Roles of Retinoic Acid and Cyp26 Inhibitors in Ovarian Follicle Development

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Roles of Retinoic Acid and Cyp26 Inhibitors in Ovarian Follicle Development

A Thesis
Presented in
Partial Fulfillment of the
Requirements for the Degree of
Master of Science

August, 2014

BY
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Introduction

The ovaries are the female reproductive organs that are located on either side of the uterus. They contain eggs and secrete hormones that are critical for female reproduction. Each follicle consists of an egg at the center, surrounded by multiple layers of somatic cells. These eggs undergo various stages of development wherein the follicular structure matures as a whole; the hormones and growth factors secreted by the somatic cells are essential for the maturation of the egg while the signaling molecules produced by the egg are crucial for somatic cell proliferation and follicle maturation and development.

Thus, mammalian ovarian follicle formation and development involves the establishment of the initial follicle pool, follicle growth, proper maturation of eggs, and timely production and release of hormones and the mature eggs. This process is essential for propagation of the species as well as for development and homeostasis of the female reproductive system[1]. Abnormalities in the regulation and development of ovarian follicles such as inadequate maturation of the follicle due to inadequate cell-cell interactions and signaling between the somatic cells and the egg can lead to infertility and ovarian diseases, for instance Premature Ovarian Failure (POF), Polycystic Ovary Syndrome (PCOS) and ovarian cancer.

These diseases lead to a loss of fertility due to the formation of immature or unhealthy follicles and/or oocyte development. Both intraovarian and extraovarian factors play a role in regulating the development of follicles. However, factors involved in follicular growth and survival are not well defined. Recently, studies have suggested that the
potent morphogen retinoic acid (RA) plays a role in the development of a healthy ovary[1, 2]. However, the underlying mechanism is not understood. Germ cells in the fetal ovary undergo sex-specific entry into meiosis, the initiation of which is thought to be mediated by selective exposure of fetal ovarian germ cells to retinoic acid[2]. Nevertheless, the timing and regulation of meiosis vary amongst the sexes. It was determined that high levels of RA were necessary for initiation of meiosis in the ovary and the testes; however, while RA is required for germ cell development in the embryonic ovary, it is required during the juvenile and adult stages of testicular functioning[3, 4]. Cyp26b1, a member of the Cyp450 family of enzymes degrades retinoic acid in the ovary and the testes thereby limiting gonadal development [1, 3, 5]. However, information about the role of RA and Cyp26b1 in the gonads is limited. It is therefore necessary to further elucidate the roles of both RA and Cyp26b1 in ovarian and follicle development.

The purpose of this study is to examine the roles of Cyp26b1 and RA in mouse ovarian follicle development. This will be done by performing in vitro follicle culture and ovary culture with treatments including retinoic acid, the Cyp26 inhibitor R115866 and the RA metabolism inhibitor Liarozole and monitoring their effects on follicular and ovary growth. We expect the findings from this study to contribute significantly to infertility treatments and disease prevention, as being able to successfully grow follicles in vitro could help the large population of women whose difficulty with conceiving is due to an immature and underdeveloped follicle pool.
Review of Background literature

Ovarian follicle development in mammals: The ovarian follicle is the functional unit of the ovary. It consists of somatic cells that surround an oocyte. The primary role of the follicle is to support oocyte development and to produce hormones that control the female reproductive cycle. The follicles undergo a process known as folliculogenesis which describes the development and maturation of ovarian follicles growing from an immature primordial stage into larger more developed follicular stages that ultimately leads to ovulation (Table 1). At birth, human ovaries contain a fixed number of immature, primordial follicles [62]. After puberty and coinciding with menarche, a small number of follicles begin to mature to larger follicles that will end in either death or ovulation. During post-pubertal follicular development, primordial follicles that have begun development undergo a multitude of changes that are morphological as well as hormonal and are essential for ovulation. This includes the primordial follicles transitioning to primary, secondary, tertiary, and antral stages of development. Tertiary and antral follicles depend on hormones that are circulated in the blood such as estrogen, progesterone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) causing a substantial increase in their growth and development. During each menstrual cycle, only one follicle is selected for ovulation. This follicle ruptures and discharges the oocyte ending its folliculogenesis[6] (Figure 1).

During folliculogenesis, the somatic cells differentiate into granulosa cells and theca cells. Both these cell types are essential for follicle growth and development. Thecal cells appear as the follicles enter into a more advanced stage of development such as
the tertiary stage. They produce androstenedione that is used as a substrate by
granulosa cells to produce estradiol, under the influence of FSH. Theca cells also
contain LH receptors while granulosa cells contain FSH receptors. The hormones
produced by these cells as well as the receptors present on them are essential for cell
interactions within the follicles that are necessary for follicle development and growth.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial</td>
<td>Small and have an oocyte surrounded by a single layer of squamous granulosa cells.</td>
</tr>
<tr>
<td>Primary</td>
<td>An enlarged oocyte surrounded by either a single layer of cuboidal granulosa cells or a single layer of mixed cuboidal and squamous granulosa cells.</td>
</tr>
<tr>
<td>Secondary</td>
<td>An enlarged oocyte surrounded by two or more layers of cuboidal granulosa cells but have no fluid-filled antrum.</td>
</tr>
<tr>
<td>Tertiary</td>
<td>An oocyte surrounded by layers of cuboidal granulosa cells that contain one or more small antra.</td>
</tr>
<tr>
<td>Antral</td>
<td>An oocyte enclosed by cumulus cells and a single large antrum surrounded by a single layer of cuboidal granulosa cells.</td>
</tr>
</tbody>
</table>

*Table 1: Different stages of follicle development in the mammalian ovary [7]*
FIG. 1: Folliculogenesis- Maturation and development of follicles until ovulation.

Ovarian follicles undergo morphologic and endocrinologic changes during the menstrual cycle. The follicles form from Primordial germ cells that undergo various stages of development that include the earlier stages of development that include the primary and secondary stages of development and the more advanced stages of follicle development like the tertiary and antral stages of follicle development before the oocyte is mature enough to be released from the follicular structure, while the follicle undergoes degradation to form the corpus luteum. *This image is adapted from: Lamb, M.M.M.D.J., Nature Medicine, 2008 [7]*
Ovarian diseases: There are several diseases that affect the ovary. These diseases lead to low ovarian stimulation that results in an immature oocyte pool that require a regimen that includes hormones, growth factors and vitamins to support the growth and development of the immature oocytes.

Among these diseases, polycystic ovary syndrome (PCOS), premature ovarian failure (POF) and ovarian cancer are three major diseases that affect a significant number of women and are the leading causes of infertility. PCOS is a common cause of anovulation and infertility among women of reproductive age. Women are usually diagnosed when in their 20s or 30s, but polycystic ovary syndrome may also affect teenage girls. The symptoms often begin when a girl's periods start [8]. It is a condition in which there is an imbalance of a woman's sex hormones. Hormones help regulate the normal development of eggs in the ovaries during each menstrual cycle. Hormonal imbalances which include excessive production of LH and testosterone result in menstrual irregularities and excessive production of testosterone also leads to acne and hirsuitism. Most, but not all, women suffering from this disorder have enlarged and containing numerous small cysts located along the outer edge of each ovary (polycystic appearance). These cysts are immature follicles that are developmentally arrested in the early antral stage due to the hormonal imbalance. These changes also contribute significantly to infertility.

POF refers to a loss of normal function of ovaries before the age of 40 which causes infertility in these patients[9]. The ovaries fail to produce normal amounts of the hormone estrogen or release eggs regularly. Infertility is a common result. This
decreased functional capacity of the ovary which is also known as ovarian hypofunction, may be caused by genetic factors such as chromosome abnormalities, or it may occur with certain autoimmune disorders that disrupt normal ovarian function[10].

