2= estradiol 17β

Δ^4= androstenedione

P_4= progesterone

DPN= ERβ agonist

PTHPP= ERβ antagonist

ICI= ERα and ERβ antagonist, fulvestrant

IGF= insulin-like growth factor

TGFβ= transforming growth factor beta

BMP15= bone morphogenic protein 15

GDF-9= growth differention factor 9

KITL= kit ligand

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CHAPTER 1: Overview

1.1 Summary

This dissertation focuses on granulosa cell function as a target of methoxychlor (MXC) and its metabolites, 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl) ethane (Mono-OH) and 1,1,1-trichloro-2,2-bis(p-hydroxyphenyl) ethane (HPTE). The overall objective of this study is to determine possible mechanisms of action of MXC in the granulosa cell. MXC, HPTE, and Mono-OH have been shown to decrease estradiol 17β (E_2) production *in vitro* through inhibition of expression of estrogen synthesizing enzymes (Basavarajappa *et al.* 2011; Craig *et al.* 2010; Zachow and Uzumcu 2006). The granulosa cell plays essential roles in the maintenance of oocytes. All nutrients and waste travels through granulosa cells to arrive and leave the oocyte. Follicle health depends on granulosa cell-associated processes such as granulosa cell proliferation and differentiation, and steroidogenesis (Barnett *et al.* 2006). Therefore, the effect of MXC on the granulosa cell may be a mechanism of action for the observed ovarian dysfunction.

In a previous report from our laboratory, HPTE was shown to inhibit the gene expression of steroidogenic enzymes in the presence of follicle-stimulating hormone (FSH) in granulosa cells; in contrast to the effect of a cAMP analog in granulosa cells (Zachow and Uzumcu 2006). HPTE's dose-dependent inhibition of steroids was concomitant with alteration in the global gene expression profiles under similar conditions (Harvey *et al.* 2009) (Chapter 4) and compared the global transcription profiles of MXC and HPTE (Chapter 5). We hypothesized that MXC and HPTE might affect cAMP production and alter the cAMP-dependent second messenger system.

Therefore, I examined the effect of MXC and HPTE on intracellular cAMP levels. Since it is well known that MXC and HPTE have antiestrogenic properties, we utilized estrogen receptor antagonists to mimic the effects of MXC and HPTE on cAMP (Chapter 3). Others have noted a connection between estrogen receptor β and cAMP production (Deroo *et al.* 2009). We evaluated the effect of MXC and HPTE on the expression of the transcription factors estrogen receptor α and β. From these results, we concluded that MXC/HPTE decreases FSH-stimulated cAMP and inhibits estrogen receptor expression, which leads to decreased estrogen signaling, which is necessary for ovulation.

1.2 Introduction

The increase in ovarian dysfunction has become an area of increased interest in the field of toxicology, the study of the adverse effects of chemicals on living systems (Klaassen and Admur 1996). As women age, normal changes in the reproductive system take place including menopause (Downs and Wise 2009; Sone *et al.* 2007) and alterations in pituitary secretion of FSH and luteinizing hormone (LH) (Huang *et al.* 1976) that coincide with a loss of fertility. In addition, production of estrogen wanes at menopause (Erlik *et al.* 1982) affecting multiple tissues in the body, leading to loss of bone (Pacifici 1996) and increased risk of congestive heart failure (Reis *et al.* 2000). Toxic agents can prematurely induce aging in the ovary through multiple mechanisms, including reduced hormone synthesis availability and altered hormone-receptor binding (reviewed in Craig *et al.* 2011). The ovary's production of estrogen is a key regulator of women's reproductive health (Erlik *et al.* 1982; Reis *et al.* 2000), supporting the main function of the ovary: the production of viable eggs for reproduction.

Diseases that inhibit ovarian function have been well documented. Two ovarian disorders of interest are premature ovarian failure (POF) which affects 1% in women by age 40 and 0.1% by age 30 (Coulam *et al.* 1986; Kalantaridou *et al.* 1998) and polycystic ovary syndrome (PCOS) that affects one in five women of reproductive age (Azziz *et al.* 2004; Teede *et al.* 2010). Diagnosis of POF is characterized by amenorrhea, hypergonadotropism, and hypoestrogenism. Characterization of PCOS is more flexibly defined with a combination of anovulation/oligovulation, excess androgens, and/or polycystic ovaries visualized by ultrasound. Since the average age of first-time mothers in 2002 has increased in the USA to 25.2 years of age from 21.4 years of age (in 1970) (National Center Health Statistics, http://www.cdc.gov/nchs/); the initiation of the reproductive window is delayed. Due to their later reproductive starts, women with PCOS or POF are more likely to be adversely effected due to their delay in becoming first time mothers unlike women in previous generations who reproduced at a younger age (Diamanti-Kandarakis *et al.* 2009). The affects of PCOS or POF may also be exacerbated by environmental factors, including exposure to endocrine disrupting-compounds (Diamanti-Kandarakis *et al.* 2009). Therefore, a better understanding of how environmental chemicals affect the ovary would be useful for developing therapeutic tools to conserve the reproductive longevity of women. This research attempts to elucidate the physiological and molecular mechanisms of the MXC-induced toxic effect in the ovary.

Exposure to xenobiotic agents has increased with advancement in industrial and technical achievements such as microprocessors, plasticizers, and food preservatives. Many of these compounds have been shown to cause detrimental effects on the female

reproductive system (reviewed in Craig *et al.* 2011; Diamanti-Kandarakis *et al.* 2009; Uzumcu and Zachow 2007). Potential effects on the ovary are of particular concern; since ova are permanent (not renewed) and irreversible insults may accumulate. Thus, the effects of exposure to environmental endocrine-disrupting chemicals can accumulate over the course of a woman's life and cause adverse effects on fertility, embryogenesis, and may have epigenetic effects on the offspring (Gorospe and Reinhard 1995; Gill *et al.* 1979; Stillman 1982; Blatt *et al.* 2003). Ovarian effects of exogenous compounds can include a decreased follicular reserve, aneuploidy, alteration in granulosa cell steroidogenesis, and POF (Diamanti-Kandarakis *et al.* 2009). A study from the World Health Organization found that environmental factors may play a role in up to 80% of diseases reported worldwide (Prüss-Üstün and Corvalán 2006); the developmental origins of health and disease paradigm extends this concept to include insults received during prenatal and early postnatal development (Hanson and Gluckman 2008). Methoxychlor (MXC) has been shown to reduce the "reproductive window" in female rodents and may have adverse effects on *in utero* exposed animals (Armenti *et al.* 2008; Gray *et al.* 1989; Uzumcu *et al.* 2006).

Methoxychlor, because of its status as a replacement pesticide for dichlorodiphenyltrichloroethane (DDT), was scrutinized as a part of the Food Quality Protection Act of 1996 (104-170 FQPA; Findlay 1993). While initially considered benign below estimated toxicity levels (LD50~6 g/kg in humans) (Kamrin 1997), sufficient evidence exists to indicate that MXC exposure causes reproductive dysfunction in multiple animal species (ATSDR 2002). These adverse effects were observed in several organs including the ovaries and uteri in females; and the testes and prostate in males

(Chapin *et al.* 1997; Gray *et al.* 1999; Gray *et al.* 1988). Because of this information and other research, MXC was placed under more scrutiny from the EPA; no commercial license to sell and distribute MXC in the United States has been approved for since expiration of its regulatory approval (EPA 2004). However, MXC has continued to be detected in the United States and abroad, which suggests that it is still in use. Methoxychlor has been detected in agriculture and soil (in Portugal) (Carvalho *et al.* 2009; Fernandes *et al.* 2009), human placentas (in Spain) (Lopez-Espinosa *et al.* 2007), herbal drug medicines (in Korea and China) (Oh 2009), and human milk (in Denmark and Finland) (Damgaard *et al.* 2006). A study from Southwestern Texas Medical Center showed that measurable levels of MXC were present in the plasma of study participants as recently as 2007 (Richardson *et al.* 2009). These studies as well as others show that exposure to MXC is still occurring throughout the world.

Therefore, understanding the mechanism of action for the observed reproductive dysfunction caused by MXC and other compounds with similar mechanisms of action is necessary to protect human health due to continued exposure to endocrine-disrupting compounds.

1.3 Preliminary Work

This document details my work as a part of a broader program designed to better understand the influence of endocrine-disrupting chemicals (EDCs) on ovarian function, female fertility and the biology of the female gonad. The long-term goal of our research is to understand the mechanisms of action of EDCs on ovarian function. Most of the current views of MXC are based on the belief that it acts as a pro-estrogenic compound whose metabolites, Mono-OH and HPTE act as selective estrogen receptor modulators

(Gaido *et al.* 1999; Gaido *et al.* 2000). MXC is of interest as a model compound due to its known affect on reproductive function and its estrogenic metabolites.

Recent results suggest that reproductive toxicity associated with MXC exposure is a direct effect on the ovarian follicle (Gupta *et al.* 2007; Gupta *et al.* 2006a; Miller *et al.* 2005). As in the case for other estrogens, exposure to MXC between gestational day 19 and postnatal day 7 can alter estrous cyclicity (Armenti *et al.* 2008). HPTE was shown to disrupt granulosa cell steroidogenesis and affect mRNA of genes encoding steroidogenic enzymes (Zachow and Uzumcu 2006). The framework for the work detailed in this dissertation was established by two previous reports from our laboratory (Armenti *et al.* 2008; Zachow and Uzumcu 2006).

1.3.1 MXC Affects Ovarian Markers *In Vivo*

Female rats exposed to 100 mg/kg MXC during neonatal development exhibited aberrant expression of ovarian markers during adulthood (Armenti *et al.* 2008). Reduced expression of ERβ was observed in the granulosa cells of antral follicles and a concurrent reduction of LHR expression was observed in the theca and interstitial cells surrounding large antral follicles (Armenti *et al.* 2008). ERβ has been reported to be necessary for FSH-stimulated granulosa cell differentiation and that LHR mRNA is reduced in ERβ knockout mice (Couse *et al.* 2005). LHR is a marker for granulosa cell differentiation and is critical for ovulation. Evidence suggests that ERβ regulates LHR expression (Deroo *et al.* 2009; Rodriguez *et al.* 2010), and disruption of ERβ signaling prevents ovulation through insufficient LHR signaling (Rodriguez *et al.* 2010). Therefore, we hypothesized that MXC directly affects the ovary through altering ERβ signaling in the granulosa cells.

1.3.2 HPTE Affects FSH-mediated Estradiol 17β Production in Granulosa Cells

Previously, our lab showed that FSH-induced E_2 production in rat granulosa cells was inhibited by HPTE in a dose-dependent fashion; while dbcAMP-induced E_2 production was resistant to the effects of HPTE. The effect of HPTE on FSH-mediated E_2 production suggested that HPTE acts on the FSH-signaling pathway prior to cAMP generation (Zachow and Uzumcu 2006). FSH-stimulated genes that encode steroidogenic proteins (i.e., CYP11A1, CYP19A1, 3-β-HSD) were also inhibited by HPTE at a concentration of 10 μM (Zachow and Uzumcu 2006). This effect of HPTE on granulosa cell steroidogenesis *in vitro* was consistent with the observations of others using MXC-treated ovaries (Cummings and Laskey 1993) and antral follicles (Basavarajappa *et al.* 2011). Collectively, these results suggest that MXC and its metabolite HPTE act through a common mechanism to disrupt E_2 production. The differential effect of HPTE on FSH- and dbcAMP-stimulated E_2 production caused us to suspect that this mechanism involves the FSH-cAMP signaling pathway. Thus, we sought further examination of the effects of MXC and its more estrogenic metabolite, HPTE, on the granulosa cell.

1.4 Hypothesis

Our hypothesis is that **MXC and HPTE share a common pathway to disrupt estradiol 17β signaling in the granulosa cell, which involves decreased production/function of cAMP leading to altered intracellular signaling and disruption of luteinization**. The main aims of these studies were to define the role of MXC on granulosa cell differentiation by analyzing the transcriptional changes induced by MXC, and defining the molecular pathways through which MXC and HPTE modulates granulosa cell function.

MXC and HPTE both inhibited granulosa cell production of E_2. Therefore, we hypothesized that MXC and HPTE may induce similar gene expression changes in granulosa cells. We examined the effect of MXC and HPTE on gene transcription using DNA microarray to determine global gene expression profiles and identify prospective candidate genes for further analysis (Harvey *et al.* 2009). Gene network analysis was performed to identify significant pathways that were affected by MXC and HPTE. Understanding the actions of these compounds can give predictive insight into the toxicity of similar compounds through comparisons of their gene expression profiles. Gene expression can also be used to identify potential biomarkers for the effects of MXC on the ovary. By understanding the mechanism of action in granulosa cell function, the potential effects of EDCs in humans can possibly be identified and avoided. Response biomarkers would be useful for developing technologies and practices for diagnosing and treating women at risk for EDCs-induced ovarian hypofunction.

1.5 Dissertation Aims

The specific objective of this work was to test the hypothesis that the granulosa cell is a target of MXC toxicity through the following aims:

1. Chapter 3
    a. To measure the effect of HPTE and MXC on intracellular cAMP and estradiol 17β.
    b. To investigate the effects of estrogen receptor antagonists on cAMP production.
    c. To study the effect of HPTE and MXC on estrogen receptor expression.

   d. To determine if molecules leading to degradation of cAMP are targets of MXC/HPTE in granulosa cells.

2. Chapter 4
   a. To determine the dose-dependent HPTE-induced gene expression changes in granulosa cells.
   b. To identify novel genes associated with granulosa cell steroidogenesis and function.

3. Chapter 5
   a. Compare morphological changes induced by MXC and HPTE.
   b. To characterize the transcriptional effects of FSH and dbcAMP in immature granulosa cells.
   c. Characterize low and high dose transcriptional changes induced by MXC and HPTE in an FSH-stimulated granulosa cells.
   d. Identify common gene networks affected by MXC and HPTE.

CHAPTER 2: Background

2.1 Female Reproductive System of the Rat:

2.1.1. Overview of the Model System

For decades, the rat has been used as an animal model system for biological research mainly because of the similarity in function between the rat and human in the cardiac, respiratory, and urinary systems (Baker *et al.* 2006), and because of their cost-effectiveness. The rat model also has been the preeminent lab animal in pharmacology and toxicology studies (Klaassen and Admur 1996). Although the advent of transgenic mice has shown their utility as research tools, recent advancements in rat transgenic techniques (Filipiak and Saunders 2006), suggest that the rat model will remain a staple in human disease research.

Rat reproductive physiology is sufficiently similar to human reproductive physiology and is often the primary animal chosen for reproductive toxicology testing. The rat model is useful in that it offers manageable animal size, large litters (~8-12 pups), short gestation period (21 days) and short reproductive cycles (4-5 days) (Davis *et al.* 2001; Gray *et al.* 2004). The placenta for rodents and primates are similar in structure and chorion proximity (Wildman *et al.* 2006).

2.1.2. Species Differences: Rats and Humans

2.1.2.1 Important Differences between Rats and Humans

While there are many similarities between rats and humans, there are also important differences. Some developmental differences between humans and rodents have proven useful for experimentation. For instance, many developmental processes that occur in the third trimester in humans take place in the postnatal rat [e.g. estrogen receptor-mediated

sexual differentiation of the brain (McCarthy *et al.* 1993) and ovarian follicle development including primordial follicle assembly and development into the antral follicle stage (McGee and Hsueh 2000)]. This provides opportunities for the investigation of critical windows of development in a relatively compressed timeframe.

During preclinical investigation into the safety of drugs and chemicals, many compounds are found to interfere with reproductive function in the female rat through disruption of the estrous cycle (Westwood 2008). The hypothalamic-pituitary-ovarian axis (HPO) regulates estrous cycle in rodents and menstrual cycle in primates. Pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus initiates the secretion by the pituitary of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which stimulate the synthesis of estrogens, testosterone, and progesterone (P_4) (Burger *et al.* 2004; Flier *et al.* 1986). These ovarian hormones form a finely synchronized interactive feedback network with the HPO that influences the cyclic secretion of GnRH, FSH, and LH (Veldhuis *et al.* 2008). Maintenance of the HPO regulatory system is critical for normal ovarian function and morphology in both rats and humans (described in section 1.2).

There are several limitations in using rodent models for understanding human reproductive diseases. One critical variable that complicates translating rodent models to humans is the increased sensitivity of rodents to estrogens during development (Clark 1998). Estrogen exposure during the critical period of development in female rats (from gestational day 18 until postnatal days 8-10) causes the reproductive cycle to fail (Christensen and Gorski 1978). This condition, referred to as persistent estrus, is characterized by an inability of the ovaries to ovulate, a constant level of serum estrogen,

and the absence of P_4 production (Bunn and Everett 1957; Clark 1998; Everett 1940; Westwood 2008; Wise 1999). The persistent estrus rat model has been used to study polycystic ovary syndrome (PCOS) disease (Krishna B 2005).

Neonatal exposure to estrogenic compounds masculinizes the female rodent brain. While exposed humans exhibit resistance to the neonatal effect of estrogens on reproductive cyclicity (McCarthy 2008). Human fetuses are exposed to micromolar concentrations of estrogens in utero, while fetal rats receive picomolar concentrations during pregnancy (Clark 1998; MacLusky and Naftolin 1981). The absence of effect of estrogens on human brains is likely due to the higher circulating levels of alpha-fetoproteins present in humans in comparison to rodents, which may protect neonatal female brain from estrogen (Clark 1998; Mizejewski 2004). Even taking into account these physiological differences, the rat is a useful model for understanding mechanisms of reproduction and has been widely used as an animal model in endocrine disruptor screening and testing (reviewed in Gray *et al.* 2004).

2.1.3 Neuroendocrine Regulation of the Reproductive System

2.1.3.1 Gonadotropin Regulation

Gonadotropins (i.e., FSH and LH) are produced in the anterior pituitary gland under the regulation of GnRH secretion (Nett *et al.* 2002). Increased rate of GnRH secretion cause an increase in FSH and LH that leads to an increase in secretion of E_2 by the ovary (Maeda *et al.* 2011). GnRH release in the hypothalamus is regulated by kisspeptin through two neuronal centers that operate through two distinct modes (Uenoyama *et al.* 2009). Kisspeptin mRNA expression by the arcuate nucleus (ARC) is negatively regulated by estrogens, while kisspeptin mRNA expression by the medial preoptic

nucleus [also known as the anteroventral periventricular nucleus (POA)] is up regulated by estrogen. Many studies present evidence that kisspeptin may regulate the GnRH release (reviewed in Uenoyama *et al.* 2009).

Pulsatile release of GnRH by the ARC induces secretory bursts of LH that are responsible for stimulation of follicle development and steroidogenesis (Maeda *et al.* 2011). The GnRH surge from the POA induces the preovulatory surge of LH required for ovulation. Around puberty, the GnRH/LH surge from the POA can be excited by binding of estrogens to estrogen receptor β. Once E_2 reaches a threshold value, a signal is sent to the hypothalamus, which decreases the frequency of the GnRH/LH pulse until the ovary is prepared for ovulation (Uenoyama *et al.* 2009). This negative feedback control between estrogen and pulsatile GnRH/LH release switches to a positive feedback system once the dominant follicle(s) mature and supports the subsequent increase in estrogen and progesterone (Goodman 1978; Naftolin *et al.* 1996).

2.1.3.2 Comparison of Estrous and Menstrual Cycle

2.1.3.2.1 Estrous Cycle

The reproductive cycle in rodents is known as the estrous cycle. It lasts between 4 and 5 days depending upon the species and strain, and is divided into four phases: proestrus, estrus, metestrus, and diestrus (Hilliard 1973). Proestrus is the preparatory period directly before the sexually receptive period known as estrus, during which the follicles enlarge and estrogen increases. During estrus, females exhibit sexually receptive behavior. Estrus ends with ovulation. Metestrus is characterized by the formation of the corpus luteum (Hilliard 1973). The final phase, diestrus, is characterized by increased progesterone

production by the corpus luteum during pregnancy or the regression of the corpus leutem in the absence of pregnancy.

2.1.3.2.2 Menstrual Cycle

Humans and most non-human primates have menstrual cycles that are characterized by shedding of their uterine lining (menses) and their production of one follicle per reproductive cycle (Mihm *et al.* 2011). Generally, human menstrual cycles are 28 days between the first day of menses to the next menses; however, menstrual cycle length may vary between individuals (Mihm *et al.* 2011; Harlow 2000). The cycle is divided into two phases, the follicular phase (estrogen-dominant) and the luteal phase (progesterone-dominant). During the menstrual cycle FSH and LH serum levels remain at a steady state until ovulation, at which point they peak following a surge in estrogen. Following ovulation, P_4 production increases while FSH and LH returns to steady state levels (Mihm *et al.* 2011; Harlow 2000). With increasing age, the number of growing follicles decreases, especially in women over 40 (reviewed in Gougeon 1998). Menses last for an average of 5 days during which the corpus luteum regresses and the endometrial tissue is shed. Animals that menstruate are able to mate at any point during the menstrual cycle, but the most fertile period is around the time of ovulation.

2.1.2.3 Role of the Hypothalamic-Pituitary in Regulating Female Reproduction

The menstrual and estrous cycles are regulated by steroid feedback; which are categorized into two phases, follicular and luteal. Rats require coital stimulation to develop a fully functional corpus luteum and exhibit a luteal phase (McCracken *et al.* 1999). In the early follicular phase, circulating levels of P_4 and E_2 are low and the surge center is inactivated. The frequency of the tonic GnRH pulse increases, resulting in rising

levels of E_2 while P_4 remains low. A negative feedback loop exists between E_2 and gonadotropins (released by the pituitary) and GnRH (secreted by the hypothalamus). E_2 increases GnRH receptor density in the anterior pituitary that amplifies the response to the GnRH surge, causing a switch from negative feedback to positive feedback (Sealfon *et al.* 1990). In pigs, this surge in GnRH coincides with an increase in P_4 that maximizes E_2 positive feedback (Christensen *et al.* 2012; Moore and Price 1932; Yen 1977).

It is well established that the preovulatory LH surge is the driving force for the rupture of the follicle to release the ova. Disruption of the hypothalamic-pituitary network prevents normal reproductive function and may cause four observable conditions in the female: (1) inhibition of oogenesis, (2) absence of steroidogenesis, (3) abnormal sexual development, and/or (4) anovulation (Mahony and Hodgen 1995). In the hypothalamus, targets of action may include the ARC or the POA (McCarthy 2008). GnRH secretions can be affected by altering transcription, translation, packaging or axonal transport (Mahony and Hodgen 1995). Neuroactive compounds also can disrupt these functions. For example, antagonists of the α-adrenergic receptor as well as opioids and chlordimeform have been observed to alter GnRH secretion (Cooper *et al.* 1998; Mahony and Hodgen 1995; Snell and Johnson 1986). Exposure to the hallucinogenic drug, phenylcyclidine (PCP) delayed vaginal opening and estrus cycle in rats injected between day 5 and day 10 (Mahony and Hodgen 1995; Sircar 1995). Estrogen and androgen antagonists can also interfere with their respective receptors located in the hypothalamus, altering normal reproductive function (Mahony and Hodgen 1995; Rempel *et al.* 2008).

Direct action by chemicals on the pituitary can lead to the alteration of proper gonadotropin production, secretion, or estrogen receptor or GnRH response (Miller *et al.* 2004; Sharara *et al.* 1998). Methylmercury can affect multiple points on the reproductive axis in fish and is known to inactivate gonadotropin production in fish (Crump and Trudeau 2009). Genistein, a phytoestrogen, has been shown to alter gonadotropin release in ewes and rats (Medigovic *et al.* 2011; Wojcik-Gladysz *et al.* 2005). The compound 2,3,7,8-tetrachlorodibenzon-*p*-dioxin (TCDD) can indirectly regulate FSH and LH secretion (Gao *et al.* 2000; Li *et al.* 1997), possibly through down regulation of ER (DeVito *et al.* 1992; Tian *et al.* 1998) and/or alteration of the GnRH neuronal system (Clements *et al.* 2009; Takeda *et al.* 2011). In addition, chemicals with antiestrogenic and antiandrogenic properties can bind to their complementary receptors located in the pituitary to prevent proper receptor response (Akingbemi and Hardy 2001).

Many chemicals, especially those that affect steroid hormone receptors, can affect multiple levels of the HPO axis and directly affect the ovary. An example of a compound that can interact with multiple foci on the HPO is TCDD. Although the exact mechanism is still unclear, TCDD interacts with the HPO through aryl hydrocarbon receptor (AHR)-mediated crosstalk with the ER (Barnett *et al.* 2007; Tian *et al.* 1998). These multi-impact effects can make it difficult to identify ovotoxic effects *in vivo*. To bridge this information gap, alternative research tools using *in vitro* assays have proved informative and transplant methods using green-fluorescent protein technology may be useful in identifying strictly ovotoxic effects (Marano *et al.* 2008).

Ovarian function is regulated by signals it receives from the pituitary (Hayden and Balen 2006) and it is important that the ovary is sensitive to these signals and responds

correctly through the production of hormones and local growth factors. For example, the mouse ovary responds to activin, a member of the transforming growth family β protein superfamily, by upregulating ER expression (Kipp *et al.* 2007) and dysfunction expression of activin is associated with ovarian pathologies such as ovarian cancer (Robertson *et al.* 2004) and PCOS (Eldar-Geva *et al.* 2001).

2.1.3 Ovary

The primary function of the ovary is the production of viable oocytes that allow for successful reproduction. This complex process is supported by the production of steroids, which are integral to both primary sexual maturity and development of secondary sexual features. The basic unit of the ovary (the ovarian follicle) is composed of a germ cell (oocyte) and somatic cells (granulosa and thecal). The process of folliculogenesis is defined as the maturation of the ovarian follicle (Figure 2.1).

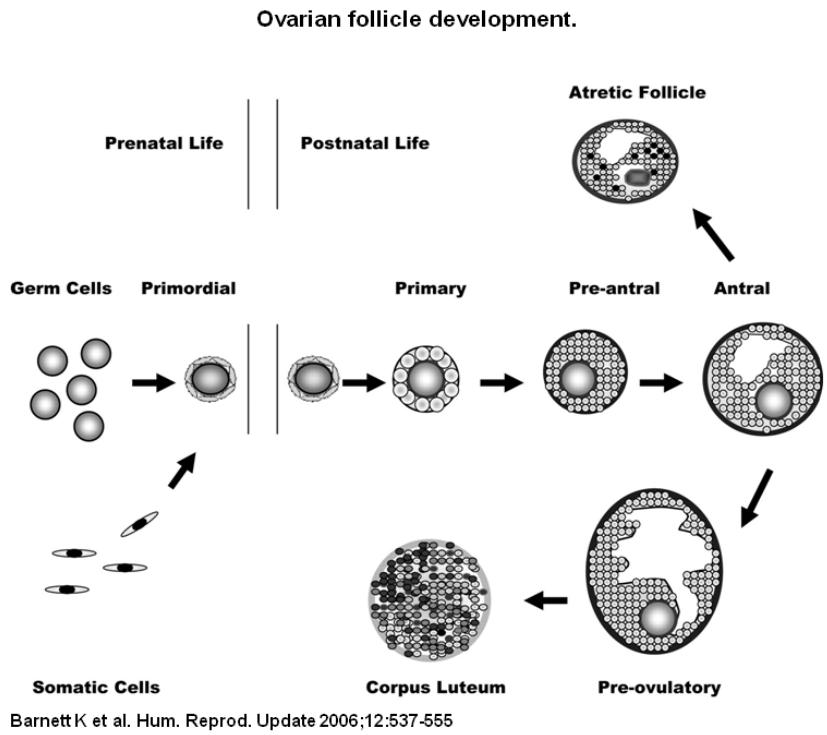


Figure 2.1: Maturation of the ovarian follicle.

Folllowing oocyte nest breakdown, germ cells (oocytes) are surrounded by somatic cells (granulosa cells) to form the finite reservoir of primordial follicles. The primordial follicles, after entry into the growing follicle pool, must undergo several developmental steps to reach the pre-ovulatory stage. First, follicles enter the growing follicle pool by becoming primary follicles. Primary follicles continue to develop through early folliculogenesis (pre-antral) stages until the antral follicle stage. At the antral stage, the follicles may become the pre-ovulatory (dominant) follicle and produce a corpus luteum. Much more likely, the antral follicle will become atreitic (undergo follicle death) (Adapted from Barnett *et al.* Hum.Reprod. Update 2006, reproduced with permission).

2.1.3.1 Folliculogenesis

2.1.3.1.1 Initial Recruitment versus Cyclic Recruitment

The process of folliculogenesis is dependent on a reservoir of primordial follicles that are established at birth (McGee and Hsueh 2000). This initial pool of primordial follicles is never supplemented by an additional source of follicles (Bristol-Gould *et al.* 2006). The term "recruitment" has frequently been used to describe the entry of dormant primordial follicles into the growing follicle pool (McGee and Hsueh 2000). "However, it has also been used by some authors to describe the movement of a cohort of antral follicles into the growing phase (McGee and Hsueh 2000)." To differentiate between the two processes, McGee and Hsueh (2000) have suggested that the first case be known as "initial" recruitment while the second case, which refers to antral follicle growth, be known as "cyclic" recruitment. In this work, we will use this convention.

2.1.3.1.2 Initial Recruitment

Primordial follicles do not all become activated at the same time. Some primordial follicles remain dormant to provide a continuous supply of growing follicles until the end of the reproductive window (months or years depending upon the species). This initial recruitment continues throughout the reproductive life of the female as needed until menopause. How follicles are activated is poorly understood, but a system of stimulatory and inhibitory hormones and local growth factors regulates primordial transition into the growing follicle population (reviewed in Fortune *et al.* 2000; Gougeon 2011). Anti-Müllerian hormone (AMH) levels have also been identified as a regulator of early follicle growth (Durlinger *et al.* 2002; Weenen *et al.* 2004).

Follicles in the growing population mature in waves of cohorts following puberty due to increased circulating levels of FSH. During this growing process, the population of the cohorts experiences an increasing failure rate and dwindles in number until only a few (rodent) or one (primate) dominant follicle reaches the preovulatory stage. The mechanism of progression of the follicles to ovulation is poorly understood, however an intricate multi-directional signaling network exists within the follicle (McGee and Hsueh 2000; Richards 2005).

Multiple significant processes must occur in order for a follicle to proceed to the preovulatory state. Local growth factors such as insulin-like growth factor 1 (IGF-1) regulate the progression of small follicles, however, the exact mechanism remain unclear (Adashi *et al.* 1985). As they mature, follicles switch from a gonadotropin-independent period to a gonadotropin-dependent phase, which allows the follicles to be regulated by gonadotropins (reviewed in Sirotkin 2012; Erickson and Shimasaki 2001). Described below are the conditions associated with early and antral folliculogenesis.

2.1.3.2 Early Follicles

2.1.3.2.1 Follicle Characterization and Structure

Early folliculogenesis is defined as the period during which the growing follicle matures and develops into the stage directly before the formation of the antral cavity (reviewed in Fortune 2004). The number of granulosa cell layers surrounding the oocyte characterizes the follicle stage. Following oocyte nest breakdown, the primordial follicles (the earliest stage follicles) are formed in the ovary. The primordial follicle is characterized by an oocyte surrounded by a single layer of squamous epithelial pre-granulosa cells (Hirshfield and Midgley 1978). Structural characterization of follicles is based on the

shape of the granulosa cells and the number of complete layers of granulosa cells encircling the oocyte, which was described in previous reports (Armenti *et al.* 2008; Zama and Uzumcu 2009).

The morphological characteristic that differentiates a primordial follicle from a primary follicle is the presence of at least two cuboidal-shaped granulosa cells in the single layer surrounding the oocyte in primary follicles. Primordial follicle assembly and development occur in the early stages of the postnatal period in the rodent and is followed by a transition into primary follicles (reviewed in Skinner 2005). While toxic agents that target primordial follicles can quickly deplete the follicular reserve and lead to permanent infertility, agents that affect only primary follicles may only temporarily affect fertility. This recovery of fertility is contingent upon removal of the toxicant and the availability of primordial follicles to replenish the stream of growing follicles (Hirshfield 1997; Skinner 2005).

An increasing number of granulosa cell layers around the oocyte and the emergence of a thecal cell layer (Uzumcu et al. 2006) characterize continued growth of the follicle. During the transition from the primary follicle stage to later more mature follicles, the zona pellucida, a clear acellular layer, forms around the oocyte and provides a nutrient/waste buffer area between the granulosa cell and the oocyte (Dunbar *et al.* 1994). Local growth factors such as IGF-1 and kit ligand (KITL) transverse this zone to regulate follicle growth (reviewed in McGee and Hsueh 2000).

2.1.3.2.2 Regulators of Early Follicle Growth

Insulin-like growth factor-1 (IGF-1) and its associated family of IGF binding proteins have long been suspected to play a role in early folliculogenesis (Adashi 1998) and have

been observed to play a role in the autocrine/paracrine regulation of intraovarian signaling (Adashi *et al.* 1985). The synergistic effect of IGF and FSH amplifies estrogen production (Adashi *et al.* 1985) and increases granulosa cell growth and differentiation (Davoren *et al.* 1986). The extent of IGF involvement in folliculogenesis appears to be species-dependent (Monget and Bondy 2000). For example, in cattle the concentrations of IGF-1 and E_2 increase in the dominant follicle, which may be an increased response to FSH and LH (Ginther *et al.* 2003). In mice, it appears that IGF-1 and its receptor are necessary for the progression of follicles to the antral stage (Baker *et al.* 1996). The exact role that the IGF system plays in follicle progression or follicle selection in humans remains unclear (Adashi 1998).

Other suspected regulators of early folliculogenesis include activins and inhibins (Findlay 1994), oocyte-derived factors BMP15 and GDF-9 (reviewed in Matzuk *et al.* 2002), and other TGFβ family members expressed by the granulosa cells (reviewed in Trombly *et al.* 2009). In addition, structural/functional changes such as FSH- and E_2-regulated increases in the number of gap junctions (Burghardt and Anderson 1981; Burghardt and Matheson 1982) can regulate granulosa cell-cell interaction and oocyte-granulosa cell communication, which can affect early folliculogenesis.

Though the gonadotropin-independent period is mainly under regulation of local growth factors, it can be influenced by FSH. The different autocrine and paracrine factors that regulate folliculogenesis may act in conjunction with FSH or alone (Gougeon 1996). For example, FSH in a whole rat ovary culture has been observed to decrease atresia in primordial follicles (Cossigny *et al.* 2011). FSH also induces progression of immature rat granulosa cells to differentiate *in vitro* through a cAMP-dependent pathway (Knecht *et*

al. 1981; Knecht *et al.* 1983). In the intact female, FSH is necessary and sufficient to generate mature, preovulatory follicles, stimulating E_2 synthesis through induction of gene expression in granulosa cells under the input of multiple signaling pathways (reviewed in Hunzicker-Dunn and Maizels 2006).

Eventually, follicles transition from gonadotropin-independent mediated growth to gonadotropin-dependent growth. The availability of the FSH receptor (FSHR) is important for hormonal regulation. Follicles grow in waves or cohorts towards the antral follicle stage under the direction of FSH. Most follicles that are in the growing follicle pool become atretic prior to the antral follicle stage (Baker and Spears 1999).

2.1.3.3 Antral Follicles

The antral follicle stage is defined by the formation of a fluid filled cavity and the increased regulation by FSH and LH (Hirshfield and Midgley 1978). At the antral follicle stage, the diameter of the follicle is used to classify them into small, medium or large sizes (Hirshfield and Midgley 1978). There are three distinct events in antral follicle growth: cyclic recruitment, selection, and dominance (McGee and Hsueh 2000). It is unclear what selection criteria are used to determine follicular growth, but FSH receptor plays an important role (reviewed in Hillier 2001). As described in the aforementioned review, cyclic recruitment occurs during small antral follicle growth and coincides with increasing production of E_2. During cyclic recruitment, the follicular environment is FSH-rich with low tonic LH levels. Selection involves medium antral follicles continuing to grow toward preovulatory follicles and continued production of E_2 with a reduction in FSH availability, an increase in LH and low levels of the FSH-inhibiting hormone, inhibin. Bao et al. (Bao *et al.* 1997) observed increased mRNA expression of *Lhcgr*,

Fshr, *Cyp19a1*, *Cyp17a1*, and *Cyp11a1* in bovine follicles during both recruitment and selection, which are ovarian markers for differentiation.

The final stage before ovulation and luteogenesis is the determination of the dominance follicle, which involves increased E_2, inhibin and LH levels as well as low levels of FSH. Once E_2 reaches threshold, the LH surge is triggered (an event which alters steroidogenesis in the follicle), E_2 levels sink and P_4 levels begin to rise. A morphological alteration characteristic of the dominant follicle stage is the internalization of the gap junctions between granulosa cells and the oocyte and the resumption of meiosis (Albertini *et al.* 1975; Burghardt and Anderson 1981; Burghardt and Matheson 1982; Sirard and Bilodeau 1990).

2.1.3.4 Ovulation and Atresia

The two ultimate outcomes of folliculogenesis are ovulation or atresia in mammals. The LH surge induces ovulation, at which time the oocyte(s) is ejected from the ovary in preparation for fertilization (reviewed in Richards 2005). LHR expression within the granulosa cell membrane coincides with the termination of proliferation of the granulosa cells and the initiation of genes that encode proteins that control ovulation and luteinization: progesterone receptor, CAAT enhanced binding protein beta, early growth regulatory factor, pituitary adenylyl cyclase activating peptide, StAR, CYP11A1, protein kinases, and others (Richards *et al.* 2002a).

Most follicles never reach ovulation. Instead, the follicle stops at a stage prior to dominance and begins to degenerate. It has been suggested that during the late growth stage, decreased availability of FSH favors FSHR rich follicles over other follicles (Zeleznik 2004). An alternative hypothesis is that certain follicles better able to utilize the

increased LH and reduce their dependence on FSH, are more likely to thrive (Zeleznik 2004). The mechanism of follicular atresia is not well understood, but may be a result of increased apoptosis of the granulosa cells of non-selected follicles (Hughes and Gorospe 1991; Chun *et al.* 1996; Peluso and Steger 1978).

2.1.3.5 Corpus Luteum

The ruptured follicle, which consists of granulosa and thecal cells, undergoes morphological and molecular changes called luteinization to form the corpus luteum. The vascularized corpus luteum produces both E_2 and P_4 and provides critical signals that maintain the early stages of pregnancy in goats (Casida and Warwick 1945), rabbits (Allen 1932), primates (Baird *et al.* 2003), rats (Nelson *et al.* 1930) and other species.

2.1.4 Granulosa Cell

2.1.4.1 Structure and Function

Granulosa cells are epithelial cells that originate from the mesoderm. They form an avascular barrier within the follicle through the formation of a basement membrane (reviewed in Brann *et al.* 1995). Squamous epithelial granulosa cells initiate breakdown of the oocyte germ cell nests into primordial follicles (Picton *et al.* 1998) and become cuboidal granulosa cells during folliculogenesis proliferating to form several layers around the oocyte. Granulosa cells differentiate into two types, the oocyte-surrounding cumulus cells and the basement membrane lining mural granulosa cells. The purpose of each type of granulosa cell is not fully understood; however, it is clear that they respond to FSH and LH, paracrine, and autocrine factors differently (Khamsi and Roberge 2001). A recent analysis of human follicular fluid suggests that granulosa cells comprise

235/426 proteins suggesting that multiple signaling pathways in the follicle are supported by granulosa cell function (Yoo *et al.* 2011).

Granulosa cells serve as the initial support cell for the follicle, and act as a barrier against toxins (Brann *et al.* 1995). As the follicle matures, the theca cells appear and are attracted to form an outer layer around the basement membrane of the granulosa cell (reviewed in Young and McNeilly 2010). The appearance of the theca cells is concurrent with the upregulation of the receptors for FSH and LH (Bukovsky *et al.* 1993; Zhang *et al.* 1997). The two-cell, two-gonadotropin theory conceptualizes steroidogenesis between granulosa and theca cells. Briefly, LH induces theca cells to produce androgens (primarily androstenedione (Δ^4)), which diffuse into the granulosa cell and aromatized into estrogens under regulation of FSH. Estrogen acts locally and enters the serum to downregulate further production of GnRH (at the hypothalamus) and FSH and LH (at the pituitary) (Liu and Hsueh 1986).

