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In Vivo Effects of a RAMBA and Vitamin A Deficiency on Ovary Development

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In Vivo Effects of a RAMBA and Vitamin A Deficiency on Ovary Development

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Abstract

The active metabolite of vitamin A, retinoic acid, is vital to several physiological functions, and is most notably associated with vision and organ development. Its role in the male reproductive system is well understood, but studies in female reproduction are lacking. Recent studies show that Cyp26b1, a retinoic acid degrading enzyme, is expressed highly in the Day1-10 mouse ovary. This time point correlates with establishment of the primordial follicle pool. *In vitro* studies show that both retinoic acid and R115866, an agent that blocks retinoic acid metabolism, increase cell proliferation. Because of this, we hypothesized that retinoic acid plays a critical role in the development of the mouse ovary.

To test this hypothesis, we placed female mice on a vitamin A deficient diet over a time course beginning from gestation. Endogenous retinoic acid levels were also increased by injection of R115866 at either 0.5 or 1.0 mg/kg during the first 7 days after birth when the levels of Cyp26b1 expression are high. At day 19, week 7, and week 15, ovaries were harvested for morphological and RT-PCR studies. Our results show that animals on a vitamin A-deficient diet or injected with R115866 developed a variety of ovarian pathologies at day 19, week 7, and week 15. Long term pathologies were also observed in mice that were placed on a vitamin A-deficient diet for 16 weeks then allowed to recover for one year. Overall, our results indicate that retinoic acid plays an important role in the development of the mouse ovary and that balanced retinoic acid levels early in development are needed for the health of ovaries. In addition, the effect of early vitamin A deficiency has long lasting consequences that cannot be corrected by a subsequent normal diet.

Introduction:

The ovary is responsible for the production of female gametes as well as hormones necessary for the regulation of development and reproduction. Aberrations in ovarian development may lead to infertility as well as other diseases. Better understanding of the process of ovarian development has potential medical applications in the treatment of such conditions.

Retinoic acid is the physiologically active form of vitamin A. Its levels are regulated physiologically by its synthesis from vitamin A and other precursors, as well as its degradation by a family of Cytochrome P450 enzymes, the Cyp26 enzymes. Recent findings suggest that retinoic acid is involved in the regulation of meiosis and ovulation. Other reports suggest it also has an effect on steroid hormone biosynthesis. In addition, *in vitro* studies have shown that increases in retinoic acid levels can induce proliferation of granulosa cells. More work must to be done to elucidate the complex role of retinoic acid during *in vivo* ovary development. To better understand the *in vivo* effects of retinoic acid, a study utilizing a mouse model with inhibition of Cyp26 enzymes, which are known to degrade retinoic acid, was conducted. This study will help to determine the biological effects of vitamin A and retinoic acid signaling in the ovary, provide a better understanding of ovarian development and pathophysiology, and provide potential treatments for infertility and other ovarian diseases.

Review of Literature

Infertility and Its Impact

It is estimated that 80 million people around the world experience impaired fertility; 10 percent of couples experience difficulties with procreation [1]. The worldwide distribution of infertility is not uniform. A higher incidence occurs in developing nations, where infectious diseases that can cause damage to reproductive organs are more common and access to fertility treatments is very limited [2, 3]. Infertility has consequences that affect relationships and psychological health. In some social groups, women who cannot conceive are ostracized, socially stigmatized, and left feeling isolated[4]. The resulting stresses on relationships may lead couples to divorce. Current treatments that address the biological causes of infertility do not always adequately address the myriad of insecurities associated with this condition.

The once intimate experience of conception now requires couples go to sterile medical facilities. The services, which are not covered by most major medical insurers, place financial burdens on couples. With an estimated 10 percent of infertility cases being unexplainable, fertility specialists offer no guarantees [5], and the financial cost becomes a huge gamble. Infertility also increases with age, ranging from 11 percent of women under the age of 29 to 27 percent of women between 40-44 years of age [6]. As more couples delay starting a family until after both partners achieve educational goals and establish careers, there are more couples that require the help of medical science to conceive [7].

Structure and Development of the Ovary:

The ovary is the female gamete-producing organ. It is also an endocrine gland, which produces several hormones. The ovary contains several cell types that form many follicles. Each ovarian follicle contains an oocyte and one or more layers of granulosa cells, which support oocyte maturation. Large ovarian follicles are also surrounded by one or two layers of theca cells. Both granulosa cells and theca cells are responsible for steroid biosynthesis. The number of follicles in the ovary is established during fetal development and the oocytes mature one by one after puberty. One follicle releases a single mature egg during ovulation in mono-ovulatory animals including human. The remaining follicular remnants then form a corpus luteum, which secretes hormones including progesterone and estradiol. Without further stimulation from a developing embryo, the corpus luteum will diminish in 14 days. This process is responsible for the cyclic changes in hormone levels that occur during the female menstrual cycle.

Follicle maturation begins when germ line cells mature into primordial follicles as a consequence of invasion by pre-granulosa cells. This process occurs early in development, and is arrested until further maturation is initiated after onset of puberty. From the primordial stage, granulosa cells develop from flat cells to cuboidal cells, indicative the BC stage. From here, the follicle moves to the secondary stage, where granulosa cells proliferate into two or more layers. By the tertiary phases, many layers of granulosa cells surround an enlarged oocyte. This then leads to the development of the antral follicle, where a cavity begins to form around the oocyte and the follicle prepares for ovulation.

Hormones Important in Female Reproduction

Hormonal cycles in females are highly coordinated through regulatory influences of the hypothalamus and pituitary glands. The hypothalamus is responsible for the pulsatile secretion of gonadotropin releasing hormone (GnRH). GnRH stimulates the anterior pituitary cells to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). LH shares similar subunits with other glycoprotein family hormones, such as thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), and human chorionic gonadotropin (hCG) [27]. FSH and LH act synergistically to regulate the development and growth of the ovary and stimulate hormone production. Increased doses of LH are required for oocyte maturation and ovulation [8]. The secretion of FSH and LH is further regulated by activin and follistatin, which will be discussed below. FSH has the most impact in the development of antral follicles, while the factors that control earlier stages of follicle development are poorly understood [9, 10].

Estrogen is primarily produced by granulosa cells within ovarian follicles and it induces the secondary sex characteristics of females, including body shape, hair growth, and breast development. Progesterone cooperates with estrogen to regulate the cycle of menses as well as the cyclic changes in the endometrial lining of the uterus. Both hormones induce pubertal maturation and facilitate the reproductive processes of the female body.

Activin is a peptide hormone that was originally isolated from the ovary and was later shown to be produced in multiple locations throughout the body. Both activin and inhibin are members if the TGF-β superfamily [10-13]. Activin acts as a ligand to a

serine/threonine kinase receptor which activates the kinase/Smad signaling pathway [14, 15]. It works in both an autocrine and paracrine fashion to stimulate the synthesis and release of FSH. Activin also triggers granulosa cell proliferation and differentiation, as well as oocyte maturation [16-18]. Activin activity is suppressed by inhibin and follistatin. Inhibin works by blocking the activin receptors, while follistatin is a soluble protein that binds to activin, thereby blocking its access to receptors [19]. Activin has also been shown to induce increased expression of estrogen receptors, while estrogen has an opposite effect on activin expression in neonatal mice [20, 21].

Effect of Nutrition on Fertility

When the nutritional needs of the mother are severely compromised, the effects to a fetus are evident. Nutritional deficits can cause developmental defects, mental retardation, and low birth weight [22-25]. The incidence of developmental defects with a dietary cause are higher in poorer countries where medical and nutritional needs, including access to vitamins, are more difficult to satisfy [26]. Certain vitamins, including vitamin A, are fat soluble and require adipose tissues for their subsequent storage. There is no current literature to address how changes in body fat composition impact the efficiency of vitamin storage and utilization, but it is known that women with low body mass index (BMI) and low body fat experience difficulty in conception as well as occurrences of amenorrhea [27]. Even otherwise healthy females with low body fat, such as competitive athletes, experience alterations in menses and fertility [28-30]. There is a further association between poor nutritional habits and infertility in both males and females [31-33].

Vitamin A and Retinoic Acid

Retinoic acid (RA) is a naturally occurring physiologically active retinoid. The term retinoid refers to any compound that is a metabolite of Vitamin A. Common dietary sources of Vitamin A are the carotenoid pigments found in a variety of plant-based foods. Animal tissues can also provide stores of vitamin A metabolites such as retinyl-esters. Retinoids can be stored in the body by esterification or oxidized to form all-trans retinoic acid (atRA). All-trans retinoic acid is believed to function in both male and female reproduction and development [34].

Retinal undergoes significant enzymatic processing in the body. The enzyme that catalyzes modification of retinal for storage is retinol acyltransferase lecithin (LRAT). Dietary retinal is dehydrogenated by alcohol dehydrogenase and retinol dehydrogenase to synthesize atRA [35]. Since Vitamin A metabolites are lipophilic, they are typically found in the serum in association with other proteins [36]. RA can also be hydrolyzed by Cytochrome P450 enzymes, including those encoded by the *Cyp26a1*, *Cyp26b1*, and *Cyp26c1* genes, rendering it inactive [37].

All-trans RA is a ligand for retinoic acid receptor (RAR) proteins, which are nuclear receptors and ligand-activated transcription factors [38]. RA also directly binds to the Retinoid-X receptors (rexinoid, RXR). RXRs are able to heterodimerize with RARs and increase their DNA binding affinity to the retinoic acid response element (RARE) [39]. Of all the retinoids, atRA has the highest binding affinity to RAR [40]. Through these interactions, Retinoids act as a gene regulators and influence cell proliferation and differentiation [41, 42].

Vitamin A deficient rats continue to ovulate on schedule, although their eggs do not appear to have matured properly, suggesting that RA may be required for normal ovarian development. RA has been shown to induce meiotic entry in cultured rat ovaries [43], and the RA responsive gene Stra8 is required for meiotic initiation in females [44]. Further studies have shown that female gonads that express Stra8 can enter meiosis without RA, suggesting that its effects can be realized independent of RA signaling [45]. When small doses of RA are added to the female diet at levels too low to support other physiological processes, 30% of oocytes will undergo meiosis. This suggests that RA may support follicle maturation and ovulation through multiple mechanisms [34], although the details of these mechanisms remain unknown. Other than the effects on follicle maturation and ovulation, female rats with severe RA deficiency have been shown to experience difficulty with uterine implantation of the embryo [46].

An investigation of the influences of isotretinoin, an acne treatment medication, on pituitary hormones may provide useful insight. Isotretinoin is a 13-cis-retinoic acid derivative that is thought to be metabolized *in vivo* to atRA, which allows it to interact with RAR. Individuals treated with isotretinoin showed decreases in LH, Prolactin, and testosterone levels [47], suggesting that it may influence reproductive processes. The study failed to measure the levels of activin, a peptide hormone that stimulates the synthesis and secretion of FSH, and has previously been implicated in modulation of RA signaling [48].

The Kipp laboratory recently conducted a pilot study of the effects of vitamin A deficiency in a mouse model of ovarian development. Preliminary data show formation of smaller than normal ovaries and increased bursa cyst development. The mechanisms by which the lack of vitamin A leads to abnormalities are not well understood. Since others have likewise reported a role for RA in ovarian development [16-18], a closer exploration of vitamin A and RA functions in the ovary may lead to better understanding of follicle development and maturation. Therefore, we propose to investigate the *in vivo* effects of inhibition of Cyp26 enzymes, of the key enzymes that degrade RA.

Cyp26b1

Cyp26 genes encode a class of cytochrome P450 enzymes responsible for the catabolism of RA [49, 50]. These enzymes convert RA to a more polar and soluble metabolite that can be more easily excreted. Degradation of RA is an important regulatory mechanism to protect the cell from excessive stimulation [51]. RA has been shown to act as a teratogen if used at high doses [52] and overstimulation by RA can manifest as cleft palate, inner ear malformations, thymic agenesis, craniosynostosis, and hypoplasia of the mandible and maxilla [53].

Tight regulation of Cyp26 gene expression determines the patterns of tissue-specific distribution of RA which are essential for embryonic development [50]. Cyp26 isoforms include *Cyp26a1*, *Cyp26b1*, and *Cyp26c1*. Cyp26a1 and Cyp26b1 proteins have high affinity for atRA, while Cyp26c1 has affinity for atRA and 9-cis-RA [37, 54, 55]. *Cyp26b1* has been shown to be expressed during early fetal development in mouse

hindbrain, limb buds, maxillary and mandibular processes [56]. In older fetuses, expression is seen in skin, precartilagenous elements and some organs [57]. *Cyp26b1* null animals exhibit craniofacial abnormalities [58] and lack of Cyp26b1 negatively impacts limb development by inhibition of chondrogenesis [59].

Cyp26b1 is expressed in the somatic cells of embryonic testes [56, 57, 60], and proper regulation of RA levels has further been shown to be critical for normal testes development [58, 60, 61]. Organ culture experiments have shown that in the presence of RA, testes are induced to express Stra8 [44]. Stra8 is also up regulated in *Cyp26b1* null mice [60], which correlates with an increase of RA levels in embryonic testes. This subsequently results in the initiation of meiosis as well as increased apoptosis of male germ cells *in utero* [61]. Embryonic testes of *Cyp26b1* null mice are largely devoid of germ cells, although the somatic cells appear unaffected [61]. Therefore, germ cells in the fetal mouse testes are initially shielded from RA-induced meiosis by the action of the Cyp26b1 enzyme [44, 60].

Recent *in vitro* studies by the Kipp laboratory have demonstrated that activin down-regulates Cyp26b1 in mouse granulosa cells [48]. These studies provide new insight into the mechanism of activin action and establish a connection between hormone regulation, ovarian development, and the importance of critical nutrients such as RA. These *in vitro* studies also showed that activin, RA, and a Cyp26 pharmacological inhibitor can each independently induce proliferation of granulosa cells [48]. Further studies show that the expression of Cyp26b1 in the neonatal mouse ovary is highest within the first 10 days

after birth. This time window correlates to the development of the primordial follicle pool [48].

Talarozole (R115866)

The highly toxic effects of exogenous RA have pressured the development of new methods to increase endogenous retinoic acid levels by targeting RA-degrading enzymes. R115866, marketed under the trade name Talarozole, is an investigational retinoic acid metabolism blocking agent (RAMBA) that works by selectively inhibiting Cyp26 isoforms. This drug is used in the treatment of skin conditions, and studies have shown that it results in increases in endogenous RA levels in epithelial and smooth muscle tissues both *in vivo* and *in vitro*. R115866 appears to work systemically in that it results in increases in serum retinoic acid levels. It has also been used to drive the differentiation of induced pluripotent mouse cell lines and embryonic stem cells into haploid gamete-like cells [62], reinforcing a role for retinoic acid in the establishment of the germ line during development.

Hypothesis

In vitro studies have shown that RA increases granulosa cell proliferation and stimulates germ cell meiosis [48]. We hypothesize that inhibition of Cyp26 during the first week after birth, when the levels of *Cyp26b1* expression are the highest in the ovary, may play an important role in *in vivo* ovarian development by increasing endogenous atRA levels. To test this hypothesis, we treated mice pups with R115866 during the first week after

birth or treated mice with a vitamin A deficient (VA-) diet and examined ovary tissues for signs of impaired development or pathology.

Methods

Mouse Breeding

In vivo studies were carried out using CD1 mice maintained on a 12 hour light/dark cycle. Food and water were supplied *ad libitum* and all animals were cared for according to all federal and institutional guidelines.

R115866 Injections

Mouse pups were injected with 0.5, or 1.0mg/kg R115866, or vehicle control, daily from day 1 to day 7 after birth. Ovaries and serum were collected at day 19, week 7, or week 15 for histology, RNA analysis, and hormone assays.

A 1.1mg aliquot of R115866 was dissolved in 5µl of DMSO, yielding a stock solution with a concentration of 0.22mg/µl. This stock solution was further diluted in corn oil to yield a final DMSO concentration of 0.23% in each working solution. Mouse pups born to mothers on a regular diet were each injected with 20ul of this preparation. This dose was based on an estimated weight of 2 grams per pup. Using a 1cc syringe with a 28G half inch needle, injections were administered subcutaneously on the lower dorsal area where a skin fold could be made. Each day, one syringe was loaded with the total R115866 preparation and used to inject 20ul into each mouse pup in sequence.

Euthanasia, Ovary Collection and Serum Collection

Mice were euthanized by CO_2 exposure in a closed chamber. Euthanasia was confirmed by cervical dislocation.

Ovaries were dissected from euthanized mice, and one ovary was fixed for histological or immunohistochemical studies (see below). The other ovary was cleaned of connective tissues in a 1x PBS solution. Cleaned ovaries were then transferred to microfuge tubes and kept on dry ice prior to storage in a -80˚C freezer for subsequent RNA analysis.

Blood was collected from freshly euthanized mice via cardiac puncture using a 3-5ml syringe with a 27G ½ inch needle. Blood was transferred to microfuge tubes and allowed to clot at room temperature for 90 minutes followed by centrifugation at 10,500 rcf for 15 minutes. Serum was then pipetted into fresh microfuge tubes and stored in a -80˚C freezer for later analysis.