Ovarian cancer is a cancerous growth arising from the ovary. Ovarian cancer is the fifth largest cause for death in women due to cancer. It has a high mortality rate due to the fact that it can only be detected at the late stage of cancer development. Measures taken to treat ovarian cancer greatly reduce the amount of the hormones estrogen and progesterone circulating in the body and affect follicle maturation and growth, which causes infertility[11].

As previously mentioned, low ovarian stimulation is often the cause of these disorders and therefore, conventional methods used to assist reproduction aren’t successful. Thus, these women need to resort to in vitro fertilization wherein an egg is fertilized by the sperm in vitro, or outside the body. This method involves the removal of a healthy and mature ovum from the woman’s ovaries to allow for successful fertilization by a sperm in a laboratory setting. However, women suffering from these diseases suffer from an immature oocyte pool and these oocytes require in vitro maturation in order to be developmentally competent for successful fertilization. Thus, traditional IVF protocols need to be modified in order to incorporate hormones, growth factors and vitamins in the medium, which are essential to bolster the competence of the immature oocytes for fertilization and embryogenesis[12].
Retinoic Acid: All trans retinoic acid, the active derivative of Retinol, is a naturally occurring retinoid responsible for growth and differentiation in mammals. Retinol, which is also known as Vitamin A is found in various foods such as carrots and spinach. It is believed that retinol, the form of retinoic acid that is found in the plasma, is converted to RA when taken up by the cells. RA can either act as a paracrine or autocrine molecule. In the paracrine model, RA is produced in one cell but exerts its effects in another target tissue, or is taken up by a non-target tissue where it is metabolized. In the autocrine model, RA is produced in the same cell where it exerts its action and is metabolized[3, 13].

RA acts as a signaling molecule and exerts its activity through binding with transcription regulatory factors, known as the RA receptors including RAR-α, RAR-β and RAR-γ. These receptors are located in nucleus. Upon binding to the RARs, the receptor-ligand complex then binds with the Retinoid X receptors (α, β, γ) to form a heterodimer that binds to the Retinoic Acid Regulatory Element within the DNA, which stimulates a cascade of events that results in the recruitment of transcriptional coactivators and can either repress or activate the transcription of target genes that encode for proteins involved in cell differentiation and development [14](Figure 2). 532 genes have been identified to be regulatory targets of RA, which include Foxa1, Egr1, Tgm2, Pck1 that are involved in differentiation and growth [67].

Although RA is required for development, it is not produced by the cells of the body at all times. Retinol is secreted by the liver and is transported to the blood via serum retinol-binding protein (RPB4) for conversion to RA. The cells also contain retinol-
binding proteins (CRBPS) that facilitate the oxidation of retinol to retinaldehyde by RDH while RALDH is responsible for the conversion of retinaldehyde to RA[14](Figure 2).

**FIG. 2: Mechanism of Retinoid signaling.** *Retinol is taken up from the blood and binds to the cellular retinol binding proteins present in the cytoplasm of the cells. Retinol dehydrogenase metabolizes retinol to retinal and retinal is oxidized to retinoic acid via cellular retinoic acid binding protein. Retinoic acid then enters the nucleus and binds to retinoic acid receptors RAR and RXR which heterodimerize and bind to the Retinoic Acid Response Element (RARE) in the DNA which turns on the transcription of genes involved in growth and differentiation[15].*
Retinoic acid is required for various biological processes such as maintaining healthy skin, regulation of apoptosis, maintenance and regulation of the immune system and the development of the placenta and embryo development. In addition to its vital role in development, RA is often used to treat patients with severe acne (due to its beneficial effects on the skin), psoriasis and certain kinds of leukemias.

Studies have shown that a slight increase in RA may lead to malformations including cleft palate, neural tube defects, heart defects[16] and limb malformations[13, 17]. On the other hand, Vitamin A deficiency decreases immunity due to impaired T cell-dependent antibody responses[18] as well as a decrease in the number and function of the natural killer cells[18, 19]. This can lead to death due to infection. RA is also required for the maintenance of proper brain function and a decrease in RA leads to memory loss and increased risk of Alzheimer's disease[20, 21].

The levels of Vitamin A within the cells are tightly regulated. If the levels become too high, it is degraded by the Cyp26 enzymes, a member of the Cyp450 family of enzymes. Cyp26a1 is present in adult and embryonic tissues and is responsible for the degradation of RA in cells such as those that make up the nervous system, the liver and skin cells, to bring it back to threshold levels. When the levels of Vitamin A drop too low, the Fibroblast growth factors (FGF) prevent the rapid degradation of RA[22]. Thus, both Cyp26a1 and FGF work in concert to keep RA concentrations at optimal levels.

*Role of Retinoic acid in the gonads:* The development of mammalian fetal germ cells along spermatogenic and oogenic pathways is controlled by signals from the
surrounding gonadal environment. Later stages of ovarian follicle development are regulated by multiple hormones, including FSH and LH that are secreted by the pituitary gland. However, a multitude of autocrine and paracrine non endocrine molecules such as C-type natriuretic peptides, insulin growth factor-1, the TGF-β family as well as fibroblast growth factors and Wnts are also essential for the maintenance of optimal follicle growth [23, 66]. An additional growth factor that has been suggested to play a role in ovarian development is retinoic acid[3].

Primordial germ cells are the embryonic precursors of sperm and eggs in the adult organism. The initiation of meiosis is thought to be mediated by selective exposure of the fetal ovarian germ cells to retinoic acid[24]. This was determined by detecting the expression of retinoid synthesizing enzymes Aldh1a2 and Aldh1a3 in fetal mouse ovaries. The expression of genes encoding RAR and RXR proteins in the ovary with localization in the nucleus further indicates the role of RA signaling in germ cell development which may be responsible for the regulation of germ cell survival or proliferation[25]

RA induces entry into meiosis in the ovary at around 13.5 days postnatal. The germ cells in the mouse ovary then stop proliferating and enter into their first meiotic division. Studies performed previously show that exogenous RA added to rat XX germ cells in ex vivo cultures accelerates their entry into meiosis, confirming the role of RA in meiosis in the mouse ovary[2, 26]. In addition, previous studies conducted in oocytes isolated from mice show that oocytes treated with RA have significantly higher meiotic competence than the control group[12]. Unlike extensive studies carried out on mouse testes, the
absence of RA and the effects of its degradation on meiosis in the ovaries have not been elucidated.

Entry into meiosis is sex-specific with respect to the timing where male mice undergo meiosis postnatally, wherein it begins at the time of reproductive development and continues until puberty and throughout adulthood. Studies have indicated that the role of RA in the reproductive health of the male is crucial and it can be detected at all times in the testis except during embryonic development. Spermatogenic arrest is observed in adult males in the absence of RA. Cyp26b1 was also found to catalyze the degradation of RA which led to the inhibition of meiosis in the embryonic testis [4, 5, 27].

It has been demonstrated that an increase of antioxidants in culture and maturation medium or embryo culture in a reduced $O_2$ atmosphere could assist in vitro survival of embryos in a variety of species. A number of studies have shown that retinoids participate in a biological anti-oxidant network and are important regulators of redox signaling pathways. Retinol derivatives can quench oxygen molecules and interact with other antioxidant compounds. It has been shown that RA could protect oocytes against oxidative stress induced by apoptosis[63, 64].

More specifically, Cyp26b1 mRNA and protein levels of Cyp26b1 were detected in primordial follicles in different stages of follicle development at the different postnatal development stages, namely days 1, 6, 10 and 20, with higher levels in the testis than the ovaries. In addition, it was seen that RA and the Cyp26b1 inhibitor, R115866, significantly stimulated the proliferation of granulosa cells dose dependently[1]. In order to study the direct effects of Cyp26b1, cells were transfected so as to overexpress
Cyp26b1. When a transient transfection efficiency of 60-90% of Cyp26b1 was achieved in granulosa cells (which was confirmed by detecting protein levels via Western Blot), a decrease in living granulosa cell numbers and an increase in apoptotic cells was observed. Conversely, an experiment was performed wherein Cyp26b1 expression was knocked down in granulosa cells using siRNA. A decrease in Cyp26b1 expression promoted granulosa cell proliferation, which was detected by quantifying BrdU incorporation in the cells. These studies further elucidate the importance of RA in ovary development, which provide credence to further studies in the field (unpublished data).