FSHR are exclusively located within membranes of the granulosa cells in the ovary. In the absence of FSH (Kumar *et al.* 1997) or FSHR (Dierich *et al.* 1998), follicles do not grow beyond the early antral stage in vivo (Adriaens *et al.* 2004; Burns *et al.* 2001). FSH stimulates the expression of *Lhcgr* mRNA in granulosa cell in the absence of the oocyte (Eppig *et al.* 1998; Eppig *et al.* 1997). Mural and cumulus cells express FSHR, but cumulus cells do not express the LH receptor; inhibition of LHR is mediated by factors secreted by the oocyte like Smad2/3 (Diaz *et al.* 2007; Eppig *et al.* 1997). However, the shift of the mural cells to the development of the LH receptor is critical to the response to the LH surge. Mural granulosa cell expression of LH receptor increases as the antral follicle grows in size (Bukovsky *et al.* 1993). An increase in LH receptor is

correlated with a decrease in FSH receptor (Eppig *et al.* 1997; Fortune *et al.* 2004; Mihm *et al.* 2006).

As the follicle matures to the antral follicle stage, the granulosa cells undergo additional processes that prepare the follicle for ovulation. As described above, the serum levels of FSH and LH and the availability of the FSH and LH receptors in the granulosa cell change leading up to ovulation. During the recruitment period, the FSHR is dominant in the granulosa cell. FSH initiates the transcription of the LH receptor in the mural granulosa cell; this period of selection results in an increase in LH receptor on the mural granulosa cells. The serum levels of FSH begin to decrease and the FSH receptor is downregulated. In the final stage, the mural granulosa cells of the large dominant follicles, now expressing LH receptors, are able to respond to the preovulatory LH surge and allow the follicle to ovulate (Cain *et al.* 1995). Cumulus cells accompany the oocyte as a part of the ejected oocyte-cumulus complex (Gilula *et al.* 1978). The remnant mural granulosa cells and thecal cells are then remodeled into the corpus luteum, which then releases signals that support pregnancy-supporting factors.

2.1.4.2 Immature Rat Granulosa Cell Culture

Primary rat ovarian granulosa cell culture is a well-established and validated model system to the study of the effects of FSH on cell differentiation (Sanders and Midgley 1982), proliferation (Rao *et al.* 1978), and steroidogenesis (Dorrington *et al.* 1975; Lunenfeld *et al.* 1975; Makris and Ryan 1977). Granulosa cell culture has been used to analyze the impact of endocrine disruptors on the ovary and modeling potential disturbances (Tiemann 2008). Examples of its use include assaying the disruptive effects

of bisphenol A (Zhou *et al.* 2008), pyrethroids (Liu *et al.* 2011), phthalates (Lovekamp and Davis 2001) and genistein (Zhang *et al.* 2011).

Preparation of primary cell culture can vary between laboratories with the use of E_2-primed animals, diethylstilbestrol (DES)-treated, equine chorionic gonadotropin or untreated primary granulosa cells. The use of E_2-and DES-primed animals increases the granulosa cell population, but can alter cellular response and produce different results from the granulosa cells of non-primed animals (Liu *et al.* 1985). For example, rat granulosa cells isolated from estrogen-primed animals exhibited a 4- to 6-fold higher basal estrogenic activity than granulosa cells isolated from untreated animals (Couse *et al.* 2006; Sharma *et al.* 1999). Granulosa cells from estrogen-primed rats did not exhibit a synergistic increase in FSH-mediated *Cyp19*a1 when co-treated with testosterone or DHT in comparison to granulosa cells from untreated rats (Fitzpatrick and Richards 1991). Therefore, proper care must be taken to understand the effects of priming on granulosa cell populations and intra- and inter-species variation. Rat granulosa cells harvested from unstimulated immature (postnatal day 21 through postnatal day 27) Sprague-Dawleys are a good source of primary cells. Unstimulated prepubescent female rats provide undifferentiated granulosa cells, which have had low exposure to gonadotropins prior to culture. This system is useful as a model for effect of exogenous chemicals on an in vivo follicle system (Tiemann 2008).

2.1.4.3 Immortalized Granulosa Cell Lines

Development of immortalized granulosa cell lines have been a focus of the ovarian biology field for more than 30 years (reviewed in Havelock *et al.* 2004). The first immortalized granulosa cell line, GC48C, was developed from primary granulosa cells

and an SV40-tranformed rat ovary line that had lost the ability to produce progesterone (Havelock *et al.* 2004; Zeleznik *et al.* 1979). None of the rodent cell lines generated exhibited the characteristics of primary cells for sustainable periods. For example, the immortalized GC48C lost steroidogenic capacity after 11 months, and did not respond to FSH (Havelock *et al.* 2004). The advent of improved granulosa cell lines could reduce the number of animals used for research and better elucidate the mechanisms involved in ovarian functions.

2.1.5 Role of the FSH-PKA Signaling Pathway in the Granulosa Cell

FSH is sufficient and necessary for the induction of ovarian follicles to a mature, pre-ovulatory phenotype (Hunzicker-Dunn and Maizels 2006; Zeleznik 2004). FSH is a glycoprotein made up of a protein dimer that consists of a 92 kDa alpha subunit that is identical LH and thyroid-stimulating hormone, and a human chorionic gonadotropin with a unique 118 kDa FSH beta unit (Ryan *et al.* 1988; Sprengel *et al.* 1990). FSH self-regulates pituitary FSH secretion through stimulation of estrogen and inhibin production by the ovary (De Jong *et al.* 1979). Binding of FSH to FSHR signals induction of physiological and transcriptional changes in the granulosa cell (Grieshaber *et al.* 2000).

2.1.5.1 FSH Receptor in the Granulosa Cell

The FSHR is a guanine nucleotide binding-protein coupled receptor (GPCR) that is made of 7 transmembrane hydrophobic α-helices which binds FSH at the N-terminal on the extracellular membrane (Griswold *et al.* 1995). FSHR is a single polypeptide glycoprotein (678 amino acid residues) (Sprengel *et al.* 1990) attached to the plasma membrane through palmitoylation, which stabilizes the protein structure and retains the position on the membrane (Uribe *et al.* 2008). Structurally, disulfide bonding within the

FSHR stabilizes its conformation (Jetly *et al.* 2003). The FSH / FSHR interaction is well studied (reviewed in Simoni *et al.* 1997); in rats FSHR appear on the granulosa cell from postnatal day 3 onward. The granulosa cell is FSH-insensitive from birth to day 3 (Sokka and Huhtaniemi 1990). *Fshr* mRNA expression and the levels of FSHR protein are strongly paralleled (Dunkel *et al.* 1994).

Binding of FSHR initiates the dissolution of the heterotrimeric G protein (Gαβγ) which frees Gα to bind to adenylyl cyclase to produce cAMP from ATP. Increased cAMP initiates downstream signaling cascades of FSH that include protein kinase A (PKA). The two regulatory units of PKA are bound by cAMP, which causes the dissociation of two catalytic subunits of PKA. The catalytic subunit then phosphorylates proteins and transcriptional activators (Hochman *et al.* 1977). Transcription factors such as cAMP responsive element-binding (CREB) and cAMP responsive element modulator (CREM) can bind to the cAMP response elements in the promoters of certain cAMP responsive genes and initiate transcription of genes such as *Cyp19a1*, *Cyp11a1*, and *Lhcgr* (Sassone-Corsi 1998).

2.1.5.2 FSH-induced Changes in Granulosa Cell Morphology

Structure-function relationships appear to play an important role in granulosa cell steroidogenesis. Unstimulated granulosa cells in culture exhibit a flattened morphology, which is altered by FSH to a nearly spherical shape (Lawrence *et al.* 1979). This change of shape is marked by reduction in surface area contact with the culture surface, decreased cell perimeter and altered microfilament distribution (Tsang *et al.* 1988). Colchicine and nocodazole (chemical agents that depolymerize microtubules) inhibited FSH-stimulated progesterone production in granulosa cells (Carnegie *et al.* 1987). The

absence of cellular projections is a morphological biomarker for inhibited steroidogenesis in cultured granulosa cells.

2.1.5.3 Intracellular Signaling in the Granulosa Cell

It is well known that a synergism exists between cAMP signaling and E_2 signaling in the cells of the ovary, uterus and breast cancer cells and other cell types (Aronica *et al.* 1994; Katzenellenbogen 1996; Richards *et al.* 2002b). Robker and Richards (Robker and Richards 1998) showed that in rats, E_2 and FSH maximize proliferation and terminal differentiation of granulosa cells through induction of the cell cycle activator, cyclin D2. This mechanism is cAMP-dependent since cAMP, the adenylyl cyclase activator forskolin (Welsh Jr *et al.* 1984), dibutryl cAMP (Erickson and Ryan 1975) and 8-bromo-cAMP, cAMP analogs (Peluso *et al.* 1993) as well as FSH, are able to induce production of E_2 in granulosa cells.

LH binding can induce activation of adenylyl cyclase and initiation of cAMP-dependent kinase cascades. Richards et al. (Richards *et al.* 1986) characterized the LH-cAMP signaling pathway in rat thecal cells. FSH and LH signaling have a shared downstream mechanism since during late folliculogenesis, the dominant follicle becomes dependent on LH (Binelli and Murphy 2009; Dekel *et al.* 1988). LH plays a critical role in folliculogenesis; but its interaction with FSH is not well understood (Zeleznik 2004). It is known that FSH stimulates *LHR* mRNA through cAMP (Eppig *et al.* 1998; Lindeberg *et al.* 2006). In addition, LH stimulates large ovarian follicle maturation independent of FSH (reviewed in Fillicori and Cognigni 2001).

FSH initiates cAMP-dependent and independent gene transcription in the granulosa cell (reviewed in George *et al.* 2010). Alternative signaling pathways, such as

extracellular signal-regulated kinases (ERK) (Cameron *et al.* 1996; Su *et al.* 2001), PI3K, and protein kinase B (AKT) (Gonzalez-Robayna *et al.* 2000; Gutkind 1998), activated by FSH have been implicated in the maturation of granulosa cell (reviewed in Hunzicker-Dunn and Maizels 2006). Mediators of FSH's effect on transcription include activin (Kishi *et al.* 1998; LaPolt *et al.* 1989; Li *et al.* 1995b), inhibin (Bicsak *et al.* 1986; Ying *et al.* 1986) and E_2 (Lei *et al.* 2010). One study indicated that AKT is required for granulosa cell differentiation (Zeleznik *et al.* 2003). Other suggested pathways include increases in intracellular calcium (Peluso *et al.* 2001) and the activation of the protein kinase C (PKC) signaling pathway (Nishizuka 1986; Peluso *et al.* 2001; Shinohara *et al.* 1985). Further studies on the roles of these cAMP-independent signaling pathways in FSH-mediated target gene expression have been suggested (Hunzicker-Dunn and Maizels 2006).

2.1.6 Ovarian Steroidogenesis

2.1.6.1 Biochemistry of Steroidogenesis

The production of E_2 in the ovary requires the coordinated action of gonadotropins and somatic cells (Section 2.1.4) in the two-cell, two-gonadotropin theory (Liu and Hsueh 1986; Johnson and Hoversland 1983; Makris and Ryan 1977). The process of E_2 production in the ovary begins with the production of cholesterol from cholesterol esters located on intracellular lipid droplets (Azhar *et al.* 1990) or low-density lipoproteins in the cellular membrane (Reaven *et al.* 1986). Cholesterol is transported into the mitochondria of the thecal cells by StAR protein, where it is converted into androgens by CYP11A1, 3 beta-HSD and CYP17. These androgens (Δ^4 and testosterone) diffuse into the granulosa cell where they are aromatized by CYP19A1 into estrogens. The increased

production of androgens and estrogens by the ovary leads to increased intra-ovarian signaling as well as increased serum levels of E_2 and P_4 (Koos *et al.* 1984).

The interaction between enzyme activity and steroid formation is linked in the ovary. The rate-limiting step for progesterone steroidogenesis is the transformation of cholesterol into pregnenolone by CYP11A1 (Richards and Hedin 1988) with subsequent dehydrogenation by 3β-HSD to progesterone. Progesterone is metabolized in the theca cells into Δ^4 by CYP17 or into testosterone (T), and diffuses into the granulosa cell. Androstenedione is either directly aromatized into estrone by CYP19A1 or converted into testosterone and then metabolized into E_2. Granulosa cells also convert cholesterol into progesterone (Figure 2.2).

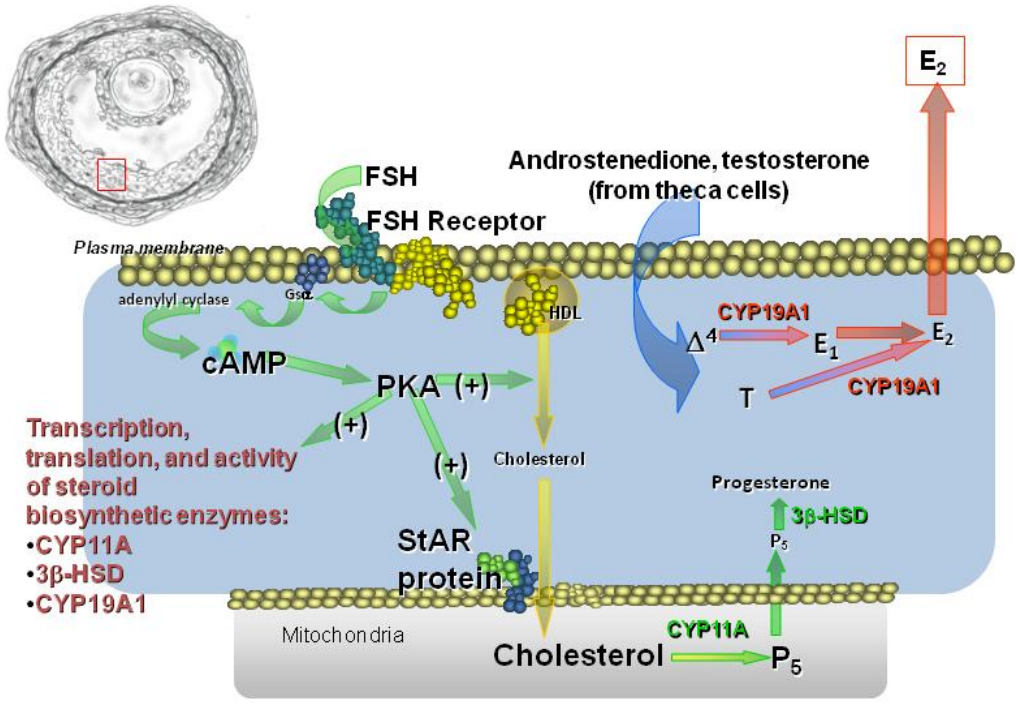


Figure 2.2: Induction of granulosa cell steroidogenesis by FSH.

FSH is activated through G-protein coupled signaling and production of cAMP and then initiates the protein kinase A signaling cascade. Many of the steroidogenic enzymes (e.g. CYP11A, CYP19A1) necessary for the conversion of theca cell-derived androgens into E_2 are downstream targets of the cAMP-PKA pathway. Cholesterol is transported into the mitochondria and metabolized into pregnenolone. Pregnenolone is converted into progesterone and can act locally or enter the serum. (Adapted from Uzumcu and Zachow 2007. Abbreviations: HDL, High-Density Lipoproteins; Gsα, guanine nucleotide-binding protein stimulatory alpha subunit; PKA, protein kinase A; StAR, steroidogenic acute regulatory protein; CYP11A, cytochrome P450 11A1; CYP19A1, cytochrome P450 19A1; 3β-HSD, 3β hydroxysteroid dehydrogenase; Δ^4, androstenedione; T, testosterone; P5, pregnolone; E1, estrone; E2, estradiol 17β).

The reaction rates of ovarian steroidogenesis are regulated by the availability of androgens and estrogen as well as FSH and LH levels (Stocco 2001). Androgens induce their own metabolism into estrogens by upregulating, in conjunction with FSH, CYP19A1 (Hillier and De Zwart 1981). Estrogens regulate their own concentrations locally through induction of E_2-catabolizing enzymes (CYP1A1 and CYP1B1) (Tsuchiya *et al.* 2004) and phase 2 conjugation processes such as sulfonation, esterases, and glucuronidation (Zhu and Conney 1998).

Estrogen signaling is critical to reproductive function. E_2, like other hormones, is able to transverse the cell membrane and enter the cytoplasm. It induces transcription in the nucleus by binding of estrogen receptor (Hall *et al.* 2001). Increasing serum E_2 induces physiological changes in the reproductive tract, prepares the female for sexual behavior, and induces the LH surge that is critical for ovulation (Fabre-Nys *et al.* 1993; Powers 1970).

2.2 Estrogen Receptors

2.2.1 Structure and Function

E_2 production in the ovary undergoes constant modulation due to local factors (e.g., IGF) and hormonal regulation (FSH, LH, and progesterone) (reviewed in Gruber *et al.* 2002). The effects of E_2 are mediated by interactions at the plasma membrane level and in the nucleus (Marino and Ascenzi 2008; Fiorelli *et al.* 1996; Szego and Davis 1967). Estrogen receptors (ER) are the specific binding sites for E_2 and act as transcription factors (Björnström and Sjöberg 2005; Sharma *et al.* 1999). Two types of ER have been identified in mammals: a surface membrane-bound estrogen receptor, GPR30, which is suspected of acting through a non-genomic mechanism (Maggiolini and Picard 2010) and

the classic nuclear ER α and β (gene identification *Esr1* and *Esr2*, respectively) which may be located in the cytosol, on organelles, or at the plasma membrane (Acconcia and Marino 2011). Historically, ERα was the first estrogen receptor to be identified in the 1950's (Jensen and Jordan 2003). ERβ was later identified in 1996 (Kuiper *et al.* 1996). Rapid response to E_2 suggested that a physiological, non-genomic effect was being observed which could not be explained by nuclear ER activation (i.e., transcription and translation) (Fiorelli *et al.* 1996; Szego and Davis 1967).

The nongenomic activation by E_2 occurs in seconds and involves alterations in calcium channels and intracellular signaling pathway (Kelly and Levin 2001). The GPR30 was identified as a binding site for E_2 on the cell surface, inducing these non-genomic changes (Hess *et al.* 2011; Madak-Erdogan *et al.* 2008). However, recent studies have identified a fraction of ERα (Acconcia *et al.* 2005; Norfleet *et al.* 1999) and ERβ (Galluzzo *et al.* 2007) bound through palmitoylation to the plasma membrane of the cell. It is hypothesized that these surface ER are the likely causes of the rapid cell signaling and cell proliferation pathways associated with E_2 (Acconcia *et al.* 2004).

The characteristics of nuclear ERs are better understood than GPR30 and membrane bound ER, however several questions remain regarding the roles of classic ERα and ERβ in reproduction. It is clear that ERα and ERβ exhibit significant overlap in tissue localization and cellular action. The transcriptional activity and difference in ligand binding also varies between the two receptors and may help explain the cell-specific effect of these compounds.

Structurally, the amino acid sequences of the ERα and ERβ are divided into 5 types of modular domains, as are all nuclear receptors (Morani *et al.* 2008). Each domain

has a specific role that fits its structure: transcriptional activation, DNA binding, nuclear localization, ligand binding, and receptor dimerization. The N-terminal (A/B), the activation function 1 (AF1) domain involved in protein-protein interactions (co-activation and co-repressor), is highly variable between ERα and ERβ (Han *et al.* 2007), which may explain the transcriptional activity difference between the two receptors. AF1 has been shown to be critical to the activity of ERα and to be cellular- and promoter-dependent (Han *et al.* 2007; Merot *et al.* 2005). The DNA-binding domain (C) is highly conserved (97%) between ERα and ERβ and possesses a weak dimerization property (Morani *et al.* 2008; Schwabe *et al.* 1993). Domain D acts as a hinge to allow nuclear localization of the receptor. The ligand-binding domain (E) is made up of 12 α-helixes which provide the folding necessary for the binding site and is involved in nuclear localization, receptor dimerization, and interaction with co-factors (Morani *et al.* 2008). C-terminal (F) activation function 2 (AF2) domain is ligand-dependent and also highly variable between ERα and ERβ. It is involved in transcriptional activation and may affect the agonist/antagonist role of the ligand (Montano *et al.* 1995; Morani *et al.* 2008).

Differences in ligand binding domain, AF1, and AF2 allow the ER to transcribe different genes. Ligand-bound ERα and ERβ homo- or heterodimerize in the cytosol and translocate to the nucleus. In the nucleus, the ER dimers can bind to the DNA at the promoter of the estrogen response element or bind to other DNA bound transcription factors such as steroid receptor coactivator-1 or CREB binding protein to form a complex to induce gene transcription (McCarthy 2008; Morani *et al.* 2008). This action likely occurs through interactions with different co-activators and co-repressors, which have different binding affinities for the ER dimer complexes (Shiau *et al.* 1998). This effect is

likely through different conformational changes induced by the compound bound to the ligand-binding domain.

Estrogen receptor subtypes appear to work together to induce normal physiological conditions; however, transgenic animal knockouts have illustrated that the two ER may supplement each other with regard to some functions including reproduction (Barnett *et al.* 2006). With the recent identification of membrane bound ER, a method for delineating the effects of surface ER and nuclear ER to understand their interaction must be further explored (Acconcia and Marino 2011).

2.2.1.1 Estrogen Receptor α and β

2.2.1.1.1 Tissue Distribution of Estrogen Receptor α and β

ERα is present in a number of tissues in the female rodent including the uterus, oviduct, cervix, vagina, ovary, pituitary, hypothalamus (specifically the ARC), bone, and heart (Couse *et al.* 1997), and ERα mRNA has shown multiple alternative splicing at the transcriptional level (Stevens and Meech 2006). However, it has widely divergent functions, which are co-activator-, tissue- and ligand-dependent and are critical for steroidogenesis. For example, in rat granulosa cells histone acetylases (i.e., CREB-binding protein 1 and coactivator 1) and peroxisome proliferator–activated receptor gamma coactivator 1-alpha interact with ERα to induce *Hsd3b* and *Cyp11a1* (Chen *et al.* 2008). Female mice lacking ERα are infertile (Barnett *et al.* 2006) indicating it is essential for reproduction.

ERβ has been observed in the mammary gland, ovary, heart, fat and parts of the brain including the POA. In the ovary, ERβ is the dominant ER subtype and is localized mainly in the granulosa cells of growing and preovulatory follicles. ERβ is

downregulated by the pre-ovulatory LH surge (Byers *et al.* 1997). Increasing expression of ERβ mRNA coincides with the initiation of mRNAs that encode proteins involved in estrogen steroidogenesis (i.e., LHR, CYP11A1 and CYP19A1 (Bao *et al.* 2000). Female ERβ knockout mice have reduced LHR expression (Rodriguez *et al.* 2010) and a reduced production of corpora lutea, resulting in subfertility (Krege *et al.* 1998). Taken together, ERβ is necessary for the regulation of follicle development to the final mature follicle stage (Armenti *et al.* 2008).

Though the ER must bind together to initiate transcriptional activity, ERα and ERβ can form a heterodimer or a homodimer, suggesting that multiple pathways may be activated through ER. This effect may explain the varied effects of estrogen receptor ligand binding in different tissue types (Kaye *et al.* 2001). Agonists and antagonists for ERα and ERβ can also alter normal E_2 signaling.

2.2.1.1.2 Tools for Analyzing the Effects of Estrogen Receptor

Classic experiments by John and Benita Katzenellenbogen have developed and categorized many of the chemicals that selectively bind to the ligand-binding pockets of ERα and ERβ (Harrington *et al.* 2003; Katzenellenbogen *et al.* 2000). These experiments have illustrated plasticity and contrasting affinities of E_2 conjugates for ER (Shiau *et al.* 2002; Sun *et al.* 1999). Ligand binding affects receptor stability and exogenous compounds reduce the receptor half-life (Long and Nephew 2006; Wijayaratne and McDonnell 2001). Fulvestrant (ICI 182,780), a non-selective ER antagonist, increases ERα degradation through alteration in receptor structure leading to activation of ubiquitin-protesome pathway (Long and Nephew 2006). Though changes in receptor stability are common, the mechanism involved varies between ligand type (E_2, selective

estrogen receptor modulator, or ICI 182,780), but ERα transcriptional activity is linked to receptor stability (Wijayaratne and McDonnell 2001). However, when properly understood these exogenous ligands are useful tools to understand the underpinning of ER-mediated signaling.

Questions remain about the nature of some compounds that have been identified as ERβ ligands. A recent study by Paruthiyil et al. (Paruthiyil *et al.* 2009) described the complexity of selective ERβ agonists, which may have some, little, or no interaction with ERα. For example, diarylpropionitrile (DPN) which was the first synthesized ERβ-selective agonist has a 70-fold higher *in vitro* binding affinity and 170-fold higher potency in transcription assays as compared to ERα (Meyers *et al.* 2001). However, DPN induces 31 differentially regulated genes that are not altered by E_2, suggesting that DPN does not totally mimic the effect of E_2. Similar differences in gene expression were seen with other ERβ selective agonists (Paruthiyil *et al.* 2009). Consequently, many studies will have to be re-analyzed to discover if what was initially considered an ERβ effect could instead also be mediated by ERα.

Other useful tools for studying ERβ include mice with homozygous deletion of ERβ ($ER\beta^{-/-}$) and the use of ERβ antibodies. However, recent issues have developed with both of these tools. Variation in the characterization of the phenotypes of $ER\beta^{-/-}$ mice has yielded conflicting published reports (reviewed in Harris 2007) and a recent report has pointed out the lack of sensitivity and significant cross-reactivity with ERα of many commercial ERβ antibodies (Wu *et al.* 2011). These confounding issues require early resolution.

The development of chemical ligands for ER has proven to be helpful in elucidating estrogen signaling and in therapeutics. For example, the partial estrogen receptor agonist, tamoxifen, was lauded as a powerful anti-breast cancer agent (Jordan 1988). However, it was discovered to have limited clinical use due to the primary and acquired resistance to its growth-inhibiting effects and the induction of tissue growth in estrogen responsive tissues (i.e., endometrium) and tumor growth in resistant patients (Robertson 2001). Subsequently, other agents such as the anti-estrogen ICI 182, 780 were developed in attempt to overcome these issues (Hu *et al.* 1993).

Antiestrogens, partial agonists, and selective estrogen receptor modulators (SERM) have helped increase our understanding of estrogen related pathologies and provided potential pharmaceutical approaches to treat diseases such as cancer (Ke *et al.* 1997) and osteoporosis (Lufkin *et al.* 1998). A number of endocrine-disrupting chemicals (EDCs) have also been observed to differentially interact with ERα and ERβ and the endocrine system (reviewed in Craig 2011).

2.3 Hormonal Birth Control

The primary mechanism for female hormonal contraception involves using exogenous steroid hormones to act on the endocrine system. These hormones are divided into two major classes. Class 1, combined contraceptives, contains both an estrogen (generally ethinyl estradiol) and a progestogen (Fruzzetti *et al.* 2012; Lawrie *et al.* 2011). Class 2 is progestogen-only which is compromised of progesterone or a synthetic analogue.

The primary mechanism of action for Class 1 and Class 2 contraception is the prevention of ovulation through the suppression of gonadotropins and changes in cervical mucus viscosity which decrease sperm motility (Rivera *et al.* 1999). Progestogen,

through negative feedback, decreases the pulse frequency of GnRH and indirectly decreases FSH and LH release by the anterior pituitary. Folliculogenesis is inhibited, and the E_2 surge is prevented, resulting in the absence of ovulation. The estrogen component of the Class 1 contraceptive pill synergizes the inhibitory effect of progestogen on FSH and reduces the amount of abnormal uterine bleeding (Rivera *et al.* 1999).

Class 2 (progestogen-only) contraceptives' mechanism of action is dependent on the inherent strength of the progestogen and its dose. In comparison to combined oral contraceptives, the amount of progestogen is less and does not consistently prevent ovulation. The secondary mechanism of action is the thickening of the cervical mucus through decreasing volume of the mucus, increasing viscosity and cell content of the mucus, and reducing the habitability of the mucus. This effect reduces sperm motility and reduces fertilization (Kesserü and Larraňaga 1971; Rivera *et al.* 1999).

A potential third class is a non-steroidal selective estrogen receptor modulator (SERM) only marketed in India (Singh 2001). This compound, Centrochroman, is a once-a-week oral contraceptive that has estrogenic and anti-estrogenic properties (Awasthi *et al.* 2007; Kriplani *et al.* 2009). Prevention of pregnancy occurs through a combination of actions including slightly increasing the rate of transport of embryos through the oviducts, accelerating blastocyst formation, and suppressing uterine endometrial proliferation and decidualization (Singh *et al.* 1986). By inducing asynchronicity between zygote development and transport, Centrochroman appears to be sufficient to alter implantation and serve as a successful birth control pill. It also may provide potential therapeutic relief for dysfunctional uterine bleeding (Kriplani *et al.* 2009; Singh 2001).

2.4 Endocrine Disruption and Disease States

Endocrine-disrupting chemicals are exogenous agents that interfere with the endocrine systems of animals and can induce pathology. EDCs are known to be significant environmental factors in human and animal diseases (Colborn *et al.* 1993).

Methoxychlor (MXC) is an organochlorine pesticide DDT-analog. Though there are no documented effects of MXC on the human reproductive system, adverse effects on the reproductive systems in multiple other animal species have been observed (Chapin *et al.* 1997; Cummings and Laskey 1993; Golub *et al.* 2003; Gray *et al.* 1989; Gill *et al.* 1979; Stillman 1982). EDCs that affect the reproductive system generally mimic the effect of sex steroids (estrogens and/or androgens), leading to irregular function (Walters *et al.* 2008).

2.4.1 Developmental Exposure Windows

The timing of exposure to endocrine disruptors is critical to the effect of the compound. Developmental windows are especially sensitive to perturbation and may affect the occurrence of the physiological onset of puberty (Mouritsen *et al.* 2010). The concept that fetal endocrine dysfunction can cause later chronic disease or adult dysfunction is referred to as the 'Barker's hypothesis' (Paneth and Susser 1995), and was invoked to describe how fetal under-nutrition during World War II in Holland was associated with increased incidence of adult coronary heart disease (Barker 1995). This hypothesis has been expanded to include other adult chronic diseases including osteoporosis (Antoniades *et al.* 2003), obesity and type 2 diabetes (Hales and Barker 2001) and breast and ovarian cancer (Barker *et al.* 2008a; Barker *et al.* 2008b). Neonatal exposure MXC has been shown to induce epigenetic reprogramming in the ovary (Zama and Uzumcu 2009).

2.4.2 Endocrine Disruption and Fertility

Recent studies have shown that the actions of many EDCs are mediated by estrogenic effects and may affect male and female reproductive systems (reviewed in Uzumcu and Zachow 2007; Gray *et al.* 1989; Gray *et al.* 1988). These compounds can be environmental compounds such as pesticides or food additives such as propyl gallate, pharmaceuticals or organic contaminants contained in innocuous household products such as detergents. For example, exposure to the organochlorine pesticide dichlorodiphenyltrichloroethane, DDT, is associated with longer time to pregnancy in female farm workers exposed while having no associated effect on male fertility (Harley *et al.* 2008). Clinically prescribed diethylstilbestrol (DES) has been associated with reproductive tract disorders (Gill *et al.* 1979; Stillman 1982), cancers (Greenberg *et al.* 1984), and fertility issues in the exposed mothers, their daughters (Blatt *et al.* 2003) and their sons (Kalfa *et al.* 2011). All of these effects are associated with reduced fecundity of reproduction through subtle adverse effects. Obvious reproductive effects have been observed with EDCs in wildlife environments. Guillette et al. (Guillette *et al.* 2000) reported altered hormone levels and reproductive tract anatomy in alligators in EDC-exposed environments in central Florida. Therefore, exogenous EDCs are ubiquitous, especially pesticides which can have subtle and acute effects on reproductive function.

2.4.2.1 Organochlorines and Fertility

Organochlorines are known to impair female fertility through altering ovarian structure and function (Tiemann 2008). For example, trichloroethylene (TCE) is an organochlorine chemical used as a degreasing agent that directly affects the rat ovary. TCE is activated

by cytochrome P450 oxidation in the ovary and formed protein adducts which may reduce oocyte fertility in rodents (Wu and Berger 2007). The impact of organochlorine compounds can also affect progeny *in utero* (Tan *et al.* 2009). Laboratory rats exposed to DDT had reduced sperm (Rhouma *et al.* 2001) and altered estrous cyclicity (Gellert and Heinrichs 1975).

Organochlorine chemicals are generally able to migrate through the placenta and reach the fetus (Bergonzi *et al.* 2011; Bergonzi *et al.* 2009). DDT levels are highly correlated in paired maternal serum and cord blood suggesting that minimal barrier exists between the maternal-fetal units (Sapbamrer *et al.* 2008). This lack of placental barrier clearly contributes to the adverse effects of organochlorines on fetuses.

2.5 MXC

Methoxychlor (MXC), an organochlorine pesticide introduced as a safer alternative to DDT, was shown to have unexpected effects on the reproductive systems in multiple animal species (ATSDR 2002; Golub *et al.* 2004; Gray *et al.* 1989; Gray *et al.* 1988; Kapoor *et al.* 1970; Miller *et al.* 2005; Tiemann 2008). In the female rat, MXC has been shown to affect the uterus and the ovary (Chapin *et al.* 1997; Jackson *et al.* 1975) with sexual behavior and neurological effects also observed (Gray *et al.* 1999; Gray *et al.* 1988). Since MXC is a DDT-analog, its mode of action as an insecticide is through depolarization of voltage-dependent sodium channels, which leads to paralysis and death (Davies *et al.* 2007).

2.5.1 Effect of MXC on Estrogen Signaling

It has been proposed that MXC acts through an estrogenic pathway (Miller *et al.* 2006). MXC itself has a relative weak affinity for the estrogen receptor subtypes (Gaido *et al.*

1999; Gaido *et al.* 2000); however, the major metabolites of MXC show a much higher affinity for these receptors, suggesting that they play a role in MXC toxicity (Gaido *et al.* 1999; Gaido *et al.* 2000). The pathway(s) through which MXC affects the ovary as well as its distribution into target tissues remain poorly characterized (Cummings and Laskey 1993; Eroschenko *et al.* 1995; Gupta *et al.* 2006a; Gupta *et al.* 2006b; Miller *et al.* 2006; Symonds *et al.* 2008; Tomic *et al.* 2006).

Female rats exposed to MXC (100 mg/kg/day) during discrete development windows (gestational day 19 and post-natal day 7) had smaller litters, fewer corpora lutea, dysfunctional estrous cyclicity, and early onset ovarian failure at adulthood (Armenti *et al.* 2008). This functional loss was associated with changes in ovarian follicle composition, including an increase in early antral follicles and primary follicles. Superovulation treatments in MXC-exposed females produced fewer oocytes than the control females, suggesting a direct effect of MXC in the ovary (Armenti *et al.* 2008). Though effects on the HPO axis may be taking place, evidence supporting the direct effect of MXC on the ovary has been generated in *in vitro* (Gupta *et al.* 2007; Gupta *et al.* 2006a; Miller *et al.* 2005) and *in vivo* systems (Armenti *et al.* 2008; Borgeest *et al.* 2004). In addition, adult ovaries from MXC-treated animals had reduced protein levels of ERβ, LHR, and CYP11A1 when compared to control ovaries (Armenti *et al.* 2008).

2.5.2 MXC and MXC Metabolite Activity

MXC is minimally bio-accumulated and rapidly eliminated in the form of two polar metabolites. The metabolites, 2-(*p*-hydroxyphenyl)-2-(*p*-methoxyphenyl)-1, 1, 1-trichloroethane (Mono-OH) and 2, 2-bis (*p*-hydroxyphenyl)-1, 1, 1-trichloroethane (HPTE) are significantly more estrogenic than MXC and have been shown to have high

affinity for the estrogen receptors α and β (Gaido *et al.* 1999). These compounds act as ERα agonists and ERβ antagonists with HPTE exhibiting greater affinity for the ER than Mono-OH (Gaido *et al.* 1999; Gaido *et al.* 2000). It has been proposed that MXC acts through its major metabolites, HPTE and Mono-OH, to affect the reproductive system through an estrogen-regulated pathway (Miller *et al.* 2006). However, the mechanisms of action for these affects remain unclear as research suggests non-classical nuclear ER-mediated pathways may be involved (Ghosh *et al.* 1999).

How MXC directly affects the follicles remains unclear. Evidence for its effect on antral follicle steroidogenesis has been presented, but a similar inhibitory effect has been observed in granulosa cells. This evidence suggests follicular toxicity may be mediated through granulosa cell dysfunction. Chemical agents can target the granulosa cells in multiple processes including alteration of estrogen synthesis/metabolism, expression of FSH and LH cell surface receptors, inhibition of steroidogenic enzyme activity, progesterone secretion, and cellular signaling (reviewed in Craig *et al.* 2011, Davis and Heindel 1998). Interference with cell signaling can occur through interruption of FSH-stimulated cAMP accumulation, a mechanism that has been demonstrated in granulosa cells exposed to mono-(2-ethylhexyl) phthalate (MEHP) (Lovekamp-Swan and Davis 2003).

MXC and its metabolites inhibit steroidogenesis, a key function of the granulosa cell. MXC may be useful as a model ovotoxicant, which we define as a xenobiotic that adversely affects ovarian function. In *in vitro* studies, HPTE has often been used as the active compound for MXC (Akgul *et al.* 2008; Zachow and Uzumcu 2006). However, a direct comparison of the effects of MXC and HPTE in granulosa cells has not been

performed. Granulosa cells assume a distinct morphology characterized by lamellipodia and fillipodia growths that form an interlaced network between the cells exposed to FSH through a cAMP-dependent pathway (Grieshaber *et al.* 2000). These granulosa cells microprocesses are associated with extracellular matrix interaction and cell-to-cell signaling (Le Clainche and Carlier 2008). This phenomenon is not observed when granulosa cells are treated with MXC/HPTE (10 μM) in the presence of FSH or cAMP (Chapter 5). The loss of the cAMP induced morphological alterations suggests that extracellular matrix interactions and cell-to-cell signaling networks are affected by MXC/HPTE. As noted in section 2.1.5, alterations in structure are associated with inhibition of function. HPTE also inhibited FSH-mediated E_2 and downregulated mRNA encoding steroidogenic enzymes (Zachow and Uzumcu 2006), and MXC inhibits FSH-mediated E_2 (Chapter 3).

Evidence suggests that HPTE targets steroidogenesis through the FSH-PKA signaling pathway (Zachow and Uzumcu 2006), and MXC may act on common gene networks to induce inhibitory action on E_2 that is observed using HPTE. Whether this interaction is a direct effect or through still vaguely defined crosstalk of parallel signaling cascades (Knight and Glister 2006) is unknown. As previously mentioned, multiple signaling pathways operate within the granulosa cell and each have some role in directing normal cellular function. Thus, a comparison of the gene expression profiles of MXC and HPTE may provide valuable information about pathological and normal granulosa cell function.

2.5.3 Analytical Measurement of MXC and MXC Metabolites

Measurement of MXC concentrations has been generally conducted in biological materials using gas chromatography with an electron-capture detector (ECD). An alternative method of analysis involves cleanup using high performance liquid chromatography (HPLC) followed by gas chromatography tandem mass spectroscopy (GC/MS/MS). The ECD method exhibits greater precision, accuracy and limit of detection values, but the GC/MS/MS method has a lower limit of detection (ATSDR 2002).