Fixation and Histology

Ovaries were fixed in 10% formalin overnight followed by 3 x 20 minute washes in 50% ethanol and 3 x 20 minute washes in 70% ethanol. The ovaries were then paraffinized and serially sectioned in 5 micrometer sections using the Northwestern University Ovarian Histology Core Center. Every other slide was stained with hematoxylin and eosin (H&E) staining for morphology studies, and unstained slides were used for immunohistochemistry studies. For young animals, slides were affixed with 5 sections in series. For one-year old animals, both ovaries were fixed, and three sections were affixed to each slide.

VA Deficient Diet and Follicle Counting

A VA deficient study was conducted using day 19, week 7, week 15, and one year female mouse ovaries. CD-1 mice were given either a VA- or control diet (Harlan Laboratories) 24 hours before introducing males for mating. Female breeders remained on either diet through gestation and until pups were weaned. Pups continued on either a VA- or regular diet until ovaries were harvested for morphological study or RNA extraction. For the one year recover study, mice remained on VA- diet to week 16 then switched to a regular diet until ovaries were harvested. Histology was completed as described above.

Images were taken of every 5th section for each series of H&E stained ovarian samples. Total follicle numbers and follicle types were estimated by multiplying follicle counts by five times to compensate by proportion of sections counted [20, 21]. Only structures containing a visibly distinct nucleus were considered to be follicles. This method minimizes the chance of double counting a follicle that spans multiple sections. Figure 1 shows the different stages of ovarian follicle development. These stages are characterized not only by the follicle size, but also by the type and number of granulosa cell layers that surround the oocyte. The primordial follicle has a characteristic single layer of flat granulosa cells. The BC follicle stage is the next progression in follicle development, and is characterized the appearance of both flat and cuboidal granulosa cells. The BC follicle stage is not shown in Figure 1, but this stage represents a transition between the primordial and primary follicle stages. The primary follicle stage is characterized by a single layer of cuboidal granulosa cells. As a primary follicle progresses to the secondary follicle stage, the granulosa cell layers multiply; by the tertiary follicle stage, several

layers of granulosa cells surround the oocyte. The tertiary follicle stage is further characterized by the appearance of multiple layers of thecal cells. The antral (Graafian) stage of follicle development has the characteristic presence of a visible cavity in the follicle as the follicle begins to prepare for ovulation. Atretic follicles are characterized by misshapen oocytes and granulosa and thecal cells that appear to atrophy. Multioocytic follicles are present when multiple oocytes are contained in indistinguishable layers of granulosa cells encapsulated in layers of thecal cells. Multinucleolar follicles were also observed, indicated by the presence of more than one dark structure in a single oocyte.

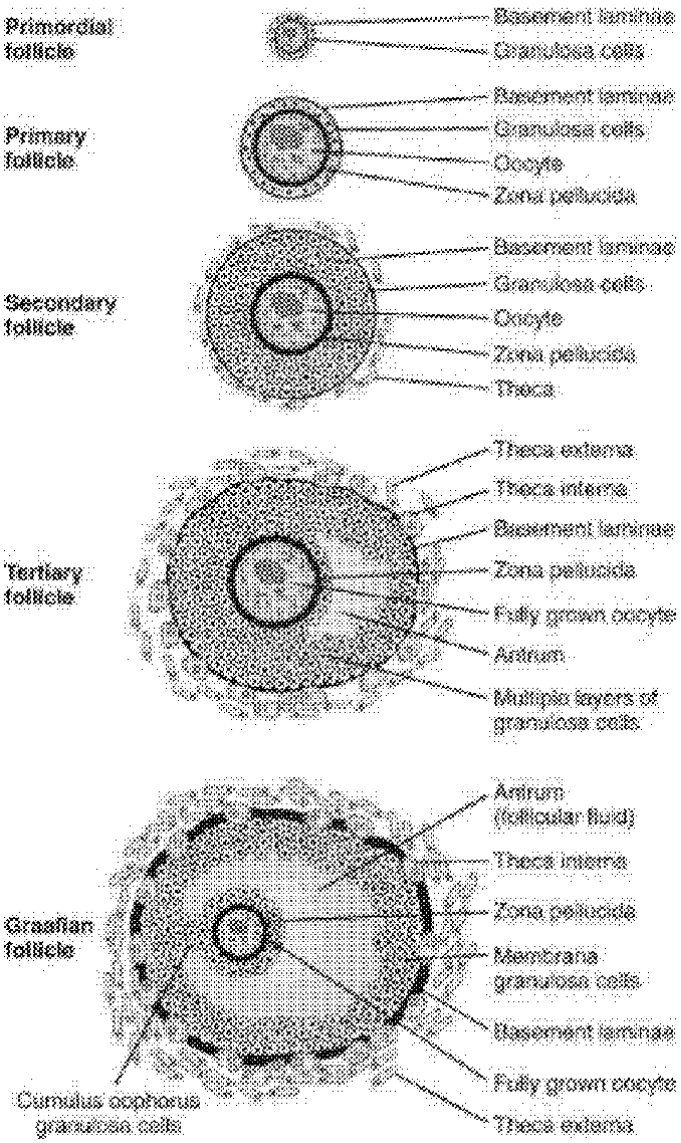


Figure 1. Diagram showing different stages of follicle maturation. [63]

Immunohistochemistry

Slides were chosen for IHC assays based on a large number of follicles apparent in series on a slide. Sections were used for TUNEL assays or stained with antibodies for either Ki67 or Cyp26b1 proteins. Assays were done on three consecutive sections for each treatment group to compare signal levels within single follicles. Slides were soaked 2 x 10 minute in citrisolv (Fisher Brand) to de-paraffinize wax. This was followed by 3 minute washes in decreasing ethanol concentrations of 100, 95, 70, and 50%. Slides then received a final wash of 3 minutes in ddH2O before antigen retrieval treatment. Slides were placed in a solution of 0.1M sodium citrate and microwaved on low for 9 minutes with attention to not allow it to boil over. The slides were left in the solution and allowed to cool for 20 minutes before 2 x15 minute washes in PBS-Tween to permeabilize the samples. Sections receiving TUNEL staining were kept moist in PBS. Sections used for Cyp26b1 and Ki67 staining were then soaked in a 3% hydrogen peroxide solution for 15 minutes followed by a brief rinse in PBS. They were then soaked in avidin for 15 minutes, briefly rinsed in PBS-Tween, soaked in biotin for 15 minutes, and then briefly rinsed in PBS-Tween. Sections were then blocked in 10% rabbit serum (Cyp26b1, Sigma) or goat serum (Ki67, Abcam) in 3% BSA-PBS for 1 hour at 4°C. Sections were incubated overnight in goat anti-mouse Ki67 (ab16667, 1:100) or rabbit anti-mouse Cyp26b1 (Sigma, 1:50) diluted in 3% BSA-TBS-10% serum at 4°C for 16 hours. Negative controls were kept in blocking solution. Slides that had been incubated with primary antibody were then rinsed 3 x 5 minutes in PBS-Tween followed by a 30 minute incubation in secondary rabbit anti-goat biotinylated IgG antibodies (Cyp26b1, 1:1000) or goat anti rabbit biotinylated IgG (Ki67, 1:100) in 3% BSA-PBS for 30 minutes at room

temperature. After treatment with secondary antibodies, slides were rinsed 3 x 5 minutes in PBS-Tween and incubated in ABC reagent for 30 minutes at room temperature followed by 5 x 5 minute washes in PBS-Tween. Sections stained for Cyp26b1 and Ki67 were kept moist in PBS while TUNEL staining was carried out on other sections. These sections were incubated in TUNEL enzyme and label for experimental assays or label solution alone to serve as negative controls. During the last five minutes of the TUNEL assay, Cyp26b1 and Ki67 samples were gently blotted with a kimwipe and incubated in TSA. Slides were rinsed 3 x 5 minutes in PBS-Tween. Slides were mounted with mounting media containing 4',6-Diamidino-2-Phenylindole (DAPI) to stain double-stranded DNA, and sealed to cover slides with clear nail polish. Tissues were visualized and imaged using a confocal microscope.

RNA Extraction

Lysate solution was added to frozen ovaries and homogenized using a Tissue Tearor™ at high speed until tissue particles were no longer visible. RNA from the homogenized samples was extracted following the RNAeasy Mini Kit (Qiagen cat # 74104) protocol.

Real Time RT-PCR

Reverse transcription PCR was done using High Capacity cDNA Reverse Transcription Kit (Applied Biosciences) in 20ul reactions. The product was then diluted 10 or 20 fold before its use in real-time PCR. Real-time PCR assays were used to quantify relative mRNA expression in each of the treatment groups. Rat ribosomal protein L19 (RPL19)

was used as a control for each assay. Assays were done in duplicate and the average cycle threshold (CT) values were used to quantify mRNA using the $2^{-\Delta ct}$ method. The cycle threshold is the value of cycles it takes for a sample signal to surpass the background signal. This determines the value of mRNA expression and is then standardized by subtracting the cycle threshold value of a constitutively active housekeeping gene (RPL19). The difference in the two CT values is the standardized difference in the number of cycles it took for the gene of interest to reach its cycle threshold. Each cycle represents a doubling of the signal or mRNA synthesis. To determine the relative expression level, the treatment groups were compared using the $2^{-\Delta ct}$ method [64]. Assays measured levels of Cyp26b1 transcripts, along with several markers of proliferation and apoptosis. Apoptotic markers, which are indicative of increases in programmed cell death, included Apaf1, BAD, BCL2 BCL121, Caspace 3, Caspace 7, and Trp53. Proliferative markers, which are indicative of increases in cell cycle entry and progression, included CyclinD1, CyclinE1, Ki67, and PCNA.

SYBR master mix was used to make 20ul assay volumes in a 96 well plate. A StepOnePlus™ Real-Time PCR system was used in conjunction with the StepOne software v. 2.1 (Applied Biosystems). The thermal cycle conditions were as follows: 1x 50°C, 2 min; 95°C, 10 min; 45x 95°C, 15 sec; 58°C, 45 sec; 72°C, 1 min; finished with 95°C for 15 sec. Duplicate ct values were checked to ensure a difference of less than 1.0 ct. If differences in duplicates were greater than 1.0, curves were analyzed for character and the curve with the expected characteristics was used without duplicate.

Statistical Analysis

Comparison of data between 2 groups was done using a one tailed student's t-test. For comparisons between more than 2 groups, a one way ANOVA was done with a Tukey-Kramer *post hoc* analysis. Data are shown as Mean±S.E. and $P \leq 0.05$ is considered significant.

Results

Effect of R115866 on Mouse Body weights

Figure 2 shows the body weight measurements taken for R115866 treatment groups and DMSO control at each time point; day 19, weeks 7 and 15. Mice receiving injections of 0.5mg/kg and 1.0mg/kg R115866 showed significant decreases in weight at day 19 and week 7 ($P<05$; n=3-5) but no differences were observed at week 15.

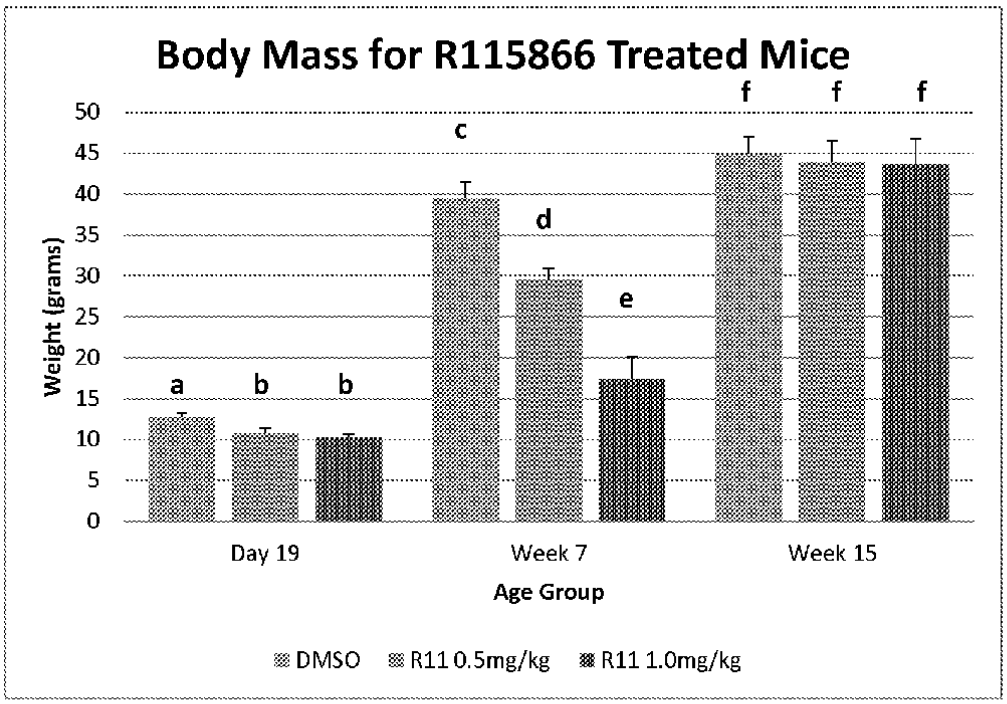


Figure 2. Body weights of control and R115866 treated mouse pups at day 19, week 7, and week 15 with different letters indicating statistically significant differences ($P<0.05$; n=3-5).

Histology

R115866 Treatment Groups

H&E staining for sections of both R115866 and VA- treatment groups show pathologies at all time points analyzed. Figure 3 shows representational pictures of day 19 DMSO control (*A*), and R115866 treated ovaries. Day 19 R115866 treated mouse ovaries show smaller ovaries as compared to vehicle (DMSO) treated ovaries along with decrease in follicle numbers. The R115866 treated ovary shows 3 follicles with multiple nuceoli, which was common amongst other samples in the R115866 treatment groups. By week 7, the decreased size of R115866 treated mouse ovaries becomes more pronounced (*Figure 4B*) as compared to DMSO treated control (*Figure 4A*). Atretic follicles are also frequently observed in the R115866 treated group (*Figure 4C*). Along with these pathologies, we observed a bilateral decrease in the presence of follicles, dilated blood vessels, and prominent bursa cysts in 100% of animals.

VA- Diet treatment Groups

Based on the results from previous studies conducted by the Kipp laboratory, similar pathologies are observed in day 19 VA- deficient diet mouse ovaries (*Figure 5B, C*) including decreased ovary size with decreased presence of follicles as compared to controls (*Figure 5A*). Other pathologies include presence of multioocytic follicles (*Figure 5B, D*). Atretic follicles are also present and represented in Figure 5C and shown enlarged in Figure 5E. Week 7 VA- ovaries (*Figure 6B*) remained smaller than control (*Figure 6A*) with the appearance of fewer follicles and reduced corpus luteum. VA-

ovaries show atretic follicles, including one with macrophage invasion observed (*Figure 6B,C*). By week 15, pathologies noted in week 7 VA- ovaries became more pronounced compared to control (*Figure 7A, B*) with smaller and fewer follicles and corpus luteum. Ovaries began to develop large bursa cysts (*Figure 7B*) and hemorrhaging follicles are noted (*Figure 7C*).

Further experiments were designed to investigate the long term effects of early VA deprivation. Mice treated with either regular or VA- diet for 16 weeks were allowed to continue a regular diet until one year. H&E staining was done on harvested ovaries (*Figure 8*). One control ovary appears to contain a follicle cyst which appears larger in serial sections (*Figure 8A*). However, this is not as dramatic as the follicle cysts and bursa cysts that appear in the VA- treated ovaries in (*Figure 8C,D*). In addition, appearance of hemorrhaging follicles is noted throughout sections of VA- ovaries (*Figure 8E*). The tissues surrounding the cyst appear to be epithelial, consistent with an ovarian origin (*Figure 8D*). A large mass of cells appears outside the bursa membrane (*Figure 8D*). This mass of cells also appears epithelial and originates from the bursa membrane (*Figure 8G, H*). It is currently unclear if this mass of cells indicates carcinoma.

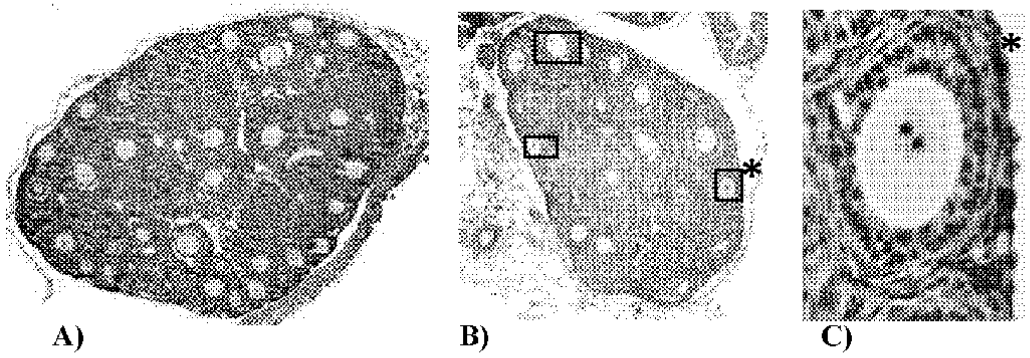


Figure 3. Histology of day 19 1.0 mg/kg R115866 treated mouse ovaries. A) DMSO Control, B) R115866 treated, C) enlarged segment showing multinucleated follicle from day 19 R115866 treated mouse.