Imbalances, Dysfunctions and the Importance of RA signaling: In order to avoid both deficiency and toxicity it is important to maintain the levels of RA within a narrow range. Addition of Vitamin A in embryos by the implantation of RA impregnated beads has been demonstrated to induce teratogenic effects and alterations in organogenesis [28]. However, rather than studying the effects of teratogenicity of RA to determine its physiological role, loss-of-function studies are more valuable in determining the normal function of RA during organogenesis.

Vitamin A deficiency in male rats causes germ cell degeneration while female rats fed with a Vitamin A deficient diet were unable to reproduce [29]. When RA is added in an organ culture system, it decreases apoptosis and increases the number of healthy oogonia, oocytes and primordial follicles. Fetal ovaries cultured without RA do not show DNA replication. These studies confirm that RA is in fact a potent stimulator of germ cell
proliferation[30]. Therefore, RA is a powerful survival factor and is a mitogen that also functions as an antiapoptotic and pro-stimulatory factor in female germ cells[30].

ALDH1 (aldehyde dehydrogenase I) is a member of the aldehyde dehydrogenase family member and is responsible for the oxidation of aldehydes to acids. It is responsible for irreversible oxidation of retinal to retinoic acid. ALDH1 expression is highest in normal human and mouse ovary and was found to be altered in malignant tumors[31], suggesting that ALDH1 may play a significant role in ovarian cancer. It was seen that ALDH1 mRNA and protein expression was lower in malignant ovarian tumors when compared to normal ovaries or benign tumors[31]. As a result, enzyme activity was also significantly reduced in malignant ovarian tissue when compared to benign ovarian tumors or normal ovaries. Microarray studies also showed a downregulation of ALDH1A1 gene expression in malignant ovarian tumors suggesting that ALDH1 plays a functional role in normal ovarian physiology. ALDH1 levels are lower in less differentiated malignant tumor cells when compared to differentiated malignant tumor cells, indicating the importance of ALDH1 and RA in normal ovarian signaling[31].

Cyp26 enzymes: Plasma and tissue levels of RA are kept under tight homeostatic control. RA signaling is negatively regulated via oxidation to an inactive metabolite, which is initiated by the 4-hydroxylation of RA. This forms inactive metabolites and is carried out by RA degrading enzymes. Although many microsomal P450 enzymes may degrade RA, their specificity for RA is low. In contrast, the Cyp26 families of enzymes have a higher specificity for RA and are responsible for its conversion to 4-hydroxy retinoic acid, 8-oxoretinoic acid and 18-hydroxy retinoic acid[32]. These enzymes contain a heme domain that act as a RA hydroxylase. Their expression is inducible by
RA and they in turn metabolize RA. The clones that were characterized and identified in humans were Cyp26A1, Cyp26B1 and Cyp26C1. Cyp26a1 and b1 have a high specificity for all trans RA [13, 33] although Cyp26c1 efficiently metabolizes both all trans RA and 9-cis RA [34, 35]. Upon overexpressing Cyp26, it is observed that there is an increase in the clearance of RA [35] while an addition of RA in vivo induces Cyp26a1 expression [36].

The discovery of the Cyp26 family was made when a strong correlation between Cyp26 expression and RARα levels was observed while Cyp26 expression was not detectable when RARα levels were very low, suggesting that Cyp26 is a direct candidate target of RARs. High RARβ levels are also associated with Cyp26 expression and RAR dependent auto-induction of RA metabolism can induce Cyp26, which results in the formation of 4-hydroxy RA as the first step in the breakdown of RA[37].

Of the three enzymes, Cyp26b1 was the most highly conserved between human, mouse and zebrafish[38]. In humans, Cyp26A1 and Cyp26C1 are located on chromosome 10 while Cyp26B1 is located on chromosome 2[39]. Cyp26A1 and Cyp26B1 are essential for animal survival as the knockouts of these genes are lethal whereas Cyp26C1 knockout animals are viable. Cyp26A1 knockout mice died mid-gestation and Cyp26B1 died shortly after birth, possibly due to respiratory failure. Both sets of knockouts showed limb and facial deformities; while Cyp26A1 knockout mice showed deformed tails, Cyp26B1 knockout mice also showed defects in limb development[40, 41]. These differences can be attributed to the expression of the enzymes during development. Cyp26A1 expression is involved in the development of the hindbrain, vertebrae and tail and Cyp26B1 m RNA is more highly expressed in the
craniofacial, hindbrain, forebrain, spinal cord, lungs, kidneys, testis and skin. In adult humans, Cyp26A1 expression is the highest in the liver, while Cyp26B1 is abundantly expressed in the placenta, ovary, testes and brain[13].

When mice were treated with RA, an up-regulation of RA metabolism was observed which is attributed to RA’s ability to auto-induce its own metabolism. Cyp26 induction has been observed in RA treated mouse liver[36]. It is believed that RARs are responsible for inducing Cyp26[13].

Oral treatment of rats with the Cyp26 inhibitor such as R115866 increases plasma and tissue levels of RA and produces effects similar to those of RA[32]. Since the ovaries have a high capacity to produce RA, it can be hypothesized that inhibition of Cyp26-mediated RA metabolism would increase the endogenous levels of retinoic acid and mimic the effects of retinoic acid in the ovary.

Retinoic Acid Metabolism Blocking Agents: The major role of Cyp26 is to degrade RA, thereby regulating RA levels in the cell[37]. RA has several therapeutic applications, which include its use in the induction of cytodifferentiation and suppressing the proliferation of promyelocytic leukemia[42], treatment of severe nodular acne[43] as well as treatment of psoriasis and some forms of ovarian cancer[44]. However, auto-induction of RA clearance during therapy leads to a clinical challenge due to Cyp26 clearance of RA. To overcome this obstacle, it is now common to co-administer Cyp450 inhibitors along with RA to decrease its clearance. This has led to the development of structural analogs of RA that were modified to inhibit RA metabolism. These drugs were
named Retinoic Acid Metabolism Blocking Agents (RAMBAs) which serve to maintain effective RA concentrations by inhibiting the metabolism of RA[13]. Two examples of RAMBAs will be discussed below.

a. **LIAROZOLE**

Liarozole is the most studied RAMBA which inhibits RA metabolism *in vitro*. It is a new imidazole derivative that has antitumor properties[45]. Liarozole has been shown to be effective against psoriasis. It has also been shown to decrease tumor growth in androgen dependent and independent rat prostate cancer. Liarozole is known to decrease testosterone levels via inhibiting aromatase[45].