Analysis of HPTE and Mono-OH has been commonly carried out using HPLC with a UV detector, GC/MS, or GC with a flame ionization detector (ATSDR 2002). GCMS was used to measure the HPTE and MXC in wastewater effluent and environmental waters (Baugros *et al.* 2008). Biological samples from channel catfish used radiolabeled isotopes and HPLC to measure the tissue distribution of HPTE and Mono-OH and their glucuronide conjugates (Nyagode *et al.* 2009). However, we are unaware of a LC/MS method for the identification/quantification of HPTE and Mono-OH in biological tissue.

2.5.4 MXC-induced CYP Activity

Induction of cytochrome P450 enzymes by MXC has been extensively studied in the liver (Bulger *et al.* 1985; Bulger *et al.* 1978; Dehal and Kupfer 1994; Li *et al.* 1995a). For example, Ohyama (Ohyama *et al.* 2004, 2005) showed that MXC-treated livers from female rats produced a higher percentage of Mono-OH (40%) versus male rats (5%).

MXC is metabolized and excreted quickly (98% within 48 hrs) in the form of the more hydrophilic metabolites, Mono-OH and HPTE (Kapoor *et al.* 1970). As described

previously, MXC metabolites are able to bind to estrogen receptors (Gaido *et al.* 1999; Gaido *et al.* 2000), and cause deleterious effects on the granulosa cells and antral follicles *in vitro* (Gupta *et al.* 2006a; Harvey *et al.* 2009; Miller *et al.* 2006). However, MXC, Mono-OH, and HPTE treatment has been shown to cause decreased gene expression of *Cyp11a1*, *Cyp19a1*, and *Cyp17a1* (Basavarajappa *et al.* 2011; Craig *et al.* 2010; Zachow and Uzumcu 2006). If Mono-OH and/or HPTE are the active agents for MXC, cells treated *in vitro* with MXC must be able to metabolize MXC into Mono-OH and HPTE.

The metabolic capabilities of the ovary have been noted in the past through modulation by EDCs (Cannady *et al.* 2003). The surface epithelium of the mouse ovary expresses the MXC-metabolizing enzyme, *Cyp2c29* (*Cyp2c7* is the rat homolog), in the presence of E_2 or MXC (Symonds *et al.* 2006). Granulosa cells are likely derived from surface epithelium (Mork *et al.* 2011) which plausibly could indicate a similar capability of gene expression for MXC metabolizing enzymes. MXC also induces the expression of the E_2-metabolizing enzyme, *Cyp1b1*, in antral follicles *in vitro* (Basavarajappa *et al.* 2011). Granulosa cells have been shown to express *Cyp1b1*, the transcript of the protein that metabolizes estrogen to the catecholestrogen metabolite (Dasmahapatra *et al.* 2002). MXC-treated granulosa cells could metabolize local E_2 levels faster than untreated granulosa cells.

2.6 Gene Expression and Regulation in the Granulosa Cells

The advent of molecular biology has broadened understanding of ovarian function and the genes that regulate ovarian physiological processes such as folliculogenesis (Barnett *et al.* 2006; Oktem and Urman 2010), luteinization (Richards *et al.* 1998), steroidogenesis (Richards 1994), and ovulation (Richards 2005). Sequencing of the rat genome along

with development of high throughput gene expression and transgenic technologies has identified important gene pathways involved in reproduction (Bonnet *et al.* 2008); however, expanded computational tools are required to understand and analyze those data. One current paradigm for data analysis is the transition from identifying individual gene regulation to a more pathway-driven method (Ovacik *et al.* 2010a). This new information may allow for the development of new models that combine biochemical and genetic techniques for better understanding the processes of reproduction.

2.7 Summary

The rat is a useful model for understanding the effects of EDCs on the female reproductive system in humans. These effects can occur at multiple foci of the HPO axis. Furthermore, the mimicry of estrogens by EDCs can cause disruption of normal ovarian reproductive functions (i.e., steroidogenesis, folliculogenesis, ovulation, etc.). Toxic insult of the granulosa cells through dysfunction of the FSH signaling pathway can be a mechanism for ovarian failure. Alternatively, disruption of ER-mediated signaling can have multiple effects on normal fertility as observed in transgenic animal experiments and *in vitro* cell-based assays using ER agonists and antagonists. The literature clearly demonstrates the impact that increased exposure to EDCs may have in animals and humans and the increased susceptibility of certain ages (*in utero* and neonatal animals) to these effects. Using MXC as a model compound to understand how EDCs affect regulators of ovarian gene expression is important for potential therapeutic solutions in susceptible populations.

CHAPTER 3: Methoxychlor and HPTE Inhibit cAMP Production and Estrogen Receptors α and β in the Rat Granulosa Cell *In Vitro*

3.1 Introduction

Methoxychlor (MXC), an organochlorine pesticide previously thought to be innocuous due to its low bioaccumulation in mammals (Kapoor *et al.* 1970), was subsequently shown to have unintended side effects on reproductive function in multiple animal species including mice, rats, and primates (Golub *et al.* 2003; Gray *et al.* 1989; Hall *et al.* 1997). MXC is a weak estrogenic compound (Gaido *et al.* 1999; Schlenk *et al.* 1998). The observed reproductive effect of MXC has been suspected to be a function of its active metabolite, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which has been shown to bind strongly to the estrogen receptors selectively as an ERα agonist and ERβ antagonist (Gaido *et al.* 1999). The specificity of receptor interaction may explain the observed tissue specific effects of MXC *in vivo.* For example, MXC acts as an ERα agonist in the mouse uterus where ERα is the primary estrogen receptor subtype, inducing tissue growth upon activation (Tiemann 2008). In contrast, MXC acts in an inhibitory manner in the rodent ovary, a tissue that primarily expresses ERβ (Drummond and Fuller 2010). However, it is likely that an action through ERα is also involved in ovarian effects as this estrogen receptor subtype is also expressed in the ovary (Miller *et al.* 2006).

In the ovary, the granulosa cell produces estradiol 17β (E_2) and progesterone and supports the oocyte to maturity. Our lab has shown that HPTE modulates the ability of *in vitro* rat granulosa cells to produce E_2 in a dose-dependent manner (Harvey *et al.* 2009; Zachow and Uzumcu 2006). We also showed that E_2 production and luteinizing hormone receptor (*Lhcgr*) and P450 cholesterol side-chain (*Cyp11a1*) expression were reduced in

FSH-stimulated granulosa cells treated with HPTE *in vitro* (Harvey *et al.* 2009; Zachow and Uzumcu 2006). Fetal and neonatal exposure to MXC resulted in adult female rats that exhibited multiple reproductive dysfunctions such as reduced litter size, early onset of puberty and of first estrous, reduced response to exogenous gonadotropin, irregular cyclicity, and reduced numbers of corpora lutea and large antral follicles (Armenti *et al.* 2008). This study also demonstrated that ERβ and LHR proteins were reduced. In addition, superovulation studies with exogenous gonadotropins showed a reduction in ovulation rates, suggesting that the effects of MXC were at least in part in the ovary (Armenti *et al.* 2008).

Estrogen receptor signaling is required for the normal development of the ovary (Drummond and Fuller 2010). HPTE binds to the estrogen receptor (Gaido *et al.* 1999) and disrupts the synthesis of E_2 (Zachow and Uzumcu 2006). E_2 synthesis is primarily stimulated by FSH binding and the activation of protein kinase A through cAMP production (reviewed in Hunzicker-Dunn and Maizels 2006; Dorrington *et al.* 1975). Recently, we observed that HPTE had a greater inhibitory effect on FSH-induced steroidogenesis and global gene expression when compared to dibutyryl cAMP-stimulated steroidogenesis and global gene expression in the granulosa cell (Harvey *et al.* 2009; Zachow and Uzumcu 2006). These observations suggest that cAMP is a target of MXC and HPTE. Estrogen receptor signaling and cAMP have been previously linked; 1 nM estrogen has been shown to induce maximal cAMP production in uterine (Aronica and Katzenellenbogen 1991) and MCF-7 cells (Aronica *et al.* 1994). Interestingly, a recent report suggested that ERβ also modulates cAMP production and *Lhcgr* expression

in ovarian granulosa cells (Deroo *et al.* 2009); leading to our hypothesis that regulation of cAMP by MXC is mediated via estrogen receptors.

Therefore, the objective of this part of our study was to examine the effects of HPTE and MXC on intracellular cAMP production and on ERα and ERβ mRNA and protein levels. In order to investigate potential estrogen receptor-related mechanisms of action in this study, we examined the effects of ICI 182,780 (ICI), an ERα and ERβ antagonist, and PHTPP, a selective ERβ antagonist, on cAMP production. Inhibition of cAMP and estrogen receptor expression offers insight into how MXC directly affects the ovary.

3.2 Materials and Methods

Animals

Ovaries from unstimulated Spraque-Dawley rats (postnatal day 21-postnatal day 27) were used to prepare granulosa cell cultures as previously described (Harvey *et al.* 2009). Animals were maintained in accordance with the Rutgers University Animal Care and Facilities Committee. Animals were allowed *ad libitum* feed and water in a room with a 14:10 h light: dark cycle at a constant temperature (~21°C).

Chemicals

Chemical agents (PHTPP; 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyri midin-3-yl]phenol, DPN; 2,3-bis(4-Hydroxyphenyl)-propionitrile, ICI; ICI 182,780; 3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1*H*-purine-2,6-dione, IBMX; 3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1*H*-purine-2,6-dione) were purchased from Tocris (Tocris Bioscience, Ellisville, Mo.). FSH (oFSH-20; 4453 IU/mg) was purchased from the National Hormone and Pituitary Program of the National Institute of Diabetes and

Digestive and Kidney Diseases (Dr A.F. Parlow, Harbor-UCLA, Torrance, CA). HPTE was synthesized using a method similar to a previously reported method (Stuchal *et al.* 2006). HPTE and MXC were confirmed to be < 98% purity by HPLC and thin layer chromatography (TLC). TLC was conducted during the reaction, post-reaction, during column separation and on a series of fractions collected to monitor the stage of the separation. Fractions were matched with expected Rf values and coupled with HPLC results; this supports the high purity of HPTE.

Granulosa Cell Culture

Cells were plated at a density of 1-1.5 x 10^6 cells in 3 ml per well in a 6-well plate for RNA, protein, and cAMP analysis or 3-4 x 10^5 in 1 ml per well in a 24-well plate for cell viability and cAMP analysis. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) /F-12 containing 5% fetal bovine serum with 100 I.U./ml of penicillin and 100 μg/ml of streptomycin. The incubator was set to an atmosphere of 5% CO_2 in air at 37° C and cultures were allowed to acclimate for at least 24 h. Media were then aspirated and replaced with serum-free DMEM/F12 containing (0.1 μM) androstenedione.

Cultures were treated with 10 μM of MXC, HPTE, or untreated for a 48 h period in the presence or absence of 3 ng/ml of FSH. Doses were selected based on previous work that showed submaximal efficacy for FSH and no cytotoxicity for MXC/HPTE in the cell culture [(Zachow and Uzumcu 2006), unpublished observations]. Concentrations of ICI (5 μM), DPN (1 μM), and PHTPP (1 μM) were chosen based on previously reported doses (Chen *et al.* 2008). The dose of IBMX (50 μM) represents the IC_{50} provided by the supplier for all phophodiesterases (Beavo *et al.* 1970). The cells were

collected for intracellular cAMP, RNA, protein isolation, and to test cell viability. Media were collected for E_2 radioimmunoassay (RIA) analysis.

Cell Viability Assay

To measure the effect of the treatments on cell viability, a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (Roche Manheim, Germany) by mitochondrial dehydrogenase was used according to the manufacturer's guidelines. The cultures were incubated at 37° C for 3 h following the 48 h treatment period. Absorbance was measured at 450 nm after a 1 min shaking period.

RIA

E_2 was measured using a COAT-A-COUNT assay kit (Siemens Healthcare Diagnostic, Deerfield, IL) according to the manufacturer's instructions. A volume of 100 μL of sample was added to each antibody coated-polypropylene tube and incubated with 1 mL of I^{125} labeled E_2 for 3 hours. The sample E_2 and radiolabeled E_2 compete for the antibody sites in the tubes. Tubes were decanted to remove the free E_2 and radioactivity was measured using a gamma counter. A calibration curve was constructed using 6 standard doses to quantify sample E_2 (in pg/ml) by comparison to the counts of the calibration.

cAMP Measurement

To measure intracellular cAMP levels, granulosa cells were lysed and assayed according to the manufacturer's instructions for the cAMP Biotrak Enzyme immunoassay (EIA) system (RPN225 Protocol 3, GE Health Life Science, Piscataway, NJ). Briefly, samples were treated with lysis buffer and incubated for 10 min with gentle shaking. Samples were then transferred into a donkey anti-rabbit IgG coated 96-well plate and incubated for 2 h in the presence of rabbit anti-cAMP antiserum. The next step was the addition of

cAMP-horseradish peroxidase conjugate for 1 h treatment followed by decanting and 4 consecutive washes. The enzyme substrate 3, 3', 4, 4'-tetramethylbenzidine/ 1% hydrogen peroxide (TMB) was used to initiate color change in the wells. Sulfuric acid was added after 30 min incubation with TMB to stop the reaction and absorbance was then measured at a wavelength of 450 nm.

Concentrations of cAMP (fmol/well) were calculated from a standard curve prepared by plotting percent bound as a function of the log of cAMP. In order to compare cAMP concentrations that are obtained from 6-well and 24-well plates (larger *vs.* smaller surface area), the latter was normalized to protein concentration (mg/ml). The cAMP values for five treatment groups (basal, FSH, FSH+HPTE, ICI, and ICI+HPTE) were compared between results from 6-well and 24-well plates and no significant difference was observed (data not shown).

RNA Isolation and Real Time PCR (qPCR)

RNA isolation was performed according to the guidelines for TRIzol® Reagent (Invitrogen, Carlsbad, CA). RNA purity and concentration was confirmed by spectrophotometry. E-Gel® Electrophoresis Precast agarose gel was used to confirm RNA integrity and clear bands were observed for both 18s and 28s rRNA using ethidium bromide dye (data not shown).

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to synthesize cDNA from 2 μg of RNA for qPCR. All transcript levels were quantitated by qPCR using Power Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA) and SteponePlus™ real-time PCR (Applied Biosystems). Primers for *Esr1*, *Esr2*, and hypoxanthine-guanine phosphoribosyltransferase *(hprt***)** were

designed using Primer-Blast (NCBI, Bethesda, MD, Table 3.1). The reference gene *hprt* was used to normalize the mRNA level for each treatment group.

Table 3.1: Real-time qPCR primer accession numbers, gene names, gene symbols, and sequences.

Accession #	*Gene Name*	*Gene Symbol*	*Forward*	*Reverse*
NM_012583.2	*Hypoxanthine-guanine phosphoribosyltransferase*	Hprt	CAGGCCAGAC TTTGTTTGGAT	CCGCTGTCTTTT AGGCTTTG
NM_012689.1	*Estrogen Receptor Alpha*	Esr1	CATCGAAAGA ACCGGAGGA	TCTGACGCTTG TGCTTCAAC
NM_012754.1	*Estrogen Receptor Beta*	Esr2	AACCGCCATG AGTATTCAGC	GTAACAGGGCT GGCACAACT

All qPCR quality control checks were done in accordance with the Minimum Information for Publication of Real-Time Quantatitive PCR Experiments. Briefly, analysis of the slope of the calibration curves indicated efficient amplification (>90%). Dissociation curves showed no evidence of primer-dimer formation. One-way ANOVA confirmed that no treatment effect was observed on hprt (data not shown). Controls including no RT product and water blanks were used to ensure specificity of amplification.

Protein Extraction and Western Blot Analysis

Granulosa cells culture wells were lysed with Complete Lysis-M kit (Roche Applied Science, Manheim, Germany). Protein concentrations in the cell lysates were measured using DC Protein Assay Kit II (Bio-Rad Life Sciences, Hercules, CA). Protein (20 µg) mixed with sample-loading buffer was heated at 70° C for 10 minutes. Proteins were run on 12% Bis–Tris–HCl-buffered denaturing polyacrylamide gels (Nu-Page, Invitrogen, Carlsbad, CA) under reducing conditions and transferred to nitrocellulose membranes. Ponceau S stain (Sigma) was used to verify the uniform and complete transfer of proteins

on to the membrane. ERα (mouse) and ERβ (rabbit) antibodies (Thermoscience Fremont, CA. Cat#MS-1071-S, and Pierce Antibodies Rockford, IL. Cat#PA1310B) and goat anti-mouse HRP conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA. Cat#sc-2302) or goat anti-rabbit HRP conjugated IgG (H+L) (Invitrogen, Cat#65-6120) were prepared (1:2,000) in 10% powdered milk and Tris-Buffered Saline Tween-20 (TBST) buffer and incubated overnight for primary antibodies, and 1 h each for secondary antibodies. Blots were washed with TBST before and after each incubation period. Visualization of the target proteins was performed using electrochemiluminescence detection system (Perkin-Elmer, Waltham, MA Cat#NEL100001EA) with β-actin used to monitor consistent protein loading. ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij/) was used to quantify band intensity.

Statistical Analysis

Enzyme immunoassay, protein, and qPCR data were analyzed statistically by one-way ANOVA (Prism 4.0 GraphPad, San Diego, CA) followed by Dunnet's or Newman-Keul multiple comparison tests. Values were presented as mean ± standard error of the mean (SEM). Correlation values between cAMP and estrogen receptor gene expression were performed using the Pearson's Correlation test in Microsoft Excel with *p*-values calculated using p-value Calculator for correlation coefficients (DanielSoper.com on May 12, 2011). Levels of significance were determined with α set at $p < 0.05$.

3.3 Results

3.3.1 MXC and HPTE inhibited FSH-stimulated cAMP production

Treatment of granulosa cells with FSH, stimulated intracellular cAMP (1953±381.1 fmol) 5-fold as compared to that in basal granulosa cells (399±175.7). HPTE (201.9±69.16) and

its parent compound, MXC, (387.1±114.4) significantly inhibited production of cAMP when FSH was present (Fig. 3.1A). HPTE treatment did not have any significant effect on cAMP levels in the absence of FSH (Fig 3.1A) and neither did MXC (data not shown).

The effect on E_2 secretion was consistent with cAMP results. FSH stimulated E_2 approximately 1000-fold, and HPTE and MXC reduced E_2 to basal levels (Fig. 3.1B). Treatment with HPTE did not affect the basal E_2 production. In order to test if HPTE and MXC treatments were affecting viability, the number of cells were estimated using WST-1 assay. The results showed no differences between treatment groups (Fig. 3.1C).

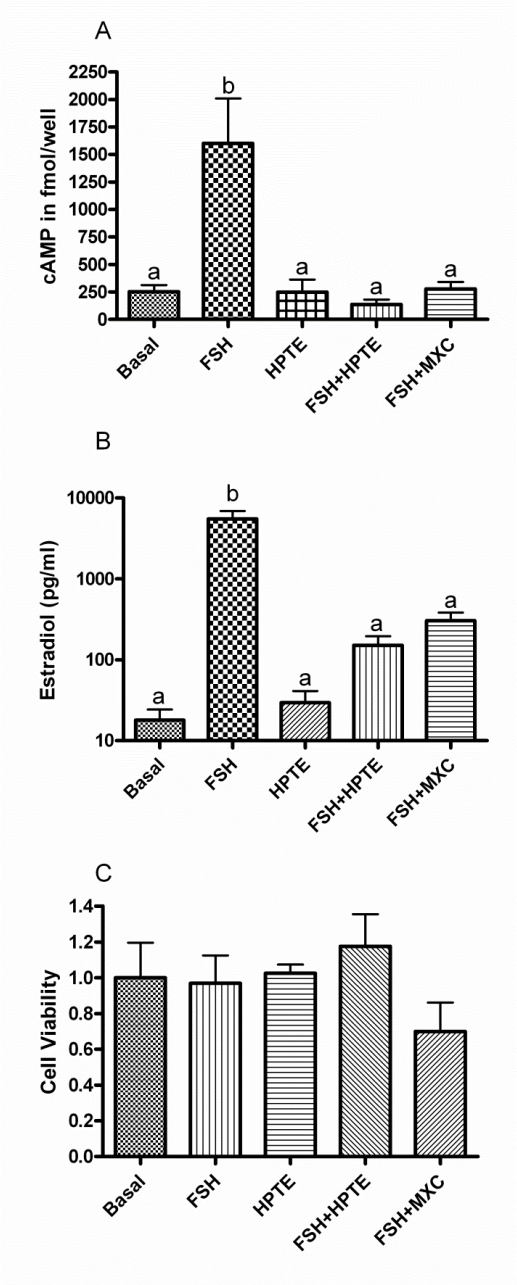


Figure 3.1: Effect of MXC and HPTE on FSH-stimulated intracellular cAMP and estradiol (E_2) production.

Granulosa cells were incubated in the absence or presence of 3ng/ml FSH, HPTE (10 μM), a combination of MXC (10 μM) and FSH, or HPTE and FSH for 48 h. Media were collected for analysis of E_2 and cells were harvested for intracellular cAMP determination or cell viability analysis. Radioimmunoassay, enzyme immunoassay and WST-1 assays were performed as described in Materials and Methods. A, cAMP was expressed as mean fmol/well ± SEM (n≥4). B, E_2 was expressed as pg/ml±SEM of culture medium (n=5). b = significantly different ($p < 0.01$) from control as determined by one-way ANOVA followed by Dunnett's Multiple Comparison test post-hoc analysis. C, Cell viability showed no difference between treatments (n=3).

3.3.2 Effect of IBMX on MXC and HPTE-mediated inhibition of cAMP

To evaluate whether degradation of cAMP was increased by MXC or HPTE, cultures were co-treated with IBMX, a non-selective phosphodiesterase inhibitor. IBMX had no effect on FSH-stimulated cAMP in the presence of MXC or HPTE (Fig. 3.2A). This result suggests that MXC or HPTE does not increase the degradation of intracellular cAMP, but instead inhibits production of cAMP. Treatment with IBMX did not prevent MXC or HPTE from inhibiting FSH-mediated E_2 production (Fig 3.2B).

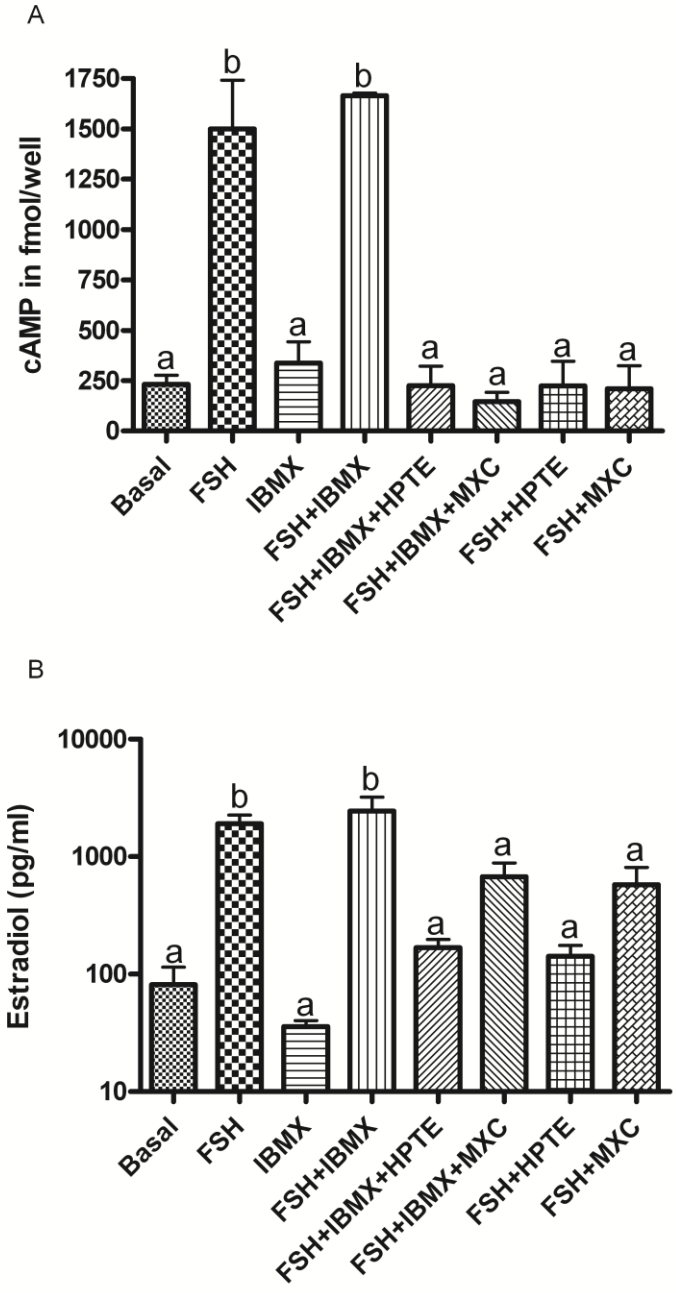


Figure 3.2: Effect of IBMX on MXC/HPTE-mediated cAMP inhibition and E_2.
Media from the granulosa cell cultures were collected for analysis of E_2 and cells were harvested for intracellular cAMP determination. Radioimmunoassay and enzyme immunoassay performed as described in Materials and Methods. Granulosa cells treated with MXC and HPTE inhibited FSH-mediated cAMP and E_2 production and in the presence or absence of IBMX (50 μM). A, cAMP was expressed as mean fmol/well ± SEM ($n \geq 3$). B, E_2 was expressed as pg/ml of culture medium ($n > 4$). Different letter indicates significant difference between treatment groups were as determined by one-way ANOVA ($p < 0.01$) followed by Dunnett's Multiple Comparison test post-hoc analysis.

3.3.3 Effects of the ESR2 antagonists and agonist FSH-mediated cAMP

Since ER and PKA signaling systems interact with each other in various systems, we tested if estrogen receptor antagonism would lead to inhibition of cAMP production. Granulosa cells were exposed to the ERα and ERβ antagonist, ICI 182,780 (ICI), in the presence or absence of FSH. FSH-stimulated cAMP was decreased by ICI to the basal level (Fig. 3.3). In comparison with FSH+HPTE treatment, FSH+ICI also inhibited cAMP production. ICI in combination with HPTE in the presence or absence of FSH had an inhibitory effect on intracellular cAMP when compared to ICI alone. In the absence of FSH, ICI treated granulosa cells produced a lesser amount of cAMP. Antagonism of the ER inhibited cAMP production in granulosa cells (Fig. 3.3).

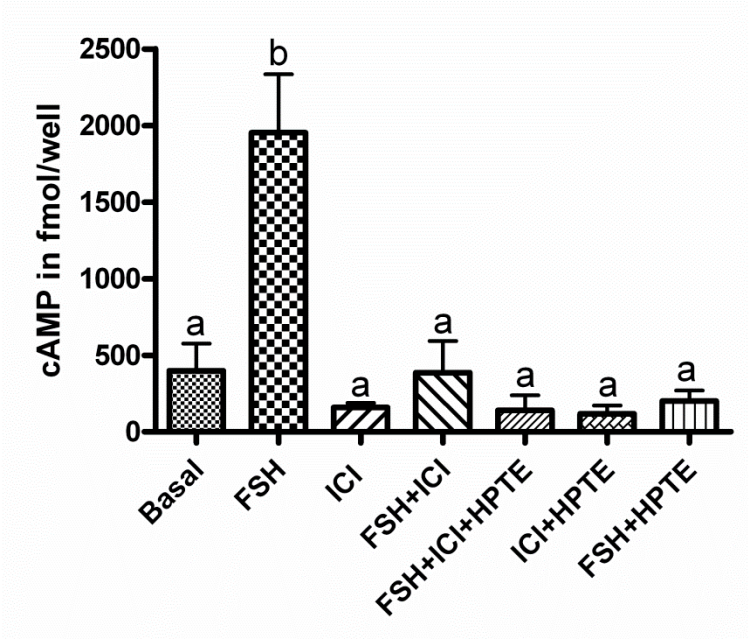


Figure 3.3: Effect of ICI 182,780 on FSH-mediated cAMP production.
Granulosa cells were treated with 5 μM ICI 182,780 (ICI) in the presence or absence of FSH. Treatment with ICI inhibited FSH-mediated cAMP production. Co-treatment of ICI with HPTE, in the absence or presence of FSH did not affect cAMP. Data were expressed as mean fmol/well ± SEM (n≥4). Analysis of cAMP was determined by enzyme immunoassay as described in Materials and Methods. Different letter indicates significant difference between treatment groups were as determined by one-way ANOVA ($p < 0.01$) followed by Dunnett's Multiple Comparison test post-hoc analysis.

Since recent studies showed that ERβ is required for maximal cAMP production (Deroo *et al.* 2009), we queried whether the effects of an ERβ antagonist, 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyri midin-3-yl]phenol (PHTPP), on cAMP production would mimic that of HPTE. PHTPP reduced FSH-mediated cAMP production by 80% (Fig. 3.4). PHTPP and HPTE co-treatment further reduced FSH-mediated cAMP by 50% over FSH+PHTPP alone. We also tested if the use of an ERβ agonist, 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN), could restore cAMP production in granulosa cells. We selected DPN based on its reported high affinity for ERβ (EC_{50} = 0.85 nM)(Meyers *et al.* 2001). The presence of DPN did not significantly alter the inhibitory effect of HPTE on the FSH-induced cAMP level (Figure 3.4). HPTE and MXC had an identical inhibitory effect on FSH-mediated cAMP in granulosa cells. Since HPTE is the purported active metabolite of MXC and acts as an ERα agonist and an ERβ antagonist: MXC was omitted from the ER antagonist and agonist studies.

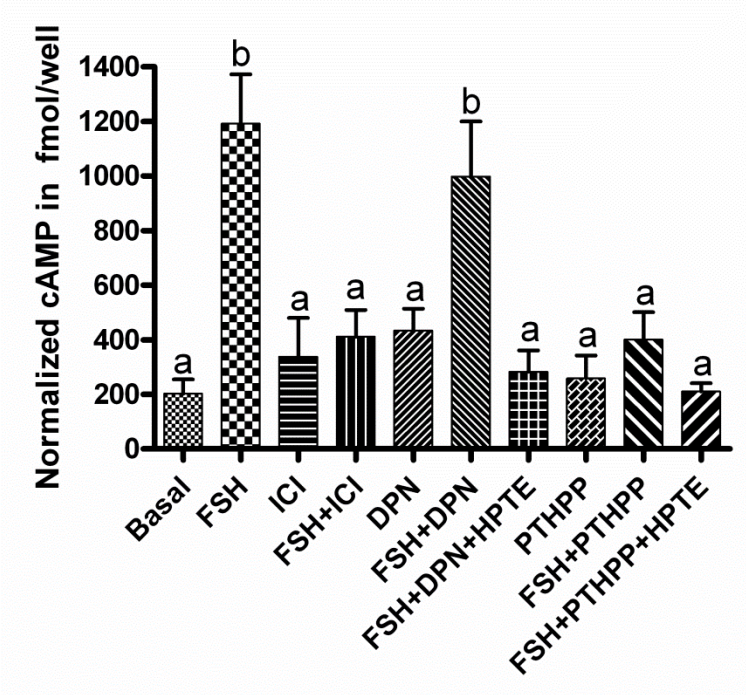
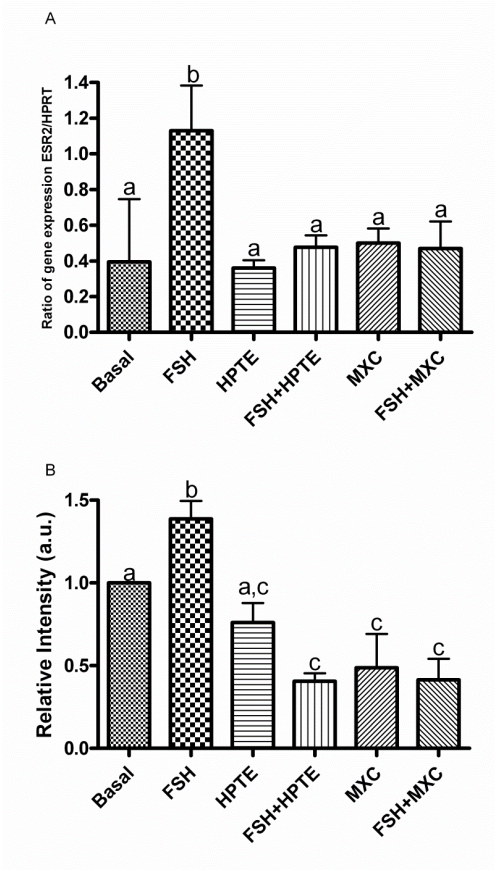


Figure 3.4: Effect of PHTPP or DPN on FSH-mediated cAMP production.
Granulosa cells were treated 1 μM 4-[2-Phenyl-5,7 bis(trifluoromethyl)pyrazolo[1,5-a]pyri midin-3-yl]phenol (PHTPP) or 1 μM 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) in the presence or absence of FSH. Treatment with PHTPP inhibited FSH-mediated cAMP production. Co-treatment of PHTPP with HPTE reduced cAMP by an additional 50%. DPN was co-administered with HPTE for 48 h in the presence or absence of FSH. DPN did not reverse the inhibitory effect of HPTE on cAMP production. Data were expressed as normalized mean fmol/well (n>4 except for FSH+ HPTE which was n=2). Analysis of cAMP was determined by enzyme immunoassay and normalized to protein concentration as described in Materials and Methods. Different letter indicates significant difference between treatment groups were as determined by one-way ANOVA ($p < 0.01$) followed by Dunnett's Multiple Comparison test post-hoc analysis.

3.3.4 MXC and HPTE inhibits *Esr2* mRNA and protein levels

We examined the effect of MXC and HPTE on *Esr2* expression in granulosa cells (Fig. 3.5A). MXC and HPTE did not alter basal levels of *Esr2* expression. FSH induced a 2.5-fold increase in *Esr2* expression, which was reversed by MXC and HPTE treatment to the basal level. We next examined the effect MXC and HPTE on protein levels using western blotting. MXC and HPTE significantly inhibited FSH-stimulated ERβ protein levels and reduced ERβ protein to 31% of FSH alone (Fig. 3.5B).



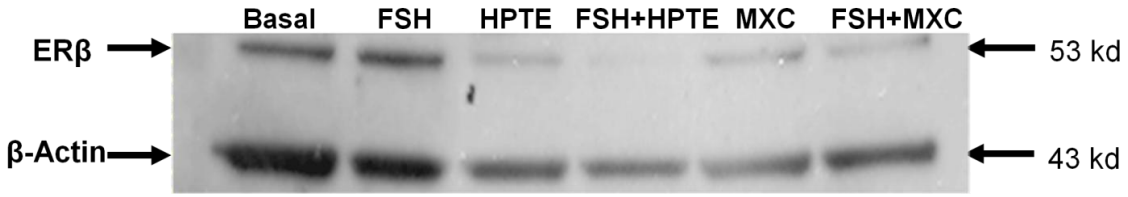


Figure 3.5: Effect of HPTE and MXC on *Esr2* mRNA and ESR2 protein levels in granulosa cells.
(A) *Esr2* mRNA was induced by FSH-treatment at least 2-fold over basal. MXC (10 μM) or HPTE (10 μM) inhibited FSH-induced *Esr2* expression. Total RNA was extracted from granulosa cells and analyzed by qPCR for *Esr2* mRNA expression as described in Materials and Methods. All expression values were normalized with *hprt* as a reference gene. Experiments were repeated 4 times. (B) Western blots confirmed the inhibitory affect of HPTE and MXC on FSH-stimulated *Esr2* and showed that FSH stimulated ESR2 and HPTE inhibited ESR2 protein levels. ImageJ Software (NIH) was used to quantify protein band intensity. β-actin was used to normalize ESR2 levels (n=3) and samples were compared to basal. Different letter indicates significant difference between treatment groups as determined by one-way ANOVA ($p < 0.01$) followed by Newman-Keul Multiple Comparison test post-hoc analysis. Representative Western blots are shown.

3.3.5 MXC and HPTE inhibits *Esr1* mRNA

Similar to *Esr2*, the pattern of expression of *Esr1* mRNA was stimulated by FSH and this FSH effect was suppressed by MXC and HPTE (Fig. 3.6A). Again, no changes were observed in *Esr1* mRNA in the absence of FSH. The relative mRNA level for *Esr1* was lower than the expression of *Esr2* (Fig. 3.6B). Western blotting did not detect ERα in our samples (data not shown).

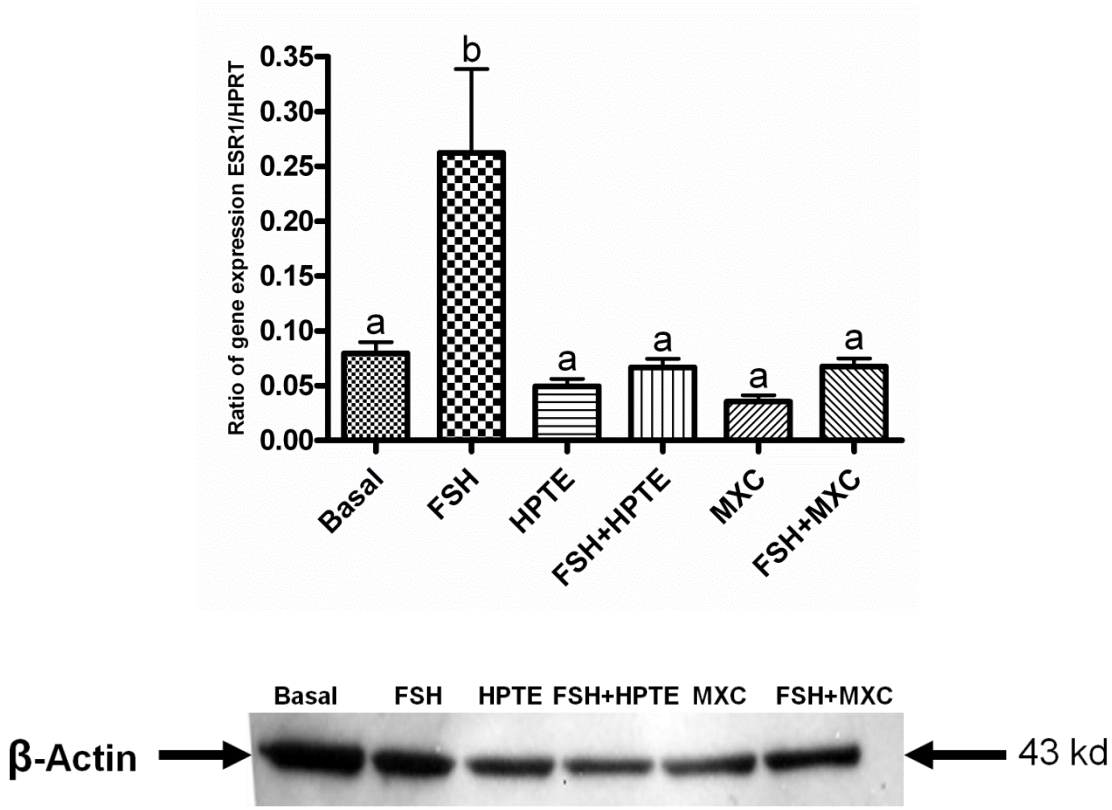


Figure 3.6: HPTE and MXC reduced *ESR1* mRNA in granulosa cells.
Gene expression of *Esr1* was induced by FSH-alone 3- to 4-fold over basal. MXC (10 µM) or HPTE (10 µM) inhibited FSH-induced *Esr1* expression. Total RNA was extracted from granulosa cells and analyzed by qPCR for *Esr1* mRNA expression as described in Materials and Methods. All expression values were normalized with *hprt* as a reference gene. Experiments were repeated 4 times. Different letters indicate significant difference between treatment groups as determined by one-way ANOVA ($p < 0.01$) followed by Dunnett's Multiple Comparison test post-hoc analysis. (B) Western blots showed that ESR1 protein levels were below detection limit (data not shown) while β-actin was observed as expected.