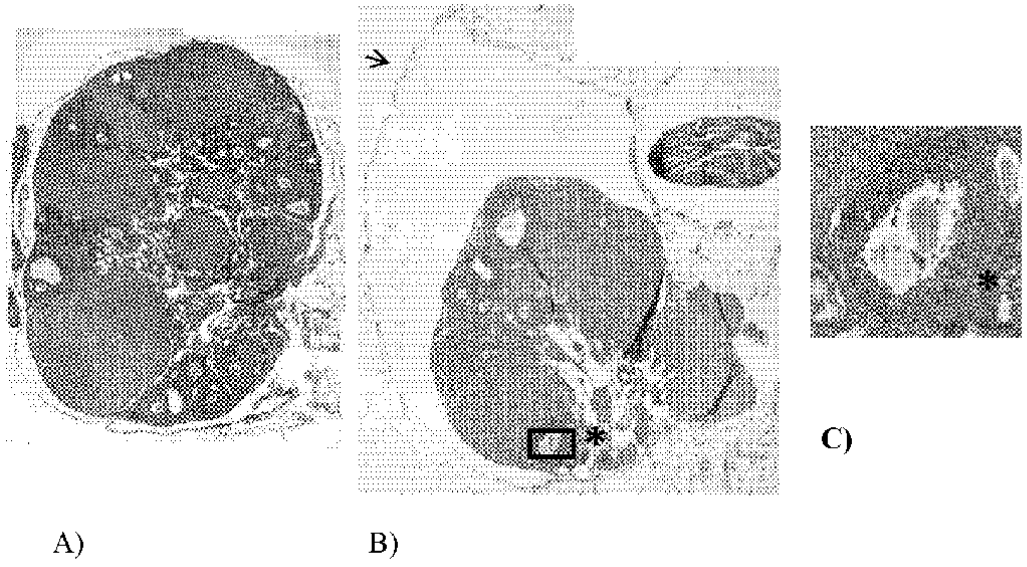


Figure 4. H&E staining of Week 7 0.5 mg/kg R115866 treated mouse ovaries. A) DMSO Control, B) R115866 treated ovary with bursa cyst, C) enlarged segment showing atretic follicle from week 7 R115866 treated mouse ovaries.

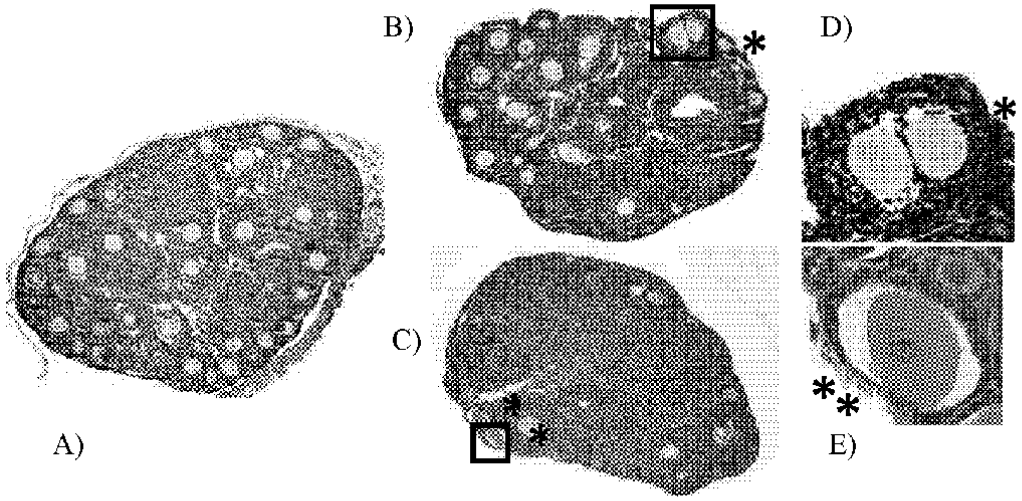
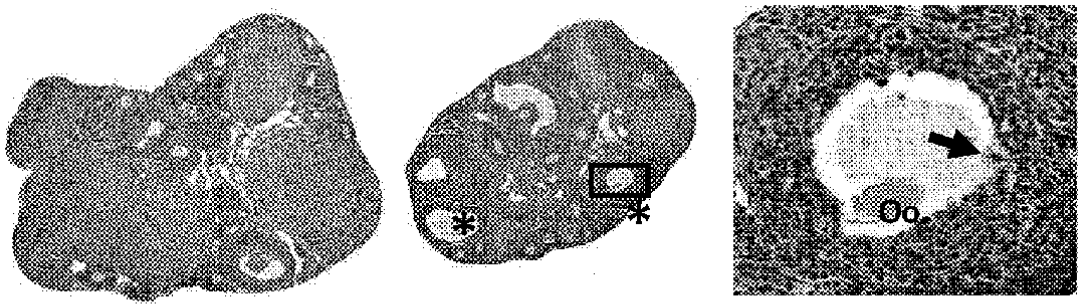
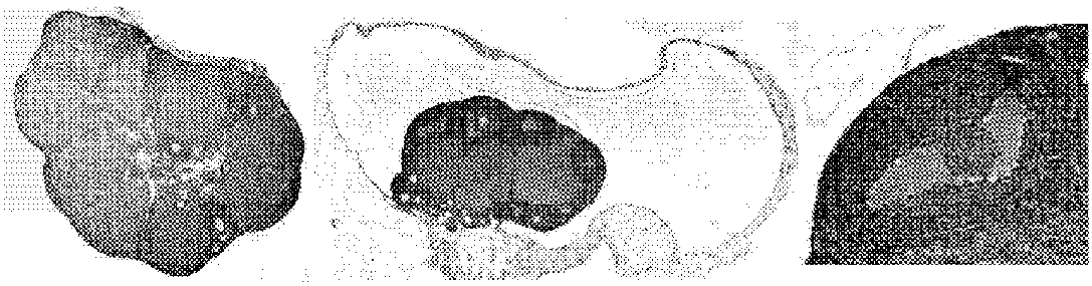


Figure 5. H&E staining of representational day 19 VA- mouse ovaries A) Control diet ovary, B &C) VA- mouse ovaries showing decreased ovary size, multiocytic follices (B) and atretic follicles (C). Enlarged segments of VA- ovaries showing multioocytic follicles (D) and atretic follicles (E). Images courtesy of Michael Demczuk and Tessa Bonney.



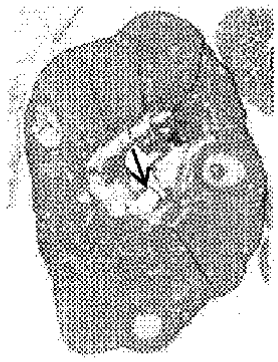
A) B) C)

Figure 6. H&E staining of representational week 7 VA- mouse ovaries A) Control diet ovary, B) VA- mouse ovaries showing decreased ovary size and atretic follices. Decreased numbers of corpus luteum are also noted. (C) Enlarged segments of VA- ovaries showing atretic follicle with presence of macrophage (arrow) indicating immune response. Images courtesy of Michael Demczuk and Tessa Bonney.



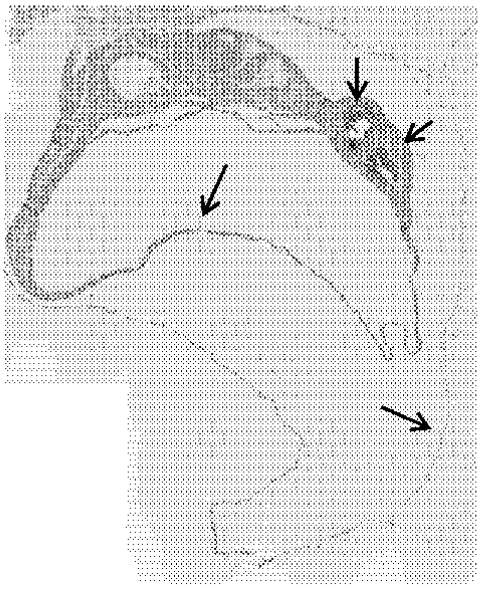
A) B) C)

Figure 7. H&E staining of representational week 15 VA- mouse ovaries A) Control diet ovary, B) VA- mouse ovaries showing decreased ovary size, atretic follices, and large bursa cyst. Decreased numbers of follicles and corpus luteum are also noted. (C) Enlarged segments of VA- ovaries showing hemorrhaging follicle. Images courtesy of Michael D. and Tessa B.

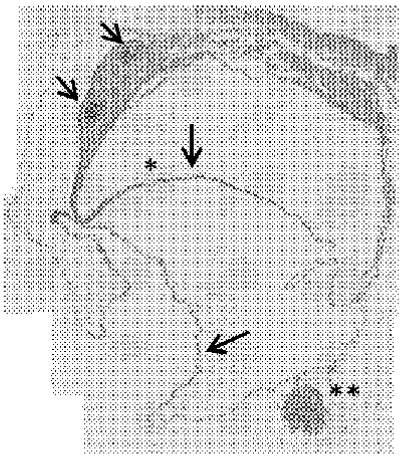


A)

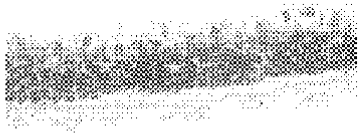
B)

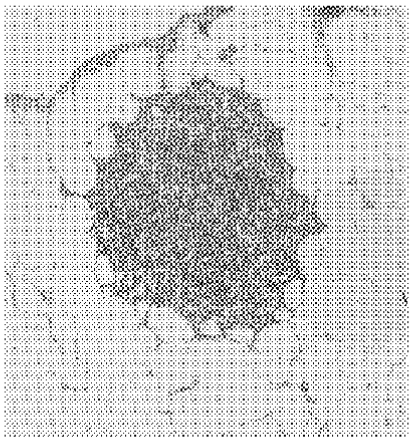


C)

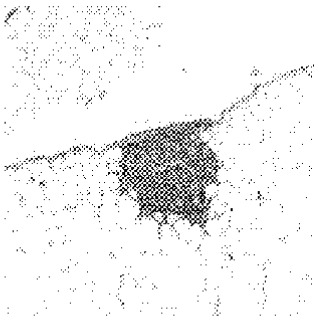


D)





E) F)



G) H)

Figure 8. H&E staining of representative ovaries from 16 week VA-mice at one year. A & B) Control with formation of a cyst present. C & D) VA-treated ovaries with several pathologies. Most noted are very large follicle and bursa cysts and hemorrhaging follicles in both sections. D) Formation of a mass of cells external and originating from the bursa membraine. E) Enlarged segment of tissues from ovary (D) surrounding the follicle cyst showing normal appearing ovarian type tissue. F) Enlarged segment of the cell mass forming outside of the bursa from ovary (D). G & H) Cell mass from from section (D) enlarged in (F) shown to originate from the bursa membrane.

Follicle Counting

R115866 Treatment Group

A count of follicle number and classification of follicle types in day 19 ovaries shows significant differences between treatment and control groups. R115866 treatment leads to a significant decrease in total follicle numbers in both treatment dose groups. ($P<0.05$; n=3-5) (*Figure 9*).

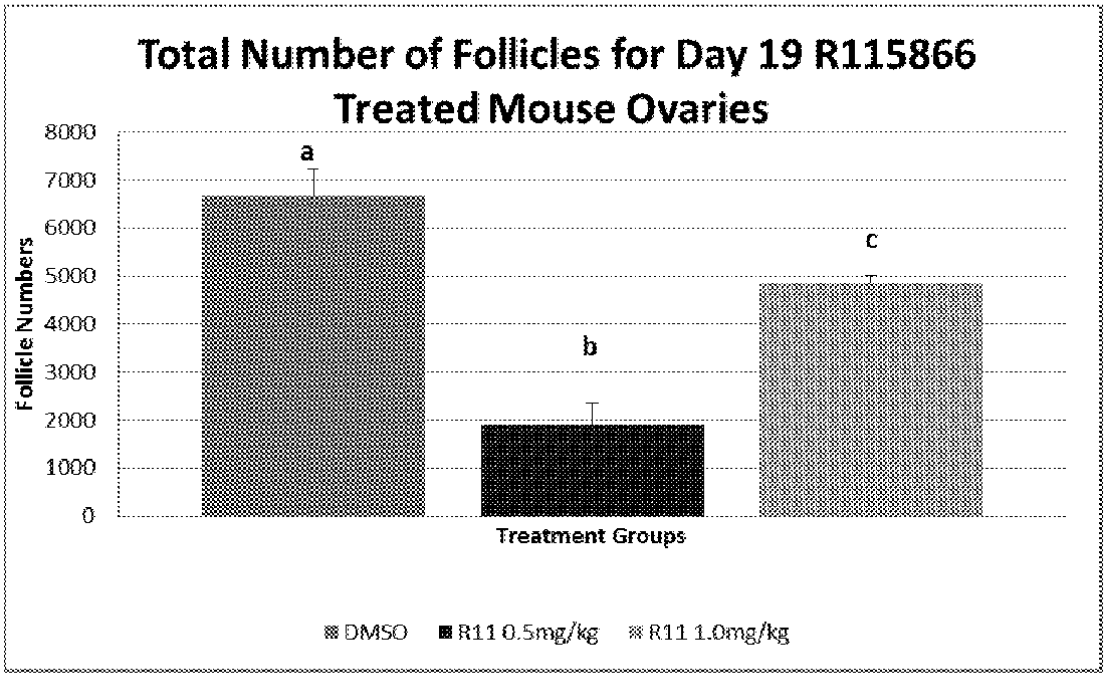


Figure 9. Total follicle numbers in ovaries harvested from day 19 control and R115866 treatment groups with different letters representing statistically significant differences. ($P<0.05$; n=3-5)

A significant difference in the numbers of primordial and BC follicles was observed in R115866 treated ovaries compared to control ovaries at day 19 for the 0.5mg/kg treatment group ($P<0.05$; n=3-5) (*Figure 10*). Although both 0.5mg/kg and 1.0mg/kg R115866 treatment groups had decreases in primordial and BC follicle counts, the higher dose did not have a statistically significant decrease in either primordial or BC follicle counts.

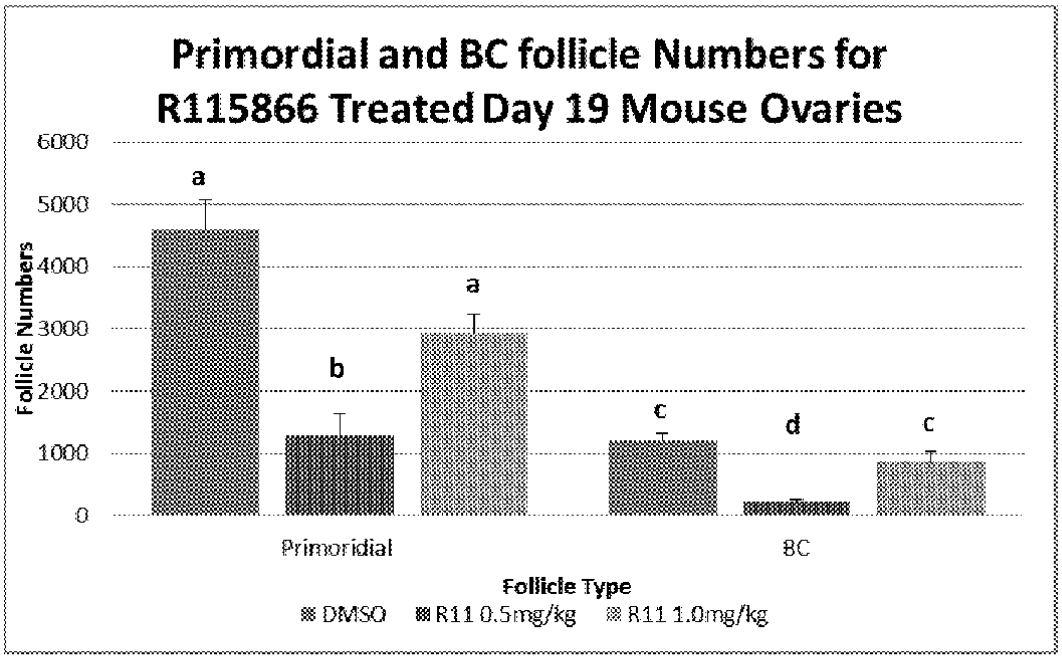


Figure 10. Primoridial and BC follicle type counts amongst ovaries harvested from day 19 control and R115866 treatment groups with different letters representing statistically significant differences. ($p<0.01$; n=3-5)

A significant decrease in primary and secondary follicles was observed in the 0.5mg/kg R115866 treatment group ($P<0.05$; n=3-5) (*Figure11*) compared to controls. Decreases in primary and secondary follicle counts for 1.0mg/kg R115866 treated ovaries were not statistically significant and for secondary follicle counts, 1.0mg/kg R115866 treated ovaries show no statistically significant difference than either control or the lower dose treatment group. Tertiary follicle counts of either the lower dose or higher dose R115866 treatment groups do not show to be statistically different compared to control, but there is a statistically significant increase in the higher dose treatment group compared to the lower dose ($P<0.05$; n=3-5). The numbers of multi-nucleoli follicles show a statistically significant increase in the higher dose R115866 treatment ($P<0.05$; n=3-5). The prevalence of other follicles types shows no significant differences.