RA plays an important role in proliferation of epithelial tissue and has antitumoral activity, and Liarozole inhibits several Cytochrome P-450 enzymes[45, 46]. Liarozole is known for especially inhibiting 4-hydroxylase, which metabolizes RA. It has been shown that when patients suffering from acute promyelocytic leukemia with retinoid resistance were pretreated with Liarozole one hour prior to ingesting RA, there was a decrease in RA catabolism and an increase in plasma RA, which may contribute to the antitumoral activity of the compound[46]. It has also been shown that Liarozole by itself did not have much of an effect on the proliferation of MCF-7 cells but it did significantly potentiate the antiproliferative effect of RA by more than 10-fold. This showed that although RA and Liarozole were active in different ways individually, in combination they showed antiproliferative activity and that the potentiation of the effect of RA on the proliferation of MCF-7 cells by Liarozole was also due to the prevention of the inactivation of RA due to metabolism [45]. However, liarozole does not show Cyp isoenzyme specificity since it
is also involved in testosterone synthesis. It is therefore important to carry out studies to
discover compounds that are Cyp26-specific inhibitors in order to mimic retinoidal
effects without the unwanted side effects associated with decreased testosterone
synthesis[32].

b. R115866

R115866 is a potent and selective inhibitor of the Cyp26 family of enzymes. The
chemical name of R115866 is (B)-N-(4-(2-ethyl-1-(1H-1,2,4 - triazol (1-yl) butyl) phenyl)
-2-benzothiazolamine. It is a selective Cyp26b inhibitor and is therefore less likely to
cause undesired side effects. The in vivo activities of R115866 include inhibition of RA
metabolism when Cyp26 enzymes were coincubated with R115866 and increased
systemic levels of RA. R115866 also illicit a dose dependent increase of hepatic Cyp26
mRNA expression. In addition, a single dose of R115866 in rats produced a discernible
surge of endogenous RA levels in the plasma, skin, kidney, spleen and fat. This
suggests that R115866 delays the metabolism of RA and increases local levels in
tissues where RA is metabolized [32]. It not only increases endogenous plasma and
tissue RA levels by delaying RA metabolism but also mimics the effects of RA [47]. Oral
treatment of R115866 in rats results in retinoidal effects like suppression of vaginal
keratinization and increased pinnal hyperplasia [47].

Conversely, studies performed to determine the effect of RA on cell lines have
demonstrated that the administration of RA to human and mouse cell lines results in
Cyp26 expression and leads to an increase in Cyp26 mRNA levels in the liver[32]. Since CypP26 mRNA and RA activity are directly related, it can also be said that RA is
a strong inducer of Cyp26 mRNA expression and enzyme activity[32]. It would therefore be interesting to study the effects of R115866 and RA in follicle development.

Mice as a model for ovarian follicle development studies: Mice are the most commonly used mammalian model for laboratory research. They are relatively easy to maintain and handle, reproduce quickly, and share a high degree of similarity with humans in terms of morphology and hormone/gene regulation. The mouse genome has been sequenced, and many mouse genes have human homologues. The mouse model has also been important in revealing some essential principles of follicle growth, assembly and the role of growth and differentiation factors.

Follicle culture as an in vitro model: Experiments in mice using the methodology of in vitro follicle culture have shown that long term follicle culture does not affect imprinting[48]. Previous studies on ovarian follicle development have made use of various in vivo and in vitro systems, and the latter includes follicle cultures and cell cultures. Some of the in vitro studies involve the use of theca, oocytes or granulosa cell cultures that eliminate the otherwise complicated in vivo effects of hormones and other growth factors that could potentially interfere with the studies. However, these studies do not take into account cell-to-cell interactions that are important for development and growth. Granulosa cells, theca cells and oocytes within the follicles show cell-cell interactions which influence the growth and viability of these follicular cells.
Nourishment and the exchange of regulatory factors from granulosa cells to the oocyte are essential for oocyte development, wherein the granulosa cells act as nurse cells[49]. Follicular somatic cells promote or arrest the progression of meiosis and determine oocyte competence to allow for fertilization. Oocyte growth is under the control of somatic cell signaling as they continue to grow once they acquire the competence to resume meiosis[50]. On the other hand, oocytes regulate the growth and development of granulosa cells. Oocytes play an integral role in the formation and the activation of the primordial germ cell pool in the ovaries [51]. Oocytes also play an important role in the development of the follicles to more advanced stages such as tertiary and antral stages of development [52, 53], and in granulosa cell proliferation and differentiation and ovulation [54-56]. Thus, the signals exchanged between the somatic cells and oocytes are crucial for the initiation and coordination of differentiation in the oocyte and somatic cells. These signals not only promote oocyte but also granulosa cell growth, thereby orchestrating follicle growth as a whole [57, 58]. Therefore, follicle culture has the advantage in knowing the effects of drugs on these cells in an environment that simulates the environment in the ovary without the interference of other hormones/growth factors.

*In vitro* follicle culture will provide us with a better understanding of the mechanisms by which follicle growth and oocyte maturation take place. It also has clinical applications such as cryopreservation of ovarian tissue that is used for the restoration or preservation of fertility. The major goals of *in vitro* culture systems include providing support to the somatic cells so as to maintain the cell-to-cell interactions between the somatic cells and the oocytes, and permeability to the media where hormones can
diffuse into the follicle and factors secreted by the follicle can be released into the media [48]. Two types of \textit{in vitro} systems follicle culture have been developed to grow follicles, -the two-dimensional and three-dimensional system.

The three-dimensional systems are successful in supporting oocyte maturation in growing follicles. It provides the mechanical support that is needed to maintain cell-to-cell interactions within the follicle. The matrix or hydrogel that is used is an alginate system, an inert algae-derived polysaccharide that undergoes gelation with calcium ions. This system has the rigidity to maintain the 3D structure and at the same time, allows for the expansion of the follicle due to oocyte growth and granulosa cell proliferation[48]. This system also mimics the environment of the ovary, which thereby supports the growth and development of the follicles isolated from a variety of animals. Till date, successful folliculogenesis has been achieved in follicles obtained from mice, primates, cows and humans using the 3D alginate system [59]. Follicles grown in the alginate system have also been proven to be meiotically competent that were readily fertilizable and resulted in the birth of viable offspring in mice[60].

Two-dimensional systems are commonly used to grow primary and secondary follicles that have been mechanically isolated from mice [59]. When the follicles are isolated from their natural environment, they are exposed to various physical restrictions such as oxygen tension, temperature and nutrient availability as well as uptake that might pose various obstacles in their growth. It is therefore necessary to provide these follicles with ambient temperature and the appropriate nutrient and oxygen requirements so as to mimic the natural environment \textit{in vivo}, thereby allowing for its growth and development.
In order to grow follicles in 2D culture, FBS is commonly used in combination with insulin, selenium and transferrin (ITS). Alpha minimal essential medium is also used predominantly along with FSH to promote follicular development and survival[48].

In vitro studies performed in 1996 that lead to the production of a live mouse from an oocyte that developed from the primordial follicle stage to a developmentally competent stage in culture gave hope to the field of in vitro follicle culture[48, 58].

Although 3D culture is extensively used to culture follicles that were meiotically competent and have provided viable offspring, I chose to use the 2D culture system for my studies as the aim of my studies is to perform a quick study on the growth profile of the follicles when treated with RA and RAMBAs and the 2D culture system is simple, fast and a more convenient method for a screening process.
Experimental Designs and Methodology

Animals

CD-1 mice were maintained in a temperature and light controlled environment and were provided with food and water at all times. Animals were maintained and bred in accordance with all federal and institutional guidelines[1].

Follicle cell culture, collection and treatment

The following method is adapted from the very well established follicle culture handbook provided by Dr. Teresa Woodruff’s Laboratory at Northwestern University, Chicago.