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3.3.6 Correlation analysis of cAMP and estrogen receptor mRNA expression

Analysis of the relationships between cAMP, *Esr1*, and *Esr2* was conducted to examine the correlation between gene expression of estrogen receptors and FSH-induced cAMP production in granulosa cell. *Esr1* showed a significant positive correlation with intracellular cAMP (r^2=0.745 and p= 0.00025). The correlation of *Esr2* with cAMP was less robust (r^2=0.513 and p= 0.025), however, a strong positive correlation between *Esr1* mRNA and *Esr2* mRNA levels (r^2=0.835 and p= 0.000008) exists.

3.4 Discussion

This study investigated the effect of MXC and its metabolite, HPTE, on FSH-mediated cAMP production and the potential role for estrogen receptors in these interactions in the granulosa cell. Treatment with HPTE or MXC reduced cAMP production, which was mimicked by estrogen receptor antagonists. MXC/HPTE also affected Esr1 and Esr2 gene expression and/or ESR2 protein levels. We also tested whether inhibition of phosphodiesterases activity would protect cAMP production from the effects of MXC or HPTE. Co-treatment of IBMX did not reverse the suppressive effect of MXC or HPTE on cAMP production which suggests that cAMP metabolism was not affected. Correlation analysis suggested an association between a reduction in cAMP production and ESR1 and ES2 levels in HPTE/MXC treated granulosa cells

MXC and HPTE inhibited cAMP production in the granulosa cell. EDCs have been shown to inhibit intracellular cAMP in reproductive tissue, however this effect has been species-and tissue-dependent (Heindel and Chapin 1989). For example, the phthalate metabolite, mono-2-ethylhexyl-phthalate, has been reported to inhibit cAMP production in rat granulosa cell (Lovekamp-Swan and Davis 2003). The observed MXC-

mediated inhibition of cAMP in the granulosa cell were expected based on our previous results (Harvey *et al.* 2009; Zachow and Uzumcu 2006), however, it is in contrast to the findings of others, who observed no effect of MXC on cAMP levels in primary porcine granulosa cells (Chedrese and Feyles 2001). This disagreement in results is likely due to variation in experimental protocols including the use of E_2 and animal species. In the ovary, the effect of FSH is dependent upon follicle stage. Granulosa cells from growing follicles respond to FSH by proliferating and differentiating, while granulosa cells of large antral follicles differentiate and express *Lhcgr* and *Cyp11a1* in response to FSH (Richards 1994). This responsiveness to gonadotropins is primarily mediated through cAMP and the initiation of the phosphorylation cascade through protein kinase A.

The effect of MXC and HPTE on intracellular cAMP production supports our hypothesis that the mechanism of action for inhibition of E_2 in the granulosa cell is upstream of initiation of cAMP generation (Zachow and Uzumcu 2006). In our previous reports, we showed the FSH-treated granulosa cells were more sensitive to HPTE than cAMP-treated granulosa cells (Harvey *et al.* 2009). This suggests that MXC inhibits FSH-stimulated cAMP production, which is the primary second messenger for FSH-induced E_2 production in the granulosa cell. Others have reported that MXC reduces E_2 levels. This effect is likely through inhibition of mRNA for genes encoding steroidogenic enzymes and increased expression of mRNA encoding *Cyp1b1*, which is an estrogen metabolizing enzyme (Basavarajappa *et al.* 2011). In addition, cAMP-induced steroidogenic enzymes and ovarian differentiation markers, *Cyp11a1* and *Lhcgr*, were down-regulated by HPTE (Harvey *et al.* 2009), signs that granulosa cell differentiation is

inhibited. The observed effect on *Cyp11a1* and *Lhcgr* expression *in vitro* was mirrored in vivo at the protein level in MXC-treated ovaries (Armenti *et al.* 2008).

Estrogen receptor and cAMP signaling pathways have been shown to interact in breast (El-Tanani and Green 1996) and uterine cells (Aronica and Katzenellenbogen 1991) using cAMP-mediated transcription of endogenous and reporter genes that contain only estrogen response elements (Lazennec *et al.* 2001). However, inhibition of cAMP decreases steroidogenesis and inhibits the synergistic actions of FSH and E_2 on CYP11A1 and other steroidogenic enzymes (Veldhuis *et al.* 1982), an effect that would hinder ovulation and oocyte maturation. MXC induced atresia in the antral follicles of mice and baboons (Gupta *et al.* 2007; Gupta *et al.* 2006a); an effect possibly mediated by granulosa cell dysfunction. Since granulosa cells are critical to support the oocyte to maturity, changes in granulosa cAMP-PKA signaling and the production of E_2 may contribute to the MXC-mediated inhibition of antral follicle growth.

Our correlation analysis suggested a significant association between cAMP and Esr2 mRNA expression. Since MXC and HPTE decrease estrogen receptor mRNA expression and cAMP production, these data suggest that gene expression (Esr1 and Esr2) and cAMP are strongly associated. Estrogen receptors and cAMP have been shown to interact to increase gene transcription of two estrogen responsive genes, Liv-1 and Ps2 in MCF-7 breast cancer cells and this effect is blocked by ICI-182,780 (El-Tanani and Green 1996). Though these results did not indicate causation, they do suggest a strong association. This association was further tested by using estrogen receptor antagonists, which mimicked the inhibitory effect of MXC and HPTE on cAMP.

Since HPTE is known to act as an ERβ antagonist, ICI and PHTPP inhibition of cAMP production in the granulosa cell was expected. Co-treatment of HPTE with DPN did not reverse the inhibitory effect on cAMP even though DPN has a greater affinity for the estrogen receptor than HPTE or MXC. The down regulation of ERβ protein caused by MXC and HPTE shown by western blotting may explain the lack of effect of DPN on cAMP, since a reduction in the binding sites for DPN would reduce its activity. MXC down regulates ERβ in the hypothalumus of female sheep (Mahoney and Padmanabhan 2010). Alternatively, these effects can be separate or indirectly related due to the absence of E_2.

MXC and HPTE also inhibited ERα mRNA expression. Some of our other observations extend beyond ERβ and require further explanation. For example, MXC and HPTE inhibited the stimulatory effect of FSH on ERα mRNA. The inhibition of ERα mRNA exhibited a higher association with cAMP production than ERβ mRNA. ERα is primarily localized in the theca and interstitial cells, but has been shown to be a component in rat granulosa cell steroidogenesis (Chen *et al.* 2008). Research with mice overexpressing ERα has shown that their antral follicles exhibit increased sensitivity to MXC and its metabolites *in vitro* and *in vivo* (Paulose *et al.* 2011; Tomic *et al.* 2006). This suggests that MXC may alter estrogen signaling through ERα. An alternative hypothesis that may explain the correlation between ER expression and cAMP levels is the level of E_2 present in the granulosa cell. In the absence of E_2, estrogen receptor expression is downregulated (Ing and Tornesi 1997).

We have previously shown that HPTE decreases *Lhcgr* mRNA in FSH-treated granulosa cells *in vitro* and the LHR protein levels in antral follicles from MXC-treated

rats (Armenti *et al.* 2008; Harvey *et al.* 2009). The follicular population of MXC-treated animals exhibits a higher number of preantral and small antral follicles, and reduced populations of pre-ovulatory (large antral follicles) and corpora lutea, suggesting a failure in follicular maturation and ovulation (Armenti *et al.* 2008). During the transition to the pre-ovulatory follicle, mural granulosa cells of the antral follicle respond to FSH by expressing LHR (Eppig *et al.* 1997; Woods *et al.* 2007), an effect which appears to be mediated through ERβ (Bao *et al.* 2000; Couse *et al.* 2005; Rodriguez *et al.* 2010), since mice lacking ERβ have deficiencies in final follicular maturation and LHR expression. The previous observations on the effect of MXC/HPTE on LHR *in vivo* and *in vitro,* combined with the current result suggest that the effect of MXC may be mediated by decreasing the LHR availability in granulosa cells and preventing follicular progression perhaps through an ERβ mechanism. In particular, the lack of response to LH (or to hCG in superovulation studies) could be explained by the observed reduction in cAMP (Armenti *et al.* 2008). Further experiments testing the effectiveness of LHR selective ligands such as human chorionic gonadotropin or other novel selective compounds (Bonger *et al.* 2009) on this system would confirm this effect.

Our results show that estrogen synthesis and estrogen receptor expression decreases in the granulosa cell when challenged by MXC and HPTE. Based on these studies and our previous work, our working hypothesis for MXC's affect on E_2 action is presented in Figure 3.7. MXC interferes or interacts with estrogen signaling at multiple points including; disruption of E_2 synthesis, increased E_2 metabolism through induction of Cyp1b1 (Appendix A), ER antagonism, ER down-regulation, and inhibition of cAMP

production. These results suggest multiple related pathways are affected; one of which is clearly cAMP signaling.

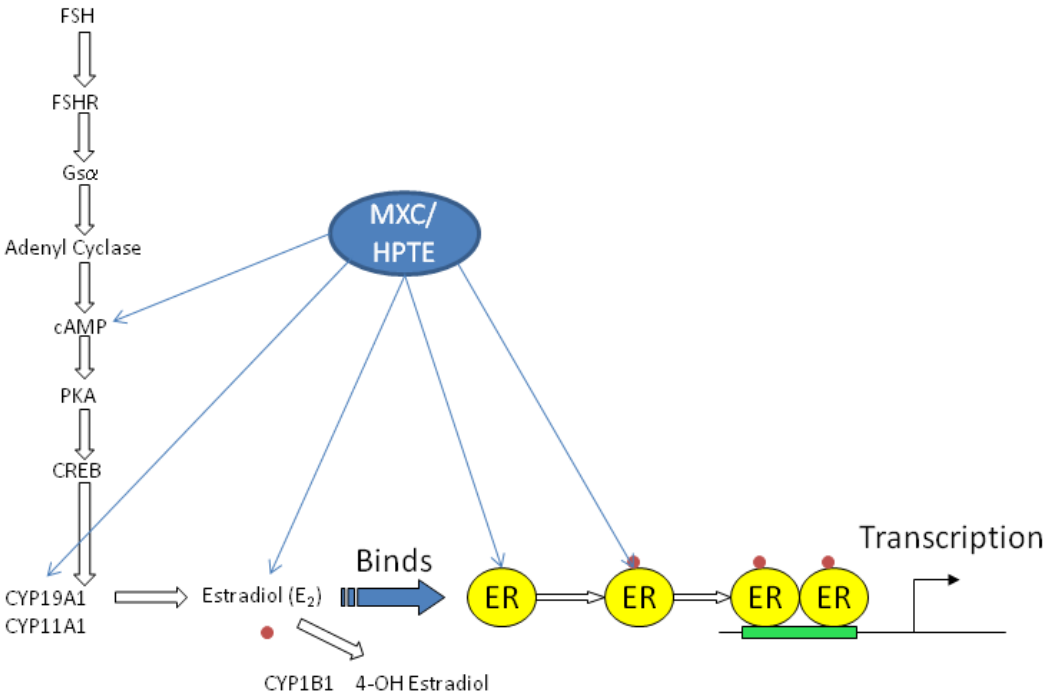


Figure 3.7: Working hypotheses for the effect of MXC on estrogen signaling in the granulosa cell *in vitro*.
Multiple estrogen-related processes are affected by MXC. MXC has been shown to inhibit cAMP production, steroidogenic enzymes CYP11A1and CYP19A1, alter E_2 synthesis, expression of genes that encode estrogen metabolizing enzymes and ER expression. MXC/HPTE has also been shown to antagonize ESR2 expression. This effect suggests that estrogen signaling and cAMP signaling are targets of MXC**.**

3.5 Summary and Conclusions

The sensitivity of the ovary to EDCs has become an area of increased interest in reproductive biology. Follicle-stimulating hormone signaling in the granulosa cell is critical for ovarian production of estradiol. The EDC methoxychlor is a weak estrogenic organochlorine pesticide that induces reproductive dysfunction. The major metabolite of MXC, 2, 2-bis-(p-hydroxyphenyl)-1, 1, 1-trichloroethane (HPTE), is not only a stronger estrogen receptor α agonist, but also acts as an estrogen receptor β antagonist. Studies show that estrogen production is inhibited by MXC *in vitro*. Estrogen synthesis is primarily mediated by FSH binding to its receptor and activation of protein kinase A through cAMP production. Recently, we observed that HPTE had a dose-dependent inhibitory effect on FSH-induced steroidogenesis in granulosa cells. Therefore, we examined the effect of MXC and HPTE on FSH-stimulated intracellular cAMP production. In order to elucidate if the effect of HPTE/MXC on cAMP production is estrogen receptor-mediated, we have utilized an ERα and ERβ antagonist, ICI, an ERβ selective-antagonist, PHTPP, and an ERβ agonist, DPN. ERα and ERβ mRNA and protein levels were examined in the granulosa cell in the presence and absence of MXC or HPTE by real time polymerase chain reaction (qPCR) and western blotting, respectively. HPTE and MXC inhibited the FSH-induced cAMP production. While the inhibitory action of HPTE was mimicked by ICI 182,780 and PHTPP, DPN did not rescue the HPTE-mediated inhibitory effects. MXC and HPTE reduced FSH-stimulated ERβ mRNA and protein to the basal level. FSH-stimulated ERα mRNA was also inhibited by MXC and HPTE. The greater inhibition on FSH-stimulated granulosa cells is likely due to reduced cAMP levels that may be mediated by estrogen receptor signaling.

This abnormal estrogen receptor expression and reduced cAMP availability in the granulosa cell could explain the inhibition of ovarian function observed in MXC-treated animals.

In conclusion, these results show the inhibitory effects of MXC and HPTE on cAMP production in granulosa cells. These results demonstrated that MXC and HPTE inhibited estrogen receptor expression, which may play a critical role in FSH-induced cAMP production in ovarian granulosa cells. Coupled with previously reported inhibitory effects of MXC/HPTE on E_2 production and steroidogenic enzymes (*Cyp19a1*) and *Lhcgr*, these results also suggest an ER-mediated mechanism for the inhibitory effects of MXC on FSH-mediated cAMP production in the granulosa cell and elucidate MXC-induced ovarian dysfunction.

CHAPTER 4: Effect of the Methoxychlor Metabolite HPTE on the Rat Ovarian Granulosa Cell Transcriptome *In Vitro*

4.1 Introduction

Methoxychlor (MXC) is an organochloride pesticide with estrogenic activities (Hall *et al.* 1997); this compound was used in the U. S. as a replacement for DDT until 2004 (Stuchal *et al.* 2006). Methoxychlor is considered to be an endocrine disrupting compound (EDC) based on the adverse reproductive effects of MXC observed in many species, including rats (Gray *et al.* 1989), mice (Eroschenko *et al.* 1995), and non-human primates (Gupta *et al.* 2007). This toxicity has been linked to the metabolism of MXC into the more potent compounds 2-(*p*-hydroxyphenyl)-2-(*p*-methoxyphenyl)-1, 1, 1-trichloroethane, also known as Mono-OH; and 2, 2-bis-(*p*-hydroxyphenyl)-1, 1, 1-trichloroethane (HPTE) via cytochrome P450 activity in the liver (Hu and Kupfer 2002). HPTE, for example, can interact with the estrogen receptor (ER) subtypes and androgen receptor (AR) (Gaido *et al.* 2000), acting as an ERα agonist and an antagonist to both ERβ and AR (Gaido *et al.* 2000). Since other prevalent EDCs show similar activities, MXC and its metabolites are good model compounds which can be used to elucidate the actions of EDCs within the reproductive system.

The ultimate function of the ovary is ovulation. The process of folliculogenesis precedes ovulation and is finely controlled by ovarian steroid hormones (Findlay *et al.* 2001) and growth factors (Skinner 2005). Folliculogenesis and ovulation are intimately linked to multi-directional communication between the oocyte, granulosa cells, and theca cells (Uzumcu and Zachow 2007).

The first somatic cell type that interacts with germ cells is granulosa cells or precursor granulosa (pre-granulosa) cells (Pepling and Spradling 2001). The nature of this interaction is bi-directionally established at the time of follicle formation, and remains active throughout the life of a follicle (Matzuk *et al.* 2002). A second population of somatic cells, the theca cells, is recruited during later stages of folliculogenesis (Skinner 2005). Granulosa cells and theca cells begin to interact with each other during the early stages of folliculogenesis through cell-to-cell signaling which is primarily mediated by local paracrine factors and juxtacrine mechanisms (Skinner 2005). Although the involvement of growth factors is still maintained, successful progression to the later stages of folliculogenesis requires actions of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Eppig and O'Brien 1996). Prior to the preovulatory follicular stage, LH targets theca cells, while FSH stimulates granulosa cells to induce steroidogenic differentiation, which enables the production of aromatizable androgens and estradiol-17β (E_2) (Palermo 2007). Within granulosa cells, FSH mobilizes numerous signaling motifs; noteworthy is the activation of the cAMP-dependent signaling pathway, which exerts profound effects on granulosa cell steroidogenesis (Hsueh *et al.* 1984).

Previous studies from our laboratory have shown HPTE inhibits FSH- and dibutyryl cAMP-stimulated steroid hormone production in granulosa cells isolated from the ovaries of immature rats (Zachow and Uzumcu 2006). In addition, these studies showed that HPTE had a greater inhibitory effect on FSH-induced granulosa cell steroidogenic enzyme mRNA levels than cAMP-induced mRNA levels. Specifically, HPTE inhibited levels of mRNAs encoding FSH-induced cytochrome P450 side-chain

cleavage (CYP11A1), 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD), and cytochrome P450 aromatase (CYP19A1), while having no effect on the level of steroidogenic acute regulatory protein (StAR) mRNA. In contrast, HPTE caused an increased accumulation of mRNA levels of cAMP-induced StAR, CYP11A1, and 3β-HSD, with a moderate reduction in CYP19A1 (Zachow and Uzumcu 2006). These differential effects suggested that at the molecular level, HPTE acts somewhere between binding of FSH to its receptor and cAMP production. Although our previous report demonstrated that HPTE regulates steroidogenesis, with changes in gene expression as a plausible target (Zachow and Uzumcu 2006) the potential mechanism of action for this regulation is unknown. Therefore, the objective of this study was to use a genomic approach to better understand the effect of HPTE in the ovary by examining its effect on FSH- and cAMP-induced gene expression in immature rat granulosa cells.

4.2 Materials and Methods

Chemicals

HPTE was generously supplied by Dr. Stephen Safe, Texas A&M University. Follicle-stimulating hormone (NIDDK-oFSH-20; 4453 IU/mg) was purchased from the National Hormone and Pituitary Program of the NIDDK (Dr. A.F. Parlow, Harbor-UCLA Torrance, CA). N6, 2′-*O*-dibutyryladenosine 3′, 5′-cyclic monophosphate (cAMP) was purchased from Sigma (St. Louis, MO).

Animals

Sprague-Dawley rats were used. The animals were maintained in a room with controlled illumination (lights on 0700-2100h), temperature (26-28°C), and humidity (30-70%) and were given free access to regular rat diet and water. All the procedures were carried out

according to guidelines provided by Rutgers University Animal Care and Facilities Committee.

Granulosa cell culture

Granulosa cells were prepared as described previously with some modifications (Zachow and Uzumcu 2006). Briefly, intact, non-primed, immature female rats (21-27 days old) were sacrificed by cervical dislocation. Ovaries were removed from the animals, cleaned free of associated fat, oviduct, and bursa ovary, and then placed in ice-cold DMEM/F-12. Granulosa cells were isolated using a non-enzymatic needle puncture method with a sterile bundle of beading needles to release the cells from follicles. Cell viability was determined by the trypan blue exclusion method. The cells were plated and cultured (24-well plates) at approximately 3-4 x 10^5 viable cells/ml/well and incubated at 37°C for 24 h in DMEM/F-12 containing FBS (5%) in an atmosphere of 5% CO_2 in air. Following the 24 h acclimation period, the medium was replaced with serum-free DMEM/F12 containing androstenedione (0.1 M) as a substrate for aromatization.

Cells were treated with increasing doses of HPTE (0, 1, 5, and 10 µM; in a final concentration of 0.1% DMSO) in the absence (basal) or presence of 3 ng FSH/ml or 1 mM cAMP for 48 h. The doses of FSH and cAMP used in this study were chosen following a preliminary dose-response curve experiment, and FSH or cAMP concentrations that caused a sub-maximal stimulation for E_2 production were selected (data not shown). HPTE concentrations were chosen based upon those previously used (Zachow and Uzumcu 2006). Samples were collected at 48 h following the addition of treatments. At this time, cell-conditioned media were collected for E_2 radioimmunoassay (RIA), and cell lysate was prepared for RNA isolation and microarray analysis.

E_2 RIA

E_2 was measured using a commercially available RIA kit, COAT-A-COUNT (Diagnostic Products Corp., Los Angeles, CA) via manufacturer's protocol. Hormone levels are expressed in pg/ml of culture medium.

Oligonucleotide microarray

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) followed by DNase I treatment. RNA quality was assessed by electrophoresis using the Agilent Bioanalyzer 2100 and spectrophotometric analysis prior to cDNA synthesis (data not shown). Forty nanograms of total RNA from each sample were used to generate a high fidelity cDNA for array hybridization using the NuGen Ovation Biotin RNA Amplification and Labeling system (NuGen). After fragmentation and biotin labeling, the samples were hybridized to Affymetrix Rat Genome 230 2.0 arrays, which have eleven pairs of oligonucleotide probes per chip. Washing and staining of all arrays were carried out in the Affymetrix fluidics module using the manufacturer's protocol. The detection and quantification of target hybridization was performed with an Affymetrix GeneChip Scanner. Three separate samples were analyzed for each of the treatment groups. A total of 36 separate arrays were used and the datasets in their entirety are available through NCBI via the Gene Expression Omnibus (GEO) data repository (http://www.ncbi.nih.gov/geo/), GEO accession number GSE13883.

Microarray data analysis and statistics

Raw data from three independent experiments were initially analyzed using Robust Multichip Analysis with a logarithmic base 2 conversion utilizing the Affymetrix ArrayAssist Suite (Affymetrix, Inc., Santa Clara, CA). One-way ANOVA of each group

(basal, FSH, or cAMP) was used to determine genes that are significantly affected by HPTE. This was followed by an unpaired *t*-test to determine changes in global gene expression between HPTE-treated groups and the control groups to determine the levels of fold change. All statistical analyses for microarray analysis were performed using $P \leq 0.005$ as the level of statistical significance. This is a level of probability consistently used in microarray analysis literature in order to minimize false positives (Moreira *et al.* 2008). Two-way completely randomized ANOVA was used to analyze the effect of HPTE on E_2 production. The results showing changes in steroidogenic enzyme level were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Statistically significant differences were confirmed at $P \leq 0.05$ for the last two parameters.

Validation of data consistency

DNA-Chip Analyzer (dCHIP), a Windows-based software package developed by the Wing Wong Laboratory (www.dchip.org), was used to determine if any outliers were present in the oligonucleotide array repeats as described (Li and Wong 2001). The arrays were shown to have no significant outliers (data not shown).

Real-time quantitative RT-PCR (QPCR)

Gene expression was examined using Taqman chemistry with probes and primers designed using the Roche Universal Probe Library (www.universalprobelibrary.com; UPL). All cDNAs were measured in a 10 µl PCR reactions containing: 5 µl of ABI 2x Universal Master Mix, 1.25 µl of each forward and reverse primers (final concentrations ranging from 200-900 nM depending on the primer set), 1 µl of the corresponding UPL probe and RNAase/DNAase free water. All QPCR reactions were performed in triplicate

on triplicate biological replicates leading to 9 QPCR data points per condition measured. The cycling parameters for ABI 7900HT were: 1 cycle of 50^{o}C (2 min) followed by 95^{o}C (10 min), 40 cycles of 95^{o}C (15 s) followed by 60^{o}C (1 min). Data were collected at every temperature phase during every cycle. Raw data were analyzed using the Sequence Detection Software (ABI, Foster City CA) while relative quantitation using the comparative threshold cycle (C_T) method was performed in Microsoft Excel (ABI Technote #2: Relative Gene Expression Quantitation). Twelve differentially expressed genes were chosen as validation targets, those that exhibited gene expression down-regulation (*LHCGR, CYP11A1, CYP19A1, INHBA, and INHA*) or upregulation (*IGF1, CASP12, TGFB2, TGFB3, IGFBP1, IGFBP5, and CASP4*). A gene that showed no changes in expression in the microarray data set *(StAR)* was used as the normalizer in the $\Delta\Delta C_T$ analysis.

Enrichment analysis

Enrichment analysis was conducted using two analysis tools: the U.S. Federal Drug Administration (FDA) supported ARRAY TRACK and the Medical College of Wisconsin's (MCW) APROPOS. ARRAY TRACK software is both a management and an analysis tool designed to collect gene expression data from microarray and xenobiotic-genomic studies (Tong *et al.* 2003). It is equipped with pathway and gene expression analysis libraries linked to the KYOTO Encyclopedia of Genes and Genomes and Ingenuity Systems Library. ARRAY TRACK has been shown effective for examining pathways enriched by different treatment groups and also for conducting statistical analyses of microarray data (Guo *et al.* 2006).

APROPOS is open source software packaged developed at the MCW (http://apropos.mcw.edu/). APROPOS, a MySQL database is built on the top of the rat, mouse, and human International Protein Index datasets (Kersey *et al.* 2004). The system cross references to the Rat Genome Database (RGD), Mouse Genome Database, Uniprot, Entrez gene and others groups to facilitate annotations of each record.

4.3 Results

4.3.1 Effects of HPTE on E_2 accumulation

Basal E_2 accumulation was not altered by HPTE (Figure 4.1). In contrast, 5 and 10 µM HPTE inhibited both FSH- and cAMP-stimulated E_2 secretion, but the effect on FSH group was much greater as compared to that on cAMP group. These data corroborated that HPTE was more effective in inhibiting FSH- than cAMP-stimulated steroidogenesis in the present experiments. We then utilized the mRNA collected from the same granulosa cells for oligonucleotide microarray analysis. During the treatment period, microscopic evaluation suggested that cell viability was comparable between the groups.

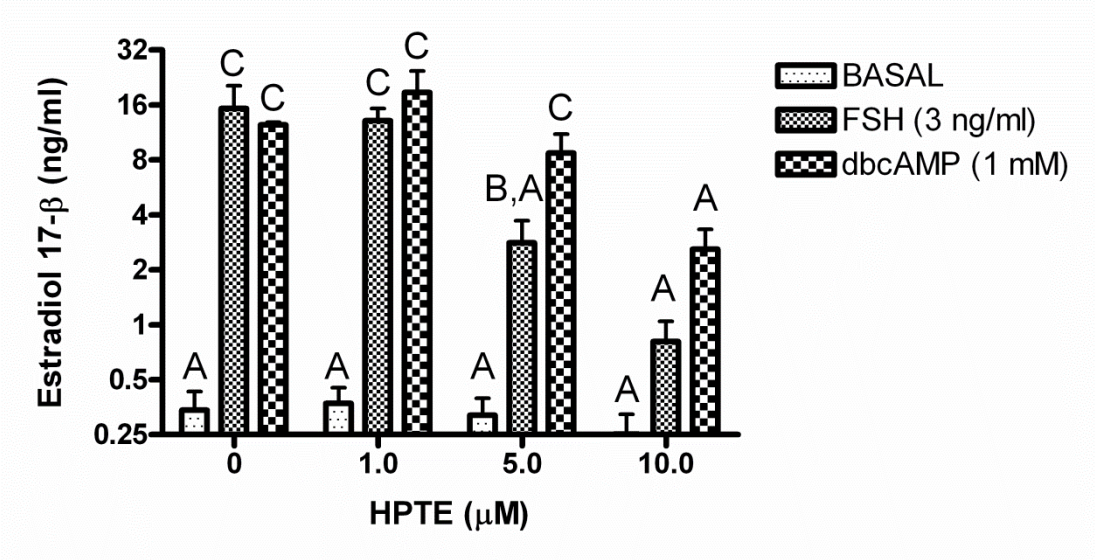


Figure 4.1: The effect of HPTE on E_2 accumulation in immature rat granulosa cells *in vitro*.
Granulosa cells were exposed to increasing doses of HPTE (0, 1, 5, or 10 μM) in the absence (basal) or presence 3 ng FSH/ml or 1 mM cAMP. After a 48 h treatment, media were collected and E_2 was measured by RIA as described in Materials and Methods. Different letters on the bars indicate statistically significant differences**.**

4.3.2 Effect of HPTE on FSH-stimulated steroidogenic pathway gene expression

Since our lab and others have previously shown that HPTE and MXC affected steroidogenesis (Akgul *et al.* 2008; Chedrese and Feyles 2001), we analyzed the microarray data to determine the effect of HPTE on the key steroidogenic enzymes. Figure 4.2 shows that HPTE (10 μM) significantly lowered the FSH-dependent expression of CYP11A1 and LH receptor (LHR; $P \leq 0.05$). In addition, 10 μM HPTE showed a tendency to inhibit FSH-induced CYP19A1 mRNA expression. In contrast, HPTE did not significantly alter the expression of 17β-hydroxysteroid dehydrogenase or StAR (Figure 4.2). Although it was not statistically significant, HPTE (5 and 10 μM) caused an upregulation in 20α-hydroxysteroid dehydrogenase (20α-HSD) mRNA in the presence of FSH.

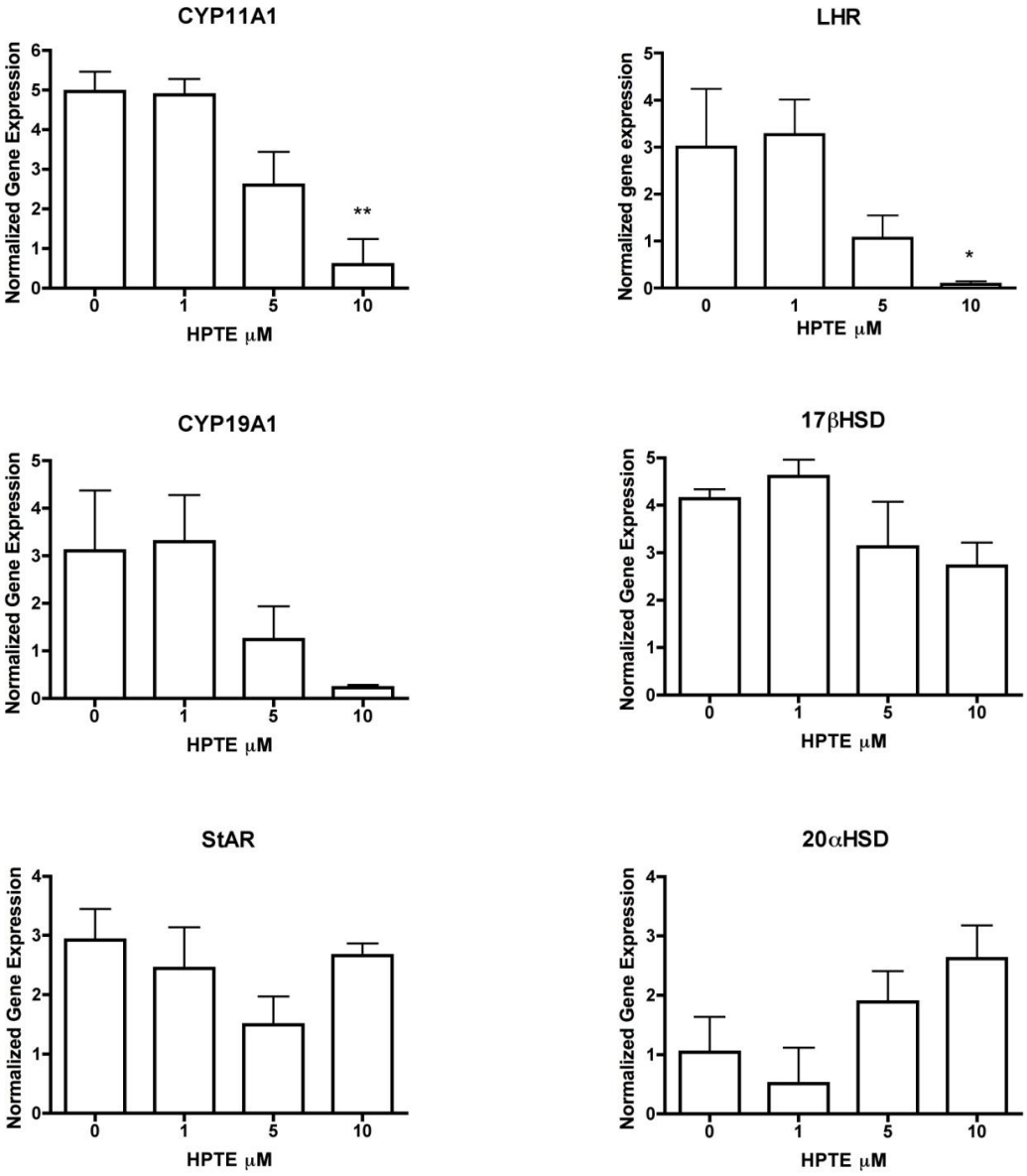


Figure 4.2: The effect of HPTE on FSH-stimulated steroidogenic pathway gene expression in granulosa cells *in vitro*.

Granulosa cells were treated with HPTE (0, 1, 5, or 10 μM) in the presence 3 ng FSH/ml. After a 48 h treatment, cell lysate was collected for mRNA isolation and microarray analysis as described in Material and Methods. CYP11A1 = P450 side-chain cleavage, LHR = luteinizing hormone receptor, CYP19A1 = P450 aromatase, 17βHSD = 17β-hydroxysteroid dehydrogenase, StAR = steroidogenic acute regulatory protein, 20αHSD = 20α-hydroxysteroid dehydrogenase. "**, $P \leq 0.01$; and *, $P \leq 0.05$.

4.3.3 Hierarchical clustering analysis reveals 3 transcriptome patterns.

Hierarchical clustering was used to compare global gene expression profiles, and to identify similar gene expression patterns between the individual treatment groups. Before analysis, the data were normalized using values from the basal (0 µM HPTE) group. Three vertical cluster patterns were observed. The highest dose of HPTE (10 µM) in the FSH-stimulated and basal groups exhibited the same pattern (Figure 4.3). The cAMP groups showed the least change across the HPTE doses used in this study as they clustered in the same node as the cells given FSH with 1 µM HPTE, and FSH alone (0 µM HPTE). Granulosa cells concomitantly incubated with FSH and 5 µM HPTE displayed an intermediary pattern. A one-way ANOVA was used in order to determine if any consistent patterns of gene expression were observed between groups. Results are presented in the next section.

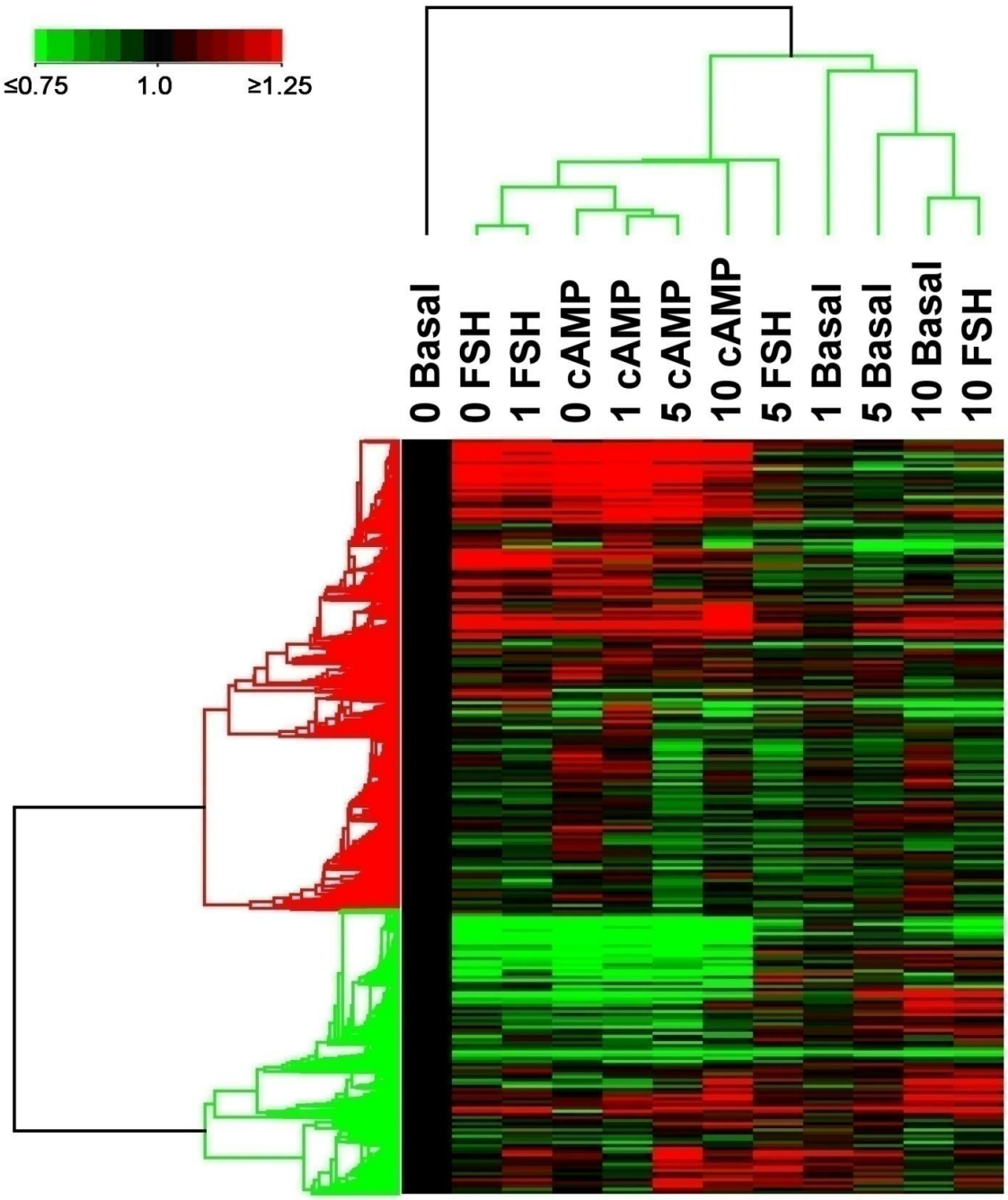


Figure 4.3: Hierarchical cluster analysis of the effect of HPTE in granulosa cells.
Three vertical patterns of expression were observed among the twelve groups delineating the effect of HPTE (0, 1, 5, or 10 μM) with and without FSH or cAMP. The legend indicates that data in 0 μM HPTE in the basal group (0 Basal) is set to 1 (black color) and used to normalize the data in the other groups. In addition, normalization values between 0.75 and 1.25 were excluded to reduce the background noise.

4.3.4 Genes that are common in basal, FSH, and cAMP groups were altered by HPTE.

Across the basal, FSH, and cAMP groups, the number of genes that showed significant changes in response to HPTE treatment (1, 5, and 10 µM) were determined (Figure 4.4). In the FSH group, 679 genes showed significant changes in response to HPTE. This is compared to the cAMP group with 356 genes; and in the basal group, 271 genes were significantly affected by HPTE. Thirty-two genes that were in common between the three groups (i.e., basal, FSH and cAMP) were affected by HPTE (Figure 4.4). Using the Apropos program, the commonly affected genes in granulosa cells that were untreated or treated with FSH or cAMP were categorized according to biologic function. Two or more genes were affected in cell cycle, multicellular organismal development, transport, amino acid transport, regulation of transcription, Wnt receptor signaling, and cell division (Table 4.1). Approximately 70 additional genes were commonly regulated between the FSH- and cAMP-treated granulosa cells (Figure 4.4; Table 4.2).