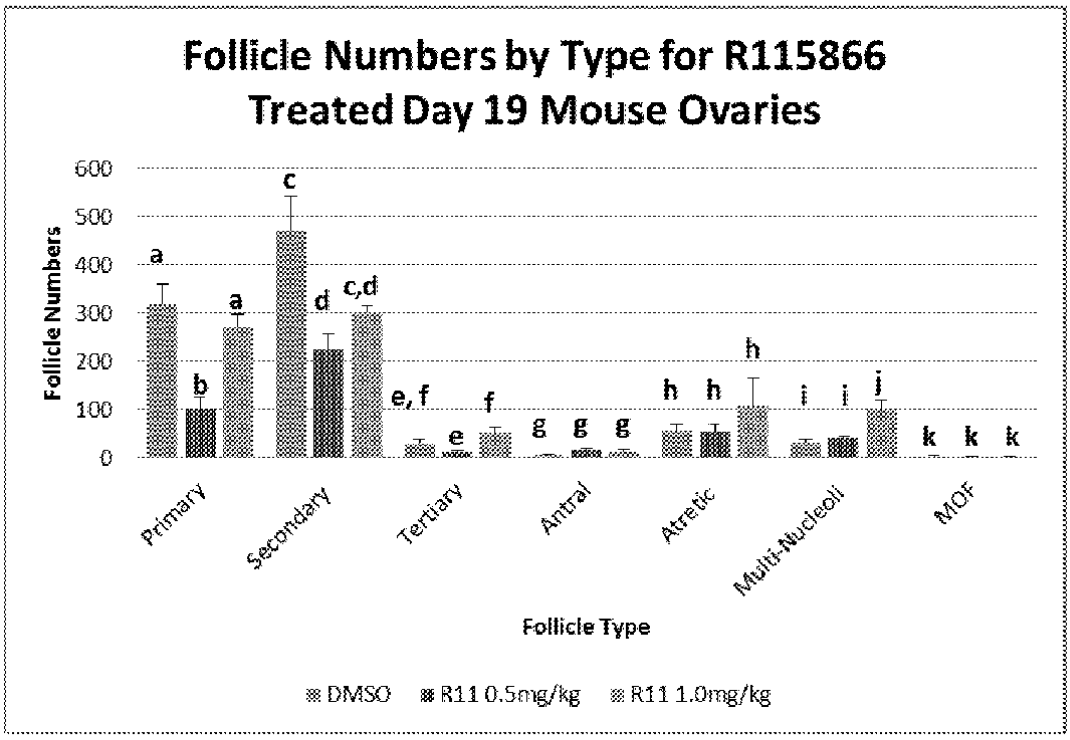


Figure 11. Primary, secondary, tertiary. antral, atretic, multi-nucleolic, and multi-oocytic follicle numbers in control and R115866 treatment groups with different letters representing statistically significant differences. ($p<0.05$; n=3-5)

VA- Follicle counts

Vitamin A deficient animals show a decrease in total follicle numbers at day 19 (*Figure 12*) (n=3), but this does not reach significance. These ovaries also show small, but not significant decreases in primordial and BC follicles (*Figure 13*). Small, but not significant differences were observed in primary, secondary, tertiary, antral, and multi-nucleolic follicles (*Figure 14*). The numbers of atretic follicles significantly increase in the VA- treated ovaries ($p=0.0238$; n=3)

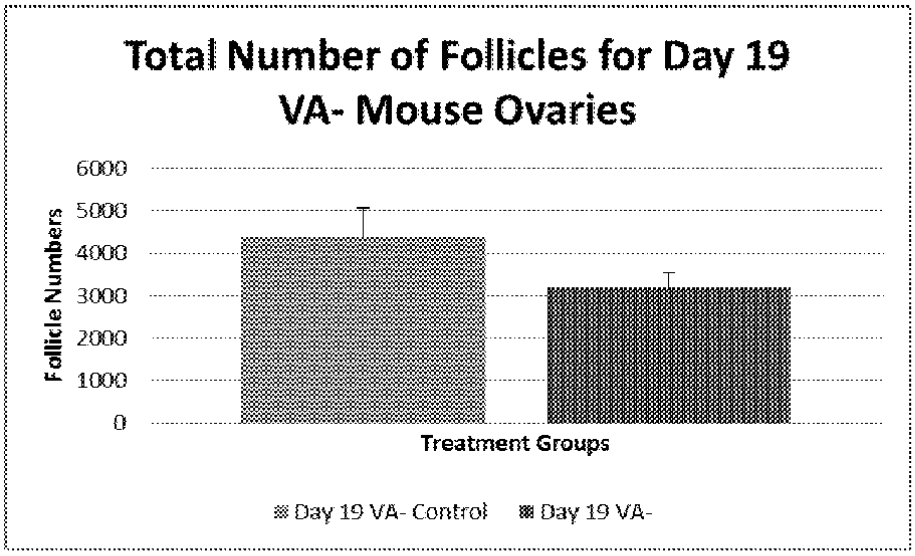


Figure 12. Total follicle numbers of ovaries harvested from day 19 control and VA- treatment groups showed no significant difference. (n=3)

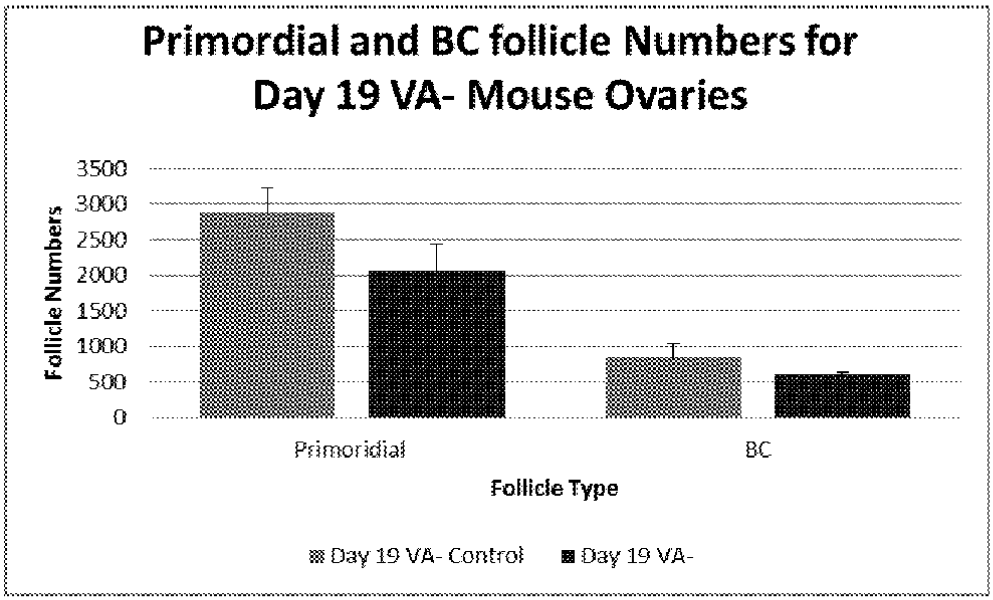


Figure 13. Primoridial and BC follicle type counts of ovaries harvested from day 19 control and VA- treatment groups showed no significant differences. (n=3)

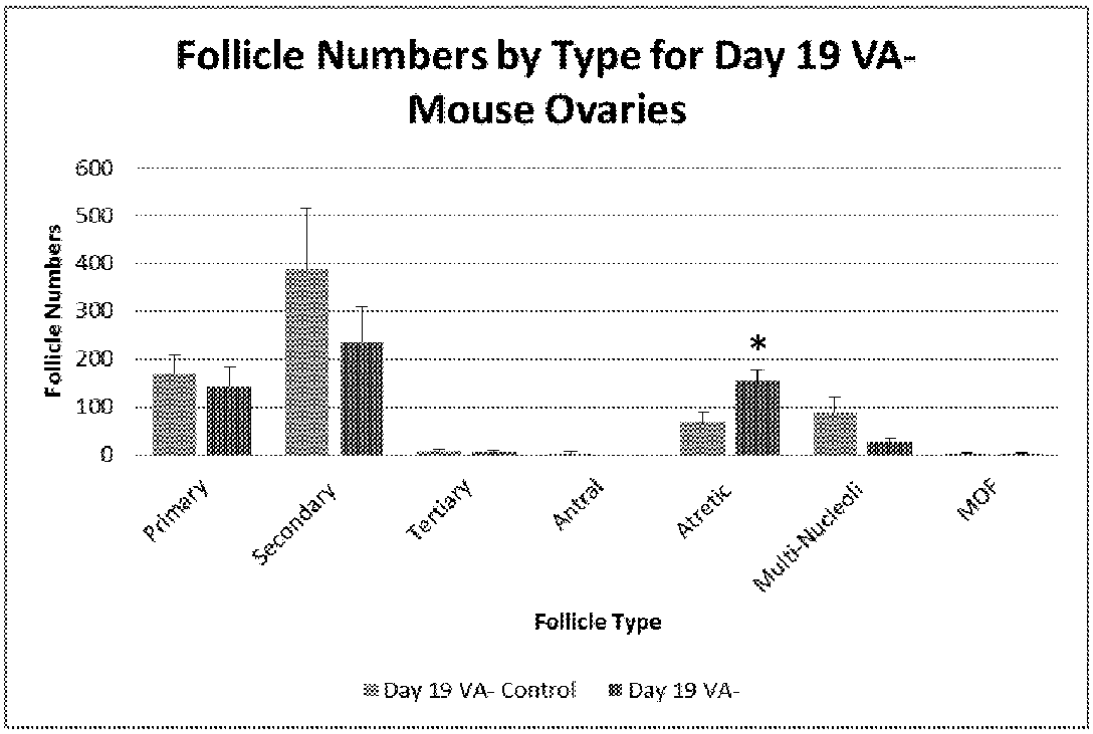


Figure 14. Primary, secondary, tertiary. antral, atretic, multi-nucleolic, and multi-oocytic follicle numbers in day 19 control and VA- treatment groups. (*$p<0.05$; n=3).

IHC

Ovarian tissue samples were stained for Cyp26b1, Ki67, and TUNEL, and images were taken using a confocal microscope. Staining for Cyp26b1 appears to be increased in R115866-treated ovaries as compared to DMSO controls (*Figure 15*). Staining for the proliferative marker Ki67 also appears to be increased in R115866-treated ovaries and shows a similar pattern seen with Cyp26b1 staining (*Figure 16*). In contrast, detection of apoptosis using a TUNEL assay shows a decrease in the appearance of apoptotic cells in R115866 treatment groups (*Figure 17*).

CYP26B1

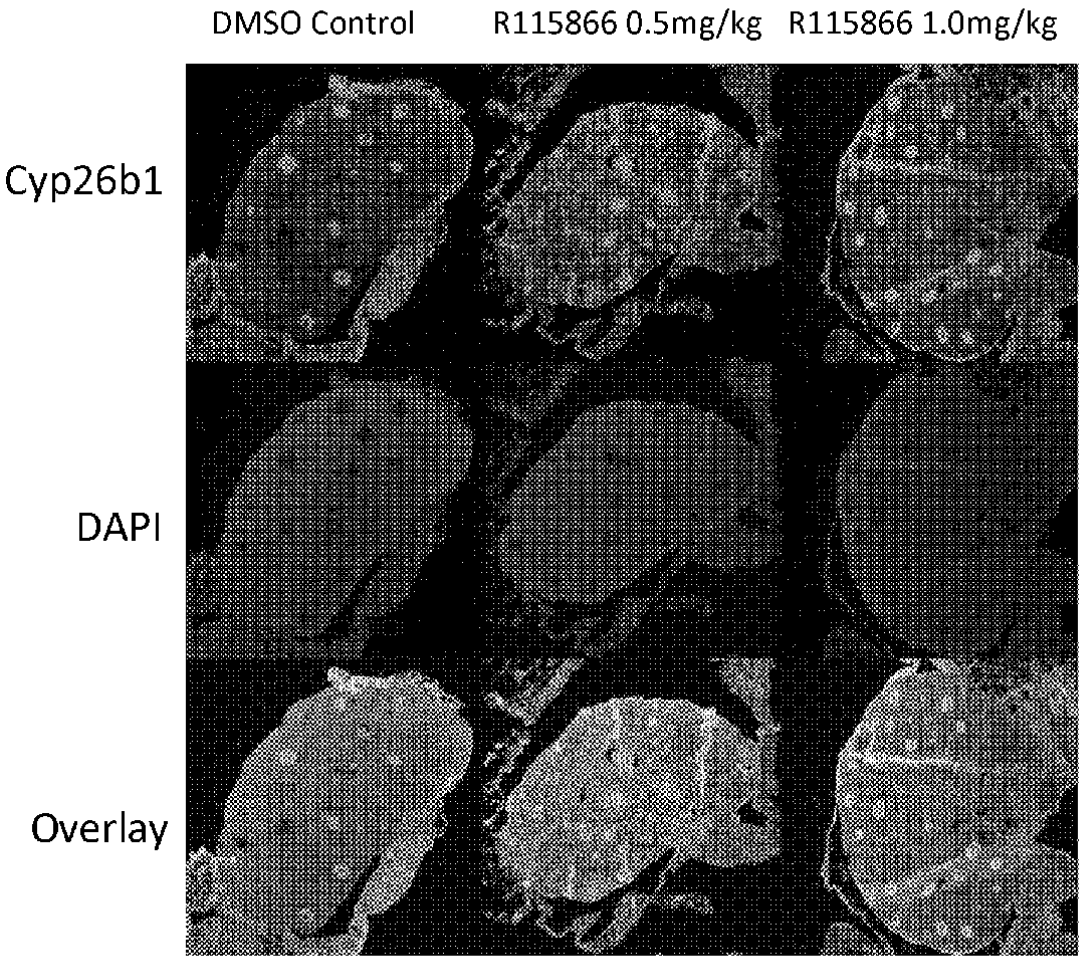


Figure 15. Immunohistochemical staining for retinoic acid degrading enzyme Cyp26b1 in control and R115866 treated day 19 ovaries.

Ki67

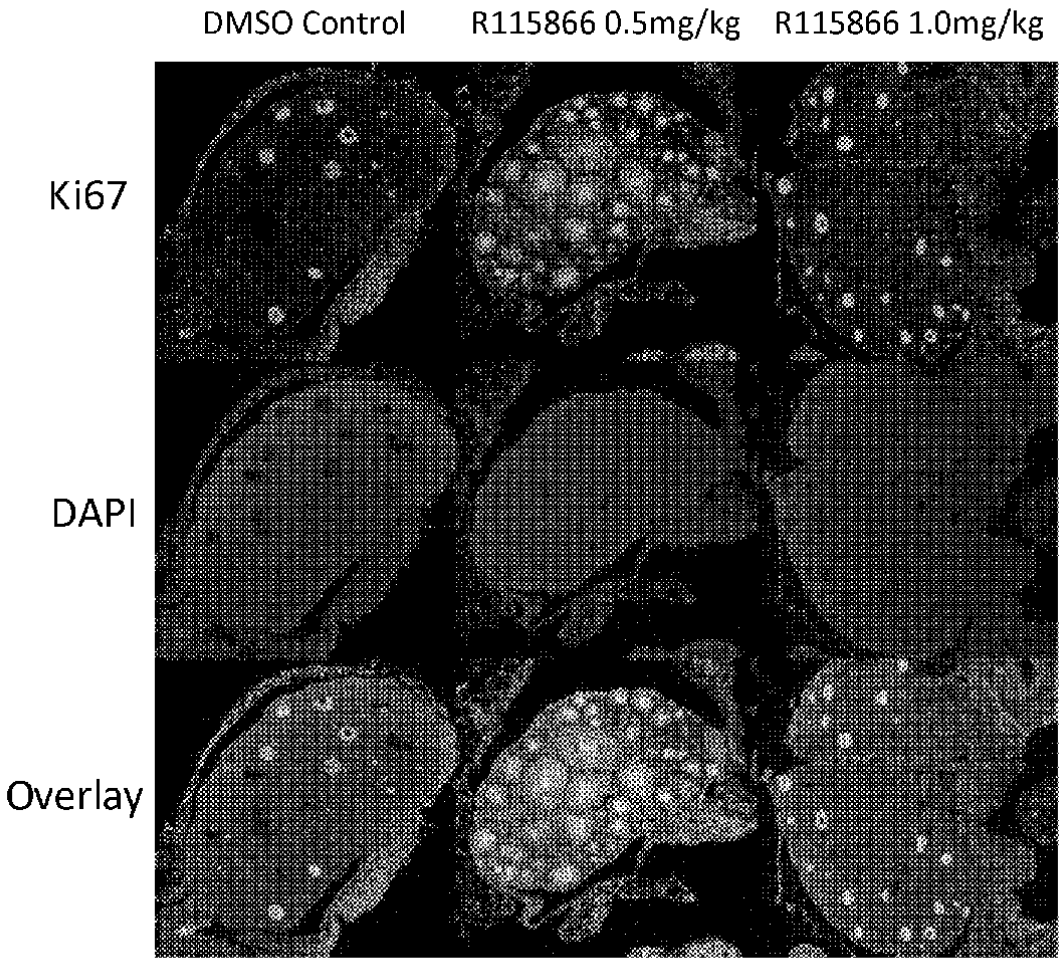


Figure 16. Immunohistochemical staining for proliferative marker Ki67 in control and R115866 treated day 19 ovaries.

