DePaul University
UNIVERSITY LIBRARIES

College of Science and Health Theses and Dissertations

College of Science and Health

Spring 6-12-2015

Retinoic Acid Regulation of Aromatase Expression in the Ovary

Sarah Persaud
DePaul University, spersau2@mail.depaul.edu

Follow this and additional works at: https://via.library.depaul.edu/csh_etd

Part of the Biology Commons

Recommended Citation

Retinoic Acid Regulation of Aromatase Expression in the Ovary

A Thesis

Presented in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

June, 2015

BY

Sarah A Persaud

Department of Biological Sciences

College of Science and Health

DePaul University

Chicago, Illinois

Table of Contents

3

Acknowledgements

I thank Dr. Jingjing Kipp for allowing me to work in her lab regardless of my lack of previous bench research experience and for serving as a mentor over the past two years. I truly appreciate the opportunities I have had to learn different laboratory techniques and to present my research at meetings and symposiums. In addition, I would like to thank Drs. Dorothy Kozlowski and Eric Norstrom for serving as my thesis committee members. I appreciate the support and ideas I received from both of them.

I would also like to thank other members of the Kipp lab for their support throughout the past two years. I thank Rebecca Ursin for introducing me to basic laboratory techniques and for genotyping the MT-α mice used in my experiments. I thank Michael Demczuk for teaching me many research skills, and for providing help on a daily basis. I also thank previous graduate students Michael Akroush and Shruti Kamath for their advice and support.

I would like to thank the College of Science and Health of DePaul University for awarding me with the Graduate Research Fund Grant to help me purchase a reagent for the western blot. I would also like to thank Dr. Kelly Mayo from Northwestern University for providing our lab with the transgenic MT-α mouse line. Finally, I would like to thank Dr. Jalene LaMontagne for her help with the statistical analysis for my experiments.

Abstract

Retinoic acid is the active derivative of vitamin-A in the body. Retinoic acid is essential in normal embryonic development, and studies from the Kipp laboratory have also demonstrated its importance in the developing ovary. Aromatase, encoded by the gene *Cyp19a1*, is an enzyme that converts androgens to estrogens, and is primarily expressed in the granulosa cells of the ovary. Previous studies have suggested that retinoic acid influences aromatase expression in a few different tissue types; however, how retinoic acid may regulate aromatase expression in the ovary is not known. Therefore, the purpose of this study was to examine the role of retinoic acid in regulating aromatase expression in granulosa cells from the mouse ovary.

Granulosa cells were isolated and treated with retinoic acid, a retinoic acid metabolism blocking agent R115866, a pan-retinoic acid receptor inhibitor AGN193109, and combinations of these treatments. Cells were treated for 24- and 72- hours, after which mRNA and proteins were collected for RT-PCR and western blot analyses, respectively. The results showed that retinoic acid or R115866 treatment increased *Cyp19a1* mRNA levels at 24-hours. The stimulatory effects were specific as they were abolished by AGN193109. AGN193109 continued to suppress *Cyp19a1* mRNA levels after 72-hours, but no stimulatory effect by retinoic acid was observed, possibly due to the short half-life of the mRNA. Retinoic acid and R115866 also increased aromatase protein expression at 24- and 72- hours. It was observed that 19-day MT-α transgenic mice had increased levels of *Cyp26b1 mRNA*, which encodes an enzyme that metabolizes retinoic acid, and decreased levels of *Cyp19a1* mRNA, suggesting retinoic acid signaling is important in aromatase expression *in vivo*. Overall, this study provides strong evidence that further supports an important role of retinoic acid in ovarian development, as it demonstrates that retinoic acid regulates ovarian aromatase expression.

Abbreviations

CRABP	Cellular Retinoic Acid Binding Protein
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
LH	Leuteinizing Hormone
MT-α	Metallothionein-I promoter
NLM	Normal Littermates
PBS	Phosphate Buffered Saline
PCOS	Polycystic Ovarian Syndrome
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RAMBA	Retinoic Acid Metabolism Blocking Agent
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Elements
RXR	Retinoid X Receptor
TGF-β	Transforming Growth Factor β

Treatments

DMSO	Dimethyl Sulfoxide
All-*trans*-retinoic acid	Active Derivative of Vitamin A
R115866	CYP26 Enzyme Inhibitor
AGN193109	Retinoic Acid Receptor Inhibitor

Genes

Cyp19a1   Aromatase

Cyp26b1   Encodes enzyme CYP26B1 which metabolizes retinoic acid

Rpl19   Rat Ribosomal Protein 19, Internal Control for Real Time RT-PCR

Introduction

The ovaries are the female reproductive organs. Ovaries contain the female gametes, oocytes, and produce hormones necessary for female development and reproduction. A follicle is the functional unit of the ovary and is composed of an egg in the center, surrounded by one or more layers of granulosa cells. As a follicle grows larger, it also acquires one or two layers of theca cells. Granulosa cells express aromatase, an enzyme that converts androgens to estrogens. Because estrogens are crucial for female development and reproduction, aromatase is very important for female health. Decreased expression of aromatase leads to developmental and reproductive abnormalities.

Retinoic acid is an active derivative of vitamin A, which is acquired from food and then oxidized into retinoic acid by synthesis enzymes. Retinoic acid can be broken down by the CYP26 family of Cytochrome P450 enzymes. Retinoic acid is critical in embryonic development and it regulates the expression of hundreds of different genes. Retinoic acid also plays an important role in meiosis and ovary development. Furthermore, retinoic acid has been shown by the Kipp Laboratory to induce granulosa cell proliferation. Retinoic acid regulates aromatase expression in some tissues and cancer cell lines, although such regulation has never been investigated in the ovary. Therefore, the purpose of this study was to examine the effect of retinoic acid on aromatase expression in the ovary. To achieve this goal, the present study utilized an *in vitro* model of granulosa cell cultures and an *in vivo* model of MT-α transgenic mice, which has been shown by the Kipp Laboratory to have reduced retinoic acid signaling. The findings from this study would help to improve our understanding of ovarian development, retinoic acid signaling,

and aromatase expression, and contribute to the prevention and treatments of infertility and reproductive diseases.

Review of Background Literature

Importance of Studying Ovary Development

Infertility is a problem that affects 11% of American couples [1]. Infertility or trouble conceiving has been associated with an increased risk of depression and can put psychological and financial stress on couples [2]. Therefore, a better understanding of how the development and functions of the reproductive system are regulated can be beneficial to people suffering from infertility, as more knowledge of the causes can lead to better prevention and treatments. On the other hand, learning more about the ovary can lead to the development of new methods of fertility control, which can help to combat many problems associated with the alarmingly increasing world population. Finally, there are many reproductive diseases that affect the ovary, including ovarian cancer and polycystic ovarian syndrome. As will be discussed later, both retinoic acid and aromatase have been demonstrated to be involved in these reproductive diseases.

Anatomy and Development of the Ovary

The ovary is the female gonad, containing different cell types aimed at the maturation of gametes and the production of hormones. The female gamete is the oocyte, which develops through a process called oogenesis. In the developing ovary, oogonia mitotically divide into primary oocytes. Primary oocytes originally are clustered together, and as the ovary develops, individual oocytes separate and become surrounded with a layer of squamous pregranulosa cells, forming the primordial follicle [3]. The primary follicle is formed next, which consists of the primary oocyte surrounded by one layer of cuboidal granulosa cells. As the secondary follicle forms, a

layer of theca cells is recruited to surround the granulosa cells. As the granulosa cells continue to divide throughout folliculogenesis (Figure 1), the process of follicle formation and development, a fluid-filled cavity referred to as the antrum begins to form within the tertiary follicle. Follicles with a fully developed antrum are called antral follicles, which are mature follicles ready for ovulation. Eventually, a surge of luteinizing hormone (LH) is released from the anterior pituitary gland in the brain, causing ovulation. The oocyte is pushed out of the follicle by the antral fluid and exits the ovary. During folliculogenesis, most follicles die, or become atretic, before they get a chance to develop enough to undergo ovulation [3]. However, for follicles that do reach ovulation, the remains of the follicles differentiate to the corpus luteum after the extrusion of oocytes. The corpus luteum functions as an endocrine structure, secreting estrogens and progesterone, and eventually degenerates within the ovary if fertilization does not occur [3]. The oocyte remains in prophase I of meiosis I before birth until ovulation. Upon ovulation, meiosis resumes, and the egg remains in metaphase II of meiosis II until fertilization, upon which meiosis II is completed [3].

The theca cells proliferate into two layers in the secondary follicle: the theca externa and the theca interna. The theca externa acts as a layer of smooth muscle, aiding in the release of the oocyte during ovulation [3]. The theca interna responds to LH to produce androgens. Granulosa cells proliferate in response to follicle stimulating hormone (FSH), and FSH is necessary for development of mature follicles and ovulation [3]. Females lacking FSH are sterile. Granulosa cells carry out many actions in the ovary. These cells nourish the developing oocyte and also produce the peptide hormones activin and inhibin [3].
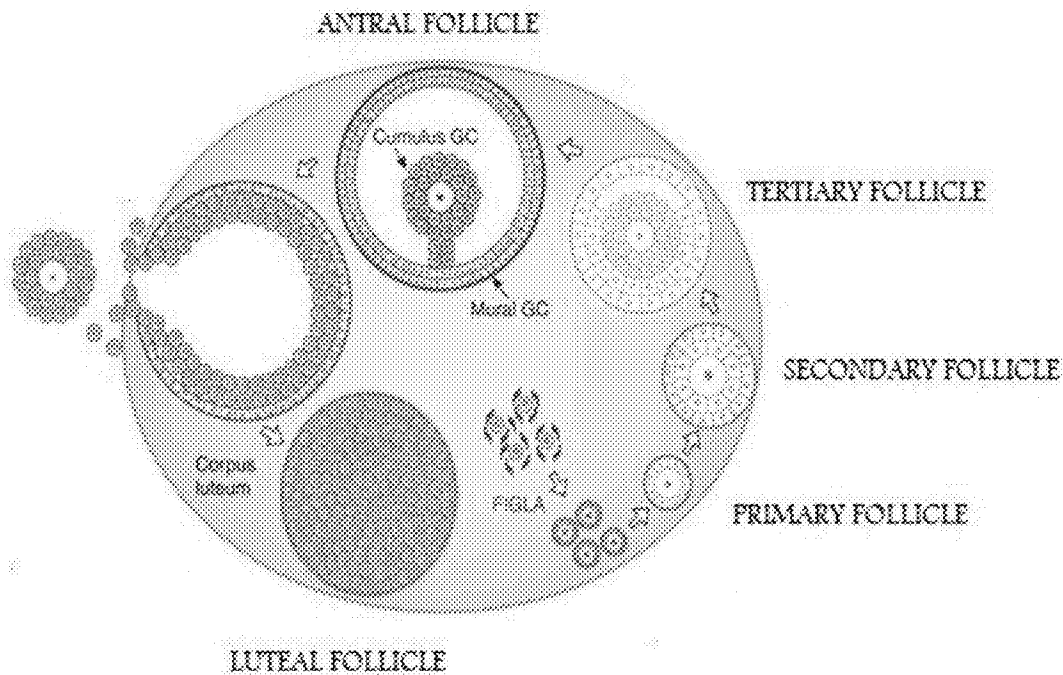
Figure 1: The stages of folliculogenesis. *Primordial follicles grow and somatic cells differentiate upon stimulation from gonadotropins. Granulosa cells, originally present in one layer in the primary follicle, divide to produce multiple layers in secondary follicles [3]. Theca cells form as the outer layer(s) of the secondary follicle [3]. The fluid-filled antrum begins to develop in the tertiary follicle [3]. A large antrum is present in antral follicles. Upon LH stimulation, the antral fluid pushes the oocyte out of the ovary during ovulation. What remains after ovulation is the luteal follicle, serving as an endocrine structure and degenerating if the egg is not fertilized. Figure adapted from Lamb, M.M.M.D.J., Nature Medicine, 2008 [4].*

Reproductive Hormones

The hypothalamic-pituitary-gonadal axis plays a crucial role in reproduction regulation and in the development of the gametes in both males and females. The hypothalamus secretes numerous peptide hormones, including gonadotropin-releasing hormone (GnRH). This hormone acts on the anterior pituitary gland, which, in turn, secretes the gonadotropins, FSH and LH. In both males and females, these peptide hormones act on gonadal cells and facilitate the development and maturation of gametes [5]. In the female ovary, granulosa cells express FSH receptors, whereas theca cells express receptors for LH. Theca cells produce androgens in response to LH stimulation, and androgens produced by theca cells provide negative feedback to the hypothalamus and anterior pituitary to regulate the production and secretion of GnRH and the gonadotropins. Granulosa cells express aromatase and therefore convert androgens produced by theca cells into estrogens. Estrogens normally provide negative feedback to the hypothalamus and anterior pituitary gland to reduce the production of GnRH, FSH, and LH; however, before ovulation, estrogens stimulate an increase in LH released from the anterior pituitary [6]. Estrogens are also responsible for the secondary sex characteristics of the female. Both estrogen receptor α (ERα) and estrogen receptor β (ERβ) are present within granulosa cells, although levels of ERβ are higher than levels of ERα in these cells [9]. Activin upregulates the expression of ERα and ERβ in granulosa cells [9].

Activin and inhibin are peptide hormones and transforming growth factor beta (TGF-β) super-family members [8]. These hormones play a role in the development of many tissues throughout the body, and both are produced by granulosa cells [8]. Activin functions as an endocrine,

paracrine, and autocrine molecule, stimulating FSH release from the anterior pituitary and regulating many important functions in the gonads [8]. Notably, activin, together with estrogens, has been shown to initiate the formation of primordial follicles [8]. Activin also increases the number of estrogen receptors on the cell membrane of granulosa cells [9]. Inhibin exerts negative feedback to the anterior pituitary, leading to decreased production of FSH. Inhibin also stimulates theca cells to increase the production of testosterone [10]. Primordial follicles produce high amounts of activin and lower amounts of inhibin, and this activin:inhibin ratio decreases as the follicle grows [11]. Decreased levels of inhibin are associated with premature ovarian failure, whereas ovarian tumors are often associated with increased levels of inhibin [12, 13]. Activins appear to suppress the growth of ovarian tumors [14].

Aromatase

One of the most notable actions of granulosa cells is the expression of aromatase. Aromatase is an enzyme encoded by the *Cyp19a1* gene [15]. Aromatase converts androstenedione to estrone and testosterone to 17-β estradiol [16] (Figure 2).