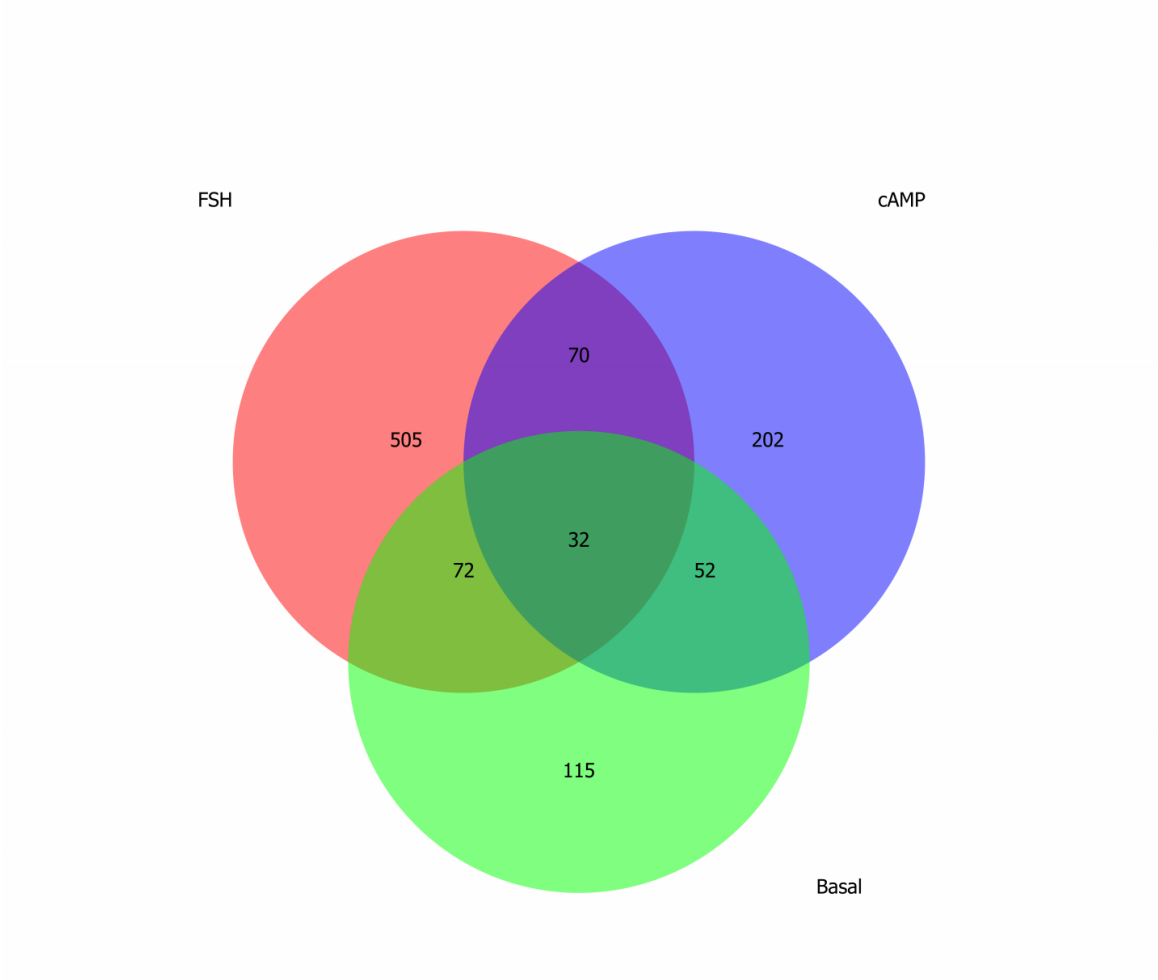


Figure 4.4: Venn diagrams of the genes under HPTE regulation in granulosa cells.
Genes that were affected by HPTE in 3 groups were analyzed by one-way ANOVA. Between the cAMP and FSH groups, 102 common genes were regulated in granulosa cells ($P \leq 0.005$).

Table 4.1: Genes that are commonly altered by HPTE in untreated and treated (FSH or cAMP) granulosa cells ($P \leq 0.005$).

Biological Processes	Genes
Multicellular Organismal Development	RGD1308165_predicted RGD1564008_predicted Cd24 Dixdc Itgb1 Angptl4
Signal Transduction	Angptl4 Ank2 Fcgr2b Hivep Igfbp5 Tas1r2
Metabolic Process	Ank2 Asns LOC3040860 Man1a_predicted Tas1r2
Regulation of Transcription, DNA-dependent	E2f8 Bhlhb2 Atf5 Hivep2 Irf6_predicted
Cell Cycle	E2F8 CDCa1 Kif11a IPI00768806 Mad2l1_predicted
Transport	Tcn2 Slc7a3 Slc6a9 Kcnd2 Cp
Cell Differentiation	Angptl4 Cd24 Ctgf RGD1308697
Cell Adhesion	Cd24 Ctgf Itgb1 Sdc1
Mitosis	Kif11 Kif15 Mad2l1_predicted Cdca1
Regulation of Progression Through Cell Cycle	Atf5 E2f8 Itgb1
Regulation of Cell Growth	Ctgf Htra1 Igfbp5
Cell Division	Cdca1 Madl1_predicted Kif11
Ion Transport	Cp Kcnd2 Tcn2
Cell Matrix Adhesion	Ctgf Itgb1
Humoral Immune Response	Cd24 Fcgr2b

Table 4.2: Genes that were commonly regulated by HPTE in FSH- and cAMP- treated granulosa cells.

Gene Title	Gene Symbol	Annotation[i]	Entrez
activating transcription factor 5	Atf5	Anti-apoptosis; regulation of transcription from RNA polymerase II promoter; regulation of cell cycle and regulation of cell proliferation.	282840
amplified in osteosarcoma	Os9	Protein binding.	362891
angiopoietin-like 4	Angptl4	Signal transduction; multicellular organismal development; negative regulation of lipoprotein lipase.	362850
ankyrin 2, neuronal	Ank2	Paranodal junction assembly; nervous system development; response to methylmercury; lipid raft; plasma membrane; synaptosome; cytoskeleton; structural constituent of cytoskeleton.	362036
beta galactoside alpha 2,6 sialyltransferase 2	St6gal2	Carbohydrate metabolic process; protein amino acid glycosylation; oligosaccharide metabolic process membrane; integral to Golgi membrane; Golgi apparatus.	301155
C1q and tumor necrosis factor related protein 5	C1qtnf5	Phosphate transport; extracellular region; cytoplasm.	315598

carbohydrate (chondroitin synthase 1 (predicted)	Chsy1_predicted		292999
Ceruloplasmin	Cp	Plasma membrane copper ion transport; response to nutrient; aging; cellular iron ion homeostasis; anchored to plasma membrane.	24268
connective tissue growth factor	Ctgf	cartilage condensation; cell differentiation; angiogenesis; angiogenesis; regulation of cell growth; fibroblast growth factor receptor signaling pathway.	64032
cystatin B	Cstb	Adult locomotory behavior; regulation of apoptosis; cysteine protease inhibitor activity.	25308
D-dopachrome tautomerase	Ddt	Melanin biosynthetic process from tyrosine; inflammatory response; dopachrome decarboxylase activity; dopachrome isomerase activity.	29318
death-associated protein	Dap	Apoptosis.	64322
EH-domain containing 2	Ehd2	Cortical actin cytoskeleton organization and biogenesis; cortical actin cytoskeleton organization and biogenesis;.	361512
enolase 2, gamma, neuronal	Eno2	Glycolysis; phosphopyruvate hydratase complex; lyase activity; phosphopyruvate hydratase activity; magnesium ion binding; phosphopyruvate hydratase activity.	24334
family with sequence similarity 20, member C	Fam20c		304334
family with sequence similarity 82, member C	Fam82c	Cell differentiation; apoptosis cytoplasm; mitochondrion; mitochondrial membrane; integral to membrane.	311328
Fc receptor, IgG, low affinity III	Fcgr3	Positive regulation of type I hypersensitivity; positive regulation of type III hypersensitivity; phagocytosis, cell surface receptor linked signal transduction.	116591
FIG4 homolog (S. cerevisiae)	Fig4		309855

GLI pathogenesis-related 2	Glipr2		679819
glycophorin C (Gerbich blood group)	Gypc		364837
glypican 3	Gpc3	Ureteric bud branching; negative regulation of cell proliferation; positive regulation of BMP signaling pathway; negative regulation of growth; anchored to membrane.	25236
Granulin	Grn	Positive regulation of epithelial cell proliferation; blastocyst hatching; lipid catabolic process; embryo implantation; phospholipid metabolic process.	29143
GTP binding protein 2	Gtpbp2		363195
guanylate nucleotide binding protein 2	Gbp2	Immune response; GTPase activity; GTP binding.	171164
histone cluster 2, H2be	Hist2h2be	Protein binding.	295274
histone deacetylase 2	Hdac2	Histone deacetylation; replication fork; nucleus; heterochromatin; enzyme binding; histone deacetylase activity; transcription factor activity.	84577
HtrA serine peptidase 1	Htra1	negative regulation of transforming growth factor beta receptor signaling pathway; regulation of cell growth; proteolysis; negative regulation of BMP signaling pathway.	65164
human immunodeficiency virus type I enhancer binding protein 2	Hivep2	Transcription; regulation of transcription, DNA-dependent; zinc ion binding; DNA binding.	29721
immediate early response 3	Ier3	Antigen processing and presentation of peptide antigen via MHC class; antigen processing and presentation; immune response; MHC class I protein complex.	294235
insulin-like growth factor binding protein 5	Igfbp5	Skeletal muscle growth; regulation of cell growth; intracellular signaling cascade; extracellular region; insulin-like growth factor binding.	25285

integrin beta 1 (fibronectin receptor beta)	Itgb1	Regulation of G-protein coupled receptor protein signaling pathway; cell adhesion; integrin-mediated signaling pathway; cell-matrix adhesion; sarcomere organization.	24511
interferon regulatory factor 6 (predicted)	Irf6_predicted	Biological process; skin development; negative regulation of cell proliferation; keratinocyte differentiation; cell development; keratinocyte proliferation; cellular component.	364081
interleukin 17 receptor E	Il17re	Membrane; integral to membrane; receptor activity.	362417
IQ motif containing GTPase activating protein 1 (predicted)	Iqgap1_predicted	Cytoplasm; leading edge; protein complex; neuron projection; protein complex binding.	361598
kinesin family member 15	Kif15	Microtubule-based movement; microtubule associated complex; growth factor activity; ATP binding; GTPase activator activity; microtubule motor activity.	353302
lamin A	Lmna	Nuclear membrane organization and biogenesis; spermatogenesis; nuclear matrix; lamin filament; intermediate filament.	60374
LOC363015 (predicted)	RGD1310444_predicted		363015
lysosomal membrane glycoprotein 1	Lamp1	Autophagy; lysosomal membrane; endosome; late endosome; melanosome; external side of plasma membrane; endosome membrane.	25328
mannosidase 1, alpha (predicted)	Man1a_predicted	ER-Golgi intermediate compartment; Golgi membrane; membrane; mannosyl-oligosaccharide 1,2-alpha-mannosidase activity; calcium ion binding.	294410
N-acetylneuraminate pyruvate lyase	Npl	Metabolic process; carbohydrate metabolic process; cytoplasm; lyase activity; N-acetylneuraminate lyase activity.	304860
N-terminal asparagine amidase	Ntan1	Memory; adult locomotory behavior; hydrolase activity; protein N-terminal asparagine amidohydrolase activity.	360462
potassium voltage gated channel, Shal-related family, member 2	Kcnd2	Potassium ion transport; regulation of action potential; protein heterooligomerization; cell surface; voltage-gated potassium.	65180
profilin 2	Pfn2	Regulation of actin filament polymerization; actin cytoskeleton organization and biogenesis; actin	81531

		cytoskeleton.	
protective protein for beta-galactosidase	Ppgb	Proteolysis; cellular component; molecular function; serine carboxypeptidase activity.	296370
protein phosphatase 1, regulatory (inhibitor subunit 14B)	Ppp1r14b	Regulation of phosphorylation cytoplasm; protein phosphatase type 1 regulator activity; protein phosphatase inhibitor activity; protein binding.	259225
RAB3B, member RAS oncogene family	Rab3b	Peptidyl-cysteine methylation; regulation of exocytosis; protein transport; small GTPase mediated signal transduction; secretory granule; synaptic vesicle.	81755
ral guanine nucleotide dissociation stimulator,-like 1 (predicted)	Rgl1_predicted	Protein binding.	289080
RNA binding motif protein 12	Rbm12	Nucleotide binding; nucleic acid binding; protein binding.	652928
S-adenosylhomocysteine hydrolase-like 1 (predicted)	Ahcyl1_predicted		362013
SH3 and PX domain containing 3 (predicted)	Sh3px3_predicted		315696
similar to 3632451O06Rik protein (predicted)	RGD1310110_predicted		361032
similar to cis-Golgi matrix protein GM130	LOC690485		690485
similar to DNA segment, Chr 4, Brigham & Womens Genetics 0951 expressed	RGD1308059		362535
similar to GA binding protein transcription factor, beta subunit 2 (GABPB2 (predicted)	RGD1560391_predicted		499883
similar to GREB1 protein isoform a (predicted)	RGD1562371_predicted		498819
similar to hypothetical protein FLJ14146	RGD1310587	Integral to membrane; membrane.	360894
similar to hypothetical protein MGC17337 (predicted)	RGD1308165_predicted	Identical protein binding.	362516
Similar to RIKEN cDNA 2610019F03	LOC498662		498662

similar to T03G11.6	LOC689540		689540
SPARC related modular calcium binding 2 (predicted)	Smoc2_predicted		292401
syndecan 1	Sdc1	Response to glucocorticoid stimulus; inflammatory response; response to hydrogen peroxide; odontogenesis; cell adhesion; cell-cell signaling; wound healing; response to toxin.	25216
taste receptor, type 1, member 2	Tas1r2	response to stimulus; G-protein coupled receptor protein signaling pathway; signal transduction; proline biosynthetic process; sensory perception of sweet taste; metabolic process.	29408
Tax1 (human T-cell leukemia virus type I binding protein 1)	Tax1bp1	Anti-apoptosis; intracellular; identical protein binding.	246244
testis expressed gene 14 (predicted)	Tex14_predicted		287603
tetratricopeptide repeat domain 6 (predicted)	Ttc6_predicted		299067
thymosin beta-like protein 1	Tmsbl1	Actin cytoskeleton organization and biogenesis; sequestering of actin monomers; cytoplasm; actin binding.	286978
transmembrane and tetratricopeptide repeat containing 4	Tmtc4		290501
ubiquitin-activating enzyme E1-domain containing 1	Ube1dc1	Ubiquitin cycle; metabolic process; cytoplasm; catalytic activity.	300968
vasohibin 1	Vash1		503052
vasohibin 2	Vash2		498309
Vesicle amine transport protein 1 homolog (T californica)	Vat1	Metabolic process; oxidoreductase activity; zinc ion binding.	287721

4.3.5 Confirmation of the limited HPTE effect within untreated and cAMP-stimulated granulosa cells

In order to determine which genes exhibited the most changes in the level of expression relative to the baseline, a comparative analysis was performed. A two-fold change was established in all groups as the cut-off criteria to filter out relatively small changes in gene expression. The result from this analysis confirmed the previous analysis; that is, the greatest numbers of genes were affected in the FSH group (669 total, 159 down & 420 up). In the basal group, 90 genes showed changes in expression; specifically, 52 genes were down-regulated and 38 genes were upregulated. HPTE affected the least number of genes in the cAMP group, with the expression of 76 genes significantly altered (16 geness down-regulated and 60 genes upregulated) (Tables 4.3A and 4.3B). These results do not include Expressed Sequence Tags.

Table 4.3A: Distribution of the down-regulatory effect of HPTE on gene expression in untreated (basal) or treated (FSH or cAMP) granulosa cells *in vitro.**

	HPTE			
	1 µM	*5 µM*	*10 µM*	***Total***
Basal	1	6	45	52
FSH	0	64	95	159
cAMP	0	0	16	16

*****Only those genes exhibiting a 2-fold or greater level of change are shown; $P \leq 0.005$.

Table 4.3B: Distribution of the upregulatory effect of HPTE on gene expression in untreated (basal) or treated (FSH or cAMP) granulosa cells *in vitro.**

	HPTE			
	1 µM	*5 µM*	*10 µM*	***Total***
Basal	0	1	37	38
FSH	0	163	257	420
cAMP	2	4	54	60

*****Only those genes exhibiting a 2-fold or greater level of change are shown; $P \leq 0.005$.

4.3.6 Analysis of genes that were affected by 10 µM HPTE

The expression of the greatest number of genes was affected by 10 µM HPTE, therefore we focused on this dose for further analysis. A list of the upregulated and down-regulated genes was compiled, and an enrichment analysis was conducted to profile the targeted genes. Analysis revealed that 257 genes were upregulated, and 95 genes were down-regulated in the FSH group. Fifty-four genes were upregulated, and 16 genes were down-regulated in the cAMP group; whereas, HPTE upregulated the expression of 37 genes, and down-regulated 45 genes in basal group. ARRAY TRACK and APROPOS software were used in order to determine the functional groups of the genes regulated by HPTE, and these are listed in Tables 4.4A and 4.4B.

Table 4.4A: Biological function of genes that are down-regulated by HPTE (10µm) in untreated (basal) or treated (FSH or cAMP) granulosa cells.

Functional Group	basal	FSH	cAMP
Signal Transduction	3	15	4
Transport	3	11	8
Ion Transport		8	3
Amino Acid Transport			3
Cell Adhesion	4	14	
Cell Differentiation		6	
Cell-Cell Signaling	2	5	
Cell Motility		4	
Regulation of Cell Growth	3	6	
Regulation of Progression Through Cell Cycle			3
Anti-apoptosis		5	
Regulation of Apoptosis		4	2
G-protein Coupled Receptor Signaling Pathway		6	
Multicellular Organismal Development	4	5	
Skeletal Development		5	
Proteolysis			4
Metabolism			3
Glucose Metabolism	3		

Table 4.4B: Biological function of genes that are upregulated by HPTE (10µm) in untreated (basal) or treated (FSH or cAMP) granulosa cells.

Functional Group	basal	FSH	cAMP
Signal Transduction	2	4	
Transport		2	
Cell Adhesion		3	
Cell Division	6		1
Cell Motility			1
Regulation of Progression Through Cell Cycle	4		
Cell Cycle	7		
Apoptosis		2	
Multicellular Organismal Development		3	
Metabolic Process		3	
Lipid Metabolism	2		
Fatty Acid Metabolism		2	

Upregulation was observed in genes associated with signal transduction, cell adhesion, and various transport functions. Down-regulation was observed in genes associated with signal transduction, transport, and cell division. In FSH-stimulated granulosa cells, HPTE induced the largest fold changes in the expression of several genes previously linked with ovarian function, and these data are shown in Table 4.5.

Table 4.5: Genes associated with ovarian function, which were significantly affected by HPTE (10 µM) in FSH-stimulated granulosa cells.
Fold-change and summary of function are included.

Gene Symbol	Gene Name	Summary of the Function[1]	Fold-Change
Lhcgr (LHR)	Luteinizing hormone/chorionic gonadotropin receptor	Receptor for both luteinizing hormone and chorionic gonadotropin; involved in reproductive development and function [RGD].	-5.3
Prlr	prolactin receptor	Expression changed during estrous cycle; may be regulated by Prolactin receptor or steroid hormones [RGD].	-4.7
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	Monooxygenase that catalyzes synthesis of cholesterol and steroids[RGD].	-4.3
Acsbg1	acyl-CoA synthetase bubblegum family member 1	May play a role in fatty acid metabolism and biosynthesis of steroid hormone precursors [RGD].	-4.1
Cyp19a1	cytochrome P450, family 19, subfamily a, polypeptide 1	Catalyzes end-step estrogen formation from androgens; aromatization of testosterone to E_2 [RGD].	-3.7
Inhba	inhibin beta-A	Subunit which can homodimerize or heterodimerize with Inhbb to form activins or heterodimerize with alpha subunit to form inhibin A to regulate FSH secretion [RGD].	-3.5
Ddit4	DNA-damage-inducible transcript 4	HIF-1-responsive gene that may protect some types of cells from hypoxia and H(2)O(2)-triggered apoptosis [RGD].	-2.9
Fabp6	fatty acid binding protein 6, ileal (gastrotropin)	May mediate metabolism of steroids in steroid hormone-producing tissues [RGD].	-2.7
Nr5a1(SF-1)	nuclear receptor subfamily 5, group A, member 1	Transcription factor; essential for adrenal and gonadal development [RGD].	-2.7
Kitl	kit ligand	Stimulates proliferation of both myeloid and lymphoid hematopoietic progenitors in bone marrow cultures and interacts with the basic fibroblast growth factor to promote the primordial to primary follicle transition in rat ovaries [RGD].	-2.5
Rasd1	RAS, dexamethasone-induced 1	Interacts with neuronal NO synthase adaptor protein CAPON and is involved in nitric oxide-mediated signaling [RGD].	-2.5
Fdxr	ferredoxin reductase	Component of electron transfer system for mitochondrial cytochrome P450; mediates production of steroid hormones and bile acids; activates vitamin D3 in steroidogenic tissues, liver and kidney [RGD].	-2.4
Stmn4	stathmin-like 4	Controls cell proliferation and activities for stathmin [RGD].	-2.4

Mrap_predicted	melanocortin 2 receptor accessory protein (predicted)	Homologous to the mouse gene that interacts with ACTH receptor [IHOP].	-2.4
Pla2g1b	phospholipase A2, group IB	Putative phospholipase A2 enzyme [RGD].	-2.4
Inha	inhibin alpha	Alpha subunit of protein hormone that heterodimerizes with one of the two alternative of beta subunits to form inhibin A and B [RGD].	-2.2
Nr5a2	nuclear receptor subfamily 5, group A, member 2	An orphan nuclear receptor that may bind DNA and activate gene transcription [RGD].	-2.2
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	Binds the CCAAT promoter motif as well as a core enhancer homology; activates transcription [RGD].	-2.2
Hsd3b1_predicted	hydroxysteroid dehydrogenase-1, delta<5>-3-beta (predicted)	A member of the C-21 steroid pathway and progesterone metabolism pathway [RGD].	-2.0
Egfr	epidermal growth factor receptor	Promotes cell proliferation and differentiation; mediates GPCR-regulated induction of protein synthesis, plays role in ovulation [RGD].	-2.0
Snf1lk	SNF1-like kinase	Inhibits CREB binding and transcription of cAMP-responsive element-dependent genes Cyp11A and StAR [RGD].	-1.9
Pgr	progesterone receptor	Receptor for progesterone; regulates glutamic acid decarboxylase expression in the hypothalamus during proestrus; may mediate production of the GnRH and LH surge, critical for ovulation [RGD].	-1.9
Nr0b1	nuclear receptor subfamily 0, group B, member 1	Human homolog is an orphan nuclear hormone receptor [RGD].	-1.7
Hsd17b7	hydroxysteroid (17-beta) dehydrogenase 7	A phosphoprotein that associates with the short form of the prolactin receptor [RGD].	-1.6
Akr1c18(20α-HSD)	aldo-keto reductase family 1, member C18	Catalyses the convariant of progesterone into 20α-dihydroprogesterone; activity is regulated by prolactin [RGD].	1.6
Igf1	insulin-like growth factor 1	Growth factor; plays a major role in mammalian growth [RGD].	1.6
Casp12	caspase 12	Cysteine-aspartic acid protease (caspase); involved with the terminal stage of apoptosis [RGD].	1.6
Pdcd4	programmed cell death 4	Acts as an inhibitor of apoptosis; has similarity to eukaryotictranslation initiation factor (eIF)4G [RGD].	1.6
Hsd11b1	hydroxysteroid 11- dehydrogenase 1	Catalyzes the interconversion of cortisol and cortisone; plays a role in glucocorticoid metabolism; may regulate blood pressure [RGD].	1.7
Ca3	carbonic anhydrase 3	Catalyzes hydration of carbon dioxide; may be involved in cellular response to oxidative stress [RGD].	1.7
Akr1cl1_predicted	aldo-keto reductase family 1, member C-like 1 (predicted)	Unknown function.	1.8

Ddit3	DNA-damage inducible transcript 3	Plays a role in the ER stress response [RGD].	2.1
Tgfb2	transforming growth factor, beta 2	Binds the transforming growth factor-β receptor; plays a role in regulation of cell growth and proliferation; may be involved in mesenchymal-epithelial cell interactions during development [RGD].	2.2
Adamts5	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)	Human homolog catalyzes the degradation of aggrecan, a component of cartilage extracellular matrix, plays role in ovulation [RGD].	2.3
Tgfb3	transforming growth factor, beta 3	Involved in epithelial and endothelial cell proliferation and differentiation during development [RGD].	2.4
Igfbp1	insulin-like growth factor binding protein 1	A modulator of a Insulin growth factor bioavailability [RGD].	2.4
Ptgis (CYP 8A)	prostaglandin I2 (prostacyclin) synthase	Catalyzes the conversion of prostaglandin H2 to prostacyclin in prostaglandin biosynthesis and appears to be important to implantation [RGD].	2.4
Igfbp5	insulin-like growth factor binding protein 5	May be involved in ovarian folliculogenesis and may play a modulatory role in type I fiber-dominated muscles [RGD].	2.5
Casp4, Casp11	caspase 4, apoptosis-related cysteine peptidase	Cysteine-aspartic acid protease (caspase); involved with the terminal stage of apoptosis; may also be involved with response to inflammation [RGD].	2.9
Grem1	gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis)	Protein may play a role in cellular growth control, viability and differentiation; high expression levels may induce apoptosis [RGD].	3.3
Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Functions in the negative regulation of bone resorption by inhibiting formation of osteoclasts [RGD].	4.2

[1]The function of the genes as described in rat genome database (RGD) or Information Hyperlinked over Proteins (IHOP).

4.3.7 Validation of microarray results for select transcripts by QPCR

Validation of microarray results was performed by examining the expression levels of 12 genes using QPCR. Similar gene expression patterns were observed for all targets measured by QPCR when compared to the results of the microarray gene expression

study (Figure 4.5); specifically, the overall stimulatory and inhibitory effects of HPTE on the expression for target genes were the same. As expected, the magnitudes of change were greater for QPCR because this approach not only is more quantitative but also represents a different probe sequence than that used to conduct array measurements. In addition to *StAR*, other normalizers were evaluated and demonstrated similar results (not shown).

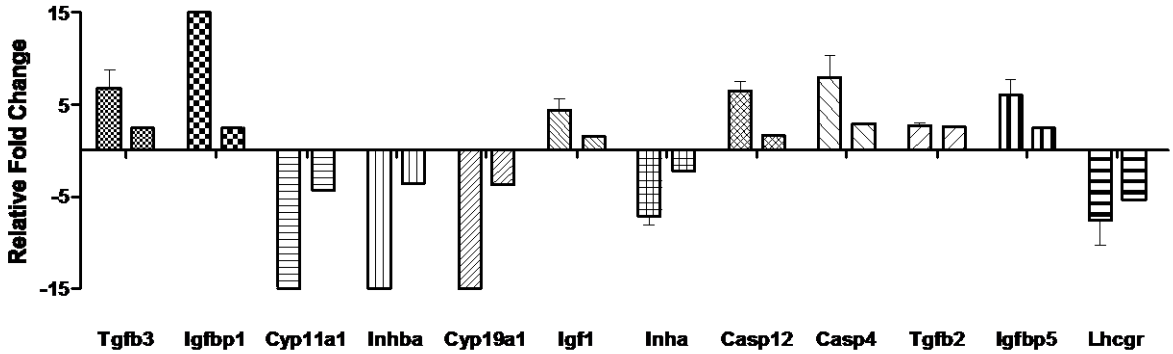


Figure 4.5: Validation of microarray data for key biomarkers by real-time quantitative PCR (QPCR).

To validate gene expression changes obtained by microarray analysis, twelve transcripts from groups given 0 or 10 µM HPTE with FSH were evaluated by QPCR using the same cDNA pool as was hybridized to the arrays. QPCR data were normalized using an assay to *StAR* whose expression levels were not significantly changed by the HPTE treatment (other normalizers were also evaluated and demonstrated similar results – data not shown). The assay protocols are described in Materials and Methods. The left bars in each bar pairs represent the QPCR results and the right bars represent microarray results. The direction of differential expression for targets measured were the same, however, as expected the magnitudes of change were greater for QPCR because this approach not only is more quantitative but also represents a different probe sequence than that used to make array measurements. All QPCR measurements are mean ± standard deviation of three separate reactions from three independent granulosa cell cultures.

4.4 Discussion

We examined the effect of the potent MXC metabolite HPTE on global gene expression in cultured immature rat granulosa cells. This study shows that HPTE exerts a stronger inhibition on FSH-induced gene expression when compared to that regulated by exogenous cAMP. In addition, these data reveal for the first time the effects of HPTE on the expression of a plethora of genes which are important to numerous vital pathways in granulosa cells, including signal transduction, transport, cell differentiation, growth, survival, and apoptosis. We also analyzed the effect of HPTE on the expression of genes associated with ovarian function, showing that HPTE affects multiple genes previously linked with the processes of folliculogenesis, steroidogenesis, and/or ovulation.

In vivo exposure to MXC reduces serum progesterone levels, and disrupts female reproductive parameters and ovarian morphology (Chapin *et al.* 1997; Gray *et al.* 1989). Transient developmental exposure to MXC can affect the level of key ovarian regulators including certain steroidogenic regulatory proteins (e.g., LHR and CYP11A1) (Armenti *et al.* 2008). Direct inhibition of CYP11A1 enzyme activity by HPTE, leading to reduced progesterone production in cultured granulosa cells, were previously observed; although, HPTE did not alter protein or mRNA levels of this enzyme (Akgul *et al.* 2008). We have previously shown that HPTE inhibits FSH- and cAMP-induced steroid hormone production. Furthermore, we demonstrated HPTE-directed changes in the mRNA levels of several steroidogenic pathway proteins/enzymes in granulosa cells (Zachow and Uzumcu 2006). While HPTE completely abrogated CYP11A11, 3β-HSD and CYP19A1 mRNA levels in FSH-treated granulosa cells, HPTE reduced CYP19A1 mRNA levels by only 50% in the presence of cAMP (Zachow and Uzumcu 2006); indicating that FSH-

stimulated cells were more sensitive to the inhibitory effects of HPTE. The current study confirmed that FSH-induced steroidogenesis is more sensitive to down-regulation by HPTE. This action was maintained on a global scale as shown by the hierarchical clustering analyses. Hierarchical clustering profiles suggest that in the presence of FSH, effects manifest above 1 µM HPTE, while granulosa cells given exogenous cAMP are more resistant to HPTE. The expression profile of FSH groups that were treated with 5 and 10 µM of HPTE resembled the gene expression profile of the unstimulated (basal) groups. However, the expression profile of the cAMP-stimulated cells remained distinct. These data and our study in Chapter 3 suggest that HPTE targets the cAMP-dependent cascade at one or more loci preceding cAMP production (e.g., FSH receptor, G proteins, and or adenylyl cyclase) in immature rat granulosa cells. This is in contrast to a study by Chedrese and Feyles, where MXC had no observable effect on the levels of FSH-induced cAMP, but still inhibited FSH-dependent steroid accumulation in porcine granulosa cells (Chedrese and Feyles 2001). This suggests that MXC exerts its effects distal to cAMP production. These apparently disparate results may be due to differences in action of MXC and HPTE *in vitro*, specific culture conditions, and/or species-specific effects.

In the present report, FSH-treated granulosa cells were more sensitive to the effects of HPTE, and FSH represents a more physiological stimulus. Therefore, the FSH group was further analyzed to determine the HPTE-dependent alterations in gene expression. We examined those genes that exhibited the largest fold changes in expression, and detected that many of these genes are associated with ovarian function (Table 4.6). HPTE suppressed the expression of several genes encoding mediators of steroidogenesis and/or ovulation (LHR, CYP11A1, HSD17B7, CYP19A1, FABP6,

ACSBG1, PGR, EGFR), transcription (SF-1, NR5A2, CEBPA,), and other aspects of ovarian function (KITL, INHβA, INHα, PRLR). In contrast, HPTE upregulated the expression of several genes encoding mediators of apoptosis (CASP11, CASP12, PDCD4), cell differentiation and growth regulation (GREM1, TGFβ2, TGFβ3, IGF-1, IGFBP1, IGFBP5), ovulation/tissue remodeling (ADAMTS5), and stress response (DDIT3 and CA3).

Some genes of interest from this list are GREM1, INHα (α subunit of inhibin), $INH\beta_A$ (β_A subunit of inhibin), TGFB2, TGFB3, ACSBG11, IGFBP5, CASP11, and CASP12. Gremlin 1 (GREM1) expression was upregulated by HPTE. GREM1 is an antagonist of BMP signaling (Merino *et al.* 1999), and is spatiotemporally expressed in the ovary (Pangas *et al.* 2004). It is primarily expressed in granulosa cells within preantral and antral follicles. In large antral follicles, GREM1 mRNA is detected in cumulus, but not in mural granulosa cells (Pangas *et al.* 2004). While its expression is stimulated by bone morphogenetic proteins (BMPs) and growth and differentiation factor-9 (GDF-9), GREM1 inhibits BMP signaling with no effect on GDF-9 signaling (Pangas *et al.* 2004). Thus, it has been speculated that this regulated expression of GREM1 may inhibit the actions of theca cell-derived BMP on granulosa cell luteinization, while allowing GDF-9 of oocytic origin to mediate cumulus expansion (Pangas *et al.* 2004). The fact that the expression of some of the luteinization markers (LHR, PGR EGFR) was inhibited by HPTE in the present study supports the above notion. Therefore, it may be worth exploring the effect of HPTE on theca cell-derived BMPs and BMP-coupled signaling molecules.

In addition, GREM1 has been shown to affect other signaling pathways that may interact with the FSH/cAMP-dependent protein kinase (PKA) signaling pathway. Over-expression of GREM1 has been observed to inhibit the activity of Wnt signaling through its connection with β-catenin (Gazzerro *et al.* 2007). Recently, β-catenin was proven to be critical for gonadtropin-directed signal transduction (specifically FSH) through the coordination of SF-1 and β-catenin (Parakh *et al.* 2006). Parakh et al. (Parakh *et al.* 2006) also showed that β-catenin selectively modified the FSH-driven production of CYP19A1 and CYP11A1 in granulosa cells. In addition, β-catenin, in conjunction with SF-1, regulated the activity of CYP19A1, and was necessary for the FSH-cAMP mediated regulation of CYP19A1. In the present study, HPTE showed an inhibitory effect on the mRNAs encoding CYP19A1 and CYP11A1. This may be a result of a downstream effect on Wnt signaling via GREM1-induced β-catenin inhibition or other possible up-stream effectors of β-catenin (Hino *et al.* 2005). The Wnt pathway, and other signaling pathways that may interact with FSH-PKA signaling, appears to be one of many novel interactions that may be affected by HPTE in granulosa cells (Figure 4.6).

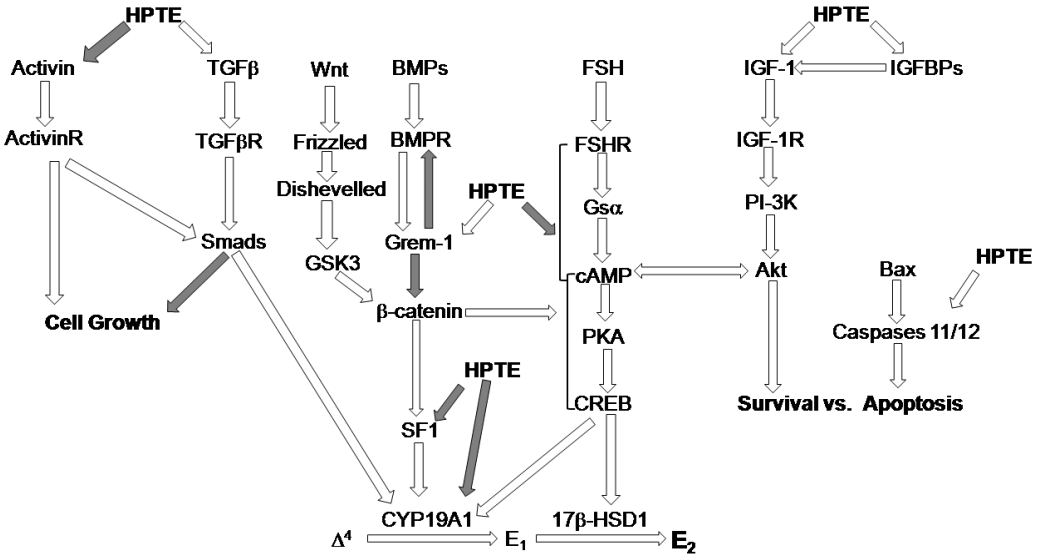


Figure 4.6: Proposed model for the effect of HPTE in the granulosa cell.
FSH binding to the FSHR initiates the protein kinase A (PKA) signaling pathway which promotes E_2 production in granulosa cells. HPTE appears to inhibit this cascade through an unknown mechanism, but effects in multiple pathways are implicated. HPTE affected the expression of several genes known to control granulosa cell function, as described in the text. Among these, activin, TGFβs, GREM1, SF1, IGF-1, IGFBPs, and caspases all have distinct effects in controlling granulosa cell growth, differentiation, survival, and steroidogenesis. White arrows indicate induction. Gray arrows indicate inhibition. Gsα, α subunit of guanine nucleotide-binding stimulatory protein; CREB, cAMP-response element binding protein; SF1, steroidogenic factor 1; Δ^4, androstenedione; E_1, estrone; E_2, estradiol-17β; BMP, bone morphogenetic protein; IGF, insulin-like growth factor; IGFBP, IGF binding protein; PI-3K, pshosphoinositide 3 kinase; TFGβ, transforming growth factor-β; GSK3, glycogen synthase kinase-3.

Down-regulation of the expression of both INHα and INHβ_A by HPTE may have significant consequences for the production of inhibins and activins in granulosa cells. Inhibins are dimers of an α subunit combined with either a β_A (inhibin A) or β_B subunit (inhibin B) (Knight and Glister 2006). Thus, HPTE inhibition of INHα could affect the level of both inhibin isoforms. In addition, activins are homodimers ($\beta_A\beta_A$, activin A or $\beta_B\beta_B$, activin B) or a heterodimer (activin AB) of β subunits (Knight and Glister 2006). Therefore, inhibition of INHβ_A would also prevent formation of activins A and AB. Collectively, activins and inhibins have important roles in granulosa cell proliferation and differentiation and folliculogenesis (Findlay 1993; Hutchinson *et al.* 1987; Kishi *et al.* 1998; Knight and Glister 2001; LaPolt *et al.* 1989; Li *et al.* 1995b). For example, activin stimulates basal and FSH-induced granulosa cell proliferation (Miro and Hillier 1996). Activin also regulates basal and gonadotropin-induced steroid production in rat granulosa cells (Miro *et al.* 1991). A modulatory role of inhibin on follicular steroidogenesis has also been reported (Smyth *et al.* 1994). As a result, inhibition of INHβ_A and INHα may be causal to the reduced steroidogenesis in HPTE-treated granulosa cells (Zachow and Uzumcu 2006).

In addition to inhibin and activin, the expression of the mRNA encoding TGFβ2 and TGFβ3 was increased by HPTE. The TGFβs have a synergistic effect on FSH-stimulated proliferation in granulosa cells, and TGFβ-directed changes in granulosa cell steroidogenesis are well documented (Hutchinson *et al.* 1987; Knight and Glister 2006; Ying *et al.* 1986). Since E_2 has been shown to impair TGFβ expression (Kleuser *et al.* 2008), a possible mechanism that explains the upregulation of TGFβs is the HPTE-

dependent decrease in E_2 and/or a direct effect of HPTE on TGFβ expression. However, further experiments are needed to determine this.

The long chain fatty acid synthase known as Acyl-CoA synthase bubblegum 1 (ACSBG1) was down-regulated in granulosa cells challenged with HPTE. ACSBG1 is normally located in theca cells and testicular Leydig cells, and has been linked to spermatogenesis (Pei *et al.* 2003). The role of ACSBG1 in folliculogenesis has not been defined, but it may function as survival factor which mediates the synthesis of steroid precursors (Pei *et al.* 2003). By inhibiting ACSBG1, HPTE may reduce steroidogenesis by directly reducing steroid precursors and/or energy availability.