TUNEL

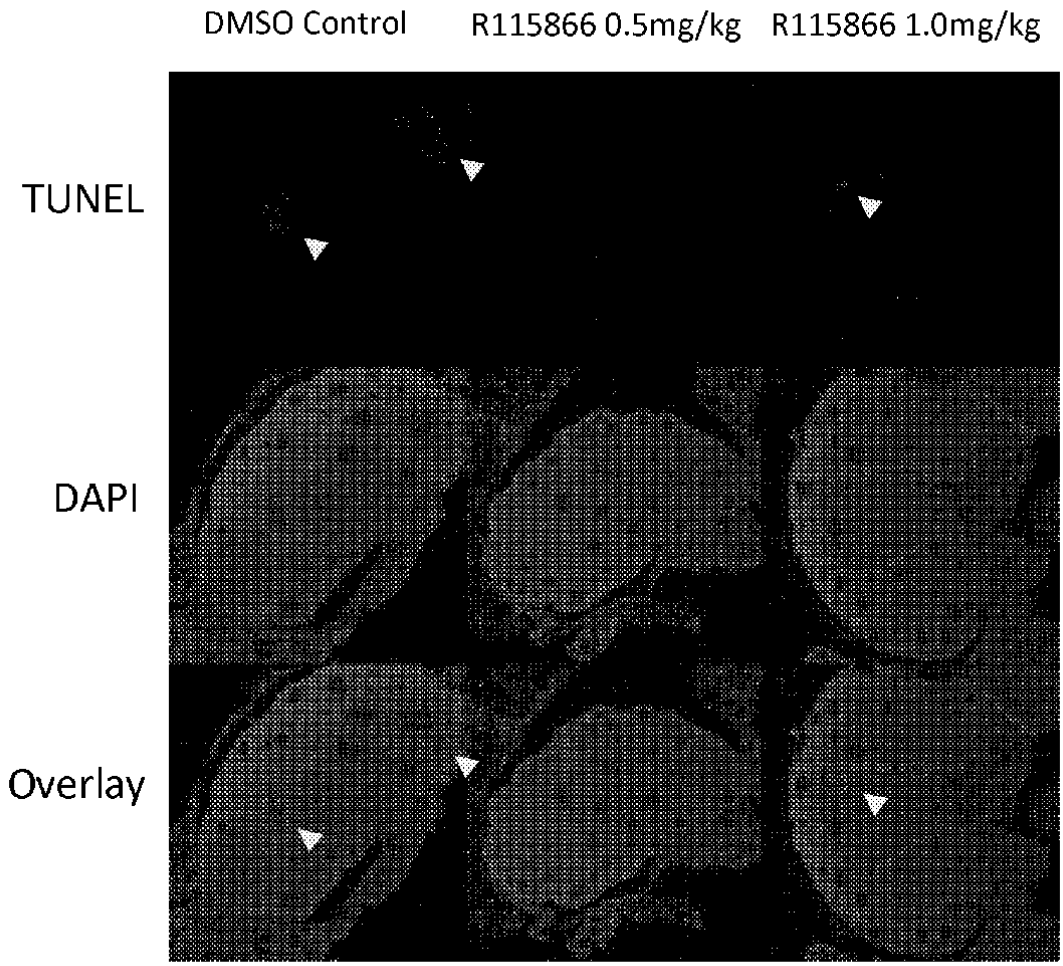


Figure 17. TUNEL staining for control and R115866 treated day 19 mouse ovaries.

Real Time PCR

To further validate and quantify the IHC results seen in day 19 R115866-treated ovaries, levels of Cyp26b1 as well as several proliferative (CyclinD1, CyclinE1, Ki67, and PCNA) and apoptotic (Apaf1, BAD, BCL121, BCL2, Caspace3, Caspace7, and Trp53) markers were assayed by rtPCR. Figure 18 shows an increase in the relative expression of Cyp26b1 in both R115866 treatment groups although this increase was not statistically significant. Interestingly, there is also an increase in proliferative markers CyclinE1, as well as PCNA which appear to be dose dependent, but these increases were also not statistically significant.

Figures 21 and 22 show the relative expression of apoptotic markers in DMSO control, 0.5mg/kg and 1.0mg/kg R115866 treated mouse ovaries. A general trend towards an increase in the relative expression of apoptotic markers is seen, with a significant increase in caspace 7 in the 1.0mg/kg R115866 treated ovaries ($P<0.05$; $n=3$-5). Increases in the relative expression of BCL121, BAD, TRP53, and BCL2 were not statisitically significant. Although TUNEL assays suggested a decrease in the presence of apoptotic cells, this suggests that it may have failed to capture the effects of the upregulation of pro-apoptotic factors, possibly due to an acceleration of the cell death process.

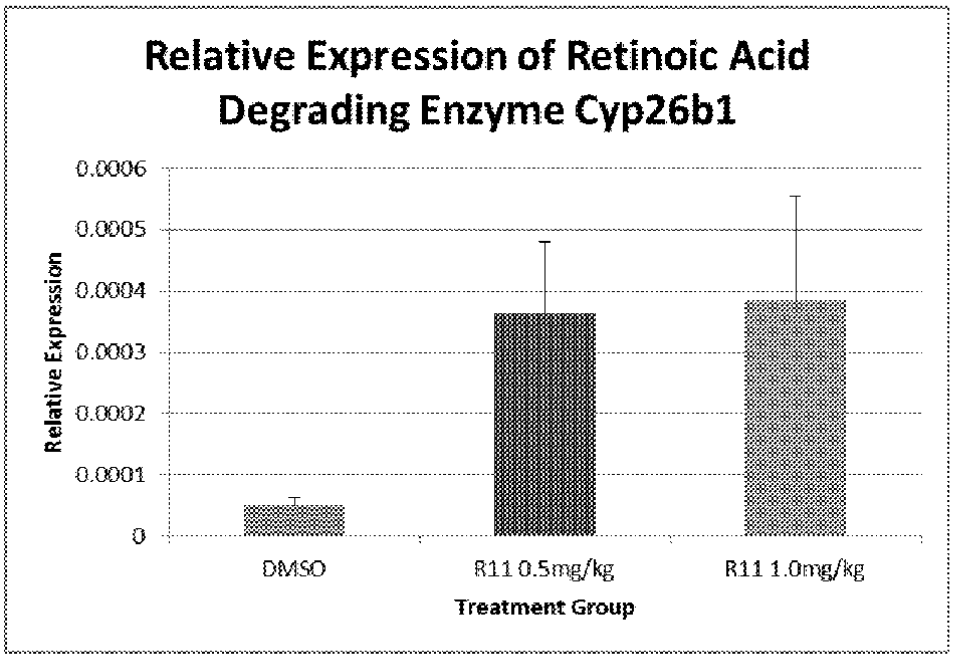


Figure 18. Relative expression of retinoic acid metabolising enzyme Cyp26b1 in day 19 control, and R115866 treated ovaries do not show a statistically significant increase in relative expression amongst R115866 treatment groups and DMSO control. (n=3-5).

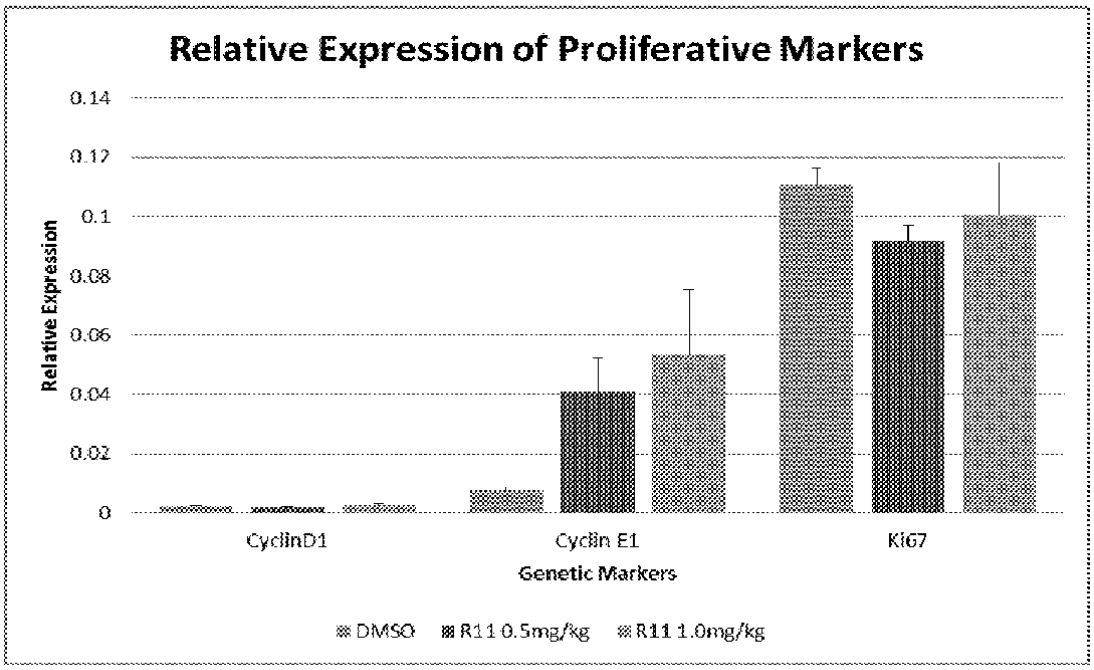


Figure 19. Relative expression of proliferative markers in day19 control and R115866 do not show statistically significant difference amongst treatment groups. (n=3-5).

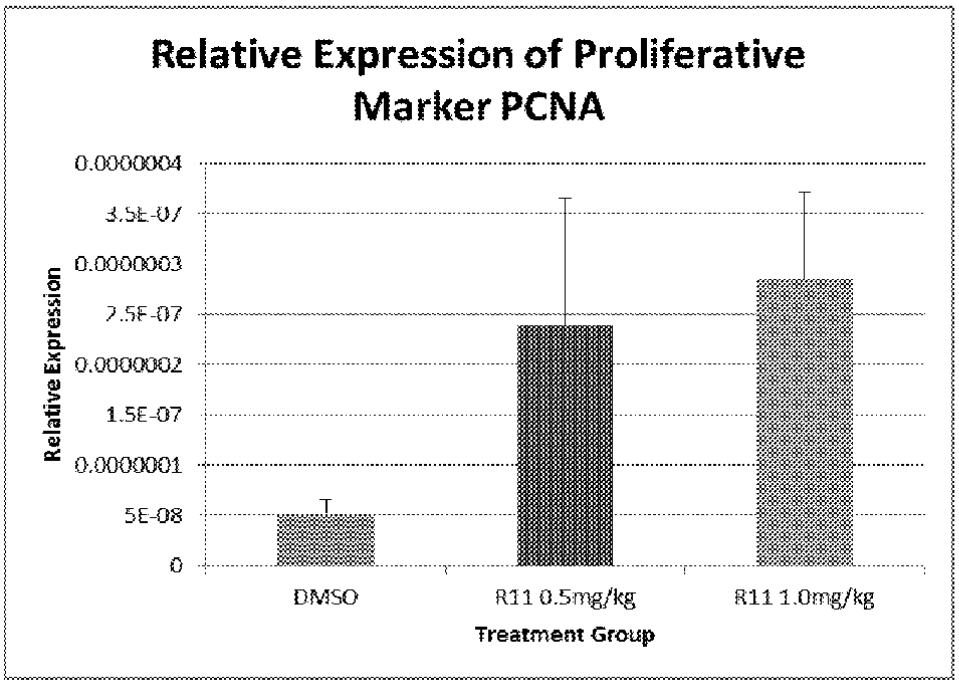


Figure 20. Relative expression of proliferative marker PCNA in day 19 control and R115866 treated ovaries do not show a statistically significant difference amongst treatment groups. (n=3-5)

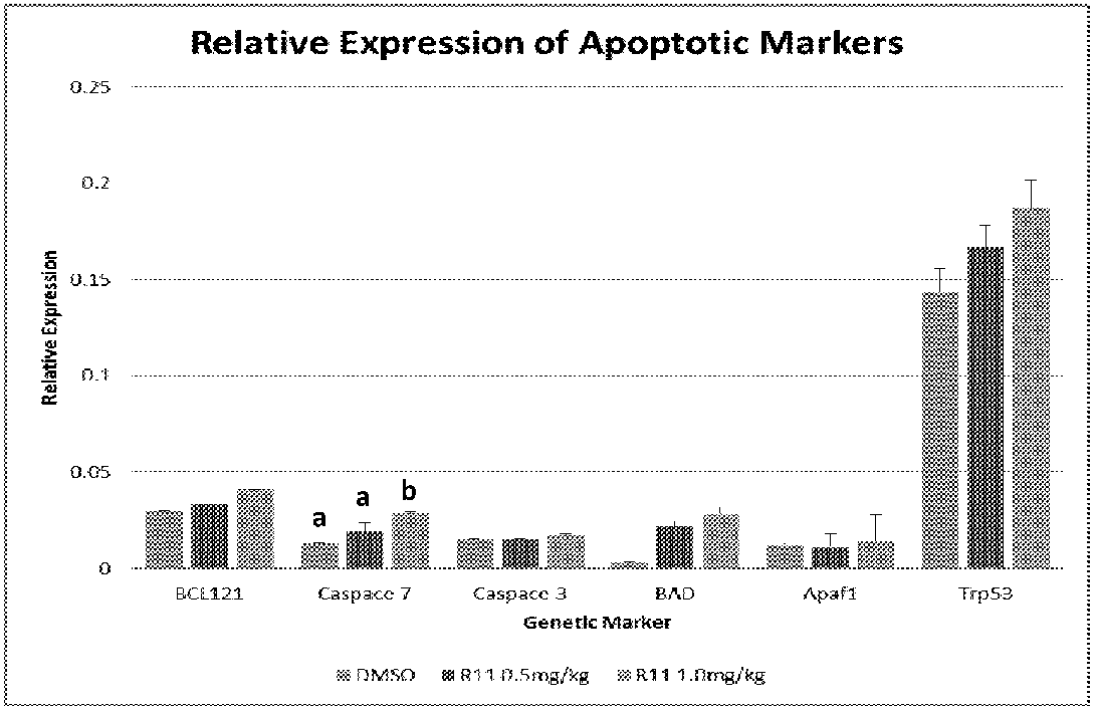


Figure 21. Relative expression of apoptotic markers in day 19 control, 0.5 and 1.0mg/kg R115866 treated ovaries with different letters representing statistically significant differences. ($P<0.05$; n=3-5).

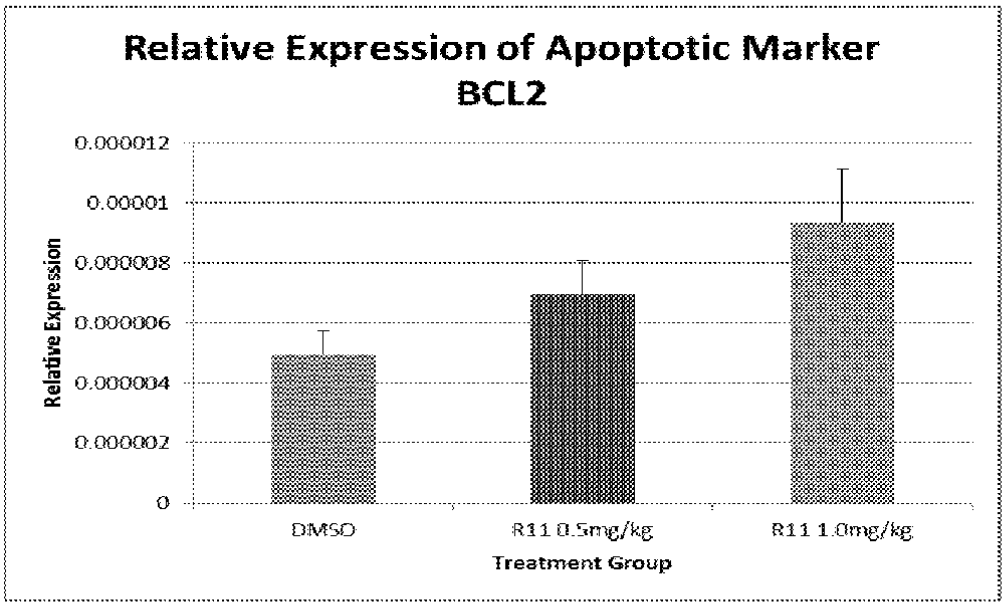


Figure 22. Relative expression of apoptotic marker BCL2 in day 19 control and R115866 treated ovaries show no statistically significant differences amongst treatment groups. (n=3-5).