Specifically, aromatase is expressed in the mural granulosa cells on the outer edge of antral follicles, as well as in the corpus luteum [17]. In addition to its production in the ovary, in the female reproductive system, aromatase is also expressed in the placenta [18]. In the male reproductive system, aromatase expression occurs in Sertoli cells and Leydig cells of the testis, as well as in the prostate [18]. In both males and females, aromatase expression occurs in many
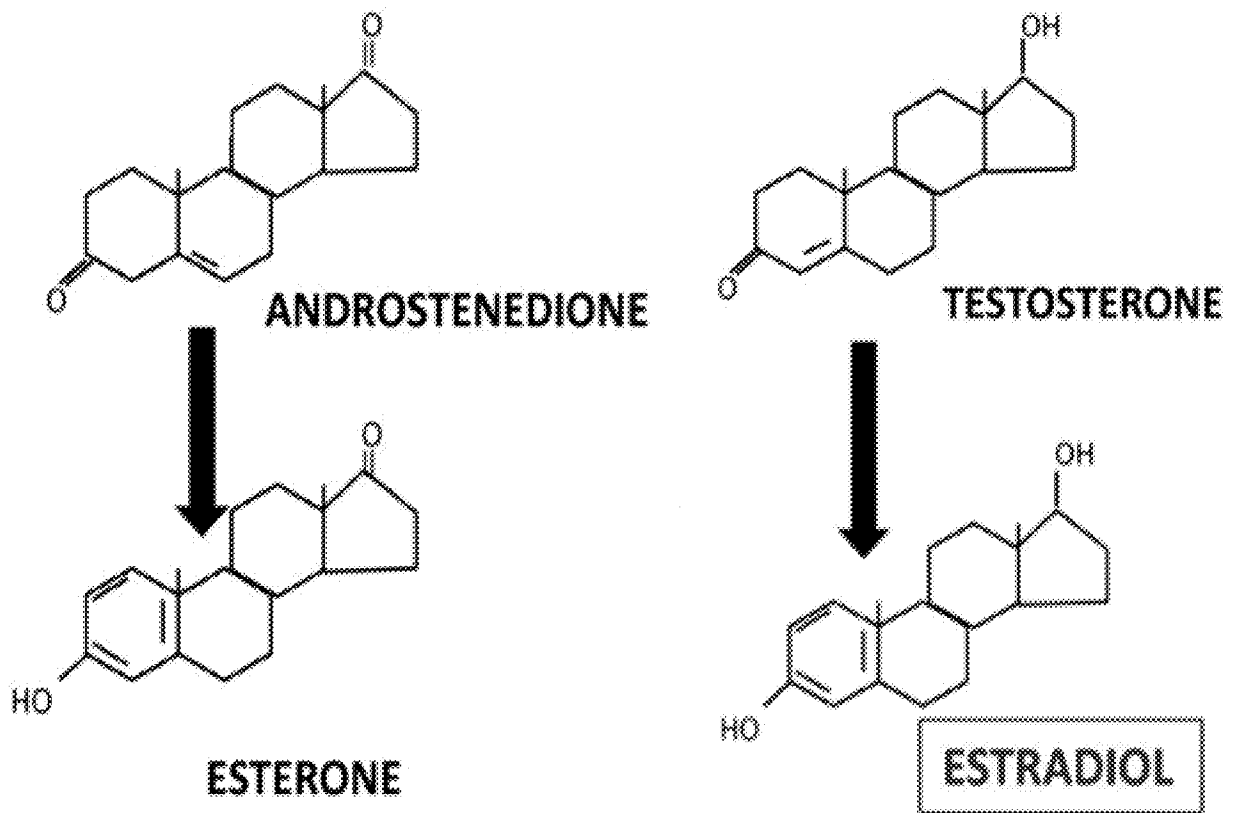
Figure 2: The conversion of androgens to estrogens by aromatase. Aromatase converts androstenedione to estrone and testosterone to estradiol, the active estrogen in humans. Figure adapted from Nothnick, W.B., Reproductive Biology and Endocrinology, 2011 [16].

locations in the central nervous system [18]. It has also been shown that adipose tissue in the abdomen, buttocks, and thighs produces aromatase at an increased rate as aging occurs [17, 19]. This could be vital in menopausal women who produce decreased amounts of estrogens in the ovary [17].

Aromatase in all tissue types is transcribed from one gene; however, the gene contains ten different promoters for tissue-specific isoforms [19, 20]. Moreover, introns and exons are spliced differently in different cells [19]. These variations lead to slightly different aromatase enzymes produced in different tissues of the body. Furthermore, methylation of different promoter regions on the *Cyp19* gene leads to decreased transcription at certain times during the reproductive cycle and, therefore, a decrease in aromatase production during these times [19].

Aromatase is responsible for the production of estrogens in fetal life. The fetus produces the androgen dehydroepiandrosterone, and aromatase from the placenta is responsible for its conversion to estrogens [15]. Aromatase is expressed in low quantities in fetal mice, and aromatase knockout mice develop normal ovaries in utero [20]. Estrogen receptor knockout mice also show no defects in embryonic ovary development, suggesting neither aromatase nor estrogens are necessary for the development of healthy ovaries in utero [20]. Aromatase expression increases in prepubertal mice, but levels remain low until FSH stimulation at puberty [20].

In granulosa cells, FSH stimulates both aromatase production and granulosa cell proliferation. FSH increases cAMP and leads to the binding of transcription factor GATA4 to the *Cyp19* promoter, and without GATA4 binding, aromatase levels decline drastically [21]. GATA4 also upregulates the expression of FSH receptors [22]. GATA4 knockout mouse ovaries show fewer antral follicles, suggesting decreased fertility [22]. FSH also leads to the removal of repressor proteins FOXO1 and FOXL2, and such removal allows for the transcription of aromatase mRNA [23, 24].

Estrogens produced by aromatase in the ovary stimulate continued granulosa cell proliferation [17]. In addition, estrogens stimulate the production of more aromatase by granulosa cells in a positive feedback relationship [25]. Testosterone also stimulates aromatase expression in granulosa cells [26]. Aromatase plays a role in initiating ovulation. When granulosa cells have proliferated and aromatase converts enough testosterone to estrogens, the estrogens feedback to the anterior pituitary and lead to the LH surge [17].

Aromatase is required before puberty for normal development of the ovary and follicles. In mice that do not express the aromatase gene, hindered folliculogenesis occurs in which follicles cease to develop and ovulation does not take place [17]. Female rats treated with an aromatase inhibitor demonstrate ovarian characteristics similar to those of polycystic ovarian syndrome (PCOS) [27]. Specifically, the rats have increased serum androgen levels, abnormal reproductive cycles, and ovarian cysts, all of which are characteristic of PCOS [27]. Furthermore, the combination of the lack of corpora lutea and absence of mature follicles indicate that ovulation is not occurring [27]. In addition, adipocytes increase in size and cells are less responsive to insulin

[27]. Thus, errors in aromatase production can lead to symptoms of PCOS and, therefore, reproductive failure. Aromatase knockout mice have macrophages in the ovaries, suggesting follicle death, which coincides with a decreased number of follicles in these mice [6]. Levels of gonadotropins in the aromatase knockout mouse are also elevated due to the lack of negative feedback on the anterior pituitary and hypothalamus by estrogens [6].

It was discovered in cells of the JEG cell line, a human placenta choriocarcinoma cell line commonly used in gene expression studies, that treatment with retinoic acid led to increased 17-β estradiol levels, increased expression of aromatase, and increased aromatase activity [15]. In addition, retinoic acid leads to granulosa cell proliferation [28]. Thus, the actions of aromatase produced by granulosa cells may be under the influence of retinoic acid.

Retinoic Acid

Retinoic acid is a derivative of the essential vitamin, vitamin A. Vitamin A is obtained through the diet from foods containing β-carotene, including vegetables, milk, and meat. The preliminary form of vitamin A is retinol, which is transported in the plasma via retinol binding protein [29]. Retinol is converted to retinyl esters by lecithin:retinol acyltransferase (LRAT). Retinyl esters are stored in lipid droplets in the liver or as retinosomes in the retina [30, 31]. A healthy adult's liver typically has a vitamin A concentration of 100 μg/g, with 20 μg/g being the minimum level that is considered safe [32]. The body controls the excretion and storage of vitamin A based on how much is available in the liver [32]. In the retina, retinal is the active derivative of vitamin A [31]. Retinol is oxidized to retinal by alcohol dehydrogenases and retinol dehydrogenases [31].

In most other parts of the body, retinoic acid is the active form of vitamin A. All-*trans*-retinoic acid is the oxidized form of retinal, a process carried out by retinaldehyde dehydrogenase enzymes [29, 31] (Figure 3).

Within the cell, retinoic acid is transported by binding to cellular retinoic acid binding proteins I or II (CRABP-I or CRABP-II). These proteins are unable to bind the precursors of all-*trans*-retinoic acid. Of these two binding proteins, CRABP-I is present in the reproductive organs of both males and females, notably the testis and the ovary, whereas CRABP-II is typically present throughout the musculoskeletal system [29]. Retinoic acid exerts its effects on gene expression by binding to intracellular receptors, RAR-α, RAR-β, or RAR-γ, expressed in many organs and during embryonic development [29]. The RARs form heterodimers with retinoid X receptors (RXRs), which are believed to aid in the binding of the proteins to the nucleic acid sequence of interest [31]. Once bound to retinoic acid, the intracellular receptors bind to retinoic acid response elements (RAREs) on DNA molecules and modify the transcription of target genes [29] (Figure 4).

Retinoic acid is essential for the formation of many different tissues during embryonic development. For example, retinoic acid plays a role in the development of the hindbrain and the neural tube, and also is vital for the formation of the spine and ribcage [31]. Retinoic acid is necessary for the formation of the lung buds, pancreas, thymus, thyroid gland, and parathyroid glands [31]. Genes induced by retinoic acid also regulate the development of the aortic arch [31]. The vitamin A derivative also activates genes crucial for the development of the forelimbs and

body patterning [31]. Overall, retinoic acid is responsible for direct or indirect regulation of 532 genes in tissues throughout the body [34].

Degradation of retinoic acid can be carried out by proteins coded by the *Cyp26* genes, including *Cyp26a1*, *Cyp26b1*, and *Cyp26c1*. The CYP26 proteins are Cytochrome P450 enzymes, and their expression is also crucial for the developing mammalian embryo [31]. The CYP26 enzymes metabolize retinoic acid to the polar molecules 4-oxo-all-*trans*-retinoic acid, 4-OH- all-*trans*-retinoic acid, and 18-OH- all-*trans*-retinoic acid, which can then be excreted [35]. Retinoic acid induces expression of CYP26 enzymes [31]. CYP26A1 and CYP26B1 catabolize all-*trans*-retinoic acid, whereas CYP26C1 breaks down both 9-cis-retinoic acid and all-*trans*-retinoic acid [36]. CYP26B1 is expressed in the heart and vasculature, brain, and gonads of the developing embryo [37, 38, 28].

Excess exposure to retinoic acid can lead to toxic effects. Specifically, overexposure to retinoic acid during embryonic development can lead to abnormal development in the brain, bones, and heart, along with immune deficiencies [39]. Abnormalities in the developing nervous system can lead to cognitive defects in children [39]. Furthermore, exposure to high levels of retinoic acid can lead to abnormal ear development or even a lack of ears, and cause spontaneous abortion in 25% of first-trimester pregnancies [39]. It is possible that retinoic acid toxicity is due to the overexpression of CYP26 enzymes in response to increased levels of retinoic acid [40]. Overexpression of CYP26 enzymes would lead to a deficiency in retinoic acid signaling.
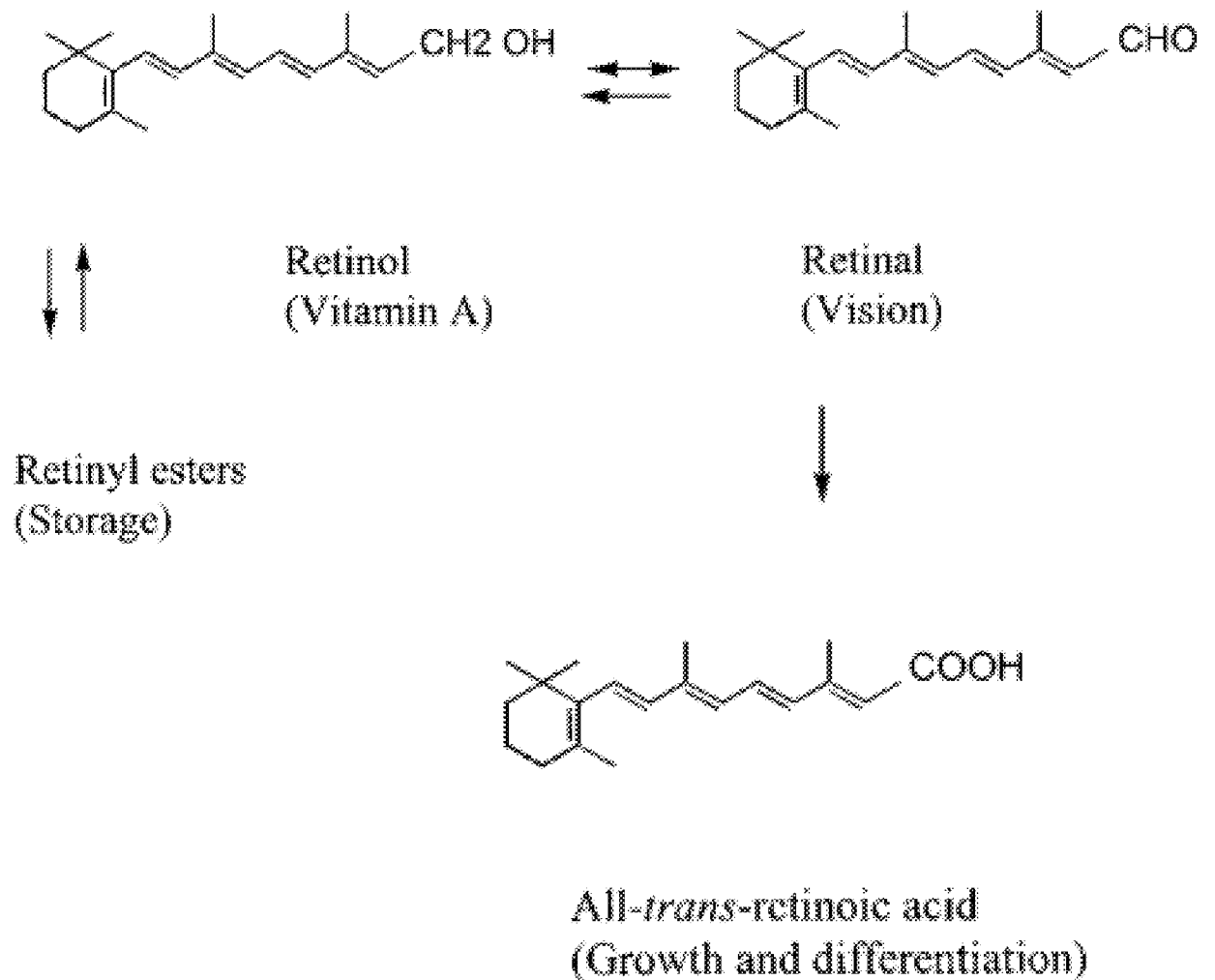
Figure 3: Chemical pathway leading to all-trans-retinoic acid synthesis. *Vitamin A is ingested from foods containing β-carotene. In the body, vitamin A is referred to as retinol. Retinol can be stored as retinyl esters or oxidized to retinal, the active derivative of vitamin A in the eye, or retinoic acid, the active derivative of vitamin A elsewhere in the body. This image is adapted from Zhang D. et al., Journal of Cellular Physiology, 2000 [29].*
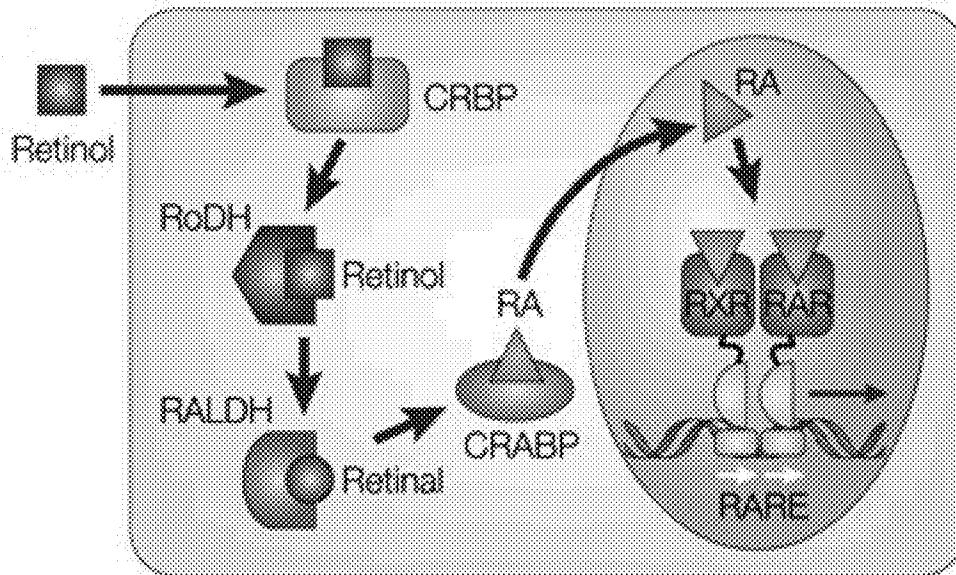
Figure 4: Retinoic acid cell signaling. *Retinol is bound to cellular retinol binding protein*