Numerous reports have shown the importance of the insulin-like growth factor (IGF) system within granulosa cells. In conjunction with FSH, IGF-I stimulates cell proliferation and steroidogenesis in granulosa cells of various species (deMoura *et al.* 1997; Mazerbourg *et al.* 2003). In contrast, IGF binding proteins (IGFBPs) can suppress FSH-induced follicular growth and differentiation, leading to atresia by possibly sequestering IGF-I protein and inhibiting its activity (Cataldo *et al.* 1993; Ui *et al.* 1989). In addition, FSH reduces IGFBP activity by stimulating proteolytic mechanisms that degrade IGFBPs (Fielder *et al.* 1993). In the present study, 10 μM HPTE stimulated the genes encoding IGF-I and IGFBPs (i.e., IGFBP-1 and IGFBP-5) in FSH-stimulated granulosa cells. Since IGFBPs bind IGF-I preventing activity, the increase in IGF-I may counteract the HPTE-directed increase in the level of IGFBPs. In addition, E_2 has been shown to inhibit IGFBP-5 in granulosa cells from large follicles (Voge *et al.* 2004); thus, the apparent up-regulation of IGFBP-5 by HPTE could be a consequence of the HPTE-dependent reduction in E_2 production. In general some of the effects on gene expression

that were observed in HPTE-treated cells may be an indirect effect due to reduced E_2 secretion as well as due to the direct effects of HPTE.

Others have noted that HPTE inhibits growth and induces atresia in antral follicles (Gupta *et al.* 2006a). HPTE upregulated caspase 11 and caspase 12 mRNAs in granulosa cells which may provide a mechanism that explain HPTE-induced atresia. Caspase 11 is classified as caspase 4 in humans and appears to be essential for activation of interleukin 1β-converting enzyme (ICE or caspase 1) (Wang *et al.* 1998). Caspase 12 mediates apoptosis driven by the endoplasmic reticulum (Liu and Baliga 2005). Both caspases are upstream initiators of apoptosis, through caspase 3 (Kang *et al.* 2000; Liu and Baliga 2005). Whether HPTE affects caspases that regulate survival in granulosa cells is currently unknown, but should be investigated.

4.5 Summary and Conclusions

Combining these observations, we have composed a working model that attempts to connect the observed gene expression changes in granulosa cells to some promising signaling mechanisms (Figure 4.6). Evidence suggests that HPTE acts on the FSH-PKA signaling pathway (Zachow and Uzumcu 2006). Whether this interaction is direct or through still vaguely defined cross-talk of parallel signaling cascades (Hunzicker-Dunn and Maizels 2006) is unknown. Multiple signaling complexes exist within granulosa cells and each has some role in directing normal cellular function (Knight and Glister 2006). Our model attempts to link these pathways with inhibition of E_2 production and steroidogenic pathway proteins/enzymes. Induction of GREM1 by HPTE is an example of multiple pathways that can be perturbed by HPTE. GREM1 is known to inhibit β catenin; β catenin is a proven stimulator of SF-1 leading to CYP19A1 activity (Gazzerro

et al. 2007; Michos *et al.* 2007). Also, GREM1 binds to BMP and therefore would block BMP activity. This provides another potential mechanism for the inhibitory effects of HPTE in granulosa cells. Finally, Wnt functions through a pathway leading to β catenin; so it is plausible that the effect of HPTE is also manifested through that cascade (Michos *et al.*, 2007). Since HPTE induced changes in the level of expression of several genes linked to granulosa cell signaling pathways, further efforts should be placed on determining the functional effects of the HPTE-stimulated alterations in gene expression. In chapter 5, we will use pathway analysis techniques to further examine and characterize the molecular mechanisms involved in HPTE toxicity in the granulosa.

In summary, the current results show that HPTE differentially affects FSH- and cAMP- stimulated gene expression in granulosa cells. In addition, these results indicate that parallel pathways, besides FSH-cAMP-PKA may be involved in the effects of HPTE on FSH-mediated steroidogenesis in granulosa cells. The discovery of the HPTE-directed involvement of these and other pathways, and perhaps cross-talk among numerous cascades, is compelling and will no doubt provide a better understanding of the effect of HPTE and MXC in the ovary.

CHAPTER 5: Pathway Activity Analysis of Granulosa Cell Microarray: Gene Network changes induced by Methoxychlor and its metabolite HPTE in the Rat Granulosa Cell

5.1 Introduction

In vivo and *in vitro* observations suggest that the ovary is a target tissue of methoxychlor (MXC) exposure (Armenti *et al.* 2008; Gupta *et al.* 2006b; Symonds *et al.* 2006). The structurally similar metabolite of MXC, 2, 2-bis-(*p*-hydroxyphenyl)-1, 1, 1-trichloroethane (HPTE) inhibits enzyme activity of steriodogenic proteins and inhibits steroidogenesis *in vitro* (Akgul *et al.* 2008; Hu *et al.* 2011; Zachow and Uzumcu 2006). HPTE acts as ERα agonist and ERβ antagonist (Gaido *et al.* 1999) which is a characteristic shared by other estrogenic endocrine-disrupting chemicals (EDCs) in the environment (Cummings and Laskey 1993). Others have shown that MXC inhibits steroidogenesis in antral follicles *in vitro* (Basavarajappa *et al.* 2011) similar to the effect of HPTE in granulosa cells (Zachow and Uzumcu 2006). Of interest is that the mechanism of action may be epigenetic-related as global methylation studies in MXC-ovaries are different from wild-type ovaries in rats (Zama and Uzumcu 2009). Therefore, MXC is a model compound for the effects of EDCs: 1) the MXC metabolite, HPTE, acts as a selective estrogen receptor modulator, 2) because it models the effect of organochlorine pesticides and their potential endocrine disrupting effects on a unique target system (i.e., the reproductive system), and 3) the mechanism of action may have components of environmental and epigenetic factors associated with disease (Crews and McLachlan 2006; Zama and Uzumcu 2010).

Immature rat primary granulosa cell cultures are a commonly used *in vitro* system for examining the direct toxic effect of EDCs in the ovary (Chedrese and Feyles 2001), for studying steroidogenesis (Lovekamp and Davis 2001; Zachow and Uzumcu 2006), as an FSH bioassay (Beers and Strickland 1978) and approximating the *in vivo* conditions of the follicle (Erickson *et al.* 1979). Granulosa cells interact bi-directionally with the oocyte to support folliculogenesis through paracrine signaling molecules (reviewed in Knight and Glister 2006) and FSH plays a key role as an essential survival hormone in early antral ovarian follicles (Chun *et al.* 1996; Hsueh *et al.* 2000) and an inducer of steroidogenesis (Erickson *et al.* 1979; Dorrington *et al.* 1975). Intercellular communication is closely associated with cytoplasmic-associated processes like cell adhesion and gap junction (Singer 1992). FSH converts flattened epithelial rat granulosa cells into spherical-shaped morphology, which maintain cytoplasmic networking *in vitro* (Lawrence *et al.* 1979; Carnegie *et al.* 1987). Thus alterations in structures that affect cell-cell communication and cell-matrix interactions may affect the availability or activity of local growth factors (Albertini *et al.* 2001).

In a recent study from our lab, we showed that HPTE altered the genes expression in FSH-stimulated granulosa cells more robustly then those of cAMP-stimulated granulosa cells (Harvey *et al.* 2009). E_2 production was reduced dose-dependently by HPTE more efficiently in FSH-treated cells than cAMP-treated cells. This effect appears to be due at least in part to the reduction in cAMP production (Chapter 3; manuscript in preparation). MXC had a similar inhibitory effect on FSH-mediated cAMP and E_2. Therefore, this suggested that common molecular and biochemical pathways between MXC and HPTE may be involved.

An alternative hypothesis for the susceptibility of FSH to MXC/HPTE-induced inhibition of steroidogenesis is that FSH may induce pathways parallel to cAMP that can affect downstream targets of cAMP through crosstalk (reviewed in Stork and Schmitt 2002). For example, FSH activates three diverse membrane-associated signaling cascades (adenylyl cyclase, RAS, and SFK) that converge downstream to activate specific kinases (PKA, ERK1/2, and PKB/FOXO1a) that control granulosa cell function and differentiation (Wayne *et al.* 2007). Although at the respective doses of FSH (3 ng/ml) and dbcAMP (1 mM) used, comparable levels of E_2 were produced, we queried whether gene expression changes and molecular pathways were similarly affected.

Microarray experiments have generated unprecedented quantities of genome-wide data on gene expression patterns (Quackenbush 2001) and new methods are being generated to help understand, store, standardized, visualize, and to analyze data (Gough and Yaffe 2011). Using a pathway activity method that was recently developed (Ovacik *et al.* 2010b), we can study the effect of treatments on granulosa cell transcriptional profiles. The main aim of this study is to understand the effect of FSH and cAMP stimulation on gene expression and try to better understand how MXC affects FSH responsiveness in granulosa cells by examining changes in cell morphology, estradiol-17β, and gene expression. Our approach is to examine the gene networks affected from the collected microarray data, and identify the difference between treatment groups. We compared gene expression between FSH and cAMP treatment using a t-test to identify the probe sets that exhibit significant difference. Next we focused on the dose-dependent effect of MXC on FSH-treated granulosa cells. Using a novel pathway-based analysis (Ovacik *et al.* 2010b), we compared the genes that were members of signaling and

metabolic pathways affected by MXC and HPTE. We also used a clustering analysis to observe pathways that behaved similarly at each treatment group. We identified novel pathways that may be involved in MXC ovarian toxicity.

5.2 Materials and Methods

Experimental Process

Data for pathway analysis were compiled from unpublished data (MXC data) and a previously published study from our lab (Harvey *et al.* 2009) and assessed using different strategies in order to examine the effects of (1) HPTE, (2) the effect of FSH and cAMP, and (3) comparison of the dose-dependent effects of MXC and HPTE on granulosa cells (Figure 5.1). Harvey et al. (Harvey *et al.* 2009) examined the gene expression alterations induced in the granulosa cell *in vitro* by HPTE and the experimental protocol is described therein. Briefly, female pre-pubertal (postnatal day 21and postnatal day 27) rats were sacrificed and their ovaries extracted for granulosa cell culture. Granulosa cells were plated at a density of 3-4 x 10^5 per well in a 24 well plate. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) /F-12 containing 5% fetal bovine serum. Media were then aspirated and replaced with serum-free DMEM/F12 and 0.1 μM androstenedione. In the experimental set-up, cells were untreated or treated with 3 ng FSH/ml or 1 mM cAMP for 48 h. Some FSH treated granulosa cell cultures were co-treated with a low (1 μM) or high (10 μM) dose of HPTE or MXC. Dose-response curves were created using FSH and cAMP to determine a comparable submaximal level of estradiol-17β production (data not shown). MXC and HPTE dosage were selected based on dose-response curve that showed a dose-dependent inhibition of estradiol-17β. Microarray data were collected from separate experiments performed simultaneously.

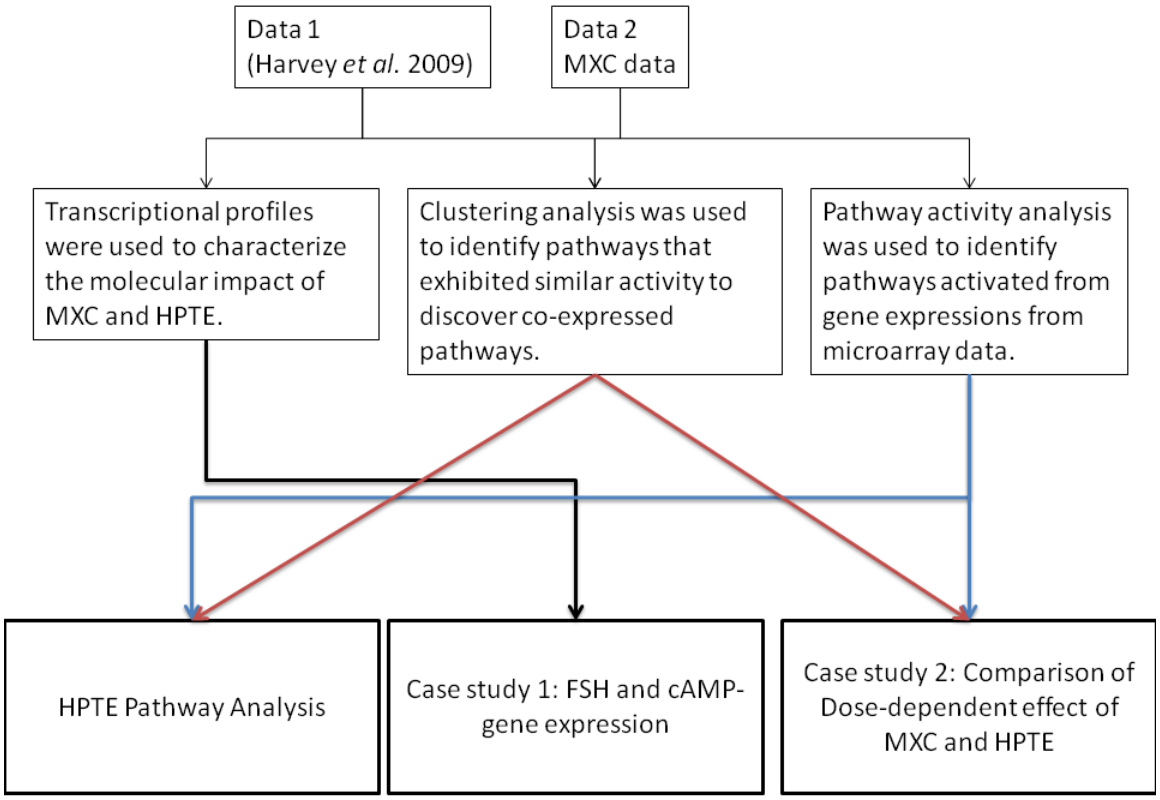


Figure 5.1: Experimental process for analysis of microarray data.
Microarray data were combined and three separate analyses were performed to study the transcriptional profiles of MXC and HPTE as described in the Materials and Methods section.

Microarray assays were performed using the same batch of reagents for both HPTE and MXC data sets to minimize variability. Total RNA was isolated from individual wells using RNeasy Mini Kit (Qiagen) followed by DNase I treatment and analyzed for purity and concentration prior to cDNA synthesis. Forty nanograms of total RNA from each sample were used to generate a high-fidelity cDNA for array hybridization using the NuGen Ovation Biotin RNA Amplification and Labeling system (NuGen Technologies). After fragmentation and biotin labeling, the samples were hybridized to Affymetrix Rat Genome 230 2.0 arrays. All datasets were available through the NCBI Gene Expression Omnibus data repository (http://www.ncbi.nih.gov/geo/).

Case Study 1

The main aim of this study is to understand the effect of FSH and cAMP stimulation on granulosa cells. We performed a t-test ($p < 0.01$) to identify the probe sets that exhibit significant difference between FSH and cAMP treatment. We defined significant changes with respect to FSH treatment since the probe sets generated from the cAMP data were a subset of the FSH probesets. For example, probe sets are defined as up regulated if the gene expression is higher in FSH treatment compared to cAMP treatment. Similarly, the probe sets are defined as downregulated if the gene expression is lower in FSH treatment compared to cAMP treatment (Figure 5.2).

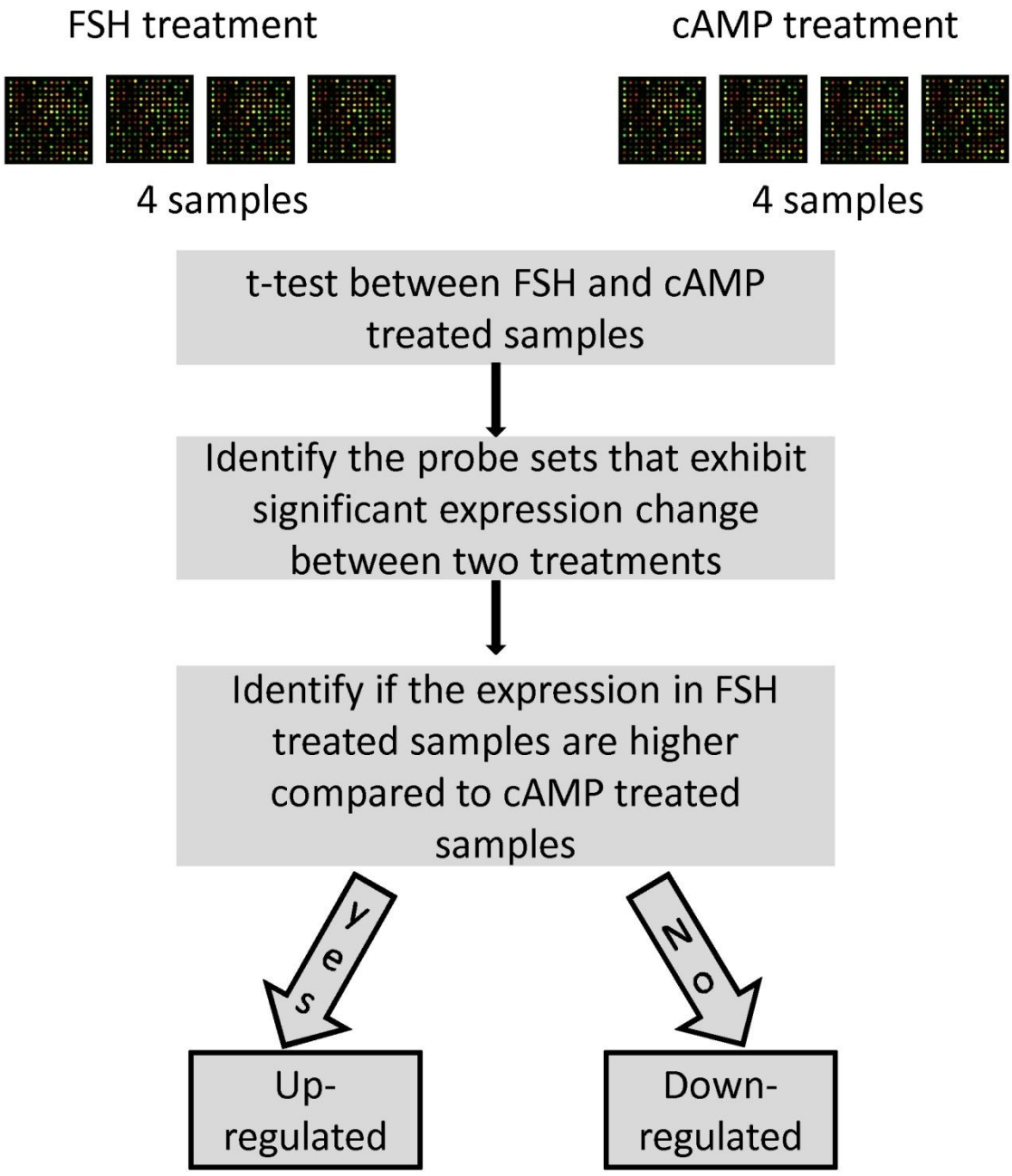


Figure 5.2: Outline for comparing genes regulated by FSH- and cAMP-treated granulosa cells.
We performed a t-test to identify the probe sets that exhibit significant difference between FSH and cAMP treatment (p-value < 0.01). We defined the significant change with respect to FSH treatment as described in the Materials and Methods section.

Case Study 2

The main aim of this study is to understand the effect of FSH stimulation in granulosa cells when the cells were exposed to HPTE and MXC at two doses, 1µM and 10µM. We performed a clustering analysis to identify the probe sets that exhibit the same response across different experimental conditions. The relationship between HPTE and MXC treated samples were compared to the control (FSH alone) samples.

Hormone radioimmunoassay

Media from granulosa cell cultures were collected to confirm the inhibitory effect of HPTE and MXC on FSH-mediated E_2 production as described previously (Harvey *et al.* 2009) using Coat-A-Count Estradiol kits (Siemens Health Diagnostic Deerfield, IL). The assay was performed according to the manufacturer's provided instructions.

Selection of significant probe sets

In order to identify significantly different probe sets, a pairwise comparison was made between probe sets. To accomplish this, a *t*-test was performed ($p< 0.01$). To determine a fair measure of significance in an analysis of this kind, it is important to account for testing a large number of hypotheses (Dudoit *et al.* 2003). The identified clusters can be present in random data, i.e., randomly permutated microarray data. A significance value can be assigned to the clusters based on their population size to identify the clusters that characterize non-random data (Iyer *et al.* 2010). Hence, microarray data were permutated 1000 times and a distribution for the cluster sizes that corresponded to random data were generated. The randomly generated cluster size distribution was then compared to the original cluster size distribution and the clusters that were likely to be generated by a

random model were filtered out. Specifically, the p-value of each cluster size for random data was modeled by Eq. (1)

$$p - value = e^{(-\lambda x)} \quad (1)$$

where x is the cluster size and λ was estimated from the distribution of random dataset (Iyer *et al.* 2010). The significance threshold used corresponds to p-value of 10^{-8}.

Functional analyses of microarray data

The functional analyses of the significant gene groups were performed through the use of Ingenuity Pathways Analysis (IPA, Ingenuity® Systems, www.ingenuity.com). To identify signaling and metabolic pathways that were overrepresented by the gene groups, the library of pathways provided by IPA was used. The significance (p-value < 0.05) of the association between the data set and the canonical pathway was measured by Fisher's exact test. Transcriptional factor analysis and identification of associated gene networks also were performed using IPA.

Clustering analysis

To understand the effect of FSH stimulation in granulosa cells when the cells were exposed to HPTE and MXC at two doses, 1µM and 10µM, we performed a clustering analysis to identify the probe sets that exhibit the same response across different experimental conditions. The relationship between HPTE and MXC treated samples were compared to the control samples.

For a given dose, the transcriptional responses of a probe set are translated to a symbolic representation with a t-test (Iyer *et al.* 2010). If two treatment conditions do not exhibit significant change, the relationship is translated as constant (C). If the two treatment conditions exhibit significant change, the relationship is translated as up

regulated (U) or down-regulated (D) depending on how the treatment condition changes with respect to basal. Symbolic translation is performed at each dose of HPTE and MXC exposure, separately. At the end, the transcriptional profile of a probe set is characterized by 4 symbolic representations. Each two consecutive symbols in this representation correspond to the relationship of HPTE-control and MXC-control, respectively. For example, a symbolic representation of UC DU corresponds to the following; 1 µM HPTE treated samples exhibit up-regulation whereas 1 µM MXC treated samples do not exhibit a significant change (the first two consecutive symbols, UC). Further, 10 µM HPTE treated samples exhibit down-regulation whereas 10 µM MXC treated samples exhibit up-regulation (the last two consecutive symbols, DU). The outline of the clustering algorithm is illustrated in Figure 5.3.

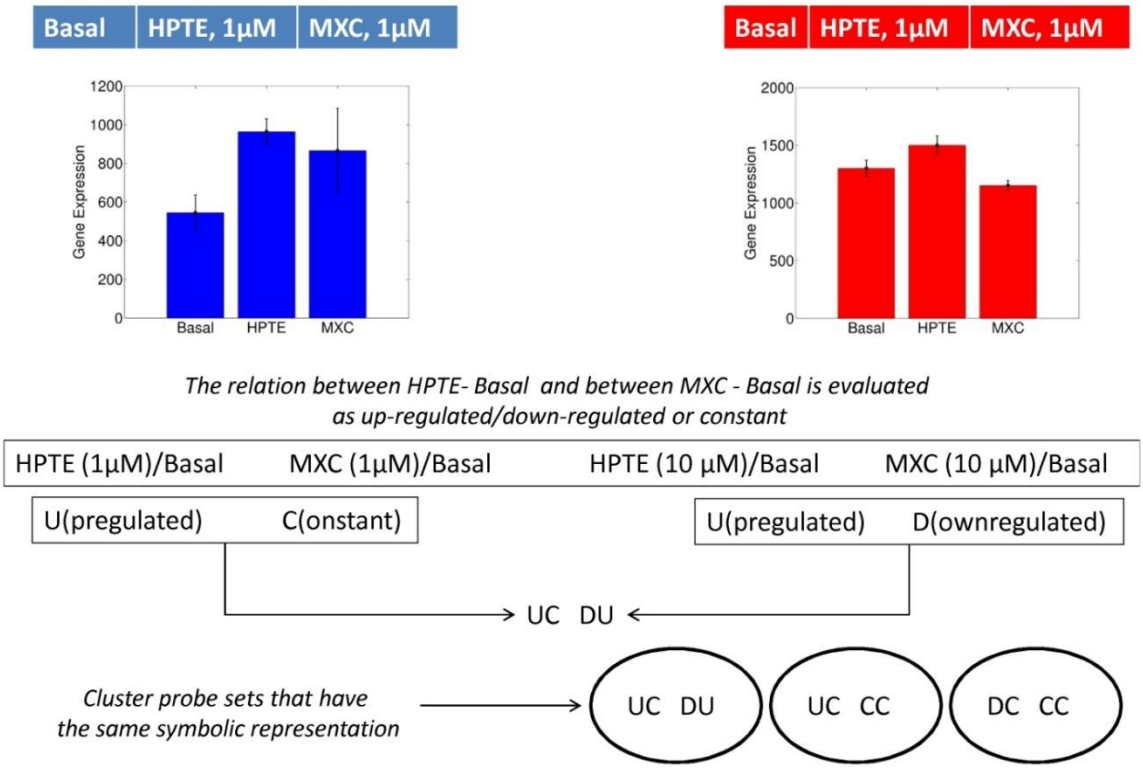


Figure 5.3 Outline of the clustering algorithm.
First, the transcriptional profiles of a probe set are translated to their corresponding symbolic representation with a t-test. Then, the probe sets that have the same symbolic representation are clustered together. Microarray analysis was conducted as described in Materials and Methods.

Pathway activity analysis

Pathway activity analysis (Ovacik *et al.* 2010b) was performed on the HPTE microarray data from Harvey et al (Harvey *et al.* 2009) and unpublished MXC microarray. Briefly, all the genes are mapped onto associated pathways and then the gene expression levels within a given pathway are reduced to a single value, pathway activity levels. The significance of pathway activity level (PAL) is then compared to randomly generated data and a probability value calculated. These PAL values are filtered and compared between treatment groups and controls (Ovacik *et al.* 2010b).

5.3 Results

We first analyzed the effect of HPTE on the global gene expression in the granulosa cell in the presence or absence of FSH and cAMP (Harvey *et al.* 2009). Microarray generates tremendous amounts of data that required computational and statistical methods for analysis (Allison *et al.* 2006). Initially, to analyze the effect of HPTE, we focused on individual gene changes and those genes that were hypothesized to be a part of ovarian function and steroidogenesis (Harvey *et al.* 2009). From this step, we next examined pathways that were affected by HPTE.

Clustering analysis results agreed with our conclusions from (Harvey *et al.* 2009) that in the absence of HPTE, FSH and cAMP-stimulated granulosa cells behaved the same. FSH and cAMP-stimulated granulosa cells were also different from untreated (basal) granulosa cells. Basal (i.e., absent FSH or cAMP) cells exhibited little effect due to increasing HPTE with only 1, 2 and 24 pathways showing significant difference from untreated cells for 1μM, 5μM and 10 μM HPTE-treated cells, respectively. However, increasing levels of HPTE caused FSH-treated cells but not cAMP-treated cells to behave

like untreated cells with increasing doses of HPTE; supporting our previous hypothesis that FSH-treated granulosa cells were more sensitive to HPTE than cAMP-treated granulosa cells (Harvey *et al.* 2009). Using the pathway activity analysis, 70 pathways were identified from the KEGG database that showed cumulative difference between FSH and cAMP when treated with different doses of HPTE (Table 5.1) and showed two principle patterns of expression that matched the gene expression patterns observed in Chapter 4 (Figure 5.4). In cluster 1, HPTE induced pathway activity in FSH-treated groups while in cluster 2, the activity of pathways normally activated by FSH were inhibited by increasing doses of HPTE.

Table 5.1: Pathways activated by HPTE in FSH- and cAMP-treated granulosa cells.

p-value	*Pathway name*	*P-value*	*Pathway name*
5.46E-05	Type I diabetes mellitus	0.000929	Neuroactive ligand-receptor interaction
5.46E-05	ECM-receptor interaction	0.000929	Glycan structures - biosynthesis 1
5.46E-05	Small cell lung cancer	0.001694	Adherens junction
5.46E-05	Axon guidance	0.002404	C21-Steroid hormone metabolism
5.46E-05	Cell adhesion molecules	0.002951	Benzoate degradation via hydroxylation
5.46E-05	Circadian rhythm	0.003224	Nitrogen metabolism
5.46E-05	MAPK signaling pathway	0.004044	Insulin signaling pathway
5.46E-05	Cytokine-cytokine receptor interaction	0.004208	Glioma
5.46E-05	mTOR signaling pathway	0.004481	D-Glutamine and D-glutamate metabolism
5.46E-05	Melanoma	0.004809	Apoptosis
5.46E-05	Cell cycle	0.005301	Glycosphingolipid biosynthesis - globoseries
5.46E-05	Focal adhesion	0.005574	Glycosaminoglycan degradation
5.46E-05	Chronic myeloid leukemia	0.005628	Glutamate metabolism
5.46E-05	Pancreatic cancer	0.00612	Fatty acid elongation in mitochondria
5.46E-05	Hematopoietic cell lineage	0.007596	Leukocyte transendothelial migration
5.46E-05	Neurodegenerative Disorders	0.007814	Glutathione metabolism
5.46E-05	Glycosphingolipid biosynthesis - ganglioseries	0.008142	Basal cell carcinoma
5.46E-05	Arginine and proline metabolism	0.009235	Keratan sulfate biosynthesis
5.46E-05	Colorectal cancer	0.010437	DNA polymerase
5.46E-05	Biotin metabolism	0.010546	Retinol metabolism
5.46E-05	Renal cell carcinoma	0.010929	Glycan structures - biosynthesis 2
5.46E-05	Toll-like receptor signaling pathway	0.01235	Glycan structures - degradation
5.46E-05	Lysine degradation	0.013661	Renin-angiotensin system
5.46E-05	Glycerolipid metabolism	0.018197	Fructose and mannose metabolism
5.46E-05	Natural killer cell mediated cytotoxicity	0.018197	Glycosphingolipid biosynthesis - lactoseries
5.46E-05	Long-term depression	0.019891	Hedgehog signaling pathway
5.46E-05	Nicotinate and nicotinamide metabolism	0.022896	ARH Signaling
0.000109	Jak-STAT signaling pathway	0.027104	N-Glycan degradation
0.000109	Bile acid biosynthesis	0.031257	Tryptophan metabolism
0.000109	1- and 2-Methylnaphthalene degradation	0.034317	Phenylalanine, tyrosine and tryptophan biosynthesis
0.000109	Carbon fixation	0.03459	Novobiocin biosynthesis
0.000328	TGF-beta signaling pathway	0.035027	Fc epsilon RI signaling pathway
0.000328	Regulation of actin cytoskeleton	0.03623	p53 signaling pathway
0.000492	Pyruvate metabolism	0.036667	Alkaloid biosynthesis I
0.000656	Chondroitin sulfate biosynthesis	0.03918	Pentose and glucuronate interconversions

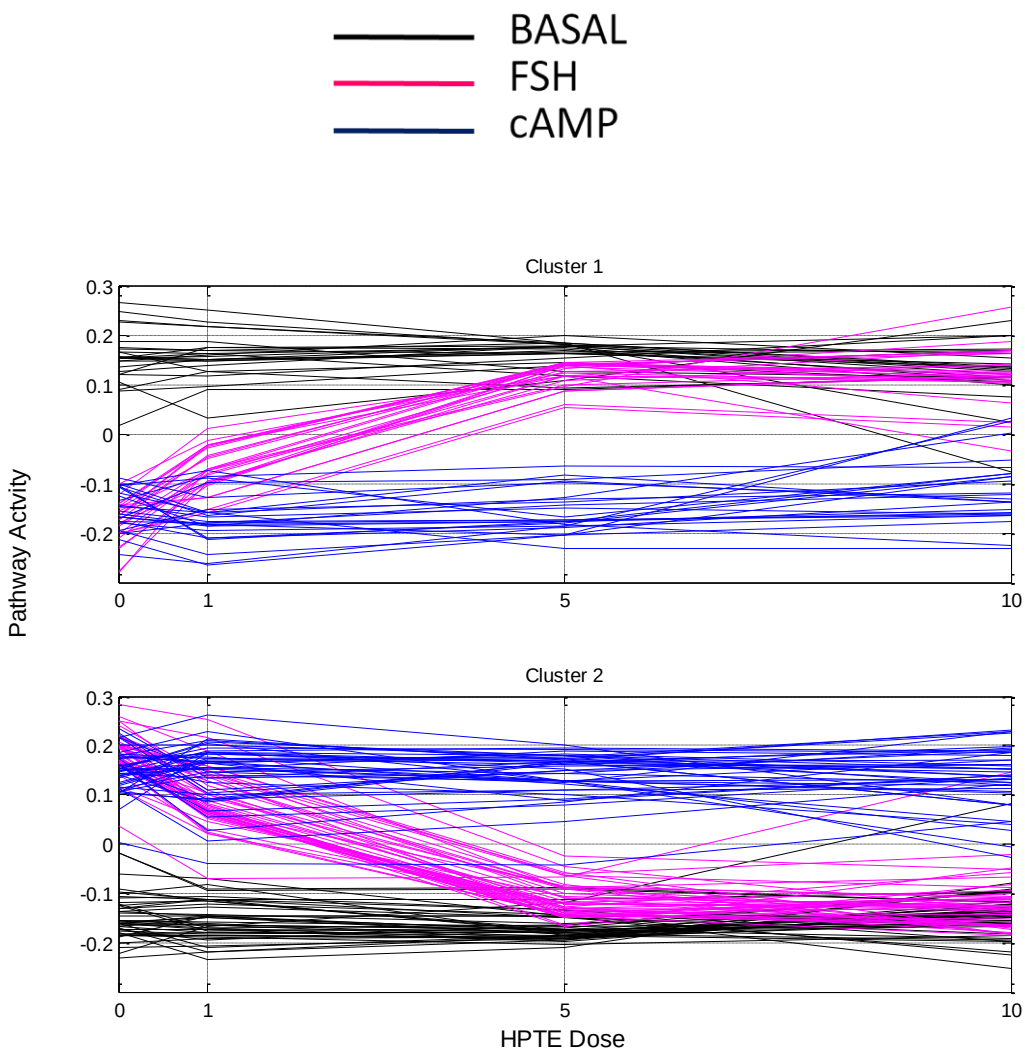


Figure 5.4: Pathway activity analysis of increasing HPTE.
Pathway activities measured across two cluster groups delineating the effect of HPTE (0, 1, 5, or 10 μM) with and without FSH or cAMP. This analysis showed that basal (black) had no effect due to HPTE. FSH (pink) and cAMP (blue) were significantly different from basal and exhibited similar activity in the absence of HPTE. With increasing HPTE (5 and 10 μM), FSH-treated granulosa cells behave like basal cells while cAMP does not change.

PAL identified pathways that are associated with folliculogenesis such as Aryl hydrocarbon receptor signaling, TGFβ signaling, p53 signaling, cell cycle, apoptosis, C-21 steroid metabolism pathways and regulation of actin cytoskeleton signaling (Figure 5.5). Pathway activity analysis is consistent between microarray results and database results in showing alterations in (1) steroidogenesis-related metabolism, (2) xenobiotic and glutathione metabolism pathways, and (3) signaling pathways related to tissue and cellular reorganization (Harvey *et al.* 2009).

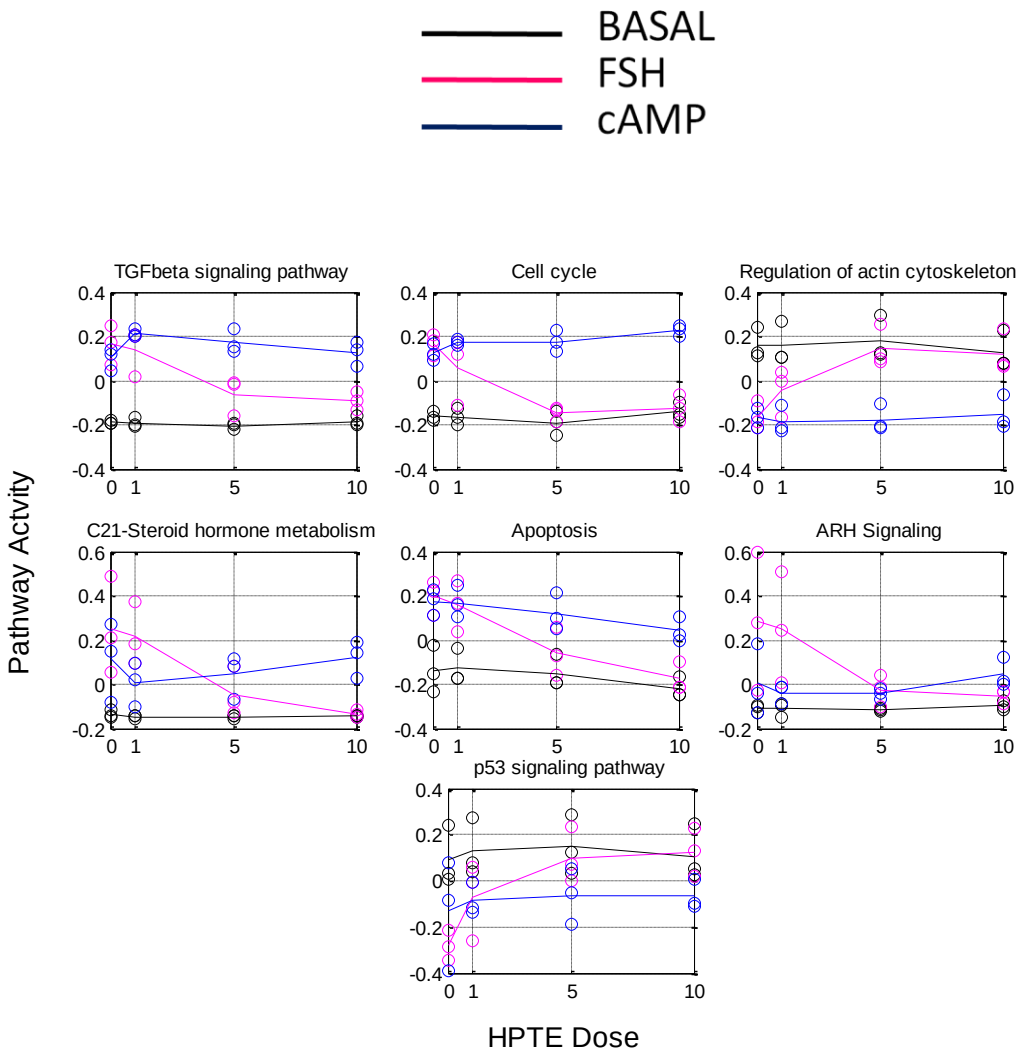
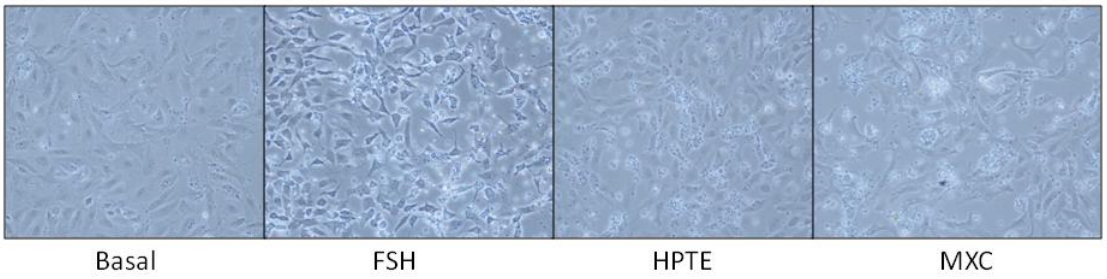


Figure 5.5: Pathway activity analysis of select key pathways.
Selected pathways of interest associated with ovarian function activated by HPTE.