Six 12-16 day old female CD-1 mice were euthanized using CO₂ asphyxiation. The ovaries were then removed from the animals. The roughly dissected ovaries were then placed into a 35mm dish with dissection media that consisted of 30 ml L15 (98.52%v/v), 150 µL of Pen (0.49%v/v) and 300 µL of FBS (0.99%v/v). The ovaries were cleaned free of fat, bursa and uterus under a dissecting microscope using dissection scissors. The cleaned ovaries were transferred into a new 35 mm dish containing maintenance media that consisted of 98.52%v/v of αMEM, 0.49%v/v of Pen-Strep and 300 µL of FBS (0.99%v/v). Follicles were mechanically isolated from the ovaries using 2 insulin syringes. Twenty to forty secondary follicles per ovary were collected. The development stage of these follicles was determined by their sizes, all of which ranged between 100-180 µm in diameter. Intact follicles were transferred to 2.5 ml of maintenance media that consisted of 98.52%v/v of αMEM, 0.49%v/v of Pen-Strep and
0.99% v/v of FBS. The health of these follicles was evaluated. The follicles that contained proper amount of granulosa cells, showed proper association between oocyte and granulosa cells, and had round oocytes were considered healthy. The follicles were maintained in growth medium. Healthy follicles were plated into a 96 well plate with one follicle per well, using only the 24 wells at the center of the plate in order to prevent evaporation of the media. Each well contained 100 µl growth media that was made by combining 27 ml αMEM (89.9 %v/v), 30 µL of ITS (0.099 %v/v), 3 ml of FBS (9.99 %v/v), and 300 mIU rhFSH. 50 µl of the media was replaced every day with fresh media. The follicles were measured and imaged within 10 minutes of being taken out of the incubator. Cultured follicles were given the following treatments; all-trans RA, 0.7 µM; R115866, 0.7 µM, FSH, 100mIU/ml or Liarozole, 1 µM. These concentrations were chosen as the physiological concentrations of RA in mouse tissues were found to be 0.05-1 µM [65]. Thus, the doses were chosen to fit within the observed range and higher concentrations were chosen to determine the effects of supplemental RA on growth and development. The treatments were given every 24 hours for 4 days for follicle growth and survival assays[1]. A second set of experiments involved the following treatments; FSH, 100mIU/ml, FSH, 100mIU/ml plus Liarozole, 1 µM, Liarozole, 0.01 µM, Liarozole, 0.1 µM, Liarozole, 1 µM and Liarozole, 10 µM. Liarozole was found to inhibit RA metabolism and promote proliferation via a retinoid mimetic pathway [45]. However, not much is known about the role of Liarozole in ovarian development. Thus, studies using a wide range of concentrations were performed in order to study the effects of the various concentrations of Liarozole in the ovary.
For all treatments, a total of 3 replicates (n=3) were carried out and for each experimental setup, controls were set up using the same number of follicles but treated with FSH, 100 mIU/ml which was used as the positive control, or DMSO, which was used as the control[1] (Fig.3).
**Ovarian Follicle Culture**

![Ovarian Follicle Culture Diagram](image)

12-16 day old CD1 mouse

Days in Culture

0 1 2 3 4

- Control
- FSH (positive control)
- RA
- R115866
- Liarozole

Monitor follicle survival and growth daily
Measure follicle diameter daily

**Fig. 3: Follicle cell culture methodology and treatment** Follicles are collected from ovaries harvested from female CD1 mice that are day 12-16 and are cultured in media for four days under the various treatment groups (as shown), wherein the follicles are imaged and measured and half the media is replaced with fresh media every 24 hours.
Monitoring follicle and oocyte growth and follicle morphology

Cultured follicles were measured daily for diameter and were monitored using dissection microscopy for any changes in size, morphology as well as the presence of oocyte in the follicle (health status). The follicles were also monitored for maintenance of the integrity of the cells within the follicle.

This was done to help establish and determine the healthy growth of the follicle and the oocyte which could serve as an additional method to establish the relationship of these drugs on follicle development [61].

Ovary culture, collection and treatment

Breeding cages were set up and a vaginal plug was observed daily until a plug was visible. Pregnant mice deliver pups between day 19-19.5 dpc. In the meantime, 50 ml of ovary culture media was prepared that consisted of 2X DMEM/F12 (50%v/v), 120 mg sodium bicarbonate, 2.5 mg ascorbic acid, 50 mg Albumax 1, 50 μl insulin (10 mg/ml), 275 μl transferrin (5 mg/ml), 500 μl BSA (100 mg/ml) and 0.25 ml Pen/Strep. The volume was then raised to 50 ml with distilled water to pH 7.3. When the pups were born, the females were removed from the litter. The pups were kept warm upon separation. Before decapitation, 5 ml of the culture media was pipetted out of the stock and aliquoted into four 1.2 ml tubes. DMSO, 7 μm, that served as the control, R115866, 7 μm, Liarozole, 1 μm and Retinoic acid, 7 μm were added to each aliquoted media tube to serve as different treatment groups. 400 μl of the media was added from each of the tubes to 3 wells each of a 24-well plate, except the tube that contained Retinoic
acid. Since Retinoic acid is light sensitive, 400 μl of the media containing RA was added to 3 wells in a separate 24-well plate and wrapped in foil to keep out the light. The plates were then placed in the incubator to warm the media (37°C, 5% CO₂). In the meantime, Millicell filters were removed under a laminar flow hood using forceps and placed in a 60 mm dish. These filters were used in order to provide a semi permeable membrane upon which the ovary was placed. Using the filter made it possible to allow for ovary growth and development by allowing for unlimited oxygen and media access. A sterile scalpel was used to cut the filters into 6 pieces. If the filters wrinkled, they were made taut by using 2 pairs of forceps. Two filters were cut in order to obtain filters good for 12 ovaries obtained from 6 females. The filters were then transferred to each of the wells containing media using forceps. The plates were then replaced in the incubator. Once the plates were ready, the culture media was transferred to 60 mm dishes. The pups were then decapitated and a deep abdominal incision was made to make lateral cuts on both sides. The pup was then placed on its back underneath a dissecting scope. Forceps were used to locate the oviduct which was used to locate the kidney and the ovary. A dissecting scissor was used to cut the area between the kidney and ovary. The ovary was then placed in the culture media. Forceps and scissors were used to remove the surrounding bursa/oviduct from the ovary to get the ovary as clean as possible. The culture plate was then removed from the incubator and 5 μl of the media from the well was added onto the floating filter, thereby creating a drop on the filter. The ovary was transferred to this drop using 22 G needles as chopsticks to pick up and transfer the ovary. This was repeated for all the ovaries. The ovaries were cultured for 4 days wherein the media was replaced every day from underneath the filter as well as the
drop on top of the filter that contains the ovaries (Fig.4). To fix the ovaries, forceps were used to place the filter on top of the plate lid. PBS was flushed gently onto the filter to dislodge the ovary from the filter which was then drawn up with a cut pipette tip. The ovary was the transferred to a vial containing 1 ml of 10% formalin solution and was fixed for 1 hour. It was then dehydrated 3 times for 20 minutes each in a tube containing 1 ml of 50% ethanol and then 2 times for 20 minutes each in 70% ethanol. The fixed ovaries were then stored in 70% ethanol at 4C. The ovaries were embedded in paraffin and histological sections in multiples of 5 were made for each ovary sample, wherein every other slide was stained for morphological studies and every fifth section from each ovary was used for counting. Sectioning and staining were performed by the Histology Core Facility at Northwestern University, Chicago, IL.
Whole Ovary Culture

Fig. 4: Methodology of Whole Ovary Development  Ovaries were dissected from newborn pups and cleaned of fat and bursa. The ovaries were then cultured for four days in growth media containing the different treatments, and the media was replaced every 24 hours. After the fourth day, the ovaries were fixed and sectioned for counting and immunohistochemistry.
Follicle Counting for Ovary Culture

Follicles were counted and identified from all the stained slides using the Image J software. Follicles that contained two or more oocytes that shared a cytoplasm and were not separated yet were considered to be oocyte nests. A follicle with one oocyte surrounded by a single layer of flattened granulosa cells was identified as primordial follicles, follicles with an oocyte surrounded by one layer of a mixture of flattened and cuboidal granulosa cells were identified as B/C cells, and a follicle that contained two oocytes was considered to be a dividing follicle. A follicle with an oocyte surrounded by one layer of cuboidal granulosa cells was considered primary, and follicles with an oocyte surrounded by two or more layers of granulosa cells were considered as secondary. Follicles that contained an oocyte that did not look healthy were identified as atretic follicles (Fig.5). Two follicles that were encapsulated within one membrane and shared cytoplasm were categorized as dividing oocytes. Only follicles with clearly stained oocyte nuclei were counted from every fifth section of each slide. The numbers for each ovary were then summed up and the sums were multiplied by 5 in order to get the total follicle count of the ovary [23].
Fig. 5: Stages of follicle development in early ovary development. The figure depicts the various stages of development that are typical and not so typical (atretic follicle) in early stages of ovarian development (follicles were imaged from ovary sections as seen under the microscope).
Immunohistochemistry