(CRBP) within the cell. Retinol is oxidized by retinol dehydrogenases (RoDH) to form retinal,

and retinal is oxidized by retinaldehyde dehydrogenases (RALDH) to produce retinoic acid.

Retinoic acid enters the nucleus and binds to RARs. RARs dimerize with RXRs and directly

regulate gene transcription (29, 31, 32). Figure adapted from Nature Reviews Neuroscience 3,

843-853, 2002 [33].

Roles of Retinoic Acid in Reproduction

Retinoic acid also plays an imperative role in the developing embryonic and postnatal gonads. Specifically, retinoic acid and R115866, a specific CYP26 inhibitor, lead to granulosa cell proliferation in developing follicles [28]. The stimulatory effect on cell proliferation was suppressed by the pan RAR inhibitor, AGN193109 [28]. In the embryonic ovary, retinoic acid activates the genes *Stra8*, *Sycp3*, and *Dmc1* [41]. *Stra8* is important for female development as it induces meiosis in germ cells, whereas *Sycp3* and *Dmc1* are also genes involved in meiosis [41]. There is no activation of these genes in embryonic male gonads [41]. In 14.5 days post-coital rat ovaries, retinoic acid induces meiosis and can lead to a 77% decrease in the number of germ cells after 9 days of retinoic acid treatment [42]. In the developing ovaries of embryonic females, oogenesis begins around 13.5 days post-coitum. In males, meiosis does not begin until after birth. CYP26B1 is present in both male and female embryos until 12.5 days post-coitum. However, after this point, the level of CYP26B1 in the developing female gonads decreases significantly, whereas it is highest in the developing male gonads at 13.5 days post-coitum [38]. Consequently, levels of retinoic acid in the developing ovaries remained high after this time point, whereas retinoic acid levels decreased significantly in the embryonic testes. Retinoic acid initiates meiosis in the germ cells; therefore, the lack of retinoic acid in the male gonads after embryonic day 12.5 prevents meiosis from occurring [42, 38].

It is believed that the retinoic acid pathway within the developing ovary is regulated by *Wnt4*, a gene expressed in somatic ovarian cells. Developing ovaries that do not express *Wnt4* contain disorganized oocytes [43]. Furthermore, these oocytes do not aggregate as in the wildtype

oocyte; rather, the individual oocytes lack cell-to-cell contact [43]. *Wnt4* deficient ovaries show

decreased expression of *Kitl*, *Figla*, and *Nobox*, genes critical for oogenesis and folliculogenesis

[43]. Moreover, *Wnt4* deficient ovaries lack sufficient expression of *Stra8*, thus preventing

meiosis from occurring [43]. In addition to containing decreased levels of *Sycp1* and increased

levels of *Cyp26b1*, these ovaries also have decreased levels of retinaldehyde dehydrogenases,

preventing the conversion of retinal to retinoic acid [43]. The *Wnt4* deficient ovaries also show

structures resembling seminiferous tubules, which contain cells similar to prespermatogonia cells

[43]. When *Wnt4* is reintroduced to these ovaries, *Cyp26b1* expression decreases, *Stra8*

expression increases, and meiosis is initiated [43]. Therefore, *Wnt4* plays a crucial role in

retinoic acid expression and the fate of the gonadal sex of the developing embryo.

In pregnant female mice, a vitamin A-deficient diet does not lead to fewer germ cells in the

ovaries of female offspring; however, there is a significant increase in the number of germ cells

that do not enter meiosis in these mice [41]. In addition, these mice display notably decreased

levels of *Stra8* expression [41]. *Stra8* expression is normally observed just before the initiation

of germ cell meiosis. In the few germ cells that are able to enter meiosis despite the vitamin A

deficient diet, meiosis progresses to the completion of prophase, at which stage the oocyte

remains until ovulation [41]. This observation implies that retinoic acid is necessary for the entry

of most gametes into meiosis, but it is not required for the germ cells to progress through the

meiotic stages after entry.

The Kipp lab has shown that mice fed a vitamin A deficient diet from gestation stage through

postnatal development exhibit numerous ovarian pathologies, including follicular and bursal

cysts and hemorrhagic follicles [Unpublished data]. The number of atretic follicles is significantly greater in vitamin A deficient mice compared to mice fed a normal diet. Furthermore, a transition to a regular diet at 16 weeks does not rescue the ovarian phenotype [Unpublished data]. Thus, retinoic acid plays a role in the development of healthy ovaries and follicles.

Importance of Studying Retinoic Acid Regulation of Aromatase Expression

Both retinoic acid and aromatase have been shown to play roles in reproductive diseases and cancers [53, 29]. One of the diseases that can lead to infertility is endometriosis, in which the endometrium, or lining of the uterus, grows in areas other than inside the uterus. In endometriosis, the cells of the endometrium are able to produce aromatase, which results in greater estrogen levels [53]. Estrogen allows the endometrium to spread further outside the uterus, therefore exacerbating the issue [53]. Retinoic acid is also involved in endometriosis, as decreased levels of retinoic acid in endometrial tissue and follicular fluid are associated with the presence of endometriosis [64, 65].

Polycystic ovarian syndrome (PCOS) is one of the leading reproductive disorders occurring in females. Characterized by ovarian cysts and elevated levels of androgens, PCOS causes infertility in some affected women [54]. Research has depicted that mutations in the gene encoding aromatase are correlated with PCOS [27]. Administration of an aromatase inhibitor to young female rats induces many symptoms of PCOS, suggesting that aromatase plays a vital role in normal development of the ovary [27].

Both aromatase and retinoic acid have been shown to play a role in the development and progression of different carcinomas in the human female. For example, an increase in aromatase production in breast tissue can lead to breast cancer [55]. Furthermore, higher levels of aromatase in breast tissue leads to growth of existing breast tumors [55]. All-*trans*-retinoic acid inhibits progression of ovarian cancer cell line CA-OV3 [29]. In addition, the ovarian cancer cell line SK-OV3, which is resistant to the proliferation-inhibitory effect of retinoic acid, also shows a reduction in cell growth after over-expression of a specific retinoic acid receptor [29]. Retinoic acid inhibits the proliferation of ovarian carcinoma cells by inhibiting AP-1, a transcription factor involved in cell growth [29]. Furthermore, AHPN/CD437, a synthetic retinoid, initiates apoptosis in ovarian carcinoma cells [29]. Research on both aromatase and retinoic acid can be beneficial to humans in terms of developing therapies for reproductive diseases and infertility.

Hypothesis

It has been demonstrated that in cancerous placental cells, an increase in retinoic acid leads to an increase in aromatase activity [15]. Retinoic acid also induces aromatase expression in adipose breast tissue [56]. Furthermore, activating retinoic acid receptors with receptor-specific ligands increases aromatase gene expression in breast cancer cells [57]. Research from the Kipp lab has demonstrated that mice fed on a vitamin A deficient diet develop ovarian pathologies similar to aromatase knockout mice, including atretic and hemorrhagic follicles and follicular cysts [Unpublished Data, 6]. Hormone profiles of vitamin A deficient mice are similar to those of the aromatase inhibitor-treated mice, including high testosterone levels [Unpublished Data, 27]. Based on the findings from previous research, the hypothesis of this study is that retinoic acid

may increase aromatase mRNA and protein expression in the ovary. To test this hypothesis, this study investigated the effect of retinoic acid on aromatase expression in primary cultured granulosa cells and in the whole ovary.

In this study, mice were used as an animal model, as the anatomy, physiology, and genome of mice are similar to that of humans, and yet they have a shorter generation time. This study utilized granulosa cell culture from normal CD-1 mice and whole ovaries from MT-α transgenic mice to investigate aromatase expression *in vitro* and *in vivo*.

Methods

Experimental Design

This study included both an *in vitro* and an *in vivo* portion. For the *in vitro* experiments, a granulosa cell culture was used (Figure 5). Ovaries were isolated from 21-22 day old mice and granulosa cells were isolated from the ovaries. Cells were treated with retinoic acid, a retinoic acid metabolism blocking agent R115866, a pan-retinoic acid receptor inhibitor AGN193109, and combinations of these treatments. Cells were cultured with treatments for either 24- or 72-hours, after which samples were collected. Total mRNA and proteins were isolated from cells for real time reverse transcriptase polymerase chain reaction (RT-PCR) or western blot purposes, respectively. Aromatase and *Cyp26b1* mRNA expression was measured using real time RT-PCR, and aromatase protein expression was measured using western blots. For the *in vivo* portion of the experiment, MT-α transgenic mice were used (Figure 6). Ovaries were isolated from MT-α transgenic mice aged 19-days, 7-weeks, and 6-months. Total mRNA and proteins were extracted from the whole ovaries for real time RT-PCR and western blot purposes, respectively. Aromatase and *Cyp26b1* mRNA expression was measured using real time RT-PCR, and aromatase protein expression was examined using western blots.
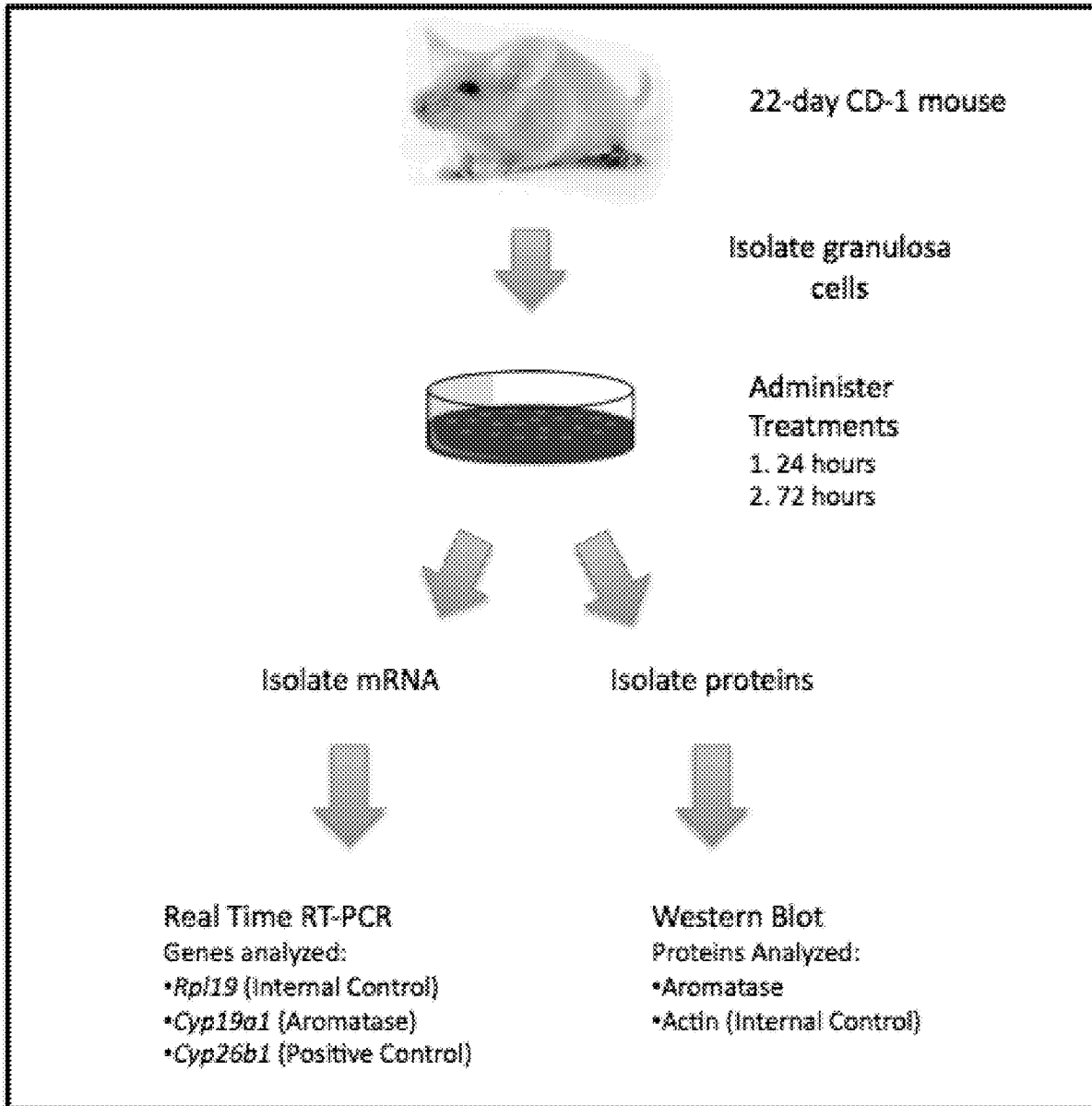
Figure 5: Overview of experimental design using granulosa cell cultures. Granulosa cells were isolated from 22-day old mouse ovaries and cultured for 24- or 72- hours. At the end of each treatment, total mRNA was isolated for real time RT-PCR. Genes analyzed included Rpl19 (internal control), Cyp19a1 (aromatase), and Cyp26b1 (positive control). Proteins were isolated for western blot purposes. Proteins analyzed included aromatase and actin (internal control).
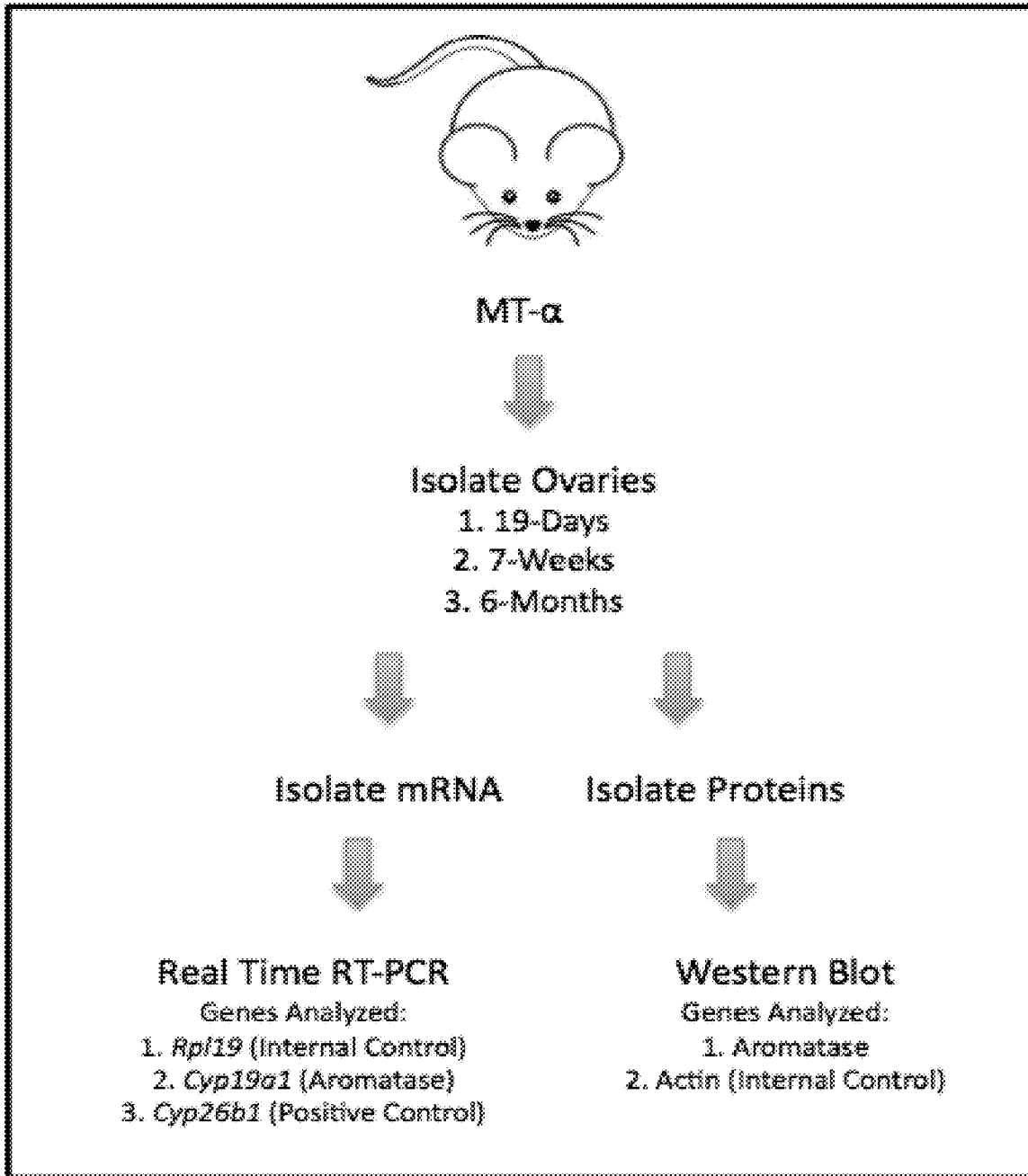
Figure 6: Overview of experimental design using MT-α transgenic mice. *Whole ovaries were isolated from 19-day, 7-week, and 6-month MT-α mice and frozen. Total mRNA was isolated for real time RT-PCR. Genes analyzed included Rpl19 (internal control), Cyp19a1 (aromatase), and Cyp26b1 (positive control). Proteins were isolated for western blot purposes. Proteins analyzed included aromatase and actin (internal control).*