Discussion

The results of this study demonstrate that imbalances in retinoic acid levels lead to improper development of the mouse ovary. Histology show pathologies in the morphology of all treatment groups. Day-19 VA-deficient and R115866 groups show differences in development; demonstrated by follicle counting and day-19 R115866 treated group show a significant difference in development assayed by IHC and quantified by real time PCR.

Injection Procedure

Previous treatment of mouse pups with retinoic acid resulted in an unacceptably high mortality rate. Due to the highly toxic effect of direct retinoic acid treatment, we instead based our strategy on the inhibition of RA degradation by R115866 treatment. Such pharmacological inhibition of RA degrading enzymes is shown to effectively increase endogenous RA levels. We still observed a high mortality rate in mouse pups injected with 2.5mg/kg R115866; it is unknown whether this was due to RA toxicity, the toxicity of the drug itself, or other unknown side effects of the drug. A lower mortality rate was seen in mice treated with 1.0mg/kg R115866, and no lethal effects were observed in mice treated with 0.5mg/kg R115866. Due to these effects, the number of individuals in this group is higher than other groups.

Subcutaneous injection of one day old mouse pups proved to be difficult, since the skin of these young pups was very thin and taut. Practice showed the best spot was the hind quarter, where a skin fold could most easily be made. As the mouse pups grew, the skin thickened, and injections became easier by day 3. The large injection volume (20ul) also caused problems with seepage from the injection site. Despite this, we maintained this volume to limit the concentration of DMSO and R115866 injected to favorable levels. This may have resulted in variability of dose within each treatment group. It is uncertain how much R115866 was effectively administered by each injection. Precaution was taken by z-tracking injections (a method of shifting the skin before injection and releasing it after injecting as the needle is retracted to shift the injection site away from the injected

liquid), injecting the solution slowly, and waiting a few seconds before retracting the needle.

Mouse Weights

Mice injected with R115866 showed a significant decrease in weight at day 19 compared to DMSO control. By week 7, we observed a more dramatic effect on weight that appears to be dose-dependent. This difference disappears as the mice age, and by week 15, there was no difference in weight between the treatment groups and DMSO control. This would suggest that there is a delay in development through puberty, but that this is resolved by adulthood. Mice treated with R115866 or VA- diets also show a different pattern of adipose remodeling. Vitamin A has been shown to be involved in adipose remodeling, and in the liver this effect may be mediated through PPAR expression [65, 66]. Since PPAR activity is assosiated with metabolic processes, an increase in PPAR activity could explain the decrease in weight seen in these mice. This effect may resolve as PPAR expression levels normalize as the mice age.

Ovary Harvesting and Follicle Counts

Ovaries harvested from R115866 treated mice appear smaller than control ovaries; however, control ovaries appeared larger than expected relative to untreated ovaries. We currently have no explanation for this, as increased ovary size due to DMSO treatment has not been previously reported. Total follicle numbers show a significant decrease in all R115866 treated groups (*Figure 9*). The 0.5mg/kg treated group shows a much more

dramatic decrease in total follicle number than the higher dose (1.0mg/kg) group. Without a direct measure of the effect of R115866 on Cyp26b1 expression or the subsequent levels of endogenous RA, it is difficult to say why lower does R115866 has a more dramatic effect. RT-PCR data shows that the observed increase in *Cyp26b1* levels appears to be dose dependant, but a statistically significant increase was not seen in either R115866 treatment groups, possibly due to a larger standard deviations in these treatment groups. However, the relative expression levels of *Cyp26b1* appear to correlate positively with a higher R115866 dose. In spite of this, endogenous RA levels were not measured due to the lack of appropriate assays. Thus, we cannot explain why lower total follicle numbers were seen with the lower dose R115866 treatment.

We expected that R115866 would cause an increase in RA levels based on *in vitro* studies in the Kipp lab showing increased proliferation of granulosa cells in response to both R115866 and RA treatment. Such stimulation of granulosa cell growth would cause follicles to prematurely progress to later stages of development. The R115866 1.0mg/kg treatment group did show a significant increase in the number of multi-nucleolar follicles. This may indicate an acceleration of cellular processes that correlate with increases in transcription; an increase in nucleolar size is further consistent with precancerous/cancerous cells **[67, 68]**. This suggests that oocytes may be directly stimulated by R115866, causing an increase in overall levels of transcription. Both CyclinE1 and PCNA proliferation markers showed an increase mRNA relative expression level; however, Ki67 did not. Apoptotic markers are also elevated, which complicates our interpretation. However, there is precendence for uncontrolled proliferation itself being an apoptotic trigger [69, 70].

We did not observe a significant difference in the number of atretic follicles in R115866 treated groups compared to control; however, there appears to be a small increase in the 1.0mg/kg R115866 treatment group. It is possible that higher dose injections cause a subsequent increase in RA to toxic levels, which may cause follicle maturation to fail. Vitamin A-deficient ovaries likewise show a significant increase in atretic follicles, which might be caused by failure of RA stimulation of follicle progression.

IHC and RT-PCR

IHC analysis showed that there is an increase in the proliferative marker Ki67 and retinoic acid metabolising enzyme Cyp26b1 upon R115866 treatment relative to control ovaries (*Figures 15,16*). It also appears that the same areas of each section exhibit increased signals for both Ki67 and Cyp26b1. This may suggest that there is an interaction between proliferation and retinoic acid degradation. In line with this, TUNEL assays done on sections show decreased apoptotic signals in these same areas (*Figure 17*). However, RT-PCR analysis of apoptotic markers shows somewhat contrary results to IHC data. Of 7 apoptotic markers, 5 show an increased trend with increased dose of R115866. Of those 5 markers, Caspase 7 showed a statistically significant increase in the 1.0mg/kg R115866 treatment group (*Figures 21, 22*). Other RT-PCR data was consistent with the IHC results. Of 4 proliferative markers, none show statistically significant increases in the R115866 treatment group, However, CyclinE1 and PCNA show an increase realtive expression with increased dose of (*Figures 19, 20*). Cyp26b1 also showed increases in the R115866 treatment group that are not statistically significant (*Figure 20*). This increase in Cyp26b1 may be as an effect of inhibition of the enzyme

causing increases in RA levels and thereby signaling in a feedback loop for higher expression of Cyp26b1.

The increases in apoptotic markers shown in the RT-PCR data, although statisitcally insignificant, would suggest increased apoposis was present, which was not detectable by the IHC methods used. This may be because of the particular sections of the ovaries chosen for staining, or apoptosis might be accelerated or interrupted by retinoic acid signaling. Based on the decrease in ovary size seen in the R115866 treatment group, we expected to detect increased apoptosis and decreased proliferation. However, the increase in some of the proliferative markers is in line with *in vitro* studies that showed an increase in granulosa cell proliferation in response to RA treatment. It is possible that the inappropriately high levels of proliferation we observed early in follicle development itself drives increased apoptosis of primoridal ovarian cells.

Overall, much of this data shows trends amongst the different treatment groups that were not statistically significant. These trends still provide insight for some of the phyotypes observed in our studies and may have not been statistically significant due to low sample size in each of the treatment groups.

Long Term Effects of Early VA- diet

One mouse placed on VA- diet from gestation to 16 weeks of age was alowed to recover for a year on regular diet to see the long term effects of early VA- diet. Morphology revealed several pathologies including bilateral follicle and bursa cysts as well as atretic and hemorrhaging follicles. In contrast, control ovaries showed an overall normal

appearance with a unilateral small follicle (*Figure 8*). It therefore appears that the role of VA in early development is vital through adulthood, and that an attempt to rescue mice kept on a VA-diet after 16 weeks was not successful. Furthermore, a growth of cells appears forming on the bursa of VA- treated ovaries (*Figure 8 F, G, H*), which may suggest the formation of carcinoma but is uncertain at this time.

Conclusion:

We hypothesized that retinoic acid plays a critical role in the development of the ovary. Treatment with R115866 causes an increase in the endogenous RA levels and we were able to observe the impact of the increase in levels of RA on ovarian morphology. Subsequently, we also treated mice with a VA- diet and observed the effect of decreased levels of RA on the ovary. We saw pathological defects in both groups, with decreased numbers of follicles, cyst formation, smaller ovaries, and atretic follicles as compared to controls. These findings suggest that RA plays a significant role in the development of the mouse ovary, and that levels of RA must be tightly regulated for healthy development. In addition, we observed that the effects of RA early in ovarian development and the pathologies involved in the improper balance of RA levels are long lasting and irreversible. This study demonstrates that RA signaling during early stages of mouse development may play an important role in establishing normal ovarian morphology and in follicle maturation.

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In Vivo Effects of a RAMBA and Vitamin A Deficiency on Ovary Development

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Abstract

The active metabolite of vitamin A, retinoic acid, is vital to several physiological functions, and is most notably associated with vision and organ development. Its role in the male reproductive system is well understood, but studies in female reproduction are lacking. Recent studies show that Cyp26b1, a retinoic acid degrading enzyme, is expressed highly in the Day1-10 mouse ovary. This time point correlates with establishment of the primordial follicle pool. *In vitro* studies show that both retinoic acid and R115866, an agent that blocks retinoic acid metabolism, increase cell proliferation. Because of this, we hypothesized that retinoic acid plays a critical role in the development of the mouse ovary.

To test this hypothesis, we placed female mice on a vitamin A deficient diet over a time course beginning from gestation. Endogenous retinoic acid levels were also increased by injection of R115866 at either 0.5 or 1.0 mg/kg during the first 7 days after birth when the levels of Cyp26b1 expression are high. At day 19, week 7, and week 15, ovaries were harvested for morphological and RT-PCR studies. Our results show that animals on a vitamin A-deficient diet or injected with R115866 developed a variety of ovarian pathologies at day 19, week 7, and week 15. Long term pathologies were also observed in mice that were placed on a vitamin A-deficient diet for 16 weeks then allowed to recover for one year. Overall, our results indicate that retinoic acid plays an important role in the development of the mouse ovary and that balanced retinoic acid levels early in development are needed for the health of ovaries. In addition, the effect of early vitamin A deficiency has long lasting consequences that cannot be corrected by a subsequent normal diet.

Introduction:

The ovary is responsible for the production of female gametes as well as hormones necessary for the regulation of development and reproduction. Aberrations in ovarian development may lead to infertility as well as other diseases. Better understanding of the process of ovarian development has potential medical applications in the treatment of such conditions.

Retinoic acid is the physiologically active form of vitamin A. Its levels are regulated physiologically by its synthesis from vitamin A and other precursors, as well as its degradation by a family of Cytochrome P450 enzymes, the Cyp26 enzymes. Recent findings suggest that retinoic acid is involved in the regulation of meiosis and ovulation. Other reports suggest it also has an effect on steroid hormone biosynthesis. In addition, *in vitro* studies have shown that increases in retinoic acid levels can induce proliferation of granulosa cells. More work must to be done to elucidate the complex role of retinoic acid during *in vivo* ovary development. To better understand the *in vivo* effects of retinoic acid, a study utilizing a mouse model with inhibition of Cyp26 enzymes, which are known to degrade retinoic acid, was conducted. This study will help to determine the biological effects of vitamin A and retinoic acid signaling in the ovary, provide a better understanding of ovarian development and pathophysiology, and provide potential treatments for infertility and other ovarian diseases.

Review of Literature

Infertility and Its Impact

It is estimated that 80 million people around the world experience impaired fertility; 10 percent of couples experience difficulties with procreation [1]. The worldwide distribution of infertility is not uniform. A higher incidence occurs in developing nations, where infectious diseases that can cause damage to reproductive organs are more common and access to fertility treatments is very limited [2, 3]. Infertility has consequences that affect relationships and psychological health. In some social groups, women who cannot conceive are ostracized, socially stigmatized, and left feeling isolated[4]. The resulting stresses on relationships may lead couples to divorce. Current treatments that address the biological causes of infertility do not always adequately address the myriad of insecurities associated with this condition.

The once intimate experience of conception now requires couples go to sterile medical facilities. The services, which are not covered by most major medical insurers, place financial burdens on couples. With an estimated 10 percent of infertility cases being unexplainable, fertility specialists offer no guarantees [5], and the financial cost becomes a huge gamble. Infertility also increases with age, ranging from 11 percent of women under the age of 29 to 27 percent of women between 40-44 years of age [6]. As more couples delay starting a family until after both partners achieve educational goals and establish careers, there are more couples that require the help of medical science to conceive [7].

Structure and Development of the Ovary:

The ovary is the female gamete-producing organ. It is also an endocrine gland, which produces several hormones. The ovary contains several cell types that form many follicles. Each ovarian follicle contains an oocyte and one or more layers of granulosa cells, which support oocyte maturation. Large ovarian follicles are also surrounded by one or two layers of theca cells. Both granulosa cells and theca cells are responsible for steroid biosynthesis. The number of follicles in the ovary is established during fetal development and the oocytes mature one by one after puberty. One follicle releases a single mature egg during ovulation in mono-ovulatory animals including human. The remaining follicular remnants then form a corpus luteum, which secretes hormones including progesterone and estradiol. Without further stimulation from a developing embryo, the corpus luteum will diminish in 14 days. This process is responsible for the cyclic changes in hormone levels that occur during the female menstrual cycle.

Follicle maturation begins when germ line cells mature into primordial follicles as a consequence of invasion by pre-granulosa cells. This process occurs early in development, and is arrested until further maturation is initiated after onset of puberty. From the primordial stage, granulosa cells develop from flat cells to cuboidal cells, indicative the BC stage. From here, the follicle moves to the secondary stage, where granulosa cells proliferate into two or more layers. By the tertiary phases, many layers of granulosa cells surround an enlarged oocyte. This then leads to the development of the antral follicle, where a cavity begins to form around the oocyte and the follicle prepares for ovulation.

Hormones Important in Female Reproduction

Hormonal cycles in females are highly coordinated through regulatory influences of the hypothalamus and pituitary glands. The hypothalamus is responsible for the pulsatile secretion of gonadotropin releasing hormone (GnRH). GnRH stimulates the anterior pituitary cells to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). LH shares similar subunits with other glycoprotein family hormones, such as thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), and human chorionic gonadotropin (hCG) [27]. FSH and LH act synergistically to regulate the development and growth of the ovary and stimulate hormone production. Increased doses of LH are required for oocyte maturation and ovulation [8]. The secretion of FSH and LH is further regulated by activin and follistatin, which will be discussed below. FSH has the most impact in the development of antral follicles, while the factors that control earlier stages of follicle development are poorly understood [9, 10].

Estrogen is primarily produced by granulosa cells within ovarian follicles and it induces the secondary sex characteristics of females, including body shape, hair growth, and breast development. Progesterone cooperates with estrogen to regulate the cycle of menses as well as the cyclic changes in the endometrial lining of the uterus. Both hormones induce pubertal maturation and facilitate the reproductive processes of the female body.

Activin is a peptide hormone that was originally isolated from the ovary and was later shown to be produced in multiple locations throughout the body. Both activin and inhibin are members if the TGF-β superfamily [10-13]. Activin acts as a ligand to a

serine/threonine kinase receptor which activates the kinase/Smad signaling pathway [14, 15]. It works in both an autocrine and paracrine fashion to stimulate the synthesis and release of FSH. Activin also triggers granulosa cell proliferation and differentiation, as well as oocyte maturation [16-18]. Activin activity is suppressed by inhibin and follistatin. Inhibin works by blocking the activin receptors, while follistatin is a soluble protein that binds to activin, thereby blocking its access to receptors [19]. Activin has also been shown to induce increased expression of estrogen receptors, while estrogen has an opposite effect on activin expression in neonatal mice [20, 21].

Effect of Nutrition on Fertility

When the nutritional needs of the mother are severely compromised, the effects to a fetus are evident. Nutritional deficits can cause developmental defects, mental retardation, and low birth weight [22-25]. The incidence of developmental defects with a dietary cause are higher in poorer countries where medical and nutritional needs, including access to vitamins, are more difficult to satisfy [26]. Certain vitamins, including vitamin A, are fat soluble and require adipose tissues for their subsequent storage. There is no current literature to address how changes in body fat composition impact the efficiency of vitamin storage and utilization, but it is known that women with low body mass index (BMI) and low body fat experience difficulty in conception as well as occurrences of amenorrhea [27]. Even otherwise healthy females with low body fat, such as competitive athletes, experience alterations in menses and fertility [28-30]. There is a further association between poor nutritional habits and infertility in both males and females [31-33].

Vitamin A and Retinoic Acid

Retinoic acid (RA) is a naturally occurring physiologically active retinoid. The term retinoid refers to any compound that is a metabolite of Vitamin A. Common dietary sources of Vitamin A are the carotenoid pigments found in a variety of plant-based foods. Animal tissues can also provide stores of vitamin A metabolites such as retinyl-esters. Retinoids can be stored in the body by esterification or oxidized to form all-trans retinoic acid (atRA). All-trans retinoic acid is believed to function in both male and female reproduction and development [34].