FSH Effect

FSH conversion of flattened epithelial rat granulosa cells into spherical-shaped morphology was observed in granulosa cells after 48 h of serum-free treatment. Co-treatment with 10 µM HPTE or 10 µM MXC caused the granulosa cells to remain flattened (Figure 5.6A). Estradiol-17β is known to be produced by FSH-stimulated granulosa cells; an effect that is inhibited by HPTE in a dose-dependent manner (Zachow and Uzumcu 2006). MXC also inhibited estradiol-17β production in a dose-dependent manner similar to HPTE (Figure 5.6B). Co-treatment with 1 µM MXC or 1 µM HPTE appeared to have no effect on FSH-mediated morphology (data not shown). Low dose (1 µM) HPTE had a slight inhibitory effect on estradiol-17β production of granulosa cells while low dose (1 µM) MXC was not significantly different from FSH-alone.

A



B

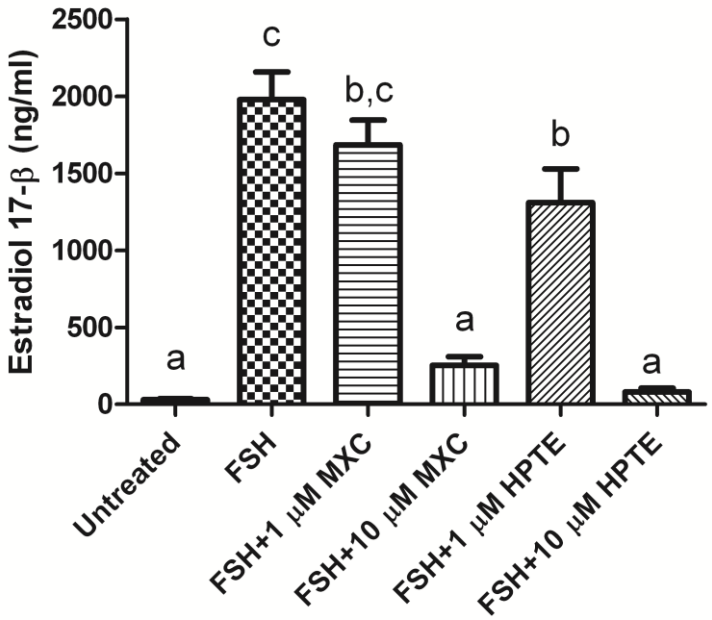


Figure 5.6: Comparison of morphology and E_2 production after treatment with MXC and HPTE on FSH-induced granulosa cells.

(A) Photographs after 48 h of granulosa cell culture with Basal, FSH (3ng/ml), FSH+10 μM MXC, and FSH+10 μM HPTE. FSH induced the granulosa cell to round up and extends fingerlike processes from the cell body while 10 μM MXC and 10 μM HPTE inhibited morphological changes. (B) Estradiol-17β was measured using a commercially available RIA kit (COAT-A-COUNT) and collected after 48 h of treatment. Hormone levels are expressed in ng/ml ± SEM of granulosa cell culture media (n=4). Different letter indicates significant difference between treatment groups as determined by one-way ANOVA ($p < 0.01$) followed by Dunnett's Multiple Comparison test post-hoc analysis.

Case Study 1: FSH cAMP-independent pathways

The similarity between FSH- and cAMP-induced estradiol-17β levels (Harvey *et al.* 2009) and morphology (unpublished observations) in the absence of HPTE suggest they impact the same gene networks. Analysis of microarray data comparing FSH-stimulated granulosa cells with cAMP-stimulated granulosa cells generated 101 probe sets (out of 33,000) unique to FSH which are shown as a heat map representing the FSH expression versus cAMP (Figure 5.7). Probe sets (61) from the first group exhibited higher expression in FSH-treated samples compared to cAMP-treated samples and 40 probe sets which exhibited lower expression in FSH-treated samples compared to cAMP-treated samples.

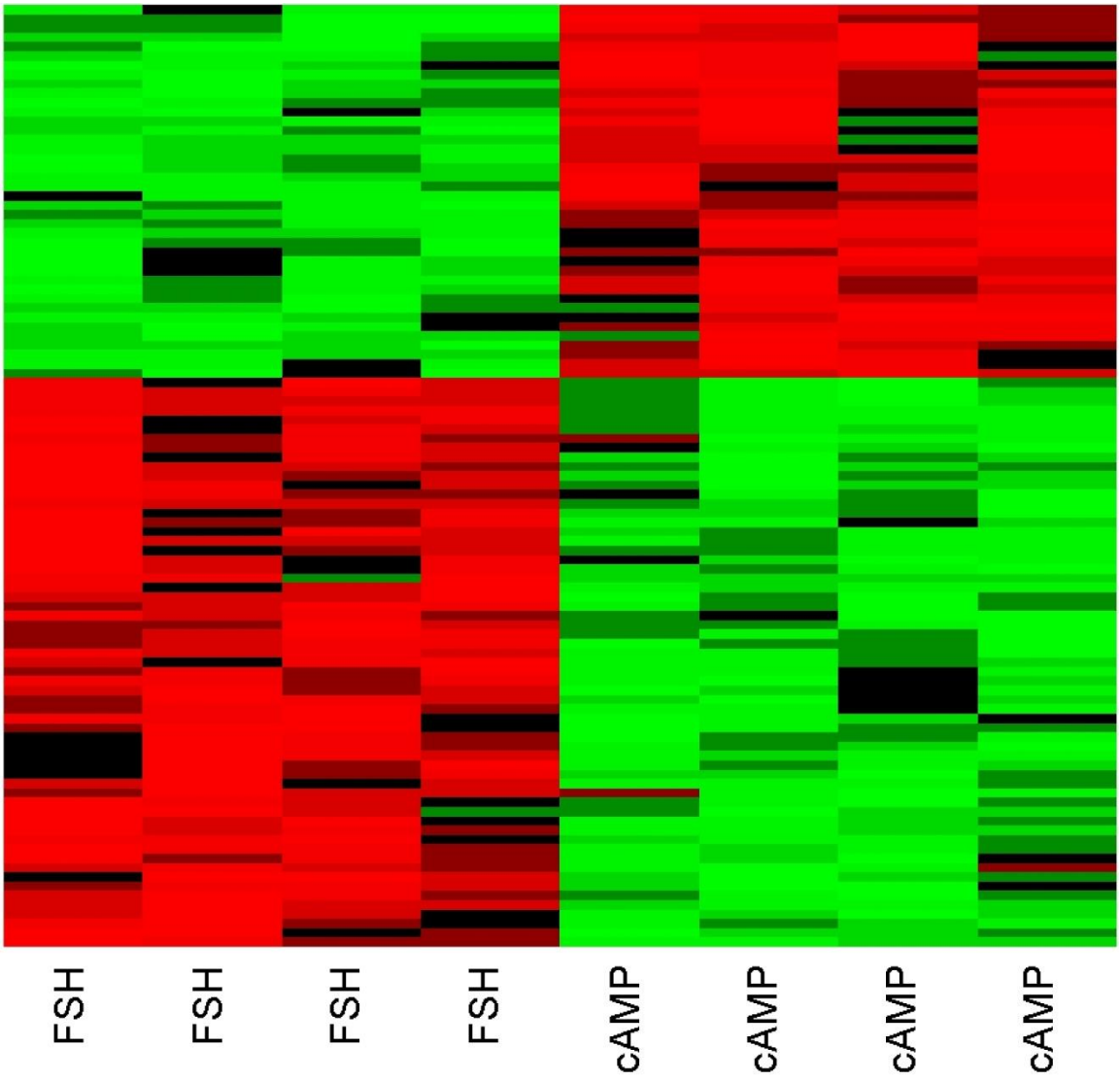


Figure 5.7: Hierarchical clustering of the 101 genes altered by FSH versus cAMP in the granulosa cell.
Probe sets (101) affected by FSH differentially with respect to cAMP. Red indicates upregulation and green indicates down regulation (black equals no change).

The selected probe sets and their associated gene names are tabulated in Table 5.2. The pathways enriched within the two groups of genes (up regulated and down-regulated) (p-value < 0.05) are given in Table 5.3. The significant pathways that were enriched by up regulated genes in FSH treatment were mostly associated with membrane traffic (e.g. Relaxin signaling, Virus Entry via Endocytic Pathways, Clathrin-mediated Endocytosis signaling, and Tight Junction signaling). In contrast, the significant pathways that were enriched by down-regulated genes in FSH treatment were associated with biosynthesis (e.g. Valine, Leucine and Isoleucine biosynthesis and Aminoacyl-tRNA biosynthesis), and cAMP and/or steroidogenesis inhibiting pathways (e.g. Protein Kinase A signaling, Endothelin-1 signaling (Calogero *et al.* 1998), Corticotropin-Releasing Hormone signaling (Kalantaridou *et al.* 2004).

Table 5.2. Genes that were differently regulated in FSH- and cAMP-treated granulosa cells (p-value < 0.05).

Up regulated in FSH group			
AFFY_PROBE_ID	ACCESSION	UNIGENE_ID	TITLE
1367907_a_at	M19262	Rn.3440	Clathrin, light polypeptide (Lcb)
1368281_at	L07316	Rn.6051	Dipeptidase 1 (renal)
1368495_at	NM_013413	Rn.9830	Relaxin 1
1369667_at	NM_033097	Rn.51354	Vacuolar protein sorting 52 (yeast)
1369765_at	NM_022384	Rn.32936	Achaete-scute complex homolog-like 1 (Drosophila)
1370793_at			
1372094_at	BG380556	Rn.100021	Suppressor of Ty 5 homolog (S. cerevisiae)
1372948_at	BG376761	Rn.22630	Bm403207
1373800_at	BF394493	Rn.172781	Transcribed locus
1373976_at	BF396534	Rn.40835	Similar to 8430411H09Rik protein (predicted)
1375055_at	AI112084	Rn.166602	Transcribed locus, moderately similar to NP_082277.1 JP-45 protein [Mus musculus]
1376364_at	BE108778	Rn.171160	Transcribed locus
1377000_at	BI297041	Rn.21208	Similar to junction-mediating and regulatory protein (predicted)
1377449_at	AI073141	Rn.20370	Similar to nectin 4 (predicted)
1386869_at	NM_012893	Rn.958	Actin, gamma 2

1386938_at	NM_031012	Rn.11132	Alanyl (membrane) aminopeptidase
1387520_at	NM_012944	Rn.10159	Dopamine receptor D4
1387582_a_at	NM_080894	Rn.81203	Phosphodiesterase 7B
1387751_at	NM_053751	Rn.9978	Whey acidic protein
1387869_s_at	AI410860	Rn.29434	Rab geranylgeranyl transferase, a subunit
1389348_at	BF282442	Rn.8860	Cleavage stimulation factor, 3' pre-RNA, subunit 1
1389567_at	BE329208	Rn.99548	SREBP cleavage activating protein (predicted)
1389581_at	BF390510	Rn.106849	Similar to RIKEN cDNA 9230117N10
1389800_at	BF420629	Rn.29879	Transcribed locus
1390041_at	AW434258	Rn.7884	SRY-box containing gene 17 (predicted)
1392407_at	AI012031	Rn.131539	Similar to RIKEN cDNA 6030405P05 gene (predicted)
1398465_at	AW526224	Rn.46210	Leucine rich repeat containing 23
1398952_at	AI233222	Rn.121465	Similar to RIKEN cDNA 2310036O22 (predicted)
1377628_at	AW523327	Rn.166492	Transcribed locus
1378203_at	BE102273	Rn.8513	Similar to RIKEN cDNA 6330509G02
1378317_at	BI282595	Rn.40671	Type II keratin Kb40
1378386_at	AA943262	Rn.105899	Similar to chromosome 9 open reading frame 7 (predicted)
1378891_at	AI578222	Rn.10894	Kinesin family member 3C
1378911_at	BF404615	Rn.62552	Transcribed locus
1379525_at	BF559514	Rn.35982	Similar to chromosome 20 open reading frame 155
1379553_s_at	AI058678	Rn.101718	Neuro-oncological ventral antigen 2 (predicted)
1379998_at			
1380421_s_at	AW532194	Rn.10935	Growth factor independent 1
1380843_at	BF414449	Rn.172619	Transcribed locus
1381129_at			
1382218_at	AI012175	Rn.18576	Hypothetical LOC298077
1382393_at	AI407100	Rn.147095	Transcribed locus, moderately similar to XP_576460.1 PREDICTED: similar to hypothetical protein PB402898.00.0 [Rattus norvegicus]
1383652_at	BF409138	Rn.22623	8-oxoguanine DNA-glycosylase 1
1383994_at	BI289342	Rn.48884	Synaptotagmin III
1384428_at	AI043872	Rn.136597	Similar to OTTHUMP00000040081 (predicted)
1384849_at	AI230851	Rn.64761	Transcribed locus
1385055_at	BF399530	Rn.59400	Similar to D0H6S2654E protein (predicted)
1385764_at	BF405897	Rn.140434	Similar to RIKEN cDNA 4933406J04
1393542_at	BF403870	Rn.119066	RGD1561556 (predicted)
1393747_at	AI009059	Rn.13875	Kazal type serine protease inhibitor 4
1394594_at	BM383974	Rn.169422	Transcribed locus
1395120_at	BF403704	Rn.2104	Amyloid beta (A4) precursor protein
1395256_at			

1396459_at	BE096723	Rn.10026	Insulin related protein 2 (islet 2)
1396525_at	BE110289	Rn.61745	Isoleucine-tRNA synthetase (predicted)
1396768_at	BF404627	Rn.52458	SET binding protein 1 (predicted)
1397185_at	BE102060	Rn.170815	Transcribed locus
1397204_s_at	BM385125	Rn.159556	Transcribed locus
1397336_at	AI168993	Rn.27992	Poly(A) polymerase gamma (predicted)
1397651_at	BF410591	Rn.44851	Symplekin
1397731_at	AA998060	Rn.165471	Transcribed locus

Downregulated in FSH group			
AFFY_PROBE_ID	ACCESSION	UNIGENE_ID	TITLE
1368872_a_at	NM_053309	Rn.30014	Homer homolog 2 (Drosophila)
1369337_at	NM_021684	Rn.42892	Adenylate cyclase 10 (soluble)
1371166_at	AJ011116	Rn.44265	Nitric oxide synthase 3, endothelial cell
1371501_at	BI291351	Rn.162451	Similar to TSG118.1 (predicted)
1371708_at	BI279097	Rn.43383	Carbonic anhydrase 6
1371821_at	BM391330	Rn.3856	Transcribed locus
1372442_at	BG673216	Rn.3887	Transcribed locus
1375118_at	BI296584	Rn.58930	Transcribed locus
1375692_at	AI229025	Rn.163633	Transcribed locus
1375859_a_at	BM386823	Rn.869	Similar to zinc finger protein 565 (predicted)
1376785_at	AI104799	Rn.34889	Synaptonemal complex protein 3
1386450_at	AI639253	Rn.137152	Outer dense fiber of sperm tails 4
1387147_at	NM_133536	Rn.11037	RAB3C, member RAS oncogene family
1389977_at			
1390081_at	AW252608	Rn.44726	Iroquois related homeobox 4 (Drosophila) (predicted)
1394175_at			
1394386_s_at	BE107955	Rn.51354	Vacuolar protein sorting 52 (yeast)
1377591_at	BE109656	Rn.35587	T-cell lymphoma invasion and metastasis 1
1377816_at	BG374857	Rn.162478	Zinc finger, RAN-binding domain containing 1 (predicted)
1379184_at	BI291332	Rn.137989	RGD1565677 (predicted)
1379731_at	BG666613	Rn.165985	Transcribed locus, weakly similar to NP_862906.1 CD59b antigen [Mus musculus]
1379834_at	BG374488	Rn.176356	Transcribed locus
1379875_at	AA957406	Rn.25493	Centaurin, gamma 2 (predicted)
1380101_at	BE109349	Rn.27992	Poly(A) polymerase gamma (predicted)
1380966_at	BE117653	Rn.16552	Transcribed locus
1382249_at	AI171858	Rn.146377	Transcribed locus
1382532_at	BF284283	Rn.11639	Elastase 3B, pancreatic (predicted)
1382812_at	BE096595	Rn.160626	Similar to Protein Njmu-R1 (predicted)
1383653_at	BG374126	Rn.13268	Sorting nexin 11

1384433_at	AI072153	Rn.148816	Transcribed locus
1385468_at	BF411864	Rn.140563	Neuronal PAS domain protein 1 (predicted)
1385860_at	BF543907	Rn.46932	Similar to SPla/RYanodine receptor SPRY (1J970) (predicted)
1386739_at	BF545627	Rn.38595	Ets variant gene 4 (E1A enhancer binding protein, E1AF) (predicted)
1392824_at	BF417701	Rn.82427	BCL2/adenovirus E1B 19kD interacting protein like (predicted)
1394241_at	BF401902	Rn.22069	Restin (Reed-Steinberg cell-expressed intermediate filament-associated protein)
1394896_at	BF545092	Rn.64274	Transcribed locus
1395018_at	BE119004	Rn.7687	Leucyl-tRNA synthetase
1395619_at	BF547002	Rn.63481	Sorbin and SH3 domain containing 3
1395773_at			
1397074_at	BF394099	Rn.194899	RAB5C, member RAS oncogene family (predicted)

Table 5.3: The FSH pathways that exhibit significant difference (p-value < 0.05).

Up regulated	Virus Entry via Endocytic Pathways, Cleavage and Polyadenylation of Pre-mRNA, Relaxin Signaling, cAMP-mediated Signaling, Clathrin-mediated Endocytosis Signaling, Tight Junction Signaling
Down regulated	Corticotropin Releasing Hormone Signaling, Relaxin Signaling , Cellular Effects of Sildenafil (Viagra), Valine, Leucine and Isoleucine Biosynthesis, RAR Activation, Endothelin-1 Signaling, Inhibition of Angiogenesis by TSP1, Protein Kinase A Signaling, Aminoacyl-tRNA Biosynthesis

Case Study 2: Comparison of dose-dependent effects of MXC and HPTE

In this study, we first identified the effects of 10 µM MXC and 1 µM MXC on gene expression. Data were then combined with gene expression profiles for 1 µM HPTE and 10 µM HPTE from Harvey et al. (Harvey *et al.* 2009) in order to increase the power of the analysis. A subset of probe sets that exhibited significant dynamic responses was identified and then analyzed via clustering analysis. Clustering analysis of the probe sets yielded 30 unique clusters. A *p*-value was estimated for each cluster based on their population size. The cluster sizes (the number of probe sets in a given cluster) and

corresponding estimated *p*-values are depicted in Figure 5.8. Based on the significance threshold (*p*-value < $10E^{-8}$), there were 8 significant clusters that are characterized by a cluster size greater than 30.

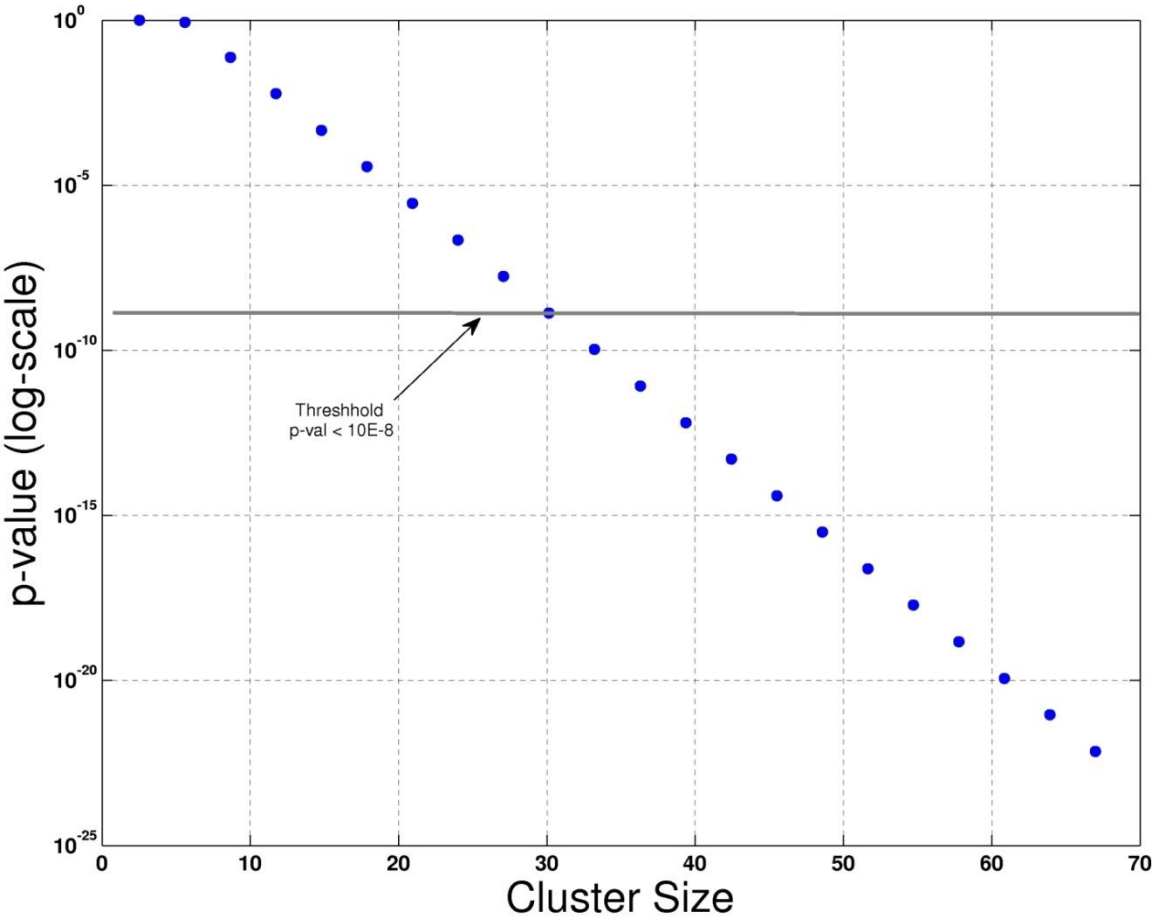


Figure 5.8: Estimated p-value vs. cluster size for pathway activity level.
Cluster size of 30 corresponds to a p-value < $10E^{-8}$.

The 8 significant clusters were arranged in four main groups based on pathway analysis results. Group 1 (pathways altered by 10 µM MXC) includes ~31 % of the selected probe sets and is the most populated group. The probe sets in group 1 exhibited either up or down regulation when the cells were treated with 10 µM of MXC and no change when cells were treated 10 µM of HPTE, 1 µM of HPTE and MXC. Group 2 includes ~18 % of the selected probe sets which exhibit change only when the cells were treated with 1 µM of MXC. Groups 3 and 4 include ~24 % of the selected probe sets where we observe significant up or down regulation only when the cells were treated with 1 µM of HPTE and 10 µM HPTE, respectively. The annotation of the biological functions of the significant clusters was determined from the IPA® library. The significant pathways (p-value < 0.05) and the representative profiles of the associated clusters are given in Table 5.4.

Table 5.4. The enriched pathways (p-value < 0.05) of each cluster group.
Each group consists of two clusters. The clusters correspond to the effect of HPTE and MXC at two different doses when the cells were treated with FSH. The first two symbols reflect the effect of 1 µM HPTE and 1 µM MXC, whereas the last two symbols reflect the effect of 10 µM HPTE and 10 µM MXC. If the gene expressions did not contain significant differential expression from control (FSH-alone) then the effect was called constant (C), while those that were significantly different were labeled either up regulated (U) or down-regulated (D).

Group 1 CC CD (308) CC CU (154)	G-Protein Coupled Receptor Signaling, cAMP-mediated Signaling, VEGF Signaling, Ovarian Cancer Signaling, Nucleotide Excision Repair Pathway, Glutamate Metabolism, Alanine and Aspartate Metabolism, FGF Signaling, ATM Signaling, Human Embryonic Stem Cell Pluripotency, N-Glycan Biosynthesis,ILK Signaling
Group 2 CU CC (198)	VDR/RXR Activation, PDGF Signaling, Intrinsic Prothrombin Activation Pathway, PTEN Signaling

CD CC (77)	
Group 3 DC CC (189) UC CC (170)	Oncostatin M Signaling, Prolactin Signaling, HIF1A Signaling, CNTF Signaling, EIF2 Signaling, PPAR Signaling, IL-6 Signaling, TREM1 Signaling, Insulin Receptor Signaling, JAK/Stat Signaling, GM-CSF Signaling, Regulation of eIF4 and p70S6K Signaling, PDGF Signaling, IL-3 Signaling, Estrogen Receptor Signaling, Glucocorticoid Receptor Signaling, TGFβ Signaling, PI3K/AKT Signaling, EGF Signaling, FAK Signaling, Cyanoamino Acid Metabolism, G Beta Gamma Signaling, Endometrial Cancer Signaling, IGF-1 Signaling, IL-2 Signaling, N-Glycan Biosynthesis, Fc Epsilon RI Signaling, Mitotic Roles of Polo-Like Kinase, Renin-Angiotensin Signaling,
Group 4 CC UC (184) CC DC (178)	Arginine and Proline Metabolism, Ceramide Signaling, IL-9 Signaling, Tryptophan Metabolism, Angiopoietin Signaling, Docosahexaenoic Acid (DHA) Signaling, EGF Signaling, Relaxin Signaling, Fatty Acid Metabolism, AMPK Signaling, PTEN Signaling, DNA Methylation and Transcriptional Repression Signaling, Cyanoamino Acid Metabolism, N-Glycan Biosynthesis, IL-15 Signaling, GM-CSF Signaling, 14-3-3-mediated Signaling, Androgen Signaling, Molecular Mechanisms of Cancer, PDGF Signaling, PI3K/AKT Signaling

Comparison of Group 1 (10 µM MXC) and Group 4 (10 µM HPTE) showed that MXC affected more probe sets than HPTE. However, high-dose MXC appears to affect fewer pathways than HPTE. In contrast, the low dose treatment effects of HPTE altered more pathways and probe sets than the low dose MXC.

MXC altered the expression of more genes than HPTE

Comparative analysis of high dose of MXC and HPTE using a 1.5 fold-change showed 34% commonality in gene expression differences compared to control (FSH-alone) (Figure 5.9). Heat map visualization revealed significant overlap in gene expression patterns with a difference nominally in expression intensity measured in fold change. Functional analysis using IPA on this subset of genes further revealed the biological

functions of the represented genes which included cellular functions such as cell death, cellular movement, and cell cycle. The physiological system development and function that were associated with these genes include tissue development, cardiovascular system development and function, organismal development and survival, and skeletal and muscular system development and function. Disease and disorders identified included cancer, reproductive disease, and neurological disorder (Table 5.5).

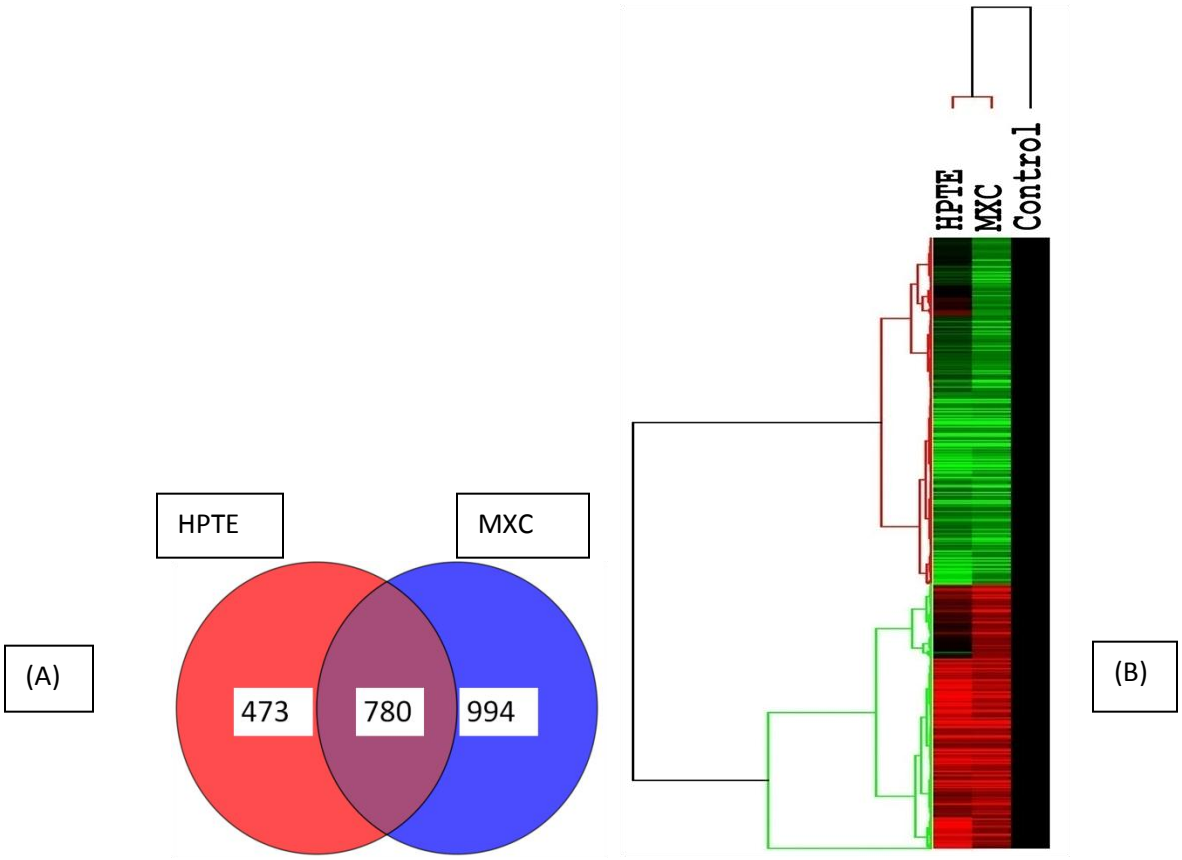


Figure 5.9: Comparison between MXC- and HPTE-induced gene expressions in a FSH-stimulated culture.

(A) is the Venn Diagram that shows that the genes effected are regulated by both HPTE (Red) and MXC (Blue). (B) is the heatmap of the genes that showed an 1.5 fold-change between the Control (FSH alone) and either 10 μM MXC or HPTE.

Table 5.5. IPA gene functions and pathways induced by MXC and HPTE in granulosa cells.

Molecular and Cellular Functions	**p-value**	**# Molecules**
Cell Death	1.12E-28 - 2.38E-04	579
Cellular Movement	1.85E-26 - 2.20E-04	406
Cellular Growth and Proliferation	5.68E-25 - 2.41E-04	616
Cell Cycle	2.13E-15 - 2.37E-04	279
Cellular Development	6.02E-15 - 1.51E-04	453
Physiological System Development and Function	**p-value**	**# Molecules**
Tissue Development	4.49E-18 - 2.41E-04	478
Cardiovascular System Development and Function	9.23E-18 - 1.98E-04	219
Organismal Development	9.23E-18 - 2.25E-04	389
Organismal Survival	4.70E-17 - 1.53E-05	261
Skeletal and Muscular System Development and Function	2.83E-13 - 2.20E-04	227
Diseases and Disorders	**p-value**	**# Molecules**
Cancer	5.39E-40 - 2.41E-04	737
Gastrointestinal Disease	1.96E-26 - 2.03E-04	633
Reproductive System Disease	5.59E-20 - 1.15E-04	437
Neurological Disease	9.45E-16 - 2.35E-04	487
Genetic Disorder	1.38E-14 - 2.03E-04	664

Top biological functions significantly affected by MXC and HPTE in FSH-stimulated granulosa cell based on the gene expression of the top 2000 altered genes using Ingenuity Pathway Analysis software.

The top canonical pathways identified from this subset of genes were associated with steroids (biosynthesis of steroids) and nuclear receptor signaling (VDR/RXR activation, AHR signaling, Retinoic Acid-mediated apoptosis signaling) (Table 5.6). Transcriptional factor analysis predicted that P53, MYC, PPARA,CTNNB, and JUN were the top five transcription regulators reported between the two compounds (Table 5.7). These results suggest that MXC and HPTE alter normal biological and physiological processes that

extend beyond steroidogenesis through activation and inhibition of transcription factors and nuclear receptors.

Table 5.6. IPA Canonical Signaling Pathways affected by MXC and HPTE.

Top Canonical Pathways	
Biosynthesis of Steroids	Propanoate Metabolism
VDR/RXR Activation	Valine, Leucine and Isoleucine Degradation
LPS/IL-1 Mediated Inhibition of RXR Function	Aryl Hydrocarbon Receptor Signaling
Hepatic Fibrosis / Hepatic Stellate Cell Activation	Retinoic acid Mediated Apoptosis Signaling

Top canonical pathways significantly changed by MXC and HPTE based on genes significantly changed in granulosa cells. This set of 2000 genes was then analyzed using Ingenuity Pathway Analysis software to determine which canonical pathways were significantly represented by this dataset. These results show that MXC is involved in multiple cellular functions associated with metabolism, transcription factors signaling, and steroidogenesis.

Table 5.7. IPA Transcription Factor analysis affected by MXC and HPTE.

Transcription Regulator	p-value of overlap	Predicted Activation State
TP53 (includes EG:22059)	2.16E-29	Activated
ATF4	4.17E-15	Activated
CDKN2A	1.45E-09	Activated
NR0B1	8.28E-04	Activated
KDM5B	2.06E-10	Activated
NFE2L2	4.95E-10	Activated
HIF1A	2.28E-08	Activated
TFEB	2.44E-04	Activated
TOB1	9.94E-03	Activated

STAT1	1.94E-05	Activated
SMARCB1	1.51E-08	Activated
CALR	1.17E-03	Activated
SMARCA4	1.05E-07	Activated
MTPN	1.53E-06	Activated
HNF1A	1.62E-03	Activated
STAT4	5.35E-08	Activated
TBX2	8.91E-09	Inhibited
RXRA	1.26E-10	Inhibited
NR5A1	2.20E-03	Inhibited
MYCN	1.64E-04	Inhibited
SREBF2	9.19E-18	Inhibited
SREBF1	8.80E-15	Inhibited
MYC	1.02E-29	Inhibited
PPARA	2.77E-18	
CTNNB	2.69E-18	
JUN	2.61E-22	

Transcriptional factor analysis identified upstream regulators that predicted to be inhibited or activated by MXC or HPTE. These transcription factors once identified can be connected through IPA's visualization networking to upstream signaling molecules or disease/biology states. Red highlight (red) indicates that the gene that encodes the transcription factor is present in the gene set. The lower the p-value indicates increased confidence of the prediction.

Finally, analysis of the Tox list, which are groups of molecules known to be associated with particular toxicity, using IPA found that the most clearly effected groups on the list were cholesterol biosynthesis, renal necrosis/cell death, hepatic fibrosis, cardiac hypertrophy, LPS/IL-1 mediated inhibition of RXR, and oxidative stress.

Regulation of cholesterol biosynthesis was clearly downregulated as 15/17 or 16/17 of the associated molecules were downregulated by HPTE or MXC, respectively. The other groups did not show a definitive directional regulation pattern, but exhibited more upregulation than down regulated genes in general. Oxidative stress, regulation of cholesterol biosynthesis, and LPS/IL-1 mediated inhibition of RXR are plausible toxicology targets of MXC and HPTE since they are associated with cholesterol and lipid metabolism which are required precursors for steroidogenesis. However, the functional annotations of renal necrosis/cell death, hepatic fibrosis, and cardiac hypotrophy appear unrelated to granulosa cell function. The networks are involved in functions that may have more general roles such as cellular movement, cell death, and organismal development. The results of the pathway-based analysis support the hypothesis that MXC and HPTE commonly alter molecular pathways that are associated with steroidogenesis as well as those that are associated with important cellular functions.

Table 5.8. IPA Tox Lists affected by MXC and HPTE.

Top Tox Lists	
Cholesterol Biosynthesis	Renal Necrosis/Cell Death
Cardiac Hypertrophy	Hepatic Fibrosis
Oxidative Stress	LPS/IL-1 Mediated Inhibition of RXR

Top Tox lists significantly changed by MXC and HPTE based on genes significantly changed in granulosa cells. This set of 2000 genes was then analyzed using Ingenuity Pathway Analysis software to determine if they were associated with a particular toxicity. These results show that MXC is involved in regulation of lipid and cholesterol synthesis as well as cellular processes involving apoptosis, and development.

Pathway activity analysis of MXC and HPTE

Pathway activity analysis was used to compare the effects of MXC and HPTE in granulosa cell. As described in (Cong *et al.* 2001), significant pathways were first identified and calculated for FSH and MXC and HPTE-treated cells. Pathways that were activated by both HPTE and MXC are listed in Table 5.9. The shared pathways were mostly associated with metabolism. Specifically, carbohydrate metabolism (e.g, pyruvate metabolism and C5 branched dibasic acid metabolism), amino acid metabolism (e.g. glycine, serine, and threonine metabolism, d-arginine and d-ornithine metabolism), and metabolism of vitamins and secondary biosynthesis (lipoic metabolism and monoterpenoid biosynthesis). The other pathways that were activated are known to be biologically related to granulosa cell morphological function and cell signaling, including extra-cellular matrix receptor interaction, regulation of actin cytoskeleton, and G-protein coupled receptor pathway. The total results from the PAL are tabulated in Table 5.10.

Table 5.9. Common pathways activated by 10 µM MXC and 10 µM HPTE identified by pathway activity analysis.

10	G-PROTEIN COUPLED RECEPTOR PATHWAY
158	GLYCINE,SERINE, AND THREONINE_METABOLISM
227	D-ARGININE AND D-ORNITHINE METABOLISM
254	PYRUVATE METABOLISM
258	1,4 DICHLOROBENZENE DEGRADATION
266	C5 BRANCHED DIBASIC ACID METABOLISM
277	LIPOIC ACID METABOLISM
283	MONOTERPENOID BIOSYNTHESIS
328	ECM RECEPTOR INTERACTION
349	REGULATION OF ACTIN CYTOSKELETON
494	PPARA PATHWAY
508	PYRUVATE METABOLISM

Table 5.10. Pathway activity analysis (p-value < 0.01) results of 10 µM MXC and 10 µM HPTE.