Animals

CD-1 mice and MT-α transgenic mice were maintained on a 12-hour light/dark cycle and were provided food and water *ad libitum*. Animals were maintained according to all federal and institutional guidelines. Mice were euthanized using carbon dioxide gas followed by cervical dislocation for tissue collection.

Metallothionein-I promoter (MT-α) transgenic mice were developed and provided by Dr. Kelly Mayo from Northwestern University. This line of mice over-express the rat inhibin-α gene. Female MT-α mice have decreased serum FSH and estrogen levels, and increased serum LH and testosterone levels [51, 52]. These hormone profiles suggest potential differences in aromatase expression between MT-α mice and their normal littermates (NLM). Furthermore, female MT-α mice exhibit ovarian pathologies such as ovarian cysts and polyovular follicles [51]. MT-α females at day 19 showed increased *Cyp26b1* expression in the ovaries, suggesting altered retinoic acid signaling [28].

Granulosa Cell Culture and Treatments

Granulosa cells were isolated from 21 or 22-day old mice as described previously [28]. Ovaries were collected and cleaned of fat and other tissues in cold 4F medium. The 4F medium was composed of Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 mix with L-Glutamine and 15 mM HEPES (CellGro), 0.5 ml 5mg/ml transferrin, 0.1 ml 10mg/ml insulin, 5

μl 4mg/ml hydrocortisone, 2.5 ml 100X Pen-Strep. Cleaned ovaries were washed twice in cold 4F media and incubated in 10 ml of pre-warmed preincubation medium at 37°C for thirty minutes. The preincubation medium was composed of 1.71 g sucrose and 0.038 g ethylene glycol teteraacetic acid (EGTA) in 4F medium and is kept at a pH of 7.4. Ovaries were then washed twice with 2 ml cold 4F media, followed by transferring into 5 ml cold 4F media. Ovaries and media were then poured into a 6 cm plate, and granulosa cells were isolated by puncturing the ovaries with a pair of 27-gauge needles for thirty minutes. Cells were centrifuged at 257xg for five minutes in 10 ml of cold 4F medium. The medium was replaced with 5 ml fresh cold 4F medium, cells were resuspended, and cells were centrifuged again at 257xg for five minutes. Medium was then replaced with another 5 ml of fresh cold 4F medium with 10% heat inactivated fetal bovine serum (FBS). Cells were resuspended and spun at 257xg for five minutes. Medium was removed and cells were resuspended in 1 ml 4F medium with 10% heat inactivated FBS. Oocytes and small follicles were filtered out using a 40 μm cell strainer. To count the cells, 10 μl of Tryptan-Blue, 80 μl of 1x PBS, and 10 μl of filtered and resuspended granulosa cells were combined, and 10 μl of the combined mixture was added to a hemocytometer. The average of four cell counts in four different quadrants was taken. This value was multiplied by 100,000 to account for dilutions to determine the total number of cells per ml of cell suspension.

Cells were either plated in a 24-well plate for mRNA isolation purposes with 250,000 cells in 500 μl 4F + 10% heat inactivated FBS per well, or plated in a 12-well plate for protein isolation purposes with 400,000 cells in 800 μl 4F + 10% heat inactivated FBS per well. After plating, cells were allowed to settle 24- to 48-hours to attach to the plate before treatments. For mRNA

level analysis, each treatment condition was administered to one well per plate, totaling one well

per experiment. For protein analysis, each treatment condition was given to one well per plate for

two plates, totaling two wells per experiment. Granulosa cells were treated for 24- and 72-hour

time points. Media with treatments were changed every 24-hours. All treatments are displayed in

Table 1. Experiments were repeated at least three times (n = 3-5) for each time point (Figure 5).

Table 1: Treatments administered to primary cultured granulosa cells.

Treatment	Expected Outcome on Aromatase Expression	Notes
DMSO	-	Vehicle Control
All-*trans* retinoic acid, 0.07 μM	↑	
All-*trans* retinoic acid, 0.7 μM	↑	
AGN193109, 5 μM	↓	Pan Retinoic Acid Receptor Inhibitor
All-*trans* retinoic acid, 0.07 μM plus AGN193109, 5 μM	↓	
All-*trans* retinoic acid, 0.7 μM plus AGN193109, 5 μM	↓	
R115866, 0.7 μM	↑	CYP26B1 Inhibitor
R115866, 0.7 μM plus AGN193109, 5 μM	↓	
All-*trans* retinoic acid, 0.07 μM plus R115866, 0.7 μM	↑	For 24-hour real time RT-PCR only. Used to examine a potential synergistic effect between treatments.
All-*trans* retinoic acid, 0.7 μM plus R115866, 0.7 μM	↑	For 24-hour real time RT-PCR only. Used to examine a potential synergistic effect between treatments.

R115866

R115866 is a retinoic acid metabolism blocking agent (RAMBA). This compound prevents the degradation of retinoic acid by CYP26 enzymes, therefore increasing endogenous levels of retinoic acid in both plasma and body tissues. Furthermore, R115866 itself induces effects similar to those of retinoic acid. One such effect is the induction of *Cyp26b1* expression by R115866, although this effect is more prominent from retinoic acid treatment [44]. R115866 has been studied as a potential treatment for skin problems and vascular diseases [45, 46]. This compound does not decrease levels of steroid hormones testosterone and estradiol, as other RAMBAs do. [44]. Within the ovary, research from the Kipp Laboratory has indicated that R115866 stimulates follicle growth, perhaps due to its induction of granulosa cell proliferation [Unpublished Data, 28]

AGN193109

AGN193109 is a pan RAR inhibitor. Specifically, this compound prevents retinoic acid from binding to RARα, RARβ, and RARγ by changing the conformation of these receptors [47]. The compound antagonizes retinoic acid effects in a dose response manner. AGN193109 is the first of its kind to inhibit all retinoic acid receptors at very low concentrations, making it an ideal compound for studying retinoic acid effects and mechanisms in regulating the expression of different genes [47]. The compound has been used in studying the role of retinoic acid in skin keratinization disorders, leukemia treatments, and embryonic vertebral development, among other applications [48-50].

Whole Ovary Collection

Whole ovaries were isolated from 3-6 MT-α transgenic mice aged 19-days, 7-weeks, and 6-months. Ovaries were frozen on dry ice and stored at -80°C until further analysis.

mRNA Extraction

At the end of treatments, granulosa cells were rinsed with 1x PBS, and total RNA was extracted following the manufacturer's protocol for the RNAeasy Mini Kit (Qiagen cat # 74104, Germantown, Maryland).

For RNA extraction from whole ovaries from MT-α transgenic mice, ovaries were frozen on dry ice and homogenized using a Tissue Tearor™. Total RNA was then extracted following the manufacturer's protocol for the RNAeasy Mini Kit (Qiagen cat # 74104, Germantown, Maryland).

Real-Time RT-PCR

Total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences, Waltham, Massachusetts) in 20 μl reactions. The resulting cDNA was diluted 10 fold and 8 μl per well of the diluted cDNA was used in real-time PCR on a StepOnePlus real-time PCR system in conjunction with the StepOne software v. 2.1

(Applied Biosystems, Waltham, Massachusetts). Rat ribosomal protein L19 (RPL19) levels were measured as controls for each assay [28]. In each experiment, gene expression was measured in duplicate for each treatment group.

Genes examined in each experiment included *Rpl19*, *Cyp26b1* (to ensure successful treatments, as *Cyp26b1* is a retinoic acid inducible gene) and *Cyp19a1* [31]. A list of primers used in this study are shown in Table 2.

Table 2: DNA primers used in this study.

Gene	PCR Primer Sequence	Product Size (bp)
Rpl19	5'-CTG AAG GTC AAA GGG AAT GTG-3' 5'-GGA CAG AGT CTT GAT GAT CTC-3'	195
Cyp26b1	5'-AGC TAG TGA GCA CCG AGT GG-3' 5'-GGG CAG GTA GCT CTC TTT CC-3'	146
Cyp19a1	5'-GAC ACA TCA TGC TGG ACA CC-3' 5'-TGC CAG GCG TTA AAG TAA CC-3'	100

For real time PCR reactions, SYBR Green master mix was used together with the diluted cDNA to make a final volume of 20 µl per well in a 96-well plate. The thermocycler cycled at: 1x 50°C, 2 minutes; 95°C, 10 minutes; 45x 95°C, 15 seconds; 58°C, 45 seconds; 72°C, 1 minute; 95°C, 15 seconds. Duplicate cycle threshold (CT) values were compared to confirm a difference of no more than 1.0 CT between the experimental duplicates. If a difference of greater than 1.0 CT was

found, only the CT value from the amplification and melt curve that looked normal was used in the analysis. To quantify mRNA using the mean CT value, the $2^{-\Delta\Delta Ct}$ value was calculated (58). Results for each treatment are expressed relative to the DMSO control.

Protein Extraction

Granulosa cells were washed twice in chilled 1x PBS and incubated on ice for ten minutes in 150 µl CelLytic MT Cell Lysis Reagent (Sigma-Aldrich, St. Louis, Missouri) per well. Protein homogenates were collected by scraping wells with a syringe plunger in the lysis reagent. Protease inhibitor cocktail (phenylmethanesulfonylfluoride, 100 mM, antipain, 1 µg/µl, aprotinin, 1 µg/µl, leupeptin, 1 µg/µl) was added to each sample at 1 µl/ml lysis reagent. Homogenized samples were frozen on dry ice and thawed at 37°C for two minutes for two cycles. Samples were then centrifuged at 4°C at 16,000xg for five minutes. The supernatant containing the protein homogenates was collected.

Whole ovaries from MT-α transgenic mice frozen on dry ice. Ovaries were homogenized using a Tissue Tearor™ in 150 µl CelLytic MT Cell Lysis Reagent (Sigma-Aldrich, St. Louis, Missouri). The solution was centrifuged at 16,000xg for five minutes at 4°C. The supernatant contained the protein homogenates.