Retinal undergoes significant enzymatic processing in the body. The enzyme that catalyzes modification of retinal for storage is retinol acyltransferase lecithin (LRAT). Dietary retinal is dehydrogenated by alcohol dehydrogenase and retinol dehydrogenase to synthesize atRA [35]. Since Vitamin A metabolites are lipophilic, they are typically found in the serum in association with other proteins [36]. RA can also be hydrolyzed by Cytochrome P450 enzymes, including those encoded by the *Cyp26a1*, *Cyp26b1*, and *Cyp26c1* genes, rendering it inactive [37].

All-trans RA is a ligand for retinoic acid receptor (RAR) proteins, which are nuclear receptors and ligand-activated transcription factors [38]. RA also directly binds to the Retinoid-X receptors (rexinoid, RXR). RXRs are able to heterodimerize with RARs and increase their DNA binding affinity to the retinoic acid response element (RARE) [39]. Of all the retinoids, atRA has the highest binding affinity to RAR [40]. Through these interactions, Retinoids act as a gene regulators and influence cell proliferation and differentiation [41, 42].

Vitamin A deficient rats continue to ovulate on schedule, although their eggs do not appear to have matured properly, suggesting that RA may be required for normal ovarian development. RA has been shown to induce meiotic entry in cultured rat ovaries [43], and the RA responsive gene Stra8 is required for meiotic initiation in females [44]. Further studies have shown that female gonads that express Stra8 can enter meiosis without RA, suggesting that its effects can be realized independent of RA signaling [45]. When small doses of RA are added to the female diet at levels too low to support other physiological processes, 30% of oocytes will undergo meiosis. This suggests that RA may support follicle maturation and ovulation through multiple mechanisms [34], although the details of these mechanisms remain unknown. Other than the effects on follicle maturation and ovulation, female rats with severe RA deficiency have been shown to experience difficulty with uterine implantation of the embryo [46].

An investigation of the influences of isotretinoin, an acne treatment medication, on pituitary hormones may provide useful insight. Isotretinoin is a 13-cis-retinoic acid derivative that is thought to be metabolized *in vivo* to atRA, which allows it to interact with RAR. Individuals treated with isotretinoin showed decreases in LH, Prolactin, and testosterone levels [47], suggesting that it may influence reproductive processes. The study failed to measure the levels of activin, a peptide hormone that stimulates the synthesis and secretion of FSH, and has previously been implicated in modulation of RA signaling [48].

The Kipp laboratory recently conducted a pilot study of the effects of vitamin A deficiency in a mouse model of ovarian development. Preliminary data show formation of smaller than normal ovaries and increased bursa cyst development. The mechanisms by which the lack of vitamin A leads to abnormalities are not well understood. Since others have likewise reported a role for RA in ovarian development [16-18], a closer exploration of vitamin A and RA functions in the ovary may lead to better understanding of follicle development and maturation. Therefore, we propose to investigate the *in vivo* effects of inhibition of Cyp26 enzymes, of the key enzymes that degrade RA.

Cyp26b1

Cyp26 genes encode a class of cytochrome P450 enzymes responsible for the catabolism of RA [49, 50]. These enzymes convert RA to a more polar and soluble metabolite that can be more easily excreted. Degradation of RA is an important regulatory mechanism to protect the cell from excessive stimulation [51]. RA has been shown to act as a teratogen if used at high doses [52] and overstimulation by RA can manifest as cleft palate, inner ear malformations, thymic agenesis, craniosynostosis, and hypoplasia of the mandible and maxilla [53].

Tight regulation of Cyp26 gene expression determines the patterns of tissue-specific distribution of RA which are essential for embryonic development [50]. Cyp26 isoforms include *Cyp26a1*, *Cyp26b1*, and *Cyp26c1*. Cyp26a1 and Cyp26b1 proteins have high affinity for atRA, while Cyp26c1 has affinity for atRA and 9-cis-RA [37, 54, 55]. *Cyp26b1* has been shown to be expressed during early fetal development in mouse

hindbrain, limb buds, maxillary and mandibular processes [56]. In older fetuses, expression is seen in skin, precartilagenous elements and some organs [57]. *Cyp26b1* null animals exhibit craniofacial abnormalities [58] and lack of Cyp26b1 negatively impacts limb development by inhibition of chondrogenesis [59].

Cyp26b1 is expressed in the somatic cells of embryonic testes [56, 57, 60], and proper regulation of RA levels has further been shown to be critical for normal testes development [58, 60, 61]. Organ culture experiments have shown that in the presence of RA, testes are induced to express Stra8 [44]. Stra8 is also up regulated in *Cyp26b1* null mice [60], which correlates with an increase of RA levels in embryonic testes. This subsequently results in the initiation of meiosis as well as increased apoptosis of male germ cells *in utero* [61]. Embryonic testes of *Cyp26b1* null mice are largely devoid of germ cells, although the somatic cells appear unaffected [61]. Therefore, germ cells in the fetal mouse testes are initially shielded from RA-induced meiosis by the action of the Cyp26b1 enzyme [44, 60].

Recent *in vitro* studies by the Kipp laboratory have demonstrated that activin down-regulates Cyp26b1 in mouse granulosa cells [48]. These studies provide new insight into the mechanism of activin action and establish a connection between hormone regulation, ovarian development, and the importance of critical nutrients such as RA. These *in vitro* studies also showed that activin, RA, and a Cyp26 pharmacological inhibitor can each independently induce proliferation of granulosa cells [48]. Further studies show that the expression of Cyp26b1 in the neonatal mouse ovary is highest within the first 10 days

after birth. This time window correlates to the development of the primordial follicle pool [48].

Talarozole (R115866)

The highly toxic effects of exogenous RA have pressured the development of new methods to increase endogenous retinoic acid levels by targeting RA-degrading enzymes. R115866, marketed under the trade name Talarozole, is an investigational retinoic acid metabolism blocking agent (RAMBA) that works by selectively inhibiting Cyp26 isoforms. This drug is used in the treatment of skin conditions, and studies have shown that it results in increases in endogenous RA levels in epithelial and smooth muscle tissues both *in vivo* and *in vitro*. R115866 appears to work systemically in that it results in increases in serum retinoic acid levels. It has also been used to drive the differentiation of induced pluripotent mouse cell lines and embryonic stem cells into haploid gamete-like cells [62], reinforcing a role for retinoic acid in the establishment of the germ line during development.

Hypothesis

In vitro studies have shown that RA increases granulosa cell proliferation and stimulates germ cell meiosis [48]. We hypothesize that inhibition of Cyp26 during the first week after birth, when the levels of *Cyp26b1* expression are the highest in the ovary, may play an important role in *in vivo* ovarian development by increasing endogenous atRA levels. To test this hypothesis, we treated mice pups with R115866 during the first week after

birth or treated mice with a vitamin A deficient (VA-) diet and examined ovary tissues for signs of impaired development or pathology.

Methods

Mouse Breeding

In vivo studies were carried out using CD1 mice maintained on a 12 hour light/dark cycle. Food and water were supplied *ad libitum* and all animals were cared for according to all federal and institutional guidelines.

R115866 Injections

Mouse pups were injected with 0.5, or 1.0mg/kg R115866, or vehicle control, daily from day 1 to day 7 after birth. Ovaries and serum were collected at day 19, week 7, or week 15 for histology, RNA analysis, and hormone assays.

A 1.1mg aliquot of R115866 was dissolved in 5µl of DMSO, yielding a stock solution with a concentration of 0.22mg/µl. This stock solution was further diluted in corn oil to yield a final DMSO concentration of 0.23% in each working solution. Mouse pups born to mothers on a regular diet were each injected with 20ul of this preparation. This dose was based on an estimated weight of 2 grams per pup. Using a 1cc syringe with a 28G half inch needle, injections were administered subcutaneously on the lower dorsal area where a skin fold could be made. Each day, one syringe was loaded with the total R115866 preparation and used to inject 20ul into each mouse pup in sequence.

Euthanasia, Ovary Collection and Serum Collection

Mice were euthanized by CO_2 exposure in a closed chamber. Euthanasia was confirmed by cervical dislocation.

Ovaries were dissected from euthanized mice, and one ovary was fixed for histological or immunohistochemical studies (see below). The other ovary was cleaned of connective tissues in a 1x PBS solution. Cleaned ovaries were then transferred to microfuge tubes and kept on dry ice prior to storage in a -80˚C freezer for subsequent RNA analysis.

Blood was collected from freshly euthanized mice via cardiac puncture using a 3-5ml syringe with a 27G ½ inch needle. Blood was transferred to microfuge tubes and allowed to clot at room temperature for 90 minutes followed by centrifugation at 10,500 rcf for 15 minutes. Serum was then pipetted into fresh microfuge tubes and stored in a -80˚C freezer for later analysis.

Fixation and Histology

Ovaries were fixed in 10% formalin overnight followed by 3 x 20 minute washes in 50% ethanol and 3 x 20 minute washes in 70% ethanol. The ovaries were then paraffinized and serially sectioned in 5 micrometer sections using the Northwestern University Ovarian Histology Core Center. Every other slide was stained with hematoxylin and eosin (H&E) staining for morphology studies, and unstained slides were used for immunohistochemistry studies. For young animals, slides were affixed with 5 sections in series. For one-year old animals, both ovaries were fixed, and three sections were affixed to each slide.

VA Deficient Diet and Follicle Counting

A VA deficient study was conducted using day 19, week 7, week 15, and one year female mouse ovaries. CD-1 mice were given either a VA- or control diet (Harlan Laboratories) 24 hours before introducing males for mating. Female breeders remained on either diet through gestation and until pups were weaned. Pups continued on either a VA- or regular diet until ovaries were harvested for morphological study or RNA extraction. For the one year recover study, mice remained on VA- diet to week 16 then switched to a regular diet until ovaries were harvested. Histology was completed as described above.

Images were taken of every 5th section for each series of H&E stained ovarian samples. Total follicle numbers and follicle types were estimated by multiplying follicle counts by five times to compensate by proportion of sections counted [20, 21]. Only structures containing a visibly distinct nucleus were considered to be follicles. This method minimizes the chance of double counting a follicle that spans multiple sections. Figure 1 shows the different stages of ovarian follicle development. These stages are characterized not only by the follicle size, but also by the type and number of granulosa cell layers that surround the oocyte. The primordial follicle has a characteristic single layer of flat granulosa cells. The BC follicle stage is the next progression in follicle development, and is characterized the appearance of both flat and cuboidal granulosa cells. The BC follicle stage is not shown in Figure 1, but this stage represents a transition between the primordial and primary follicle stages. The primary follicle stage is characterized by a single layer of cuboidal granulosa cells. As a primary follicle progresses to the secondary follicle stage, the granulosa cell layers multiply; by the tertiary follicle stage, several

layers of granulosa cells surround the oocyte. The tertiary follicle stage is further characterized by the appearance of multiple layers of thecal cells. The antral (Graafian) stage of follicle development has the characteristic presence of a visible cavity in the follicle as the follicle begins to prepare for ovulation. Atretic follicles are characterized by misshapen oocytes and granulosa and thecal cells that appear to atrophy. Multioocytic follicles are present when multiple oocytes are contained in indistinguishable layers of granulosa cells encapsulated in layers of thecal cells. Multinucleolar follicles were also observed, indicated by the presence of more than one dark structure in a single oocyte.

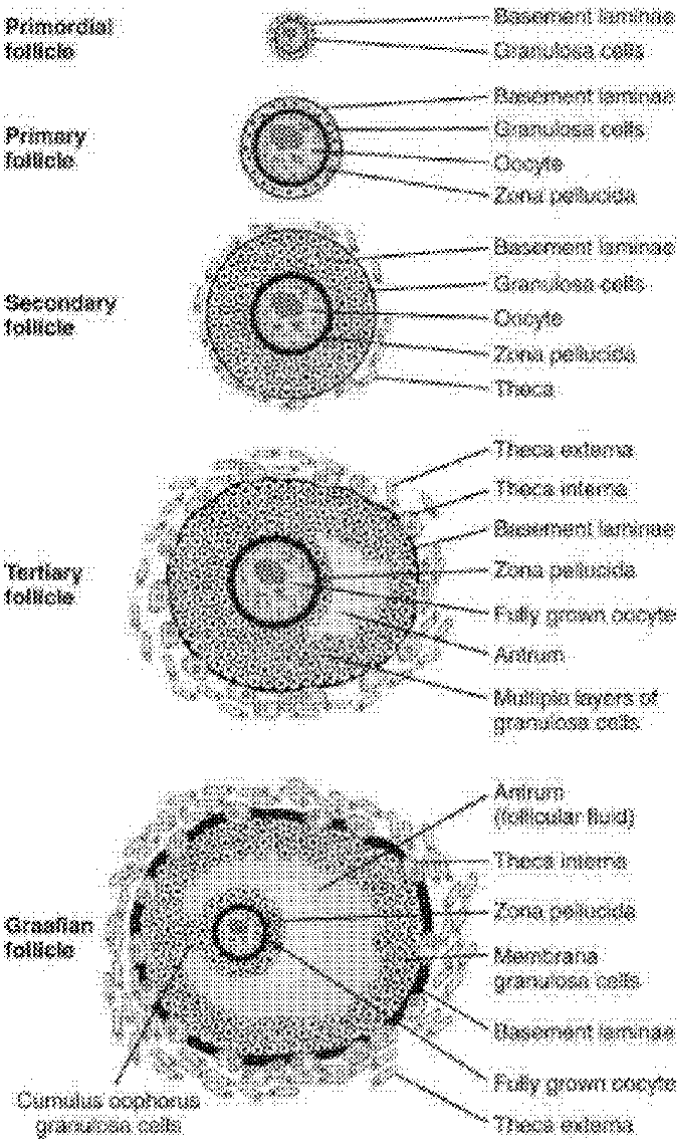


Figure 1. Diagram showing different stages of follicle maturation. [63]

Immunohistochemistry

Slides were chosen for IHC assays based on a large number of follicles apparent in series on a slide. Sections were used for TUNEL assays or stained with antibodies for either Ki67 or Cyp26b1 proteins. Assays were done on three consecutive sections for each treatment group to compare signal levels within single follicles. Slides were soaked 2 x 10 minute in citrisolv (Fisher Brand) to de-paraffinize wax. This was followed by 3 minute washes in decreasing ethanol concentrations of 100, 95, 70, and 50%. Slides then received a final wash of 3 minutes in ddH2O before antigen retrieval treatment. Slides were placed in a solution of 0.1M sodium citrate and microwaved on low for 9 minutes with attention to not allow it to boil over. The slides were left in the solution and allowed to cool for 20 minutes before 2 x15 minute washes in PBS-Tween to permeabilize the samples. Sections receiving TUNEL staining were kept moist in PBS. Sections used for Cyp26b1 and Ki67 staining were then soaked in a 3% hydrogen peroxide solution for 15 minutes followed by a brief rinse in PBS. They were then soaked in avidin for 15 minutes, briefly rinsed in PBS-Tween, soaked in biotin for 15 minutes, and then briefly rinsed in PBS-Tween. Sections were then blocked in 10% rabbit serum (Cyp26b1, Sigma) or goat serum (Ki67, Abcam) in 3% BSA-PBS for 1 hour at 4°C. Sections were incubated overnight in goat anti-mouse Ki67 (ab16667, 1:100) or rabbit anti-mouse Cyp26b1 (Sigma, 1:50) diluted in 3% BSA-TBS-10% serum at 4°C for 16 hours. Negative controls were kept in blocking solution. Slides that had been incubated with primary antibody were then rinsed 3 x 5 minutes in PBS-Tween followed by a 30 minute incubation in secondary rabbit anti-goat biotinylated IgG antibodies (Cyp26b1, 1:1000) or goat anti rabbit biotinylated IgG (Ki67, 1:100) in 3% BSA-PBS for 30 minutes at room

temperature. After treatment with secondary antibodies, slides were rinsed 3 x 5 minutes in PBS-Tween and incubated in ABC reagent for 30 minutes at room temperature followed by 5 x 5 minute washes in PBS-Tween. Sections stained for Cyp26b1 and Ki67 were kept moist in PBS while TUNEL staining was carried out on other sections. These sections were incubated in TUNEL enzyme and label for experimental assays or label solution alone to serve as negative controls. During the last five minutes of the TUNEL assay, Cyp26b1 and Ki67 samples were gently blotted with a kimwipe and incubated in TSA. Slides were rinsed 3 x 5 minutes in PBS-Tween. Slides were mounted with mounting media containing 4',6-Diamidino-2-Phenylindole (DAPI) to stain double-stranded DNA, and sealed to cover slides with clear nail polish. Tissues were visualized and imaged using a confocal microscope.

RNA Extraction

Lysate solution was added to frozen ovaries and homogenized using a Tissue Tearor™ at high speed until tissue particles were no longer visible. RNA from the homogenized samples was extracted following the RNAeasy Mini Kit (Qiagen cat # 74104) protocol.