Slides were deparaffinized twice for ten minutes in xylenes. The slides were then rehydrated for three minutes each in 100% Ethanol, 95% ethanol, 70% ethanol, 50% ethanol and ddH₂O. Antigen retrieval was performed in a 0.1M sodium citrate solution by microwaving for two minutes on high and seven minutes on low and then cooling in the retrieval solution for twenty minutes. The slides were washed twice for fifteen minutes in PBS-Tween to permeabilize and the sections were then encircled using a PAP pen. The sections were then divided to different groups: experimental Ki67, experimental CYP, negative control CYP, experimental TUNEL and negative control TUNEL. The slides were incubated in 3% Hydrogen peroxide in PBS for fifteen minutes (the negative and experimental TUNEL samples were kept moist in PBS). The slides were then rinsed in PBS briefly and the samples were blocked in Avidin for fifteen minutes. Again, the negative and experimental TUNEL samples were kept moist in PBS. The slides were rinsed briefly in PBS and the sections were blocked in Biotin for fifteen minutes wherein the negative and experimental TUNEL samples were kept moist in PBS. Upon completion of the fifteen minutes, the slides were rinsed briefly in PBS. The experimental sections were then blocked in 10% serum in 3% BSA-PBS (rabbit serum for CYP26b1 (Sigma) and goat serum for Ki67 assay (ab1667)) for one hour at 4C (negative and experimental TUNEL samples are kept moist in PBS). The samples were incubated overnight in primary antibody diluted in 3% BSA-TBS-10% serum at 4C (1:100 dilution for goat antibody for Ki67 sample and 1:50 dilution for rabbit antibody from Sigma for experimental CYP sample). Negative CYP was incubated with the Blocking serum used previously and negative and experimental TUNEL samples were
kept moist in PBS. The next day, the ABC reagent was then prepared by diluting 2 drops A and 2 drops B into 5 ml PBS and the reagent was kept on ice. The slides were then rinsed three times for five minutes each in PBS-Tween. The slides were incubated in secondary antibody conjugated to BIOTIN in 3% BSA-PBS for thirty minutes (1:1000 diluted biotinylated rabbit anti goat IgG for CYP26b1 and biotinylated goat anti rabbit IgG for Ki67. Negative and experimental TUNEL samples were kept moist in PBS) after which the slides were rinsed three times for five minutes each in PBS-Tween. Diluted ABC reagents was added to CYP and Ki67 samples and incubated for thirty minutes and negative and experimental TUNEL samples were kept moist in PBS for this period. The slides were rinsed five times for five minutes each in PBS-Tween. The TUNEL samples were incubated in label solution (negative control) and enzyme in label solution (experimental sample) for one hour at 37C wherein negative and experimental CYP and Ki67 samples were kept moist in PBS. In the last five minutes of the TUNNEL assay, the PBS was wiped off from the CYP and Ki67 samples and these samples were incubated with TSA. The samples were rinsed off three times for five minutes each in PBS-Tween. The slides were then pat dry and DAPI was added as the mounting medium. The slides were then sealed using a coverslip and nail polish.

**Statistics**

Follicle counting data are presented as the means ± se. For comparisons between the treatment group and the negative control, a Student’s two-tailed t test was used. $P < 0.05$ was considered significant[1].
Hypothesis

In order to determine the role of RA and Cyp26b1 on ovarian follicle development, I propose to look at the effects of RA and RAMBAs Liarozole and R115866, in isolated follicles, using the 2D culture system and monitoring the growth of the cultured follicles daily. I hypothesize an increase in follicle growth in all treatment groups, with a greater extent of growth in the groups treated with RAMBAs than RA treated follicles.

I also propose to look at the effects of RA, R115866 and Liarozole in whole ovary culture wherein ovaries would be harvested from newborn mice and cultured for four days in media containing RA, R115866 and Liarozole. As previously stated, Liarozole was found to inhibit RA degradation and increase plasma RA [45]. It also potentiates the effect of RA on proliferating MCF-7 cells [45] and although it does not show CYP isoenzyme specificity, it would be interesting to determine the effects of Liarozole on follicle growth and development.

A morphological and immunohistochemical analysis is used to gauge the effects of the drugs on whole ovary development. I hypothesize effects similar to those that are expected using the 2D follicle culture system, wherein ovaries treated with RAMBAs would show a stronger development profile than ovaries treated with RA.
RESULTS

Effects of RA, R115866 and Liarozole on the growth of in vitro cultured follicles

When follicles were isolated from day 15 CD-1 mice and grown in media for 4 days, most follicles maintained their structural integrity in the growth media. Survival rates did not differ significantly among different treatment groups during the first 2 days; however, after 4 days of culture, follicle survival rate was decreased in the groups treated with Liarozole and Retinoic acid when compared to the control (Fig. 6). The follicles that were treated with 1 μM Liarozole, 0.7 μM Retinoic acid, 0.7 μM R115866 or 100 mIU/ml FSH had linear growth curves and were significantly larger than follicles grown in control media (Fig. 7). Follicles treated with Liarozole and FSH showed a significant increase in diameter while follicles treated with Retinoic acid showed a less, although significant, increase in diameter.

The change in follicle growth could be attributed to variability within the assay. However, the assay was validated by performing various replicates and pilot studies wherein variability within replicates was minimized. It is important to note however, that individual difference within pups and initial follicle diameter could cause variations, but since follicle growth showed a similar trend for all replicates, the results can interpreted to be biologically significant.

In order to further study the effects of these drugs on follicle growth, additional studies were carried out wherein follicles were treated with varying concentrations of these drugs to determine the dose-response of the drugs on follicle development.
FIG. 6: Survival rates of follicles treated with RA and Retinoic Acid Metabolism Blocking Agents

Secondary follicles were isolated from mice at day 15 and cultured for 4 days with media changes every day. The follicles were treated with 100 mIU/ml FSH, 0.7 μM R115866, 1 μM Liarozole and 0.7 μM RA. Follicle survival was monitored daily.
FIG. 7: Growth Curves of follicles treated with Retinoic Acid and Retinoic Acid Metabolism Blocking Agents

Secondary follicles were isolated from mice at day 15 and cultured for 4 days with media changes every day. The follicles were treated with 1 μM Liarozole, 0.7 μM R115866, 0.7 μM Retinoic acid or 100 mIU/ml FSH and follicle diameters were monitored daily. Statistical significance was observed between different treatment groups as compared to the control groups (*P <0.05, **P<0.005, n=24)
EFFECT OF RA ON THE GROWTH OF *IN VITRO* CULTURED FOLLICLES

First, follicles were cultured and treated with 0.07 μM RA, 0.21 μM RA, 0.7 μM RA, 2.1 μM RA or 100 mIU/ml FSH. These follicles had linear growth curves and showed variable and moderate growth as compared to follicles grown in control media (Fig. 8). Follicles treated with 0.07 μM of RA showed a lower growth profile than the control. Follicles treated with 0.21 μM RA and 0.7 μM of RA showed the best follicular growth than the control that was significant at Days 3 and 4. A higher dose of RA (2.1 μM) seemed to have a positive effect on follicle growth; however, the follicles showed a decrease in growth that was comparable to that of the control on Day 4. As expected, follicles treated with FSH showed significantly larger diameters (Fig. 8).
FIG. 8: Growth Curves of follicles treated with Retinoic Acid

Secondary follicles were isolated from mice at day 15 and cultured for 4 days with media changes every day. The follicles were treated with increasing doses of Retinoic acid (0.07-2.1 μM) or 100 mIU/ml FSH and follicle diameters were monitored daily. Statistical significance was observed between different treatment groups and the control groups (*P < 0.05, **P < 0.005, n=24).
EFFECT OF R115866 ON THE GROWTH OF IN VITRO CULTURED FOLLICLES

Most follicles that were isolated from day 15 CD-1 mice and grown in media containing varying concentrations of R115866 for 4 days maintained their structural integrity in the growth media. Survival rates didn’t differ significantly among the different groups during the 4 days of culture; however, follicle survival was less in the group treated with 0.21 μM of R115866 when compared to the control (Fig. 9).