Western Blot

Polyacrylamide gels were used for western blotting. The resolving layer was made using 3.6 ml 30% Acrylamide/Bis, 2.8 ml 1 M Tris pH 8.8, 1.6 ml ddH$_2$O, 46.75 μl 20% sodium dodecyl sulfate (SDS), 25 μl 20% ammonium persulfate (APS), and 5 μl Temed. After the resolving layer hardened, the stacking layer made of 0.574 ml 30% Acrylamide/Bis, 0.626 ml 1M Tris pH 6.8, 3.85 ml ddH$_2$O, 25 μl 20% SDS, 12.5 μl 20% APS, and 7 μl Temed was poured on top of the resolving layer. Gels were kept at 4°C overnight before use.

For western blotting of proteins from cultured granulosa cells, 100 μg protein per treatment condition was mixed with loading buffer and 1% betamercaptoethanol per 1x loading buffer. For western blotting of proteins from whole ovaries, 50 μg protein was mixed with loading buffer and 1% betamercaptoethanol per 1x loading buffer (Table 3).

Table 3: Preparation of sample loading buffers.

	2x Loading buffer	3x Loading Buffer	6x Loading Buffer
1 M Tris pH 6.8	1 ml	1.5 ml	3.5 ml
Glycerol (100%)	2 ml	3 ml	2.6 ml
SDS (20%)	2 ml	3 ml	200 μl
ddH$_2$O	4 ml	1.5 ml	3.5 ml
Bromphenol Blue	6 mg	6 mg	6 mg

The above prepared, mixed samples were boiled for five minutes and then cooled to room temperature. Samples were then loaded onto the polyacrylymide gel and electrophoresed in 1x Page Buffer (10x Page Buffer: 30.2 g Tris base 188 g Glycine, 50 ml 20% SDS) at 170 V for 90 minutes. After electrophoresis, proteins were transferred to a nitrocellulose membrane in Transfer Buffer (3.133 g Tris base, 14.4 g Glycine, 1 g SDS, 0.2 L methanol, 0.8 L ddH$_2$O) at 50 V for 120 minutes. Membranes were then soaked in blocking solution (100 ml 1x PBS, 100 μl Tween, 50 g milk protein) for one hour or overnight at 4°C. Membranes were washed with two changes of wash buffer (25 ml 20x PBS, 475 ml deionized water, and 500 μl Tween) and then incubated with an aromatase goat anti-mouse primary antibody (1:500, Santa Cruz, Dallas, Texas) in blocking solution overnight at 4°C. Membranes were rinsed in wash buffer for 15-minutes, followed by three five-minute washes. Membranes were then incubated with a horseradish peroxidase labeled rabbit anti-goat secondary antibody (1:8000, Life Technologies, Carlsbad, California) in blocking solution for 60 minutes at room temperature. Membranes were rinsed in wash buffer for fifteen minutes, followed by three five-minute washes. Membranes were then incubated with ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) for five minutes at room temperature and were visualized using chemiluminescence. Membranes were stripped of antibodies using ReBlot Plus Strong Antibody Stripping Solution, 10x (1:10, Millipore, Billerica, Massachusetts) for thirty minutes at room temperature. Membranes were then washed with two changes of wash buffer for ten minutes each and then blocked with blocking solution for one hour or overnight at 4°C. Membranes were then rinsed with two changes of wash buffer and incubated in rabbit anti-actin primary antibody (1:2000, Sigma Aldrich, St. Louis, Missouri) in blocking solution for one hour at room temperature. The actin served as the control protein. Membranes were washed with wash

buffer for fifteen minutes, followed by three five-minute washes. Membranes were then

incubated in goat anti-rabbit secondary antibody (1:2500, Promega, Madison, Wisconsin) for one

hour at room temperature. Membranes were washed with wash buffer for fifteen minutes,

followed by three five-minute washes. Membranes were then incubated in ECL Western Blotting

Detection Reagent (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) for five

minutes at room temperature and were visualized using chemiluminescence.

Blots were analyzed by densitrometry using ImageJ version 2.0. Relative band intensity from

each experiment were calculated by taking the ratio of aromatase band intensity over the band

intensity of the loading control, actin. Results are presented relative to the DMSO control. The

average fold changes from four experiments were then determined for each treatment condition

and presented.

Statistical Analysis

Data are presented as mean +/- S.E. For comparison of data between one treatment group and the

control, an independent samples *t* test was used. For comparison between two treatment groups,

a Student's one-tailed *t* test was used. The Dunn-Sidak Correction was used to calculate α values

when multiple *t* tests were used in one experiment. $p < 0.05$ was considered significant in each

experiment.

Results

Treatment Effects on the mRNA Levels of Cyp26b1 and Cyp19a1 in Primary Cultured Granulosa Cells

Levels of *Cyp19a1* mRNA in granulosa cells were measured by real time RT-PCR. Levels of *Cyp26b1* mRNA in granulosa cells were also measured as a positive control, as *Cyp26b1* expression is inducible by retinoic acid [31]. After 24-hour treatments, both 0.07 μM and 0.7 μM retinoic acid induced an increase in *Cyp26b1* expression, although this was not statistically significant when compared to DMSO using an independent samples *t* test (Figure 7). Increases in *Cyp26b1* mRNA levels were also observed after 24-hour treatments with 0.7 μM R115866, 0.7 μM R115866 plus 0.07 μM retinoic acid, or 0.7 μM R115866 plus 0.7 μM retinoic acid, although these were not statistically significant compared to DMSO. Trends indicate that 5 μM AGN193109 suppressed the stimulatory effect on *Cyp26b1* mRNA induced by 0.07 μM retinoic acid, 0.7 μM retinoic acid, and 0.7 μM R115866 on *Cyp26b1* expression, although these inhibitory effects did not reach statistical significance when compared using a two sample *t* test. It was observed that treatment with 5 μM AGN193109 suppressed *Cyp26b1* mRNA levels compared to DMSO, although this was not statistically significant.

After 72-hour treatments, 0.7 μM retinoic acid induced a statistically significant increase in *Cyp26b1* expression compared to DMSO ($p < 0.05$) (Figure 8), and, although not statistically significant, 0.07 μM retinoic acid and 0.7 μM R115866 also stimulated *Cyp26b1* expression. In addition, a statistically significant suppression in *Cyp26b1* mRNA levels compared to DMSO was observed after 72-hour treatment with 5 μM AGN193109 ($p < 0.05$), suggesting endogenous retinoic acid signaling through RARs. When cells were treated with 0.07 μM retinoic acid plus 5

μM AGN193109, a statistically significant decrease in *Cyp26b1* expression compared to cells treated with 0.07 μM retinoic acid alone was observed (p<0.05). When the cells were treated with 0.7 μM retinoic acid plus 5 μM AGN193109, retinoic acid induction of *Cyp26b1* mRNA levels was decreased, but not completely suppressed (p<0.05). Trends indicate that treatment with 0.7 μM R115866 plus 5 μM AGN193109 suppressed *Cyp26b1* mRNA levels compared to treatment with 0.7 μM R115866 alone.

After 24-hours, treatment with 0.07 μM retinoic acid induced a statistically significant increase in *Cyp19a1* expression compared to DMSO (p<0.05) (Figure 9). Furthermore, this *Cyp19a1* induction by 0.07 μM retinoic acid was significantly suppressed in granulosa cells treated with 0.07 μM retinoic acid plus 5 μM AGN193109 (p<0.05). Trends indicate that 0.7 μM retinoic acid, 0.7 μM R115866, 0.07 μM retinoic acid plus 0.7 μM R115866, or 0.7 μM retinoic acid plus 0.7 μM R115866 treatments induce *Cyp19a1* expression, although these stimulatory effects were not statistically significant. Trends also indicate that treatment with 5 μM AGN193109 suppressed *Cyp19a1* expression, and treatment with 0.7 μM retinoic acid plus 5 μM AGN193109 or 0.7 μM R115866 plus 5 μM AGN193109 appeared to suppress *Cyp19a1* expression compared to treatment with 0.7 μM retinoic acid alone or 0.7 μM R115866 alone, respectively. Cells treated with 5 μM AGN193109 for 72-hours demonstrated a statistically significant suppression in *Cyp19a1* expression compared to granulosa cells treated with DMSO (p<0.05) (Figure 10), suggesting endogenous retinoic acid signaling. The *Cyp19a1* mRNA level induction by retinoic acid did not reach statistical significance after 72-hour treatments. However, trends indicate that treatment with 0.7 μM R115866 induces *Cyp19a1* mRNA levels after 72-hour treatments, and this induction was significantly suppressed by treatment with 5 μM

AGN193109 (p<0.05). Treatment with either dose of retinoic acid plus 5 μM AGN193109

appeared to suppress *Cyp19a1* expression compared to treatment with retinoic acid alone,

although such suppression was statistically insignificant.
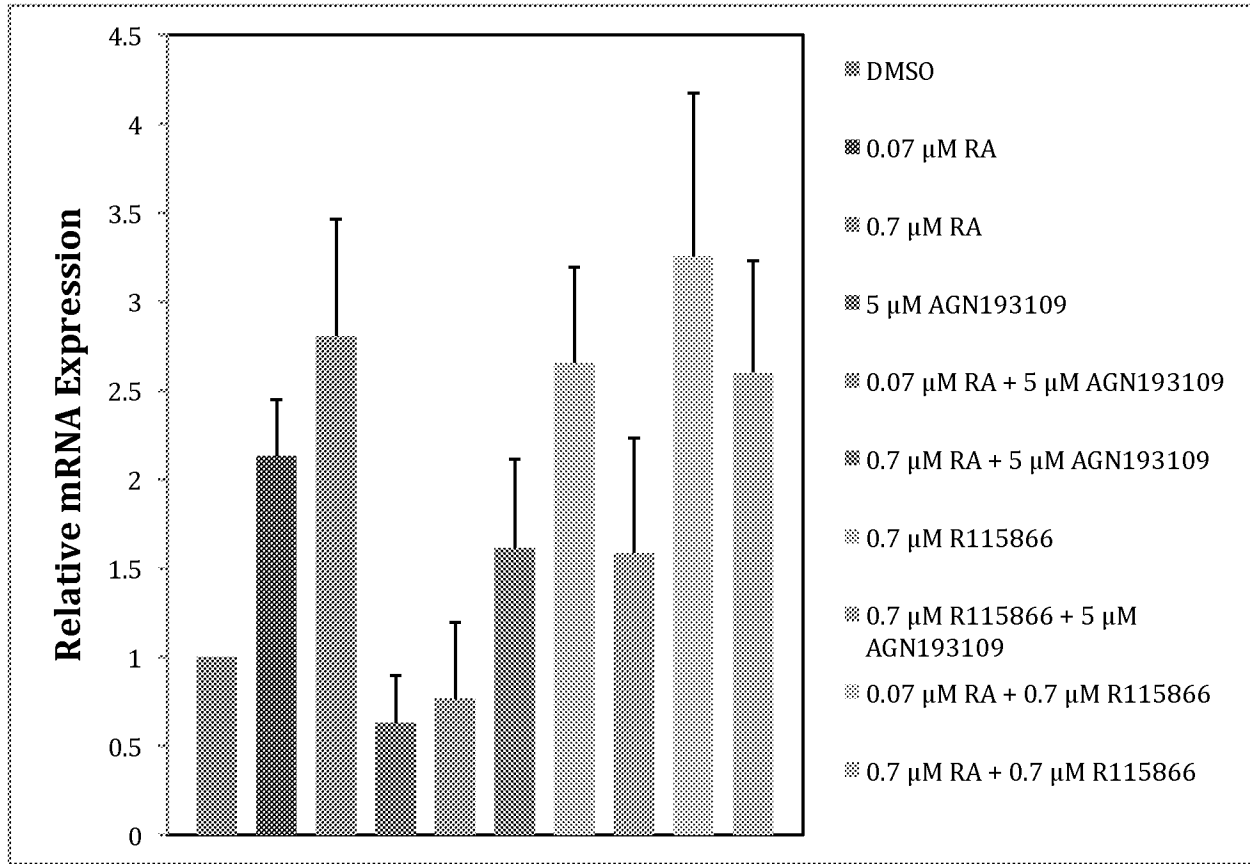
Figure 7: Relative *Cyp26b1* mRNA levels in primary cultured granulosa cells after 24-hour treatments. *Cyp26b1* levels were measured as positive controls, as *Cyp26b1* is a retinoic acid inducible gene [31]. DMSO was used as a vehicle control. Data are presented as mean +/- S.E. n = 3-5.
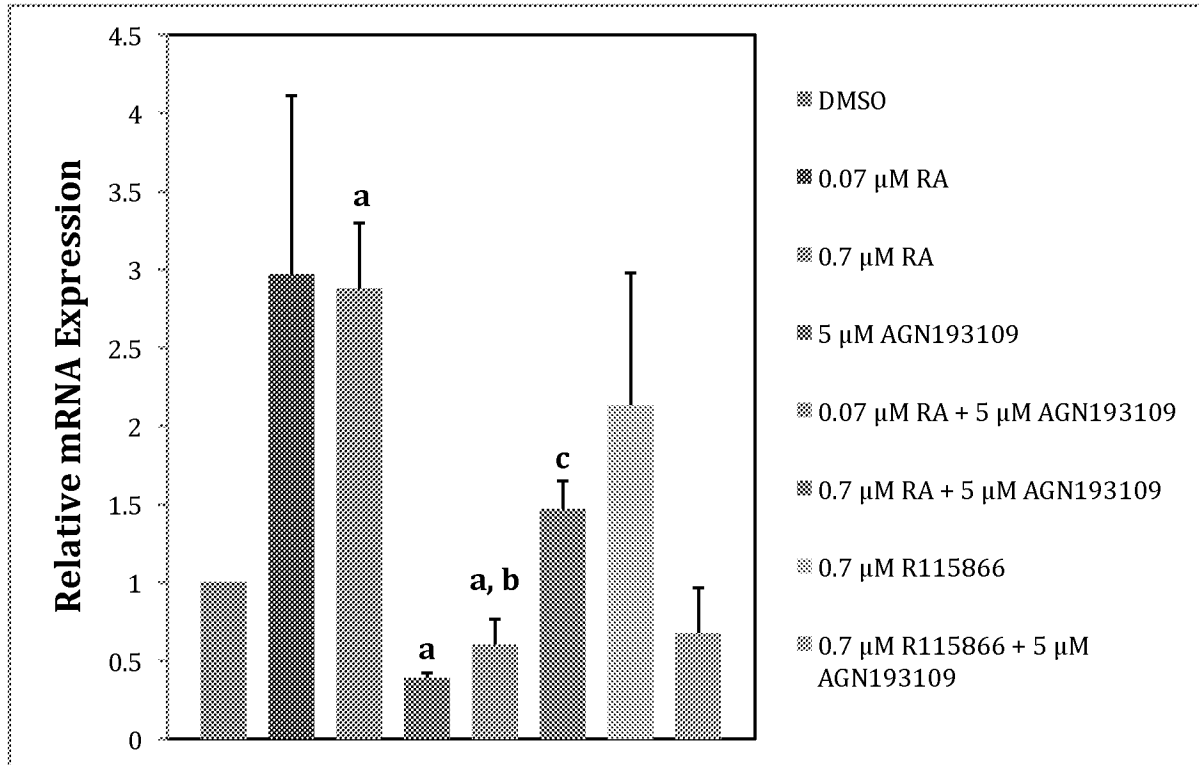
Figure 8: Relative *Cyp26b1* mRNA levels in primary cultured granulosa cells after 72-hour

treatments. *Cyp26b1* levels were measured as positive controls, as *Cyp26b1* is a retinoic acid

inducible gene [31]. DSMO was used as a vehicle control. Data are presented as mean +/- S.E.

a: $p < 0.05$ compared to DMSO, b: $p < 0.05$ compared to 0.07 μM retinoic acid, c: $p < 0.05$

compared to 0.7 μM retinoic acid.

n = 3-5.