MXC		HPTE	
Pathway ID #	Pathway Title	Pathway ID #	Pathway Title
10	AGPCRPATHWAY	10	AGPCRPATHWAY
22	ANDROGEN_AND_ESTROGEN_METABOLISM	158	GLYCINE_SERINE_AND_THREONINE_METABOLISM
23	APOPTOSIS	227	HSA00472_D_ARGININE_AND_D_ORNITHINE_METABOLISM
24	APOPTOSIS_GENMAPP	254	HSA00620_PYRUVATE_METABOLISM
25	APOPTOSIS_KEGG	258	"HSA00627_14_DICHLOROBENZENE_DEGRADATION"
26	ARAPPATHWAY	266	HSA00660_C5_BRANCHED_DIBASIC_ACID_METABOLISM
28	ARFPATHWAY	277	HSA00785_LIPOIC_ACID_METABOLISM
33	AT1RPATHWAY	283	HSA00902_MONOTERPENOID_BIOSYNTHESIS
34	ATMPATHWAY	328	HSA04512_ECM_RECEPTOR_INTERACTION
36	ATRBRCAPATHWAY	349	HSA04810_REGULATION_OF_ACTIN_CYTOSKELETON
39	BCRPATHWAY	494	PPARAPATHWAY
52	BREAST_CANCER_ESTROGEN_SIGNALING	508	PYRUVATE_METABOLISM
57	CALCINEURINPATHWAY	53	BUTANOATE_METABOLISM
58	CALCIUM_REGULATION_IN_CARDIAC_CELLS	130	FIBRINOLYSISPATHWAY
66	CCR3PATHWAY	147	GATA3PATHWAY
67	CCR5PATHWAY	205	HSA00260_GLYCINE_SERINE_AND_THREONINE_METABOLISM
75	CELLCYCLEPATHWAY	211	HSA00310_LYSINE_DEGRADATION
78	CHEMICALPATHWAY	226	HSA00471_D_GLUTAMINE_AND_D_GLUTAMATE_METABOLISM
84	CITRATE_CYCLE_TCA_CYCLE	261	HSA00640_PROPANOATE_METABOLISM
94	CXCR4PATHWAY	265	HSA00650_BUTANOATE_METABOLISM
96	CYSTEINE_METABOLISM	285	HSA00910_NITROGEN_METABOLISM
102	DNA_REPLICATION_REACTOME	297	HSA01430_CELL_COMMUNICATION
103	DNAFRAGMENTPATHWAY	333	HSA04610_COMPLEMENT_AND_COAGULATION_CASCADES
105	ECMPATHWAY	407	INTRINSICPATHWAY

106	EDG1PATHWAY	418	LONGEVITYPATHWAY
112	EIF4PATHWAY	451	NITROGEN_METABOLISM
118	ERBB4PATHWAY	475	PDGFPATHWAY
120	ERKPATHWAY	544	SHHPATHWAY
128	FCER1PATHWAY	598	STRIATED_MUSCLE_CONTRACTION
MXC-Only Pathways			
Pathway ID #		Pathway ID	
133		FMLPPATHWAY	
139		G1_TO_S_CELL_CYCLE_REACTOME	
140		G1PATHWAY	
141		G2PATHWAY	
142		G_PROTEIN_SIGNALING	
148		GCRPATHWAY	
153		GLUCONEOGENESIS	
158		GLYCINE_SERINE_AND_THREONINE_METABOLISM	
159		GLYCOLYSIS	
160		GLYCOLYSIS_AND_GLUCONEOGENESIS	
164		GLYOXYLATE_AND_DICARBOXYLATE_METABOLISM	
170		GPCRPATHWAY	
174		HCMVPATHWAY	
175		HDACPATHWAY	
179		HISTIDINE_METABOLISM	
180		HIVNEFPATHWAY	
181		HSA00010_GLYCOLYSIS_AND_GLUCONEOGENESIS	
182		HSA00020_CITRATE_CYCLE	
185		HSA00040_PENTOSE_AND_GLUCURONATE_INTERCONVERSIONS	
191		HSA00071_FATTY_ACID_METABOLISM	
197		HSA00150_ANDROGEN_AND_ESTROGEN_METABOLISM	
198		HSA00190_OXIDATIVE_PHOSPHORYLATION	
199		HSA00220_UREA_CYCLE_AND_METABOLISM_OF_AMINO_GROUPS	
200		HSA00230_PURINE_METABOLISM	
202		HSA00240_PYRIMIDINE_METABOLISM	
208		HSA00280_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION	

213	HSA00340_HISTIDINE_METABOLISM
214	HSA00350_TYROSINE_METABOLISM
218	HSA00380_TRYPTOPHAN_METABOLISM
227	HSA00472_D_ARGININE_AND_D_ORNITHINE_METABOLISM
229	HSA00500_STARCH_AND_SUCROSE_METABOLISM
242	HSA00562_INOSITOL_PHOSPHATE_METABOLISM
254	HSA00620_PYRUVATE_METABOLISM
258	"HSA00627_14_DICHLOROBENZENE_DEGRADATION"
259	HSA00630_GLYOXYLATE_AND_DICARBOXYLATE_METABOLISM
260	HSA00632_BENZOATE_DEGRADATION_VIA_COA_LIGATION
266	HSA00660_C5_BRANCHED_DIBASIC_ACID_METABOLISM
277	HSA00785_LIPOIC_ACID_METABOLISM
281	HSA00860_PORPHYRIN_AND_CHLOROPHYLL_METABOLISM
283	HSA00902_MONOTERPENOID_BIOSYNTHESIS
291	HSA00970_AMINOACYL_TRNA_BIOSYNTHESIS
298	HSA01510_NEURODEGENERATIVE_DISEASES
300	HSA03010_RIBOSOME
304	HSA03050_PROTEASOME
306	HSA03320_PPAR_SIGNALING_PATHWAY
307	HSA04010_MAPK_SIGNALING_PATHWAY
308	HSA04012_ERBB_SIGNALING_PATHWAY
311	HSA04070_PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM
313	HSA04110_CELL_CYCLE
314	HSA04115_P53_SIGNALING_PATHWAY
315	HSA04120_UBIQUITIN_MEDIATED_PROTEOLYSIS
319	HSA04210_APOPTOSIS
320	HSA04310_WNT_SIGNALING_PATHWAY
324	HSA04350_TGF_BETA_SIGNALING_PATHWAY
325	HSA04360_AXON_GUIDANCE
326	HSA04370_VEGF_SIGNALING_PATHWAY
327	HSA04510_FOCAL_ADHESION

328	HSA04512_ECM_RECEPTOR_INTERACTION
330	HSA04520_ADHERENS_JUNCTION
331	HSA04530_TIGHT_JUNCTION
332	HSA04540_GAP_JUNCTION
336	HSA04620_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY
339	HSA04650_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY
341	HSA04662_B_CELL_RECEPTOR_SIGNALING_PATHWAY
342	HSA04664_FC_EPSILON_RI_SIGNALING_PATHWAY
343	HSA04670_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION
345	HSA04720_LONG_TERM_POTENTIATION
346	HSA04730_LONG_TERM_DEPRESSION
349	HSA04810_REGULATION_OF_ACTIN_CYTOSKELETON
350	HSA04910_INSULIN_SIGNALING_PATHWAY
357	HSA05010_ALZHEIMERS_DISEASE
358	HSA05020_PARKINSONS_DISEASE
359	HSA05030_AMYOTROPHIC_LATERAL_SCLEROSIS
360	HSA05040_HUNTINGTONS_DISEASE
363	HSA05110_CHOLERA_INFECTION
364	HSA05120_EPITHELIAL_CELL_SIGNALING_IN_HELICOBACTER_PYLORI_INFECTION
365	HSA05130_PATHOGENIC_ESCHERICHIA_COLI_INFECTION_EHEC
366	HSA05131_PATHOGENIC_ESCHERICHIA_COLI_INFECTION_EPEC
367	HSA05210_COLORECTAL_CANCER
368	HSA05211_RENAL_CELL_CARCINOMA
369	HSA05212_PANCREATIC_CANCER
370	HSA05213_ENDOMETRIAL_CANCER
371	HSA05214_GLIOMA
372	HSA05215_PROSTATE_CANCER
373	HSA05216_THYROID_CANCER
375	HSA05218_MELANOMA
376	HSA05219_BLADDER_CANCER
377	HSA05220_CHRONIC_MYELOID_LEUKEMIA
379	HSA05222_SMALL_CELL_LUNG_CANCER
380	HSA05223_NON_SMALL_CELL_LUNG_CANCER
385	IGF1MTORPATHWAY
403	INOSITOL_PHOSPHATE_METABOLISM

405	INTEGRIN_MEDIATED_CELL_ADHESION_KEGG
406	INTEGRINPATHWAY
411	KERATINOCYTEPATHWAY
423	MAPKPATHWAY
425	MEF2DPATHWAY
429	METPATHWAY
436	MRNA_PROCESSING_REACTOME
440	MTORPATHWAY
444	NDKDYNAMINPATHWAY
447	NFATPATHWAY
456	NOS1PATHWAY
466	OXIDATIVE_PHOSPHORYLATION
467	P27PATHWAY
470	P53HYPOXIAPATHWAY
471	P53PATHWAY
484	PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM
491	PLK3PATHWAY
492	PMLPATHWAY
493	PORPHYRIN_AND_CHLOROPHYLL_METABOLISM
494	PPARAPATHWAY
496	PROPANOATE_METABOLISM
500	PROTEASOMEPATHWAY
503	PTDINSPATHWAY
504	PTENPATHWAY
505	PURINE_METABOLISM
506	PYK2PATHWAY
508	PYRUVATE_METABOLISM
511	RACCYCDPATHWAY
515	RASPATHWAY
516	RBPATHWAY
523	RIBOSOMAL_PROTEINS
526	RNAPATHWAY
533	SA_G1_AND_S_PHASES
543	SETPATHWAY
549	SIG_INSULIN_RECEPTOR_PATHWAY_IN_CARDIAC_MYOCYTES
551	SIG_PIP3_SIGNALING_IN_CARDIAC_MYOCTES
556	SMOOTH_MUSCLE_CONTRACTION
566	ST_FAS_SIGNALING_PATHWAY
569	ST_GA12_PATHWAY
574	ST_INTEGRIN_SIGNALING_PATHWAY
579	ST_JNK_MAPK_PATHWAY

581	ST_P38_MAPK_PATHWAY
590	STARCH_AND_SUCROSE_METABOLISM
605	TCRPATHWAY
610	TERTPATHWAY
614	TIDPATHWAY
619	TPOPATHWAY
620	TRANSLATION_FACTORS
28	UBIQUITIN_MEDIATED_PROTEOLYSIS
630	UREA_CYCLE_AND_METABOLISM_OF_AMINO_GROUPS
633	VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION
634	VEGFPATHWAY
635	VIPPATHWAY
638	WNT_SIGNALING

5.4 Discussion

FSH is critical to granulosa cell differentiation and folliculogenesis during the ovulatory cycle, and *in vitro* the early events of granulosa cell differentiation involve changes in cell morphology and cell-cell interactions (Grieshaber *et al.* 2000). Previous studies have shown that microtubule and microfilaments projections are involved in the gonadotropin regulation of steroidogenesis in rat granulosa cells (Carnegie *et al.* 1987; Carnegie and Tsang 1988) and compounds (e.g. cadmium and rat thymic epithelial culture media) that alter microtubule projections also regulate estradiol-17β (Massanyi *et al.* 2000; Paksy *et al.* 1992; Uzumcu *et al.* 1998). Induction of this FSH effect is likely mediated through a cAMP-dependent pathway (Grieshaber *et al.* 2000) which is supported by the similarity of cell morphology and estradiol-17β production between FSH and cAMP treated granulosa cells.

Our microarray results supported this conclusion since the global gene expression profile of cAMP-treated granulosa cells were a subset of FSH-treated granulosa cells. Therefore, we attempted to identify the cAMP-independent pathways initiated by FSH

since these pathways may be more susceptible to endocrine disrupting chemicals. Up regulated pathways that were identified were associated with intercellular communication and internalization of the membrane bound FSH receptor-FSH complex. FSH down regulated pathways that inhibit cAMP and estradiol-17β production. The fact that gene expression changes induced by cAMP were also induced by FSH suggests that the affect of MXC and HPTE are most likely due to inhibition of cAMP (Chapter 3).

Comparison of HPTE and MXC Treatment in Granulosa Cell

In this current study, PAL was performed on data from our previous study (Figure 5.4) (Harvey *et al.* 2009) to identify dynamic pathways affected by endocrine-disrupting chemicals in the granulosa cells. The conclusions from our current analysis support our hypothesis that FSH-treated granulosa cells were more sensitive than cAMP-treated granulosa cells to HPTE, and that those cells behave more like basal cells with increasing HPTE. Notably, though HPTE is considered the more "estrogenic" chemical in comparison to its parent compound MXC and HPTE slightly inhibits estradiol-17β level at 1 μM, it is clearly MXC which has the more robust effect on gene expression. HPTE (10 μM) altered only 17 unique pathways while MXC (10 μM) affected 163 unique pathways. This result is plausible since MXC is metabolized into HPTE in the liver (Kapoor *et al.* 1970) which may indicate HPTE has a lower overall toxicity.

While the morphology of FSH-treated granulosa cultures co-treated with MXC or HPTE appears similar, pathway activity analysis shows only 10 pathways in common. One pathway represented that is directly related to FSH-cAMP signaling is the G-Protein coupled receptor (GPCR) signaling. Attenuation of GPCR signaling provides an essential physiological feedback that protects against acute and chronic overstimulation of

receptors (Ferguson 2007); MXC and HPTE may alter GCPR signaling. So one possible mechanism of action is that MXC and HPTE could reduce the availability of FSH receptors in granulosa cells. Female mice lacking FSH receptors are sterile due to a block on folliculogenesis before the antral stage (Dierich *et al.* 1998). It is plausible that MXC and HPTE blocks progression of the ovarian follicle to the antral stage through disruption of the FSH receptor, which would also affect steroidogenesis. Since we have identified a shared subset of pathways that are affected by MXC and HPTE, further efforts should be concentrated on confirming the functional effects of MXC on the GPCR pathway *in vitro* and *in vivo*.

Clustering analysis showed pathways (Table 5.9) that followed a similar pattern for each treatment group. It is plausible that some of these pathways share common members. For example, genes that encode proteins involved in cellular metabolism pathways that affect amino acid metabolism (e.g. glutamate metabolism and alanine and aspartate metabolism) have common enzymes (e.g. glutamate synthase and glutaminase).

Alternatively, certain pathways may be downstream or commonly regulated which would be reflected in their clustering pattern. FSH-mediated signaling pathways have several downstream pathways including GPCR, cAMP-mediated signaling, and VEGF signaling which would predictably follow a similar pattern.

Increasing Effect of MXC on Pathway Activity

HPTE at both 1 µM and 10 µM levels showed a similar effect on probe set number and number of pathways affected. In contrast, 1 µM of MXC induced fewer probe sets than 10µM of MXC and affected fewer pathways as well. Alternatively, granulosa cells may metabolize MXC into HPTE, and HPTE causes the observed inhibition of estradiol-17β.

There is no current evidence to support the MXC-metabolizing capability of granulosa cells. However, the ovarian surface epithelium, which has a common embryonic origin with granulosa cells, does express cytochrome P450s that metabolize MXC in culture (Symonds *et al.* 2006). Therefore, it is plausible that the granulosa cell also expresses MXC-metabolizing enzymes. Characterization of the MXC-metabolizing capability of rat granulosa cells would answer the question of whether MXC and/or HPTE is the active agent.

Effect of MXC and HPTE on Cholesterol Biosynthesis

Steroidogenesis is dependent upon on cholesterol as a substrate. In the ovary, transportation of lipoprotein via endocytic-mechanisms, de novo synthesis of cholesterol, and hydrolysis from the cell membrane are critical steps in early steroidogenesis (Miller and Bose 2011). Cholesterol is necessary for the proper maturation of hedgehog signaling proteins (Porter and Herman 2011) which are produced in the granulosa cell and induce target gene expression in the theca cell (Wijgerde *et al.* 2005). Inhibition of steroidogenesis would downregulate cholesterol formation. Martinez and Swartz observed increased lipid accumulation on interstitial and thecal cells using ultrastructural techniques (Martinez and Swartz 1992). The presence of lipid accumulation on the surface of steroidogenic cells is indicative of impaired steroidogenesis (Ishii *et al.* 2002). Martinez and Swartz further hypothesized that the observed lipid accumulation is due to gonadotropin-insensitivity. The effect of MXC on cholesterol biosynthesis enzymes may be a direct effect or a result of inhibition of steroidogenesis.

5.5 Summary and Conclusions

This report is the first study to characterize the global gene expression profiles between FSH and cAMP-treated granulosa cells *in vitro* and compare the affects of MXC and HPTE on FSH-treated granulosa cells. The pathway analysis tool allows us to reduce multiple gene expression values into a single value, which was used to evaluate differences between treatment groups (Ovacik *et al.* 2010b). Our results suggest that MXC and HPTE may inhibit steroid hormone levels through a shared mechanism. MXC more robustly effects granulosa cell gene expression and affects more pathways than HPTE. The novel pathways induced by MXC and HPTE presented here may be causal to the reduced steroidogenesis (Harvey *et al.* 2009; Zachow and Uzumcu 2006), cAMP production (Chapter 3), and future experiments will focus on clarifying the role of MXC in these pathways.

CHAPTER 6: Summary, Conclusions and Future Directions

6.1 Summary

Overview

Granulosa cells are significant components of the oocyte support system and whose signaling pathways are tightly regulated; therefore, granulosa cell dysfunction can block follicular development. Exposure to exogenous compounds can also alter local hormone production, nuclear receptor expression, and cellular signaling pathways. For example, Lovekamp and Davis (Lovekamp and Davis 2001) showed that the phthalate, di-(2-ethylhexyl) phthalate, DEHP, acts through its metabolite, monoethylhexyl phthalate, MEHP to inhibit cAMP and activated peroxisome proliferator-activated receptors (PPARs), which suppressed aromatase expression. Previously, we have shown that the MXC metabolite HPTE inhibits FSH-mediated production of estradiol-17β in an immature granulosa cell model while dbcAMP protected against the inhibitory effect of HPTE. This effect suggested that MXC reduced FSH-stimulated cAMP production.

Our goals were to elucidate the effect of MXC on normal granulosa cell functions including cAMP signaling, estrogen receptor expression, and a comparison of the impact of MXC and HPTE on granulosa cell E_2 secretion, morphology, and transcriptional profiles. This series of studies was important for several reasons. First, the impact of endocrine-disrupting chemicals must be continued to be studied due to their increased prevalence in the environment, and their still unclear effect on human health after fetal and prepubertal exposure (Rasier *et al.* 2006). Secondly, accurate estimates of the biotic level of MXC are unknown, however it can be assumed based on recent reports that exposure continues to occur in the U.S.A. (Richardson *et al.* 2009) and abroad (Oh 2009).

Other chemicals may have similar interactions with estrogen and androgen receptors as MXC and its metabolites.

Pesticides and other chemical compounds can alter hormonal function through mechanisms beyond ligand binding; including hormone synthesis, hormone release and storage, hormone transport and clearance, hormone receptor recognition and binding, and hormone receptor post-receptor activation (reviewed in Bretveld *et al.* 2006; Craig *et al.* 2010). There are few studies that have examined the effects of MXC on human populations. However, studies have detected the presence of MXC in agriculture products and in humans (Carvalho *et al.* 2009; Richardson *et al.* 2009). Evidence from non-human primates suggests that MXC decreases the length of the follicular phase; shortening of the follicular phase in humans is associated with infertility (Golub *et al.* 2003) which has also been observed in rats (Chapin *et al.* 1997). FSH-responsiveness is critical to granulosa cell and ovarian follicle function (Thomas *et al.* 2005). Using transcriptomics, we examined the effects of MXC/HPTE on FSH-stimulated gene expression in rat granulosa cells and connect it to the chemical and physical insults observed (e.g. inhibition of steroidogenesis and morphological changes). Understanding the modes or mechanism of action of MXC (and other endocrine-disrupting chemicals) in the ovary will help define the actions of multiple chemicals and their toxicity, and is a useful tool for developing therapeutics and prophylactics.

6.1.1 Summary Chapter 3: Methoxychlor and HPTE Inhibit cAMP Production and Estrogen Receptors α and β

In chapter 3, we detailed a series of experiments designed to follow-up the results of our previous observations, which showed that estrogen receptor expression was altered (*in*

vivo) and FSH-mediated steroidogenesis was inhibited (*in vitro)* by MXC and HPTE. Our objective was to investigate the effect of MXC and HPTE on cAMP levels and estrogen receptor expression, the role of estrogen receptor antagonism on cAMP, and the association between estrogen receptor expression and cAMP.

To determine if MXC/HPTE affected cAMP production, we completed a study that measured intracellular cAMP levels. Briefly, rat granulosa cells were cultured in the presence of FSH with MXC or HPTE (10 μM) for 48 h and analyzed for intracellular cAMP by enzyme-linked immunosorbent assay. MXC and HPTE inhibited FSH-stimulated cAMP production. Studies have shown that there is a close regulation between ER signaling pathways and cAMP/PKA signaling (Lazennec *et al.* 2001; Madak-Erdogan *et al.* 2008). MXC and HPTE significantly inhibited FSH-stimulated estrogen receptor α and β mRNA expression. Furthermore, western blot analysis reflected the same pattern in estrogen receptor β when challenged with MXC or HPTE on basal or FSH-stimulated granulosa cell.

Estrogen receptor antagonists, ICI-182, 780, a non-selective estrogen receptor antagonist, and PTHPP, an ERβ antagonist, mimicked the effect of HPTE and MXC on cAMP. However, when HPTE was co-treated with an ERβ agonist, DPN, this effect was not alleviated; which may be a result of HPTE-mediated down regulation of ERβ. The down-regulation of estrogen receptor expression was highly correlated to cAMP inhibition, but further investigation is required to determine what level of causation (if any) exists between the two events.

6.1.2 Summary Chapter 4: Effect of the Methoxychlor Metabolite HPTE on the Rat Ovarian Granulosa Cell Transcriptome in vitro

Previously, we have shown that hormone synthesis in FSH-activated granulosa cells were disrupted by HPTE *in vitro* and that biosynthetic steroid-producing enzymes are down regulated after HPTE exposure (Zachow and Uzumcu 2006). These results encouraged us to examine the effect of MXC or HPTE on global gene expression of granulosa cells using a microarray study.

Analysis of the HPTE data indicated that metabolism pathways associated with steroid biosynthesis, energy metabolism, apoptosis, and cell cycle signaling pathways were down regulated. More gene expression alterations (both up- and down regulation) were observed in the FSH-treated granulosa cells than in cAMP-treated or untreated granulosa cells. HPTE returned FSH-mediated gene expression to basal level in a dose-dependent fashion, consistent with the observed effect on E_2 levels. The sensitivity of FSH-treated granulosa cells *in vitro*, coupled with our previous work *in vivo*, which showed reduced responsiveness to exogeneous gonadotropin (Armenti *et al.* 2008); suggested that MXC/HPTE inhibited FSH-responsiveness in the follicle.

6.1.3 Summary Chapter 5: Gene Network changes induced in the Rat Granulosa Cell

Using pathway activity analysis, we characterized the dose-dependent effects of MXC and HPTE on gene expression data. This allowed us to identify the difference between the treatment profiles of MXC and HPTE. Furthermore, the pathway activity method allowed us to quantify the pathway changes using an unsupervised approach. We identified transcription factors and gene networks to characterize the molecular networks underlying cellular behavior, which included changes in DNA replication, energy

metabolism. There was notable overlap between MXC and HPTE as would be expected, but MXC had an increased effect on genes than did HPTE.

We report that MXC and HPTE alter essential steroidogenic associated pathways including inhibition of cholesterol synthesis and disruption of the GPCR signaling pathway. In addition, these results also implicate MXC and HPTE with potential roles in cancer and suggest potential transcription factors that are affected including PPAR α, P53, CTTNB, and JUN. The pathways that were shared by MXC and HPTE are associated with steroidogenesis and gonadotropin-sensitivity; which may explain the observed disruption of granulosa cell function.

6.2 Conclusions

The achievements of this dissertation are:

- Demonstrated that MXC and HPTE inhibited E_2 and estrogen receptor availability in the granulosa cell. Granulosa cells hormone levels may be disturbed by MXC and HPTE, which can cause issues with female fertility. Abnormal expression of estrogen receptors could cause poor follicular development, reduced gonadotropin responsiveness, and/or ovarian dysfunction as observed in ESR2 *(Esr2$^{-/-}$)* knockout mice (Barnett *et al.* 2006; Krege *et al.* 1998). Further work *in vivo* in pre-pubertal females exposed to MXC and HPTE with analysis of ER levels would extend these results. We have developed a working hypothesis for how MXC could affect estrogen signaling (Figure 6.1).
- Exposure to FSH initiates a morphological change (Figure 5.3) in the granulosa cell that is typified by a rounding up of the cell (Lawrence *et al.* 1979). As the dose of HPTE increases, FSH-treated cells resemble (stay flattened) and behave

much like untreated cells. Basal cells remain mostly unchanged in the presence of HPTE (not shown). Coupled with the inhibitory effect of HPTE on FSH-stimulated cAMP production these results suggest that HPTE affects the FSH-PKA signaling, the major signaling pathway in the granulosa cell.

- Pathway activity analysis enabled us to identify the molecular profiles of MXC and HPTE-treated FSH-activated granulosa cells across different doses. Characterization of these molecular fingerprints is important information for understanding the complex relationship between organochlorine pesticides exposure and granulosa cell function.

MXC alters E_2 signaling

Others have reported that MXC reduces E_2 levels; likely through inhibition of mRNA expression for genes encoding steroidogenic enzymes and increased expression of mRNA encoding *Cyp1b1*, an estrogen metabolizing enzyme (Basavarajappa *et al.* 2011).We have observed similar effects in granulosa cells treated with HPTE and MXC (Harvey *et al.* 2009; Zachow and Uzumcu 2006). Receptor availability (expression and binding sites) are also affected by MXC and HPTE (Chapter 3). In addition, FSH-cAMP-induced steroidogenic enzymes and ovarian differentiation markers, $P450_{scc}$ and *Lhr*, were down-regulated by HPTE (Harvey *et al.* 2009), a sign that granulosa cell differentiation is inhibited and that FSH-cAMP-PKA signaling is altered (Chapter 3+4). The effect observed on $P450_{scc}$ and *Lhr* gene expression *in vitro* was also confirmed at the protein level in MXC-treated ovaries (Armenti *et al.* 2008). We have summarized the observed effects of MXC/HPTE on estrogen signaling in Figure 6.1.

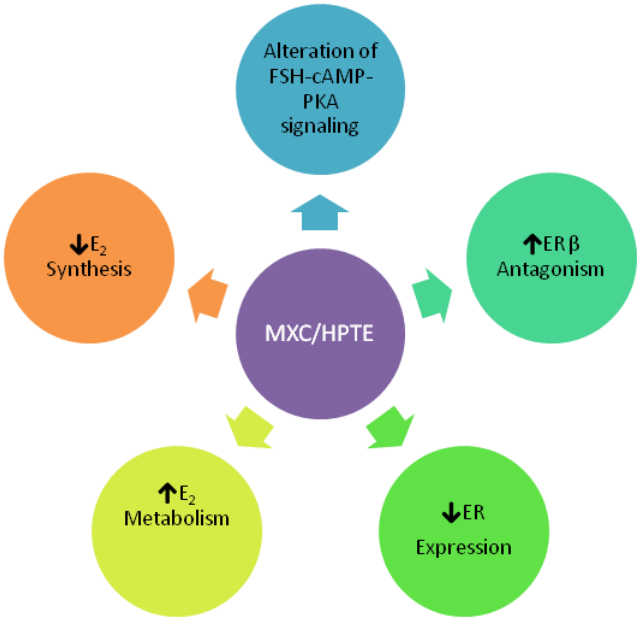


Figure 6.1: Proposed mechanism of MXC/HPTE effect on estrogen signaling.
E_2 synthesis, metabolism and its receptor expression and binding are affected be MXC and HPTE. In addition, alterations in other cell signaling pathways (e.g. kinase cascades) may have crosstalk effects on estrogen signaling.

Working hypothesis: MXC inhibits granulosa cell differentiation

Combining the results from our current study with previous observations from our lab, we have proposed a novel mechanism of action for MXC's effect on ovulation. HPTE decreased *Lhr* mRNA (a marker for granulosa cell differentiation) in FSH-treated granulosa cells *in vitro* and in the protein levels of antral follicles from MXC-treated rats (Armenti *et al.* 2008; Harvey *et al.* 2009). During the transition to the pre-ovulatory follicle, mural granulosa cells of the antral follicle respond to FSH by expressing LHR (Eppig *et al.* 1997; Woods *et al.* 2007), an effect which appears to be mediated through ESR2 (Bao *et al.* 2000; Couse *et al.* 2005; Rodriguez *et al.* 2010). Without expression of LHR, FSH-mediated follicle development and ovulation are obstructed and stop at the antral follicle stage (Pakarainen *et al.* 2005). Absent continued growth, follicles enter atresia. This is consistent with what others have reported; that MXC-treated antral follicles from mice and baboons have higher rates of atresia than controls and antral follicle growth is inhibited (Gupta *et al.* 2007; Gupta *et al.* 2006a). MXC-treated animals exhibit a reduced ability to ovulate; an effect possibly mediated by the reduced expression of LHR. This hypothesis is supported by the reduced response to exogenous gonadotropins previously observed (Armenti *et al.* 2008). Connecting these results, we suggest that MXC may be able to disrupt ovulation by decreasing the LHR availability in the granulosa cells and decreasing the ability of the granulosa cell to respond to gonadotropins.

We have identified the molecular signature of MXC/HPTE in the granulosa cells *in vitro* using microarray analysis. MXC has previously been shown to inhibit steroidogenesis. Our study shows that FSH-cAMP-PKA signaling is disrupted by MXC

through inhibition of cAMP, which may inhibit follicular differentiation and steroidogenesis. Associated with the loss of signaling was a change in morphology and E_2 production between FSH-stimulated granulosa cells and those co-treated with MXC and HTPE. Though MXC and HPTE bind to both ERα and ERβ, we showed that MXC and HPTE also down regulate expression of ERα and ERβ. Furthermore, we propose that MXC affects several aspects of estrogen signaling which are necessary for ovarian function. The mechanisms causing ER down regulation, E_2 inhibition, and cAMP disruption need further investigation.

6.3 Future Directions

This dissertation has produced several questions to be further investigated. In this section, we detail potential avenues of research into the mechanism of action of MXC in the ovary. Much of this research has focused on transcriptomic data that was designed to better understand the global view of the granulosa cells' actions. However, gene expression data coupled with observed physiological phenomena (e.g. cell morphology and cAMP levels) are important information for understanding the cells' machinery.

Ovarian Metabolism of MXC

MXC itself is metabolized and excreted quickly (98% within 48 hrs); in the form of its more hydrophilic metabolites, Mono-OH and HPTE (Kapoor *et al.* 1970). *In vitro* results suggest that these metabolites are able to bind to estrogen receptors (Gaido *et al.* 2000) and cause deleterious effects on the granulosa cell and antral follicles (Gupta *et al.* 2006a; Harvey *et al.* 2009; Miller *et al.* 2006). However, MXC, Mono-OH, and HPTE treatment causes decreased gene expression of *Cyp11a1*, *Cyp19a1*, and *Cyp17a1 in vitro* (Basavarajappa *et al.* 2011; Craig *et al.* 2010; Harvey *et al.* 2009; Zachow and Uzumcu

2006). The similarity in effect of MXC and its metabolites suggests that if metabolism of MXC is necessary for ovarian toxicity; extra-hepatic metabolism of MXC must occur.

Possible sites of metabolism for MXC include the ovary and the granulosa cell. The bioactivation capabilities of the ovary has been noted in the past (Cannady *et al.* 2003).The surface epithelium of the mouse ovary has been observed to express the MXC-metabolizing enzyme, *Cyp2c29*, in the presence of E_2 or MXC (Symonds *et al.* 2006). The granulosa cell has been shown to express *Cyp1b1*, the transcript of the protein that metabolizes estrogen to the catecholestrogen (Dasmahapatra *et al.* 2002). We have also observed increased expression of *Cyp1b1* in rat granulosa cell *in vitro* and induction of *Cyp2c7 (*rat homolog to *Cyp2c29*), a MXC metabolizing enzyme in the rat, when exposed to MXC (Appendix). To test the hypothesis, that MXC is metabolized by the granulosa cell, cell culture treated with MXC will be analyzed for the presence of Mono-OH and HPTE. Alternatively, quantification of Mono-OH and HPTE levels in an ovary culture treated with MXC could be performed to determine if metabolism has occurred. Evaluating the ovary's capacity to metabolize MXC would be powerful evidence for confirming the active agent of MXC.

Analysis of the presence of Mono-OH and HPTE has other applications for our research; determination of fetal exposure in utero to MXC metabolites can be made. Measurement of tissue localization of MXC, Mono-OH, and HPTE should also be performed in neonatal, prepubertal, and mature animals to compare the effect of age on MXC's absorption, distribution, metabolism and excretion.

FSH-induced cAMP Alters Cholesterol Synthesis

We showed the FSH-treated granulosa cells were more sensitive to HPTE than cAMP-treated granulosa cells (Chapter 4), and that cAMP levels were reduced by MXC and HPTE (Chapter 3). This suggests that MXC inhibits FSH-responsiveness in the granulosa cell through ablation of cAMP production, therefore down regulating steroidogenesis. We found that MXC and HPTE down regulated cholesterol biosynthesis associated molecules. Other endocrine disruptors such as bisphenol A and nonylphenol have been shown to alter lipid accumulation levels in adipocytes and human hepatoma cells. A similar phenomenon was observed in E_2- and MXC-treated adult female mice, where Martinez and Swartz observed increased lipid accumulation on interstitial and thecal cells using ultrastructural techniques (Martinez and Swartz 1992). The presence of lipid accumulation on the surface of steroidogenic cells is indicative of impaired steroidogenesis (Ishii *et al.* 2002). Dysfunctional cholesterol synthesis is associated with ovarian disfunction such as anovulation (Mumford *et al.* 2011) and PCOS (Wild 2012).

Identification of Novel Biomarkers for MXC-induced Ovarian Failure

Historically, signs of ovarian disease have been difficult to identify until progression of the pathology had reached an irreversible stage (McCorkle *et al.* 2003; Hiei *et al.* 2010; Kalantaridou *et al.* 1998). For example, ovarian cancers had been referred to as the silent killer due to its often late stage identification which can limit treatment options and efficacy (McCorkle *et al.* 2003). This difficulty in identification of ovarian pathologies can extend to ovarian failure and disease (Kalantaridou *et al.* 1998). Our microarray data can be useful for identifying potential biomarkers that can reveal potential reproductive dysfunction. For example, anti-müllerian hormone is useful marker for testing the quality

of the ovarian reserve (Barad *et al.* 2009), and were up regulated in MXC-treated rats (Uzumcu *et al.* 2006). Characterization of novel molecules affected by MXC/HPTE may provide a better diagnostic for determining potential issues with the ovarian reserve or as potential targets for therapeutics.

Investigation of GPCR signaling in MXC-treated granulosa cells

G-protein coupled receptors (GPCR) are one of the most common drugs targets in the pharmaceutical field (Overington *et al.* 2006; Insel *et al.* 2007). FSH and LHR are GPCR receptors which are necessary in the granulosa cell to promote folliculogenesis. We found that MXC and HPTE alter GPCR signaling pathway. These results suggest that inhibition of GPCR may be the mechanism of action of MXC in the granulosa cell. We should further explore the GPCR complex to determine if MXC and HPTE alter the FSH-cAMP second messenger system.

Effect of LHR agonists on MXC-treated granulosa cells

LHR is a crucial marker of granulosa cell differentiation, and has shown to be reduced in the absence of ERβ. MXC and HPTE also strongly inhibit *Lhcgr* expression *in vitro*. The absence of LH may be due to the MXC-induced inhibition of E_2. Using LHR agonists, we can attempt to induce *Lhcgr* expression in granulosa cells and rescue granulosa cell function.

Determine if MXC binds to plasma membrane-bound estrogen receptors

It is necessary to determine if the physiological effects of MXC and HPTE occur within the timeframe of a rapid (non-genomic ~minutes) effect or the more traditional (genomic ~ hours) effects. Recent identification of plasma membrane-estrogen receptors suggests

potential novel targets for MXC and HPTE to act. Observations using a time course analysis would provide useful information about these effects.

Investigate the effect of HPTE on β-catenin signaling pathway

The *β-catenin* (CTTNB) signaling pathway is critical to multiple cellular processes including cell growth (Stockinger *et al.* 2001) and adhesion between cells (reviewed in Clevers 2006). It also interacts with the WNT signaling pathway (Willert and Nusse 1998) and can serve as a transcription factor CTTNB. The gene that encodes CTTNB also is an oncogene that is associated with epithelial cancers such as basal carcinoma (Saldanha *et al.* 2004), colorectal (van de Wetering *et al.* 2002), and ovarian cancers (Yoshioka *et al.* 2012). This potential relationship between CTTNB and MXC should be further explored.

Time Course Effect of MXC In Vivo

Many endocrine-disrupting chemicals appear to have low-dose effects that differ from the doses used in classic toxicology experiments (reviewed in Welshons *et al.* 2003). Transient, fetal and neonatal exposure to MXC has been observed to alter ovarian morphology at postnatal day 7 (unpublished results) and at postnatal days 50-60 (Armenti *et al.* 2008; Zama and Uzumcu 2009), leading to ovarian dysfunction as well as alteration in follicular composition. In addition, certain ovarian markers (e.g., ERβ, luteinizing hormone receptor (LHR), CYP11A1) were altered. However, the effects on ovarian morphology and biomarkers have not been categorized at discrete significant postnatal events between postnatal day 7 and adulthood. Identifying if these expression patterns are different throughout pre-pubertal growth would offer insight into whether these effects are immediate and direct. An alternative hypothesis is that MXC's effects are a

consequence of alterations in LH and FSH secretion patterns at the hypothalamus and pituitary levels caused by MXC. Of critical interest is the collection points at postnatal day 12, postnatal day 21, and vaginal opening day since ovaries collected on postnatal day 7 were less severely affected than similarly treated animals at postnatal day 60 (unpublished observations). This is significant because at postnatal day 7 paracrine factors regulate ovarian function, and at adulthood the endocrine system is more involved in regulating ovarian function. This study will help fill in the observational gaps of the ovarian response to MXC.

One challenge in this experiment is using proper positive controls and experimental design (vom Saal *et al.* 2010). Current safe levels of exposure may need to be re-examined to better understand the fundamental effects of these compounds. Three issues that have been presented by vom Saal include 1) extrapolation to low-dose ranges of bioactivity of high dose toxic agents, 2) the incorrect assumption that dose response receptor binding is linear and that receptor-mediated actions do not saturate, and 3) receptor-mediated actions can also act non-monotonically (vom Saal and Welshons 2006). In the past we have used E_2 as a positive control, but we currently use an experimentally effective high dose of MXC (100 mg/kg/day) as our positive control for our in vivo experiments, which should help us avoid these issues.

APPENDIX A

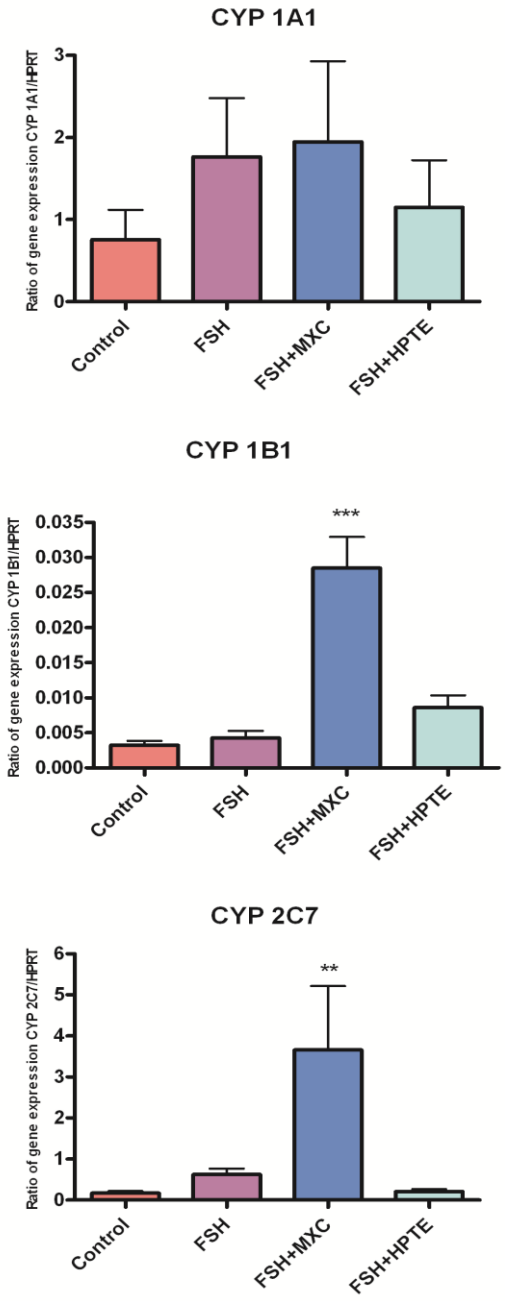


Figure A: Effect of MXC and HPTE on the regulation of genes that encode estrogen-metabolizing and MXC-metabolizing enzymes in granulosa cells.
Experiments were performed in triplicate at least three separate biological samples (** and *** $p < 0.01$ and $p < 0.001$, respectively).

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