The follicles were cultured with 0.07 μM R115866, 0.21 μM R115866, 0.7 μM R115866 or 100 mIU/ml FSH. These follicles had linear dose-dependent growth curves and showed significant growth when compared to the follicles grown in the control media (Fig. 10). Follicles treated with 0.21 μM of R115866 showed the highest growth profile amongst all treatment groups. As expected, FSH grew to more advanced stages of growth with a significantly larger diameter (Fig. 10).
Secondary follicles were isolated from mice at day 15 and cultured for 4 days with media changes every day. The follicles were treated with 100 mIU/ml FSH, 0.07 μM R115866, 0.21 μM R115866 and 0.7 μM R115866. Follicle survival was monitored daily.
Secondary follicles were isolated from mice at day 15 and cultured for 4 days with media changes every day. The follicles were treated with increasing doses of R115866 (0.07-0.7 μM) or 100 mIU/ml FSH and follicle diameters were monitored daily. Statistical significance was observed between different treatment groups (*P <0.05, **P<0.005, n=24)
EFFECT OF LIAROZOLE ON THE GROWTH OF IN VITRO CULTURED FOLLICLES

Most follicles that were isolated from day 15 CD-1 mice and grown in media containing varying concentrations of Liarozole for 4 days maintained their structural integrity in the growth media. Survival rates didn’t change significantly among the different groups during the 4 days of culture; follicle survival was less in the groups treated with 1 μM, 10 μM of Liarozole and a combination of 1 μM Liarozole and 100mIU/ml of FSH, when compared to the control after 4 days of culture (Fig. 11).

The follicles were also cultured and treated with 0.01 μM Liarozole, 0.1 μM Liarozole, 1 μM Liarozole, 10 μM Liarozole and 100 mIU/ml FSH. These follicles had linear dose dependent growth curves and showed significant growth when compared to the follicles grown in the control media (Fig. 12). What was interesting to note was that follicles treated with the higher doses (specify what does that you are talking about here) of Liarozole had a significantly larger diameter than FSH, which acted as a positive control.

In order to determine if Liarozole and FSH have a synergistic effect on follicle growth, a combination of both drugs was administered to the follicles. It was seen that there was a synergistic effect of the combination wherein a combination of 1 μM Liarozole and 100 mIU/ml FSH had a greater effect on the growth of the follicles than did either drugs on their own (Fig. 12).
FIG. 11: Survival rates of follicles treated with Retinoic Acid Metabolism Blocking Agent, Liarozole.

Secondary follicles were isolated from mice at day 15 and cultured for 4 days with media changes every day. The follicles were treated with increasing doses of Liarozole (0.01-10 μM), Liarozole (1 μM) and FSH (100 mIU/ml) or 100 mIU/ml FSH, and follicle survival was monitored daily.
FIG. 12: Growth Curves of follicles treated with Retinoic Acid Metabolism Blocking Agent, Liarozole.

Secondary follicles were isolated from mice at day 15 and cultured for 4 days with media changes every day. The follicles were treated with increasing doses of Liarozole (0.01-10 μM), Liarozole (1 μM) and FSH (100 mIU/ml) or 100 mIU/ml FSH, and follicle diameters were monitored daily. Statistical significance was observed between different treatment groups (*P <0.05, **P<0.005, n=24)
Effects of RA, R115866 and Liarozole on \textit{in vitro} cultured ovaries

FOLLICLE COUNT

In order to study the effects of Retinoic acid and RAMBAs on whole ovary development, ovarian explants cultures were used to investigate the actions of these drugs. Individual ovaries from newborn mice were cultured for 4 days with 1 \(\mu\text{M}\) Liarozole, 0.7 \(\mu\text{M}\) Retinoic acid, 7 \(\mu\text{M}\) Retinoic acid or 0.7 \(\mu\text{M}\) R115866 with media changes every day. Histological analysis was then conducted on the ovaries from each treatment group. As shown in Fig. 13, Liarozole and 0.7 \(\mu\text{M}\) Retinoic acid promoted the growth and development of follicles to more advanced stages of development such as primary and secondary follicles. In addition, the follicles were larger in the ovaries treated with Liarozole than the control or any other group.

Follicle counting showed a significant decrease in oocyte nests in the groups treated with R115866, Liarozole and RA as compared to the control. 0.7 \(\mu\text{M}\) RA and Liarozole treated ovaries showed a significant decrease in primordial follicles and ovaries treated with 0.7 \(\mu\text{M}\) RA showed a significant decrease in B/C follicles (Fig. 14, 15). However, it was observed that ovaries cultured with 0.7 \(\mu\text{M}\) RA showed a significant increase in primary follicles and ovaries treated with 0.7 \(\mu\text{M}\) RA, R115866 and Liarozole showed a significant increase in secondary follicles secondary. What was interesting to note was that atretic follicles were only observed in varies treated with Liarozole. Lastly, the ovaries treated with Liarozole and 0.7 \(\mu\text{M}\) RA showed a significant increase in the total number of follicles compared to the control (Fig. 14, 15).
FIG. 13: Morphological Analysis in newborn whole ovary sections

Individual ovaries from newborn mice were cultured with 0.7 μM DMSO (A), 1 μM Liarozole (B), 0.7 μM R115866 (C) and 0.7 μM RA (D) for 4 days with media changes every day. Ovarian Histology (20X magnification) was determined at the end of culture.
Follicle counts were determined on the newborn ovaries at the end of 4 days in culture. Statistical significance was observed between different treatment groups (*P <0.05, **P<0.005). Data represented according to different follicle development stages.

FIG.14: Identification of follicular stages of development newborn whole ovary sections
FIG.15: Stages of development categorized by treatment groups

Follicle counts were determined on the newborn ovaries at the end of 4 days in culture. Statistical significance was observed between different treatment groups (*P < 0.05, **P < 0.005). Data represented according to different treatment groups.
CYP26B1 Expression

Previous studies have shown that RA is required for growth and differentiation in the ovary[1]. However, RA induces the expression of CYP26B1 in the ovaries. In order to better understand and visualize the expression of CYP26B1 during follicle growth and development in the newborn, whole ovary sections were stained for CYP26B1 expression. For this purpose, ovaries were collected from newborn female mice and were cultured for 4 days in media containing DMSO that acted as the control, 0.7 μM R115866, 0.7 μM RA and 1 μM Liarozole. The extent of CYP26B1 expression was detected using an anti-CYP antibody and the results were monitored and compared with the negative controls set up for each treatment group as well as with the expression seen in the other treatment groups.