Figure 9: Relative *Cyp19a1* mRNA levels in primary cultured granulosa cells after 24-hour

treatments. DMSO was used as a vehicle control. Data are presented as mean +/- S.E.

a: p<0.05 compared to DMSO, b: p<0.05 compared to 0.07 µM retinoic acid.
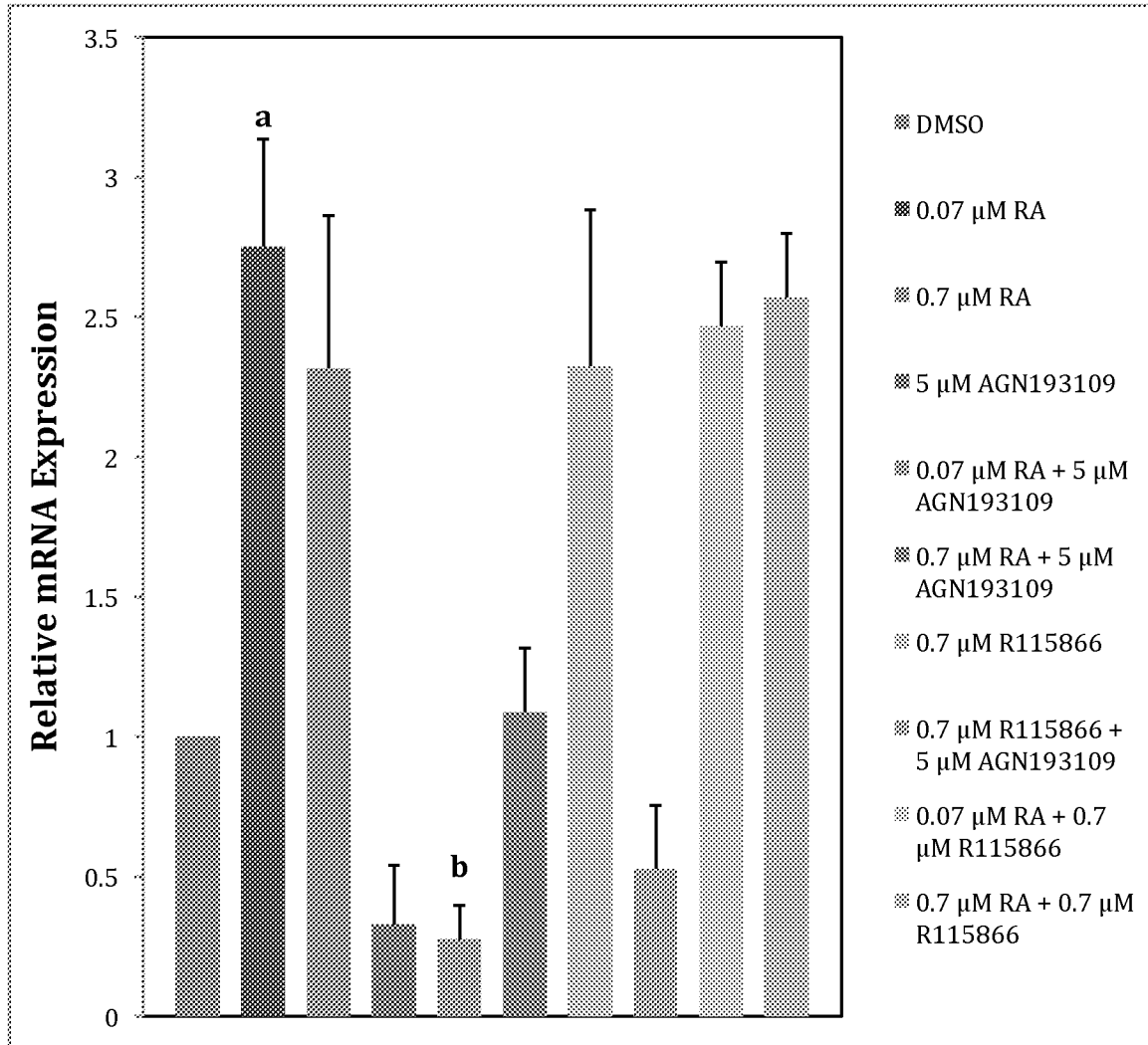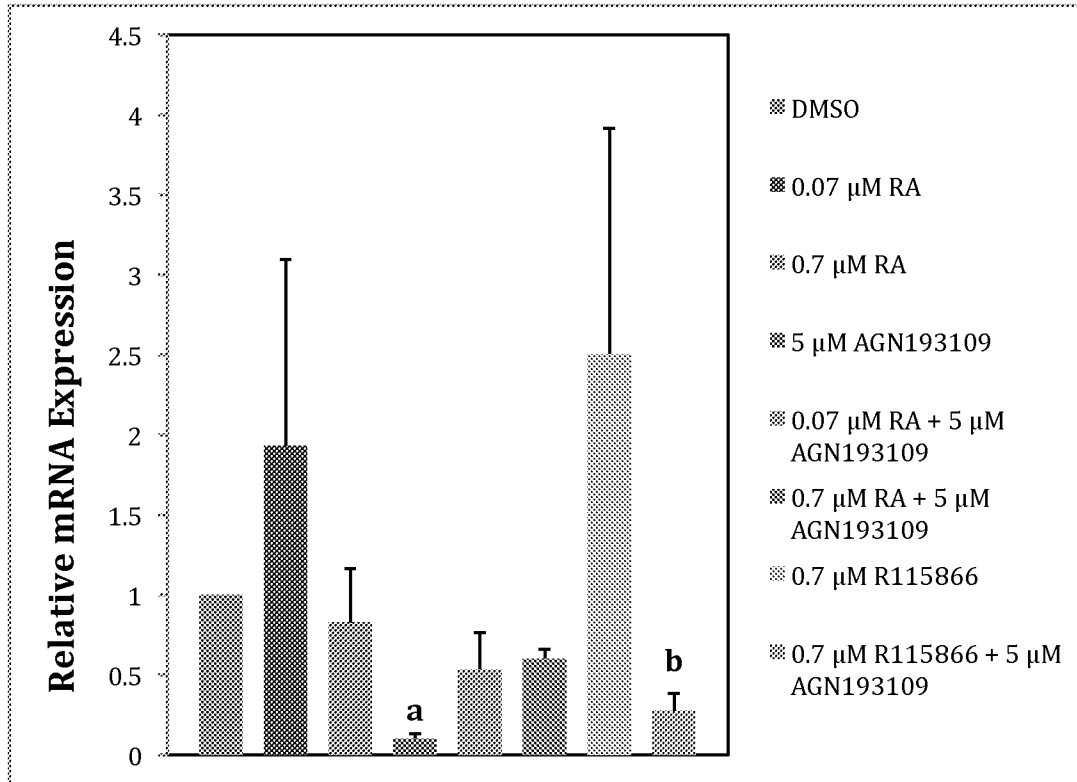
n = 3-5.

Figure 10: Relative *Cyp19a1* mRNA levels in primary cultured granulosa cells after 72-hour treatments. DSMO was used as a vehicle control. Data are presented as mean +/- S.E. a: $p < 0.05$ compared to DMSO, b: $p<0.05$ compared to 0.7 μM R115866. n = 3-5.

***Treatment Effects on the Protein Levels of Aromatase in Primary Cultured
Granulosa Cells***

Proteins were extracted from granulosa cells after 24- and 72-hour treatments. Western blotting

was used to measure aromatase expression after treatments. Relative band intensity from each

experiment was calculated by taking the ratio of aromatase band intensity over the band intensity

of the loading control, actin, to obtain fold changes. After 24-hours, treatment with 0.7 μM

retinoic acid plus 5 μM AGN193109 significantly suppressed aromatase protein levels when

compared to DMSO using an independent samples *t* test ($p < 0.05$) (Figures 11 A & B).

Furthermore, cells treated with 0.7 μM R115866 plus 5 μM AGN193109 had significantly lower

levels of aromatase protein than cells treated with 0.7 μM R115866 alone when compared using

a two sample *t* test ($p < 0.05$). Treatment with 5 μM AGN193109 alone did not suppress

aromatase protein expression compared to DMSO. Retinoic acid and R115866 stimulated

aromatase protein expression after 24-hour treatments, although these stimulatory effects were

not statistically significant. Treatment with 0.7 μM retinoic acid plus 5 μM AGN193109

appeared to inhibit the stimulatory effect in aromatase expression observed after treatment with

0.7 μM retinoic acid alone. After 72-hour treatments, retinoic acid and R115866 stimulated

aromatase protein expression, although these effects were not statistically significant (Figures 12

A & B). Treatment with 5 μM AGN193109 did not suppress aromatase protein levels compared

to DMSO, although trends indicate that treatment with either 0.07 μM retinoic acid plus 5 μM

AGN193109 or 0.7 μM R115866 plus 5 μM AGN193109 suppressed aromatase protein

expression compared to treatment with 0.07 μM retinoic acid or 0.7 μM R115866 alone,

respectively. Interestingly, it was observed that cells treated with 0.7 μM retinoic acid plus 5 μM

AGN193109 had increased aromatase protein levels compared to 0.7 μM retinoic acid, although

this effect was not statistically significant (p>0.05).

A)

| | DMSO | R115866 (0.7 μM) | RA (0.7 μM) | RA (0.07 μM) | AGN193109 (5 μM) | R115866 (0.7 μM) + AGN193109 (5 μM) | RA (0.7 μM) + AGN193109 (5 μM) | RA (0.07 μM) + AGN193109 (5 μM) |

Aromatase

Actin

B)

Legend:
- DMSO
- 0.7 μM R115866
- 0.07 μM RA
- 0.7 μM RA
- 5 μM AGN193109
- 0.7 μM R115866 + 5 μM AGN193109
- 0.07 μM RA + 5 μM AGN193109

Figure 11 A: An example picture of western blots showing aromatase and actin protein bands obtained from primary cultured granulosa cells after 24-hour treatments. Pictures are representatives of four experimental replicates. **10 B:** Quantification of aromatase protein levels in primary cultured granulosa cells after 24-hour treatments. Band intensity values were calculated by taking the ratio of aromatase band intensity over the band intensity of the loading control, actin, to obtain fold changes. DSMO was used as a vehicle control. Data are presented as mean +/- S.E. a: $p < 0.05$ compared to DMSO, b: $p<0.05$ compared to 0.7 μM R115866. n=4.

A)

| DMSO | R115866 (0.7 µM) | RA (0.07 µM) | RA (0.7 µM) | AGN193109 (5 µM) | R115866 (0.7 µM) + AGN193109 (5 µM) | RA (0.7 µM) + AGN193109 (5 µM) | RA(0.07 µM) +AGN193109 (5 µM) |

Aromatase

Actin

B)

Relative Intensity

6
5
4
3
2
1
0

- DMSO
- 0.7 µM R115866
- 0.07 µM RA
- 0.7 µM RA
- 5 µM AGN193109
- 0.7 µM R115866 + 5 µM AGN193109
- 0.07 µM RA + 5 µM AGN193109
- 0.7 µM RA + 5 µM AGN193109

Figure 12 A: An example picture of western blots showing aromatase and actin protein bands obtained from primary cultured granulosa cells after 72-hour treatments. Pictures are representatives of four experimental replicates. **B:** Quantification of aromatase protein levels in primary cultured granulosa cells after 72-hour treatments. Band intensity values were calculated by taking the ratio of aromatase band intensity over the band intensity of the loading control, actin, to obtain fold changes. DSMO was used as a vehicle control. Data are presented as mean +/- S.E. n = 4.

Aromatase Expression in MT-α Transgenic Mice

To investigate a possible *in vivo* regulation of aromatase expression by retinoic acid, whole ovaries from MT-α transgenic mice and their normal littermates (NLM) were used for analysis of *Cyp26b1* (to serve as an indicator of retinoic acid signaling) and *Cyp19a1* mRNA levels. It was observed that ovaries from 19-day MT-α mice had increased levels of *Cyp26b1* and decreased levels of *Cyp19a1* compared to their NLM when using an independent samples *t* test ($p < 0.05$) (Figure 13). In whole ovaries isolated from 7-week MT-α mice, *Cyp26b1* mRNA levels were decreased compared to their NLM ($p < 0.05$). *Cyp19a1* mRNA levels were not significantly different in MT-α mice compared to their NLM (Figure 14). Ovaries from 6-month MT-α mice also showed a statistically significant decrease in *Cyp26b1* mRNA levels compared to their NLM ($p < 0.05$), but no significant change in *Cyp19a1* mRNA levels (Figure 15). Western blot analysis was also used to investigate aromatase protein levels in 6-month MT-α mice. There was no significant difference in aromatase protein between 6-month MT-α mice and their NLM (Figures 16 A & B).

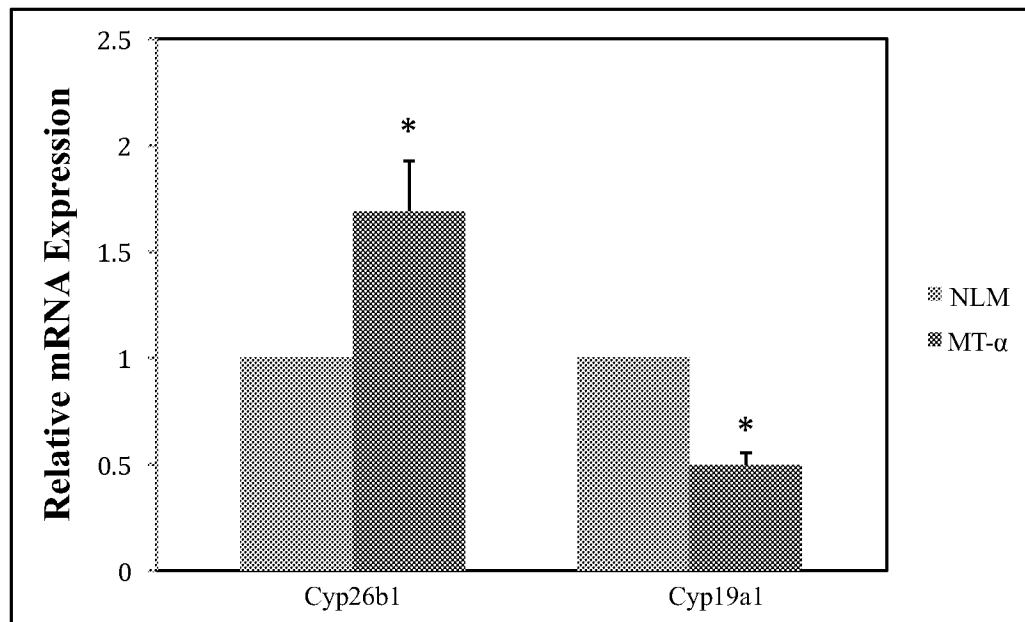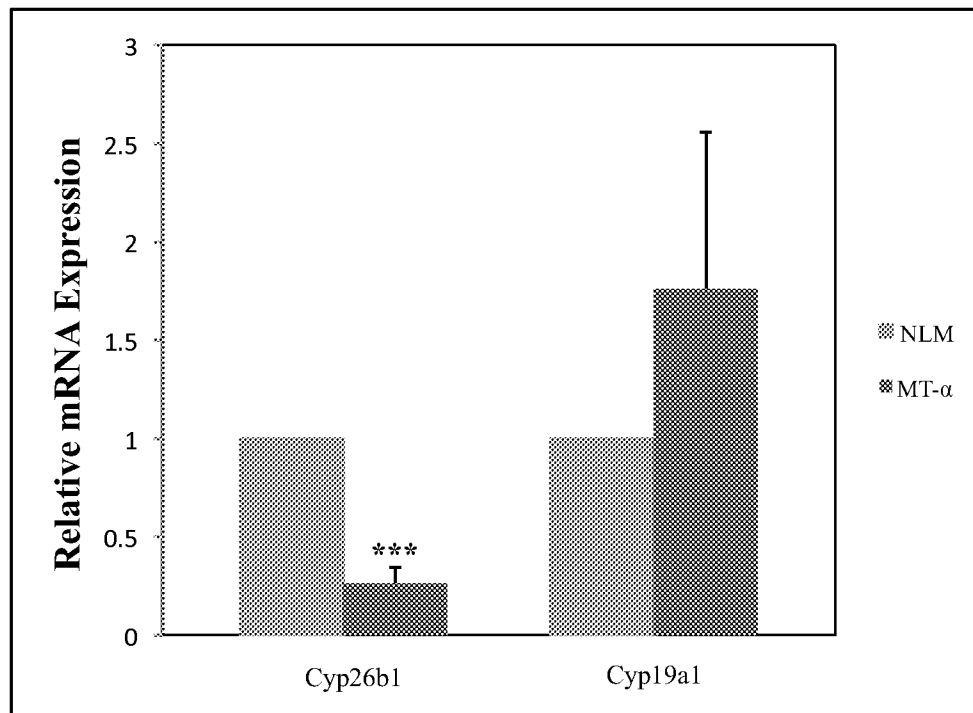**Figure 13:** Relative mRNA levels in whole ovaries from 19-day MT-α transgenic mice and their NLM. *p < 0.05, n = 3.

Figure 14: Relative mRNA levels in whole ovaries from 7-week MT-α transgenic mice and

their NLM.

*** p < 0.0005, n = 3.

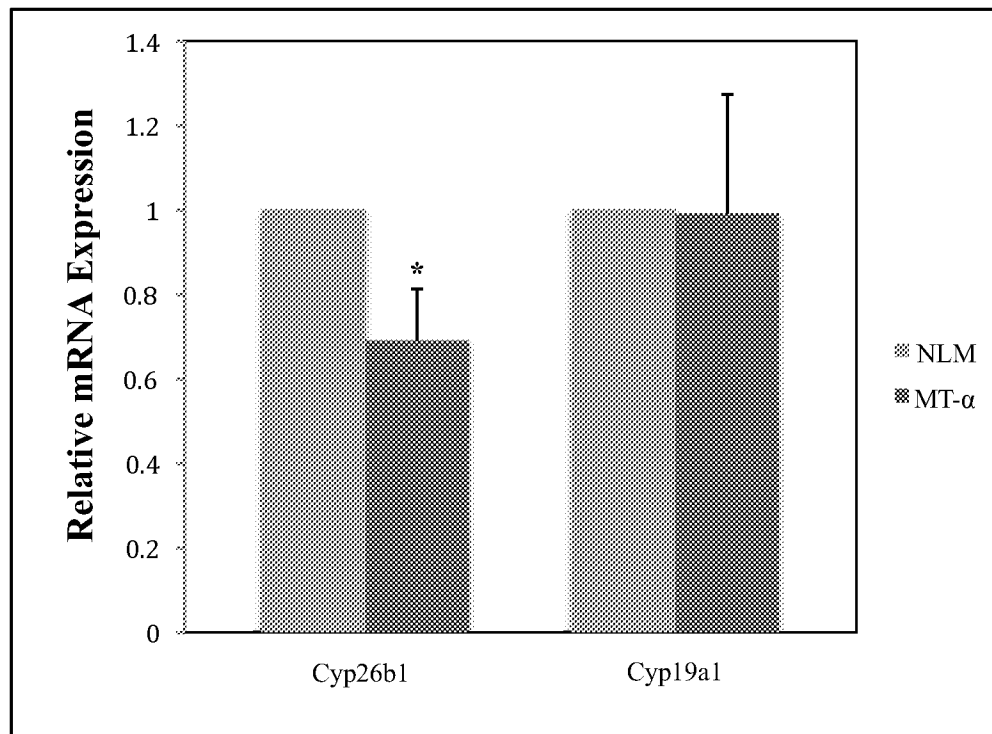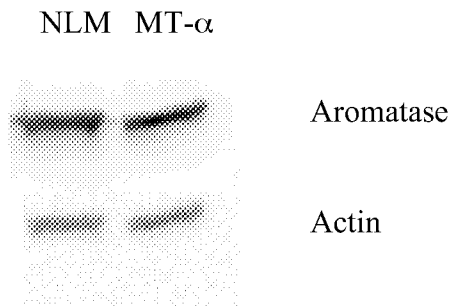**Figure 15:** Relative mRNA levels in whole ovaries from 6-month MT-α transgenic mice and

their NLM. *p < 0.05, n = 6.
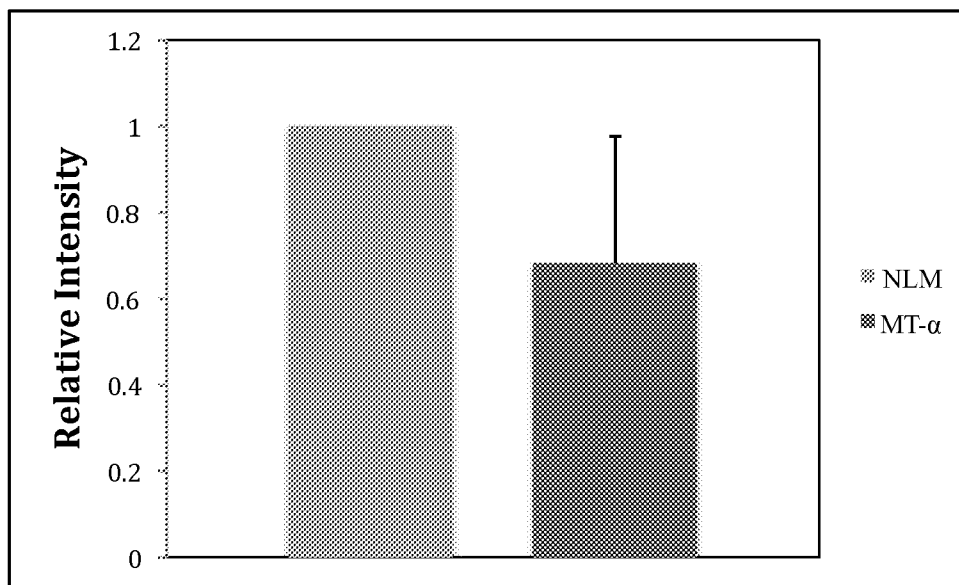
A)

NLM   MT-α

Aromatase

Actin

B)

Figure 16 A: Western blot pictures of aromatase and actin protein bands obtained from the

whole ovaries of 6-month MT-α mice and their NLM. Pictures are representatives of six

experimental replicates. **B:** Quantification of aromatase protein levels in whole ovaries from 6-

month MT-α transgenic mice and their NLM. n = 6.

Discussion

Previous research has indicated that retinoic acid regulates *Cyp19a1* expression in cancerous placental cells, breast cancer cells, and adipose breast tissue [15, 56, 57]. Furthermore, mice treated with an aromatase inhibitor exhibit similar ovarian pathologies as mice fed on a vitamin A deficient diet, suggesting a potential converging pathway [27, Unpublished Data]. This study demonstrates that retinoic acid induces *Cyp19a1* expression within the developing postnatal ovary *in vitro*. Retinoic acid stimulated a significant increase in *Cyp19a1* mRNA levels, and trends indicate that retinoic acid also stimulates aromatase protein levels.

Treatment Effects on the mRNA Levels of Cyp26b1 and Cyp19a1 in Primary Cultured Granulosa Cells

Retinoic acid induces *Cyp26b1* expression; therefore, *Cyp26b1* levels were measured for each treatment group in each experiment to confirm the success of treatments [31]. After 24-hour treatments, 0.07 µM retinoic acid increased *Cyp19a1* mRNA levels, and trends indicate that 0.7 µM retinoic acid also increased *Cyp19a1* mRNA levels. These results correlate with previous research in other tissues [15, 56, 57]. Furthermore, the stimulatory effect of retinoic acid was abolished in the cells treated with retinoic acid plus AGN193109. This observation suggests that the effect of retinoic acid was mediated through retinoic acid receptors, since AGN193109 is a pan-RAR inhibitor that prevents retinoic acid from binding to its cellular receptors [47]. Trends indicate that treatment with 5 µM AGN193109 alone led to a decrease in *Cyp19a1* mRNA levels.

R115866 also led to an increase in *Cyp19a1* mRNA levels after 24-hour treatments, although this stimulatory effect was not statistically significant. R115866 prevents retinoic acid degradation by inhibiting CYP26B1 [44]. Additionally, R115866 itself displays retinoid activity [44]. Therefore, the increase in *Cyp19a1* mRNA levels is consistent with the expected results. Not surprisingly, AGN193109 appeared to abolish this stimulatory effect when cells were co-treated with R115866, as AGN193109 blocked the RARs within the granulosa cells.

To examine if the effect of retinoic acid may be augmented in the presence of the CYP26 inhibitor, R115866, granulosa cells were also treated for 24-hours with R115866 plus retinoic acid. While not statistically significant, trends indicate that these treatments led to an increase in *Cyp19a1* mRNA levels. The increase in *Cyp19a1* mRNA levels was not statistically different from mRNA levels from cells treated with either retinoic acid or R115866 alone. This could mean that the induction in *Cyp19a1* expression seen after treatment with retinoic acid and R115866 was the maximal effect that can be exerted through the retinoic acid pathway. This would explain the lack of synergistic effect of the co-treatments of retinoic acid and R115866.

After 72-hour treatments, AGN193109 significantly suppressed *Cyp19a1* mRNA levels. Furthermore, treatment with R115866 plus AGN193109 significantly suppressed *Cyp19a1* mRNA levels compared to R115866. This suggests that *Cyp19a1* expression is regulated through RARs. Neither retinoic acid nor R115866 significantly influenced *Cyp19a1* mRNA levels, although trends indicate that either 0.07 µM retinoic acid or R115866 stimulate *Cyp19a1* mRNA levels. The decreased response at 72-hours versus 24-hours is probably related to the half-life of *Cyp26b1* mRNA or decreased sensitivity after long-term treatments. The half-life of *Cyp19a1*

mRNA is only 3-hours [59]. This short half-life could mean much of the mRNA had degraded by the 72-hour time point. If cells had adapted to retinoic acid stimulation, and the *Cyp19a1* mRNA degraded quickly, less *Cyp19a1* mRNA would be expected at the 72-hour time point. The suppression in *Cyp19a1* mRNA levels by AGN193109 implies that while cells may have become less sensitive to retinoic acid stimulation, retinoic acid still was regulating gene expression to an extent after 72-hours, and the inhibition in retinoic acid signaling by AGN193109 prevented *Cyp19a1* expression.

The large standard error values and lack of statistical significance in many treatment groups could be due to the instability of the compound. Retinoic acid isomerizes after exposure to light or oxygen [60]. While care was taken to prevent exposure to light, treating cells requires both light and oxygen exposure, which may have led to the breakdown of retinoic acid. In addition, the use of primary cultured cells leads to a heterogeneous cell population and, therefore, variability between experiments. This could be remedied by use of a cell line, which are more homogenous and would therefore exhibit less variation between experiments. Measuring aromatase mRNA and protein levels in a vitamin A deficient mouse model *in vivo* would directly demonstrate the effect of lack of retinoic acid signaling on aromatase expression without the potential degradation of retinoic acid that could be an issue *in vitro*.

Treatment Effects on the Protein Levels of Aromatase in Primary Cultured Granulosa Cells

Granulosa cells treated for 72-hours with 0.7 μM retinoic acid plus 5 μM AGN193109 had significantly decreased aromatase protein levels compared to DMSO, suggesting aromatase protein expression is regulated through RARs. No other treatment groups showed a significant change in relative aromatase protein levels compared to DMSO after 24- or 72- hour treatments. This could potentially be explained by the time it takes for *Cyp19a1* mRNA to be transcribed and for aromatase protein to be translated. After treatment with FSH, which is known to stimulate *Cyp19a1* expression, *Cyp19a1* mRNA levels in rat granulosa cells increase after 24-hours, and reach the maximum level after 48-hour treatments [61]. In these cells, aromatase enzyme activity is increased after 48-hour treatments, and the protein has a half-life of 28.2 hours [61, 62, 63]. Therefore, it could be that the 24-hour time period is too short to see much of a change in protein levels. It is also possible that retinoic acid regulates *Cyp19a1* expression at the mRNA level, but that it does not influence protein expression to the same extent, as protein expression is also tightly regulated, and upregulation of mRNA expression does not always lead to an upregulation of protein expression. On the other hand, trends do indicate that retinoic acid and R115866 stimulate aromatase protein expression, especially after 72-hour treatments. Large variation between experiments could have prevented the treatments from having statistically significant effects on aromatase expression. Furthermore, real time PCR is a more sensitive test than western blots, which could explain the more dramatic effects seen at the mRNA level. AGN193109 did suppress aromatase protein levels when combined with certain doses of retinoic acid or R115866 compared to these treatments alone, confirming results from mRNA measurements

Aromatase Expression in MT-α Transgenic Mice

Ovaries from MT-α transgenic mice were used to analyze *Cyp19a1* expression *in vivo*. Previous studies have indicated that ovaries from MT-α mice at 19-days have increased *Cyp26b1* mRNA and protein levels, suggesting decreased retinoic acid signaling in the ovary [28]. These results were confirmed in this study. In addition, at 19-days, *Cyp19a1* mRNA levels were decreased in MT-α mice compared to their NLM, suggesting that the decrease in retinoic acid signaling suppresses *Cyp19a1* mRNA expression, consistent with the major findings of this study. However, at 7-weeks, MT-α mice showed decreased levels of *Cyp26b1* mRNA, with no significant change in *Cyp19a1* levels. MT-α mice at 6-months also showed decreased *Cyp26b1* mRNA levels, but had no significant change in *Cyp19a1* mRNA or aromatase protein levels. Because retinoic acid stimulates *Cyp26b1* mRNA expression, it may be that after 19-days, increased *Cyp26b1* levels metabolize retinoic acid to such low levels that retinoic acid, in turn, no longer induces *Cyp26b1* expression later in life [31]. Long-term overexpresison of inhibin and potentially decreased retinoic acid signaling leads to significant ovarian pathologies, which could alter the gene expression of cells within the ovary [51, 52].