As expected, CYP26B1 expression was greater in the ovaries treated with 0.7 μM RA, 1 μM Liarozole and 0.7 μM R115866 than the ovaries treated with DMSO (control group)(Fig. 16). However, ovaries treated with 7 μM of RA showed a lesser extent of Cyp26b1 than did any other group (data not shown), suggesting that higher doses of Retinoic acid could be toxic to the ovary.
FIG. 16: CYP26B1 expression in cultured newborn whole ovary sections

Ovaries collected from newborn mice were treated with 0.7 μM DMSO (A), 1 μM Liarozole (B), 0.7 μM R115866 (C) or 0.7 μM RA (D) in culture media for 4 days. The expression of CYP26B1 (green) was determined by staining paraffin-embedded ovary sections with anti-CYP26b1 antibody.
PRESENCE OF APOPTOSIS

When the ovaries are treated to yield an increase in endogenous RA or administering exogenous RA, we would expect more cell proliferation and less apoptosis. In order to test this hypothesis during follicle growth and development in the newborn, whole ovary sections were stained for apoptosis and cell proliferation, however the cell proliferation assay didn’t work. In order to evaluate the extent of apoptosis, ovaries were collected from newborn female mice and were cultured for 4 days in media containing DMSO that acted as the control, 0.7 μM R115866, 1 μM Liarozole, 0.7 μM RA and 7 μM RA. The extent of apoptosis was detected using the TUNEL assay and the results were monitored and compared with the negative controls set up for each treatment group as well as with the expression seen in the other treatment groups.

It was observed that apoptosis was approximately the same in the ovaries treated with 0.7 μM RA, Liarozole and R115866 as compared to the ovaries treated with DMSO (control group) (Fig. 17). However, ovaries treated with 7 μM of RA showed a greater extent of apoptosis than did any other group (data not shown), confirming that higher doses of Retinoic acid are toxic to the ovary.
FIG. 17: Apoptosis in newborn whole ovary sections

Ovaries collected from newborn mice were treated with 0.7 μM DMSO (A), 1 μM Liarozole (B), 0.7 μM R115866 (C) and 0.7 μM RA (D) in culture media for 4 days. The extent of apoptosis (red) was determined by staining paraffin-embedded ovary sections with the TUNEL kit, for the detection of apoptosis.
Discussion

Extensive studies have been performed where it has been demonstrated that both hormonal and non hormonal factors are necessary for ovarian health and development. One of these factors has been identified to be Retinoic Acid. Previous studies have demonstrated that Retinoic acid is required for granulosa cell proliferation and that an overexpression of Cyp26b1 decreases granulosa cell numbers and induce apoptosis[1]. Conversely, treating cells with a CyP26B1 Inhibitor R115866 shows an increase in cell proliferation, suggesting the importance of Retinoic acid in the ovary[1].

RA has a moderate effect on follicle development: To further understand the role of RA in the ovary and on follicle development, studies were performed on follicles isolated from day 15 ovaries and were treated with RA or RA metabolism blocking agents to determine the effects of RA on ovary development. By treating the follicles with RA metabolism blocking agents, endogenous levels of RA can be increased by preventing its degradation. Studies conducted on follicles showed that direct administration of RA and indirect increase of RA in culture plays a significant role on follicle growth. It was seen that RA plays a significant role in follicle development; however, higher doses are toxic to the growth which is consistent with previous studies of RA. Moderate doses of RA showed the best growth profile which again is consistent with previous studies. Stimulatory effects of RA have previously been seen on granulosa cells and the ovary, however, this study is one of the first to reveal the stimulatory effects of RA on isolated follicles.
R115866 showed a significant effect on follicle development: When the follicles were treated with RA metabolism blocking agent R115866, it was seen that the follicles showed a dose dependent increase in follicle growth when treated with R115866. The reason for this increase in follicle growth would be that Cyp26B1 which is responsible for the degradation of RA in the ovaries is blocked by R115866, thereby increasing the endogenous RA levels in the ovary. This again is consistent with previous findings on granulosa cells and supports the findings that Cyp26B1 inhibition leads to increased growth and proliferation in mouse ovaries.

Liarozole showed a strong stimulatory effect on follicle growth and development. In addition to R115866, the effect of Liarozole was tested on follicle growth. It was seen that Liarozole increases follicle growth in a dose dependent manner as well. This was the first time the effect of Liarozole was tested on ovarian development and compared to the effects of R115866 on follicle growth. What was interesting to note was that higher doses of Liarozole had a stronger effect on follicle growth than FSH, a known stimulator of follicle growth. Since Liarozole showed a positive effect on follicle growth, it was interesting to perform follow up studies on Liarozole and FSH together on follicle growth. What was also interesting to note that FSH and Liarozole administered in conjugation had a synergistic effect on follicle growth. Liarozole is an aromatase inhibitor and a non selective CYP26 inhibitor. This dual action of Liarozole could be a possible reason for significant follicle growth in culture. Treatment with Liarozole along with RAR antagonist AGN193109 would help further distinguish the extent to which RA signaling contributes to Liarozole stimulation of follicle growth.
Stimulatory effects of RA and Cyp26b1 Inhibitors on whole ovary development: In vitro studies were also performed on whole newborn ovaries to determine the effects of RA on newborn ovaries. Morphological studies showed that 0.7 μM Retinoic acid and 1 μm Liarozole have a greater stimulatory effect on follicle growth when compared to the ovaries compared to R115866 and the control. The groups treated with Retinoic acid and Liarozole showed significantly higher numbers of primary and secondary follicles while those ovaries treated with Liarozole were the only ovaries that showed atretic follicles. This suggests that RA and Liarozole have a strong stimulatory effect on follicle development. What was surprising to note was that R115866 did not have as strong an effect as did the other 2 treatment groups, which can be explained by the fact that Liarozole has aromatase activity in addition to its Cyp26b1 inhibitory activity which could potentially be contributing to its action. The results obtained by administering a direct dose of RA were contradictory to the results obtained via follicle culture, where the direct administration of RA had a moderate effect on follicle growth; however, this could be due to the effects of the surrounding granulosa cells that have previously been shown to undergo proliferation and cell growth upon administering RA. This effect might have a consequence on the follicle growth as numerous studies have shown the necessity of regulatory signals passed from the granulosa cells to the follicles for a healthy growth status.

Cyp26b1 expression was seen throughout the ovary: In order to determine the effects of RA in the whole cultured ovaries, Cyp26B1 expression was carried out in RA, R115866 and Liarozole treated ovaries. Cyp26B1 expression was high in ovaries treated with 1
μM Liarozole and ovaries treated with 0.7 μM R115866 and 0.7 μM RA showed similar expression signals. This can be explained by the fact that the presence of RA induces the expression of Cyp26B1 in the body for its degradation, because of which Cyp26B1 expression is expected to be high in all groups, which is what can be seen and is supported by the results.

Apoptosis was measured amongst treatment groups: In order to determine the extent of cell death in the three treatment groups, a TUNEL assay was performed on the ovaries. It was seen that the treated ovaries showed the same extent cell death as the control. These results were expected as all treatment groups, either directly or indirectly, have increased levels of RA that have been known to increase cell proliferation and decrease cell death[1].

High doses of RA are toxic to follicles and granulosa cells: Studies were also performed wherein ovaries were treated with 7 μM RA (higher dose). These ovaries showed significantly greater cell death, suggesting that the high dose of RA administered to the ovary was in fact toxic to the ovary. This could also be a reason why the RA treated ovaries showed low Cyp26B1 expression. These results confirmed previous studies that 7 μM RA administered to the ovaries was in fact, toxic and led to the production of unhealthy ovaries.

Several studies have demonstrated the role of RA in the ovaries. The studies have mostly concentrated on studying the effects of RA on meiosis and the presence of RA in
the ovaries and the testes. However, this study provides new insights into the function and role of RA in individual follicles as well as whole ovaries. In addition, this study also studies the effects of Cyp26b1 inhibitors such as R115866 and Liarozole, and provides a comparison of the effects of the drugs on ovary and follicle development. This study demonstrates that RA is required for follicle development and growth as early as day 0 as well as days 12-16. It also provides further evidence that Cyp26b1 is expressed in the newborn ovary and that inhibition of Cyp26b1 also promotes cell proliferation and decreases cell death in the ovary, thereby supporting previous studies of the role of RA in the ovary.
References:


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