To further determine the effect of retinoic acid on aromatase expression *in vivo*, future studies could also analyze ovaries from mice fed on a vitamin-A deficient diet. A decrease in aromatase levels in vitamin A deficient mice compared to mice fed on a normal diet would imply that vitamin-A is necessary for normal aromatase expression. Mice could also be injected with R115866, which can increase endogenous retinoic acid levels, and ovaries from these mice could be investigated for changes in aromatase expression compared to control mice. An increase in

aromatase expression in these ovaries would indicate that retinoic acid stimulates aromatase expression *in vivo*.

It has previously been shown that retinoic acid influences aromatase levels in different tissues of the body. This study demonstrates that retinoic acid stimulates aromatase expression in granulosa cells of the mouse ovary. Retinoic acid was shown to play a role in regulation of *Cyp19a1* mRNA and aromatase protein at two different time-points. While future studies should continue investigating the role of vitamin-A on aromatase expression *in vivo*, this study does demonstrate one of many roles that retinoic acid plays in the developing ovary. Knowledge of retinoic acid's regulation of aromatase expression could be used in treatments for infertility, as well as in treatments for reproductive diseases such as polycystic ovarian syndrome, endometriosis, and breast cancer.

References

1. Chandra, A., C.E. Copen, E.H. Stephen. *Infertility and impaired fecundity in the United States, 1982-2010: Data from the national survey of family growth*, 2013. CDC.

2. Moura-Ramos, M., S. Gameiri, M.C. Canavarro, and I. Soares. *Assessing infertility stress: re-examining the factor structure of the fertility inventory*. Human Reproduction, 2012. **27**(2): 496-505.

3. Edson M.A., A.K. Nagaraja, M.M. Matzuk. *The mammalian ovary from genesis to revelation*. Endocrinology Review, 2009. **30**: p. 624-712

4. Lamb, M.M.M.D.J., *The biology of infertility: research advances and clinical challenges*. Nature Medicine, 2008. **14**: p. 1197-213.

5. Rivier, C. and S. Rivest. *Effect of stress on the activity of the hypothalamic-pituitary gonadal axis: peripheral and central mechanisms*. Biology of Reproduction, 1991. **45**(4): p. 523-32.

6. Britt, K.L., et al. *The ovarian phenotype of the aromatase knockout (ArKO) mouse*. Journal of Steroid Biochemistry and Molecular Biology, 2001. **79**: p. 181-5.

7. Richards, J.S. *Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation*. Physiology Review, 1980. **60**(1): p. 51–89

8. Shelling, A.N. *Mutations in inhibin and activin genes associated with human disease*. Molecular and Cellular Endocrinology, 2012. **359**(1-2): p. 113-20.

9. Kipp, J.L., et al. *Activin regulates estrogen receptor gene expression in the mouse ovary*. The Journal of Biological Chemistry, 2007. **282**(50): p. 36755-65.

10. Findlay, J.K. *An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis*. Biology of Reproduction, 1993. **48**: p. 15-23.

11. Hillier, S.G. *Regulatory functions for inhibin and activin in human ovaries*. Journal of Endocrinology, 1991. **131**: p. 171-5.

12. Munz, W., et al. *Serum inhibin A, inhibin B, pro-alphaC, and activin A levels in women with idiopathic premature ovarian failure*. Fertility and Sterility, 2004. **82**(3): p. 760-2.

13. Healy, D.L., et al. *Elevated serum inhibin concentrations in postmenopausal women with ovarian tumors*. The New England Journal of Medicine, 1993. **329**: p. 1539-42.

14. Ramachandran, A., et al. *Activin is a potent growth suppressor of epithelial ovarian cancer cells*. Cancer Letters, 2009. **285**(2): p. 157-65.

15. Zhu, S.J., et al. *Retinoic acids promote the action of aromatase and 17β hydroxysteroid dehydrogenase type 1 on the biosynthesis of 17β-estradiol in placental cells.* Journal of Endocrinology, 2002. **172**: p. 31-43.

16. Nothnick, W.B. *The emerging use of aromatase inhibitors for endometriosis treatment.* Reproductive Biology and Endocrinology, 2011 **9**(87).

17. Turner, K.J., et al. *Development and validation of a new monoclonal antibody to mammalian aromatase.* Journal of Endocrinology, 2002. **172**(1): p. 21-30.

18. Stocco, C. *Tissue physiology and pathology of aromatase.* Steroids, 2013. **77**(1-2): p. 27-35.

19. Demura, M. and S.E. Bulun. *CpG dinucleotide methylation of the CYP19 I.3/II promoter modulates cAMP-stimulated aromatase activity.* Molecular and Cellular Endocrinology, 2008. **283**(1-2): p. 127-32.

20. Stocco, C. *Aromatase expression in the ovary: Hormonal and molecular regulation.* Steroids, 2008. **73**(5): p. 473-87.

21. Kwintkiewics, J., Z. Cai, and C. Stocco. *Follicle-stimulating hormone induced activation of Gata4 contributes in the up-regulation of Cyp19 expression in rat granulosa cells.* Molecular Endocrinology, 2007. **21**(4): p. 933-47.

22. Bennett, J., et al. *Loss of GATA-6 and GATA-4 in granulosa cells blocks folliculogenesis, ovulation, and follicle stimulating hormone receptor expression leading to female infertility.* Endocrinology, 2012. **153**(5): 2474-85.

23. Park, Y., et al. *Induction of cyclin D2 in rat granulosa cells requires FSH-dependent relief from FOXO1 repression coupled with positive signals from smad.* Journal of Biological Chemistry, 2005. **280**(10): p. 9135-48.

24. Bentsi-Barnes, I.K., et al. *Human forkhead L2 represses key genes in granulosa cell differentiation including aromatase, P450scc, and cyclin D2.* Fertility and Sterility, 2010. **94**(1): p. 353-6.

25. Adashi, E.Y., Hsueh, A.J.W. *Estrogens augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured rat granulosa cells.* Journal of Biological Chemistry, 1982. **257**: 6077-83.

26. Wu, Y-G., et al. *Testosterone, not 5α-Dihydrotestosterone, stimulates LRH-1 leading to FSH-independent expression of Cyp19 and P450scc in granulosa cells.* Molecular Endocrinology, 2011. **25**(4): p. 656-68.

27. Maliqueo, M., et al. (2013). *Continuous administration of a P450 aromatase inhibitor induces polycystic ovary syndrome with a metabolic and endocrine phenotype in female rats at adult age.* Endocrinology, 2013. **154**(1): p. 434-45.

28. Kipp, J.L., et al. *Gene expression profiling reveals Cyp26b1 to be an activin regulated gene involved in ovarian granulosa cell proliferation.* Endocrinology, 2011. **152**(1): p. 303-12.

29. Zhang, D., et al.. *Retinoids and ovarian cancer.* Journal of Cellular Physiology, 2000. **185**: p. 1–20.

30. Moise, A.L., et al. *Topology and membrane association of Lecithin: retinol acyltransferase.* Journal of Biological Chemistry, 2007. **282**: p. 2081-90.

31. Rhinn, M. and P. Dolle. *Retinoic acid signaling during development.* Development, 2012. **139**(5): p. 843-58.

32. Olson, J.A. *Serum levels of vitamin A and caretenoids as reflectors of nutritional status.* Journal of the National Cancer Institute, 1984. **73**(6): p.1439-44.

33. Maden, M. *Retinoid signalling in the development of the central nervous system.* Nature Reviews Neuroscience. **3**: p. 843-853.

34. Balmer, J. E. *Gene expression regulation by retinoic acid.* The Journal of Lipid Research, 2002. **43**(11): p. 1773-808.

35. White J.A., et al. *Identification of the human cytochrome P450, P450RAI-2, which is predominantly expressed in the adult cerebellum and is responsible for all-trans-retinoic acid metabolism.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(12): p. 6403-8.

36. Taimi, M., et al. *A novel human cytochrome P450, Cyp26C1, involved in metabolism of 9-cis and all-trans isomers of retinoic acid.* Journal of Biological Chemistry, 2004. **290**(2): p. 77-85.

37. Reijntjes, S., et al. *The control of morphogen signaling: Regulation of the synthesis and catabolism of retinoic acid in the developing embryo.* Developmental Biology, 2005. **285**(1): p. 224-37.

38. Bowles, J., et al. *Retinoid signaling determines germ cell fate in mice.* Science, 2006. **312**(5773): p. 596-600.

39. Hand, J.L. *Retinoic acid: A familiar teratogen.* Pediatric Dermatology, 2012. **29**(6): p. 774-6.

40. Lee, L.M.Y., et al. *A paradoxical teratogenic mechanism for retinoic acid.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(34): p. 13668-73.

41. Li, H. and M. Clagett-Dame. *Vitamin A deficiency blocks the initiation of meiosis of germ cells in the developing rat ovary in vivo.* Biology of Reproduction, 2009. **81**(5): 996-1001.

42. Livera, G., et al. *Effects of retinoids on the meiosis in the fetal rat ovary in culture.* Molecular and Cellular Endocrinology, 2000. **165**(1-2): p. 225-31.

43. Naillat, F., et al. *Wnt4/5a signalling coordinates cell adhesion and entry into meiosis during presumptive ovarian follicle development.* Human Molecular Genetics, 2010. **19**(8): p. 1539-50.

44. Stoppie, P., et al. *R115866 inhibits all-trans-retinoic acid metabolism and exerts retinoidal effects in rodents.* The Journal of Pharmacology and Experimental Therapeutics, 2000. **293**(1): p. 304-12.

45. Pavez, L., et al. *Topical treatment with CYP26 inhibitor talarozole (R115866) dose dependently alters the expression of retinoid-regulated genes in normal human epidermis.* British Journal of Dermatology, 2009. **160**: p. 26-36.

46. Ocaya, P., et al. *Cyp26 inhibitor R115866 increases retinoid signaling in intimal smooth muscle cells.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2007. **27**: p. 1542-1548.

47. Johnson, A.T., et al. *Synthesis and characterization of a highly potent and effective antagonist of retinoic acid receptors.* Journal of Medicinal Chemistry, 1995. **38**(24): p. 4764-7.

48. Virtanen, M., A. Sirsjö, A. Vahlquist, and H. Törmä. *Keratins 2 and 4/13 in reconstituted human skin are reciprocally regulated by retinoids binding to nuclear receptor RARα.* Experimental Dermatology, 2010. **19**: 674–681.

49. Manikam, S.T. and J. Stanslas. *Andrographolide inhibits growth of acute promyelocytic leukaemia cells by inducing retinoic acid receptor-independent cell differentiation and apoptosis.* Journal of Pharmacy and Pharmacology, 2009. **61**: p. 69-78.

50. Lee, Y.J., et al. *Growth differentiation factor 11 signaling controls retinoic acid activity for axial vertebral development.* Developmental Biology, 2010. **347**(1): p. 195-203.

51. McMullen, M.L., et al. *Gonadal pathologies in transgenic mice expressing the rat inhibin α-subunit.* Endocrinology, 2001. **142**(11): p. 5005-5014.

52. Cho, B-N, et al. *Reproductive deficiencies in transgenic mice expressing the rat inhibin α-subunit gene.* Endocrinology, 2001. **142**(11): p. 4994-5004.

53. Bulun S.E., et al. *Review role of aromatase in endometrial disease.* The Journal of Steroid Biochemistry and Molecular Biology, 2001. **79**(1-5): p. 19-25.

54. Franks, S. *Polycystic ovary syndrome.* New England Journal of Medicine, 1995. **333**(13): p. 853-61.

55. Bulun S.E., et al. *Review Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment.* Pharmacological Reviews, 2005. **57**(3): p. 359-83.

56. Wilde, J. et al. *Aromatase activity induction in human adipose fibroblasts by retinoic acids via retinoic acid receptor α.* Journal of Molecular Endocrinology, 2013. **51**: p. 247-60.

57. Mu, Y., et al. *A nuclear receptor system constituted by RAR and RXR induces aromatase activity in MCF-7 human breast cancer cells.* Molecular and Cellular Endocrinology, 2000. **166**(2): p. 137-45.

58. Livak, K.J.a.S., T. D, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**: p. 402-8.

59. Sahmi, M., E.S. Nicola, and C.A. Price. *Hormonal regulation of cytochrome P450 aromatase mRNA stability in non-luteinizing bovine granulosa cells in vitro.* Journal of Endocrinology, 2006. **190**(1): p. 107-15.

60. Olsen, J.A. *The biosynthesis and metabolism of carotenoids and retinol (Vitamin A).* Journal of Lipid Research, 1964. **5**: p. 281-99.

61. Fitzpatrick, S.L., and J.S. Richards. *Regulation of cytocrhome P450 aromatase messenger ribonucleic acid and activity by steroids and gonadotropins in rat granulosa cells.* Endocrinology, 1991. **129**(3): p. 1452-622.

62. George, F.W. and S.R. Ojeda. *Vasoactive intestinal peptide enhances aromatase activity in the neonatal rat ovary before development of primary follicles or responsiveness to follicle stimulating hormone.* Proceedings of the National Academy of Sciences of the United States of America, 1987. **84**: p. 5803-5807.

63. Wang, X. and S. Chen. *Aromatase destabilizer: novel action of exemestane, a Food and Drug Administration-approved aromatase inhibitor.* Cancer Research, 2006. **66**(21): 10281-6.

64. Pierzchalski, K., et al. *Retinoic acid biosynthesis is impaired in human and murine endometriosis.* Biology of Reproduction, 2014. **91**(4): p. 1-9.

65. Pauli, S.A., et al. *Analysis of follicular fluid retinoids in women undergoing in vitro fertilization: retinoic acid influences embryo quality and is reduced in women with endometriosis.* Reproductive Sciences, 2013. **20**(9): p. 1116-24.