Real Time RT-PCR

Reverse transcription PCR was done using High Capacity cDNA Reverse Transcription Kit (Applied Biosciences) in 20ul reactions. The product was then diluted 10 or 20 fold before its use in real-time PCR. Real-time PCR assays were used to quantify relative mRNA expression in each of the treatment groups. Rat ribosomal protein L19 (RPL19)

was used as a control for each assay. Assays were done in duplicate and the average cycle threshold (CT) values were used to quantify mRNA using the $2^{-\Delta ct}$ method. The cycle threshold is the value of cycles it takes for a sample signal to surpass the background signal. This determines the value of mRNA expression and is then standardized by subtracting the cycle threshold value of a constitutively active housekeeping gene (RPL19). The difference in the two CT values is the standardized difference in the number of cycles it took for the gene of interest to reach its cycle threshold. Each cycle represents a doubling of the signal or mRNA synthesis. To determine the relative expression level, the treatment groups were compared using the $2^{-\Delta ct}$ method [64]. Assays measured levels of Cyp26b1 transcripts, along with several markers of proliferation and apoptosis. Apoptotic markers, which are indicative of increases in programmed cell death, included Apaf1, BAD, BCL2 BCL121, Caspace 3, Caspace 7, and Trp53. Proliferative markers, which are indicative of increases in cell cycle entry and progression, included CyclinD1, CyclinE1, Ki67, and PCNA.

SYBR master mix was used to make 20ul assay volumes in a 96 well plate. A StepOnePlus™ Real-Time PCR system was used in conjunction with the StepOne software v. 2.1 (Applied Biosystems). The thermal cycle conditions were as follows: 1x 50°C, 2 min; 95°C, 10 min; 45x 95°C, 15 sec; 58°C, 45 sec; 72°C, 1 min; finished with 95°C for 15 sec. Duplicate ct values were checked to ensure a difference of less than 1.0 ct. If differences in duplicates were greater than 1.0, curves were analyzed for character and the curve with the expected characteristics was used without duplicate.

Statistical Analysis

Comparison of data between 2 groups was done using a one tailed student's t-test. For comparisons between more than 2 groups, a one way ANOVA was done with a Tukey-Kramer *post hoc* analysis. Data are shown as Mean±S.E. and $P \leq 0.05$ is considered significant.

Results

Effect of R115866 on Mouse Body weights

Figure 2 shows the body weight measurements taken for R115866 treatment groups and DMSO control at each time point; day 19, weeks 7 and 15. Mice receiving injections of 0.5mg/kg and 1.0mg/kg R115866 showed significant decreases in weight at day 19 and week 7 ($P<05$; n=3-5) but no differences were observed at week 15.

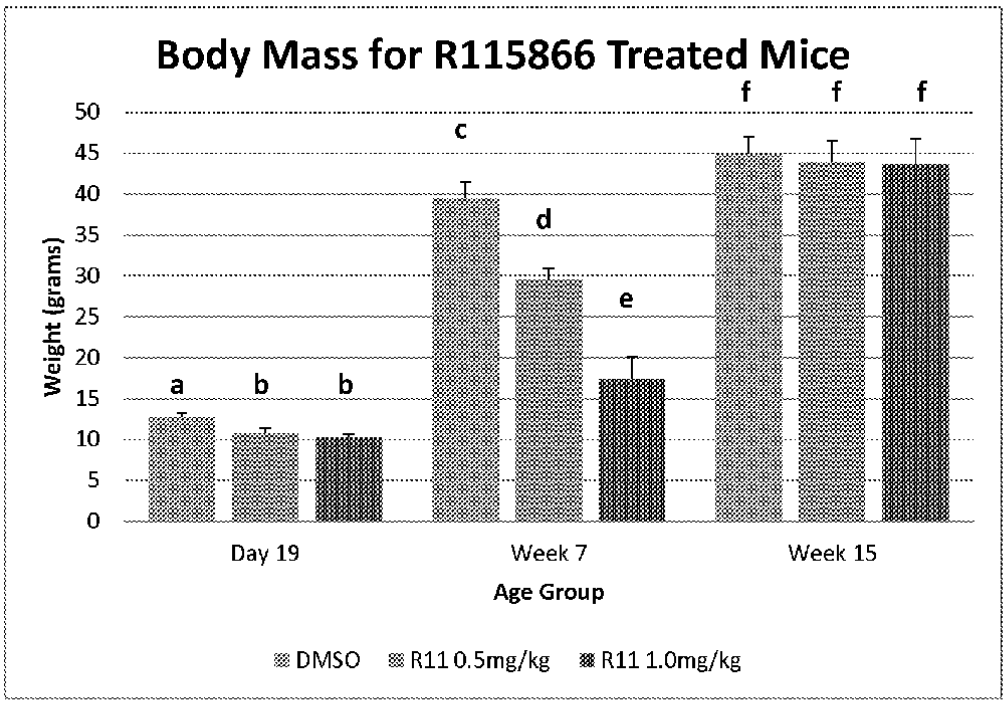


Figure 2. Body weights of control and R115866 treated mouse pups at day 19, week 7, and week 15 with different letters indicating statistically significant differences ($P<0.05$; n=3-5).

Histology

R115866 Treatment Groups

H&E staining for sections of both R115866 and VA- treatment groups show pathologies at all time points analyzed. Figure 3 shows representational pictures of day 19 DMSO control (*A*), and R115866 treated ovaries. Day 19 R115866 treated mouse ovaries show smaller ovaries as compared to vehicle (DMSO) treated ovaries along with decrease in follicle numbers. The R115866 treated ovary shows 3 follicles with multiple nuceoli, which was common amongst other samples in the R115866 treatment groups. By week 7, the decreased size of R115866 treated mouse ovaries becomes more pronounced (*Figure 4B*) as compared to DMSO treated control (*Figure 4A*). Atretic follicles are also frequently observed in the R115866 treated group (*Figure 4C*). Along with these pathologies, we observed a bilateral decrease in the presence of follicles, dilated blood vessels, and prominent bursa cysts in 100% of animals.

VA- Diet treatment Groups

Based on the results from previous studies conducted by the Kipp laboratory, similar pathologies are observed in day 19 VA- deficient diet mouse ovaries (*Figure 5B, C*) including decreased ovary size with decreased presence of follicles as compared to controls (*Figure 5A*). Other pathologies include presence of multioocytic follicles (*Figure 5B, D*). Atretic follicles are also present and represented in Figure 5C and shown enlarged in Figure 5E. Week 7 VA- ovaries (*Figure 6B*) remained smaller than control (*Figure 6A*) with the appearance of fewer follicles and reduced corpus luteum. VA-

ovaries show atretic follicles, including one with macrophage invasion observed (*Figure 6B,C*). By week 15, pathologies noted in week 7 VA- ovaries became more pronounced compared to control (*Figure 7A, B*) with smaller and fewer follicles and corpus luteum. Ovaries began to develop large bursa cysts (*Figure 7B*) and hemorrhaging follicles are noted (*Figure 7C*).

Further experiments were designed to investigate the long term effects of early VA deprivation. Mice treated with either regular or VA- diet for 16 weeks were allowed to continue a regular diet until one year. H&E staining was done on harvested ovaries (*Figure 8*). One control ovary appears to contain a follicle cyst which appears larger in serial sections (*Figure 8A*). However, this is not as dramatic as the follicle cysts and bursa cysts that appear in the VA- treated ovaries in (*Figure 8C,D*). In addition, appearance of hemorrhaging follicles is noted throughout sections of VA- ovaries (*Figure 8E*). The tissues surrounding the cyst appear to be epithelial, consistent with an ovarian origin (*Figure 8D*). A large mass of cells appears outside the bursa membrane (*Figure 8D*). This mass of cells also appears epithelial and originates from the bursa membrane (*Figure 8G, H*). It is currently unclear if this mass of cells indicates carcinoma.

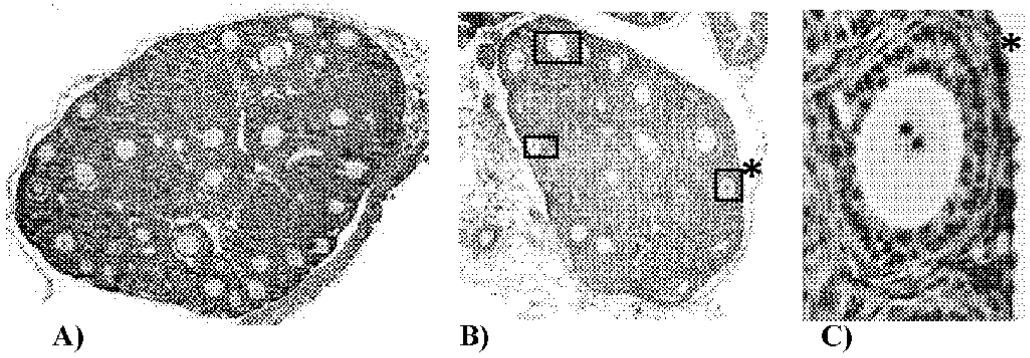


Figure 3. Histology of day 19 1.0 mg/kg R115866 treated mouse ovaries. A) DMSO Control, B) R115866 treated, C) enlarged segment showing multinucleated follicle from day 19 R115866 treated mouse.

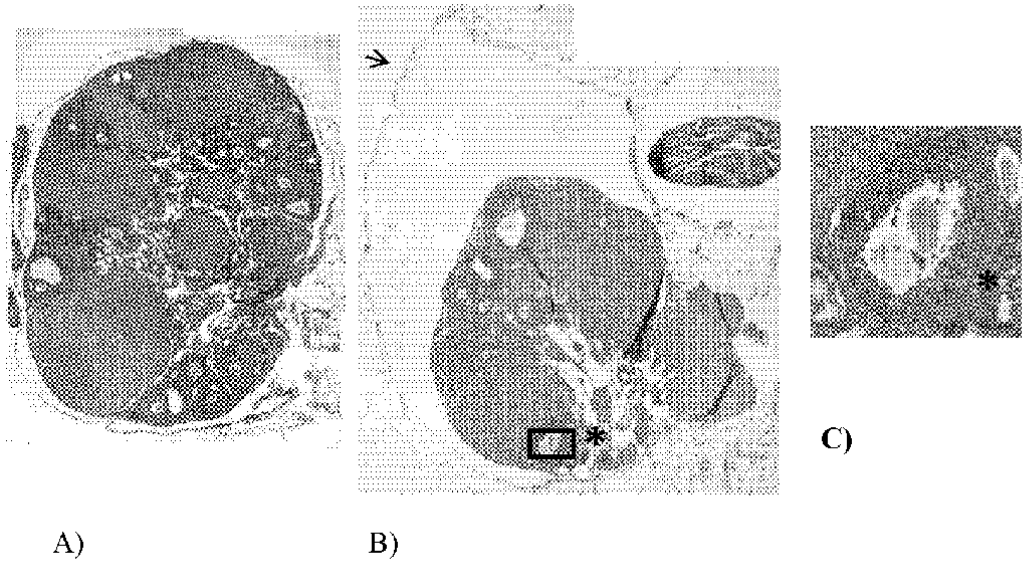


Figure 4. H&E staining of Week 7 0.5 mg/kg R115866 treated mouse ovaries. A) DMSO Control, B) R115866 treated ovary with bursa cyst, C) enlarged segment showing atretic follicle from week 7 R115866 treated mouse ovaries.

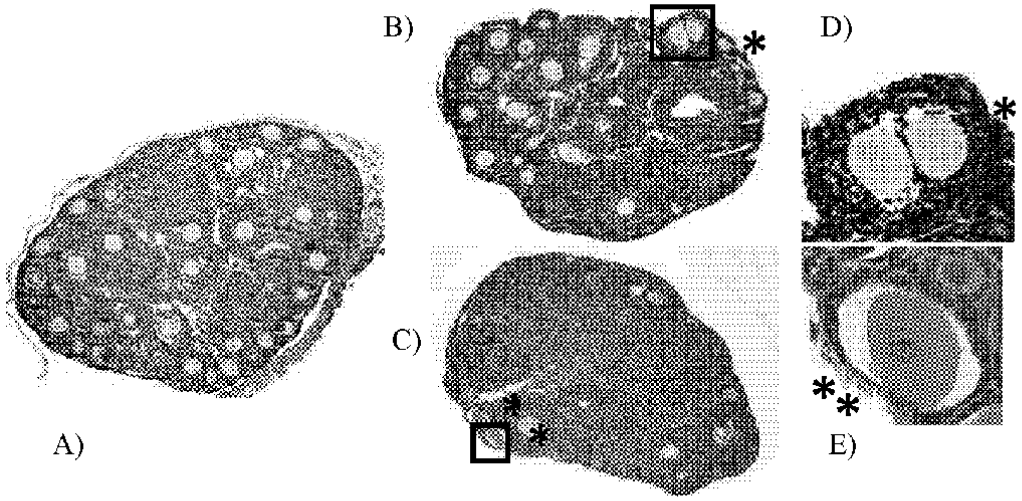
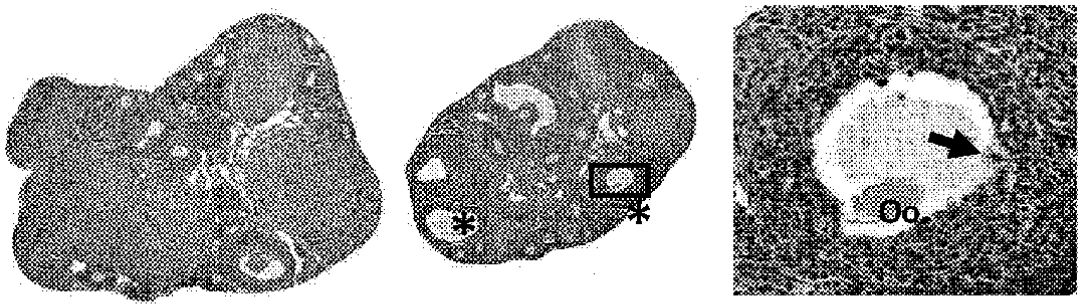
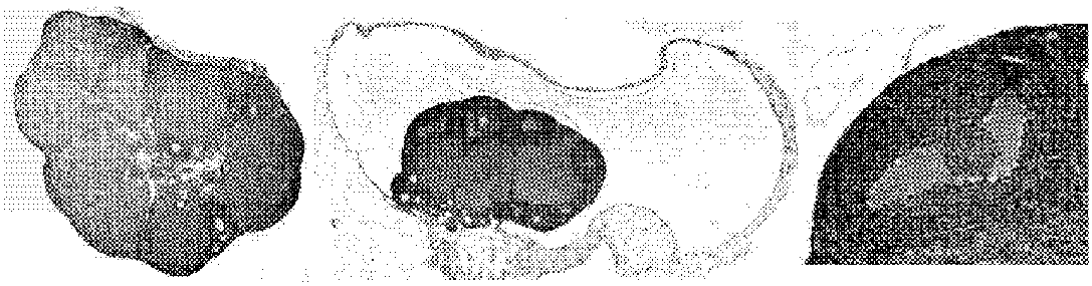


Figure 5. H&E staining of representational day 19 VA- mouse ovaries A) Control diet ovary, B &C) VA- mouse ovaries showing decreased ovary size, multiocytic follices (B) and atretic follicles (C). Enlarged segments of VA- ovaries showing multioocytic follicles (D) and atretic follicles (E). Images courtesy of Michael Demczuk and Tessa Bonney.



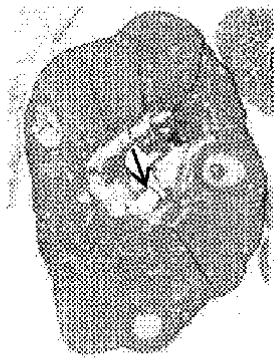
A) B) C)

Figure 6. H&E staining of representational week 7 VA- mouse ovaries A) Control diet ovary, B) VA- mouse ovaries showing decreased ovary size and atretic follices. Decreased numbers of corpus luteum are also noted. (C) Enlarged segments of VA- ovaries showing atretic follicle with presence of macrophage (arrow) indicating immune response. Images courtesy of Michael Demczuk and Tessa Bonney.



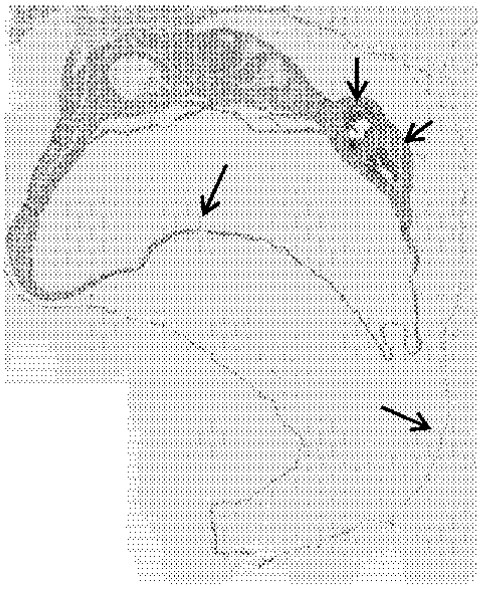
A) B) C)

Figure 7. H&E staining of representational week 15 VA- mouse ovaries A) Control diet ovary, B) VA- mouse ovaries showing decreased ovary size, atretic follices, and large bursa cyst. Decreased numbers of follicles and corpus luteum are also noted. (C) Enlarged segments of VA- ovaries showing hemorrhaging follicle. Images courtesy of Michael D. and Tessa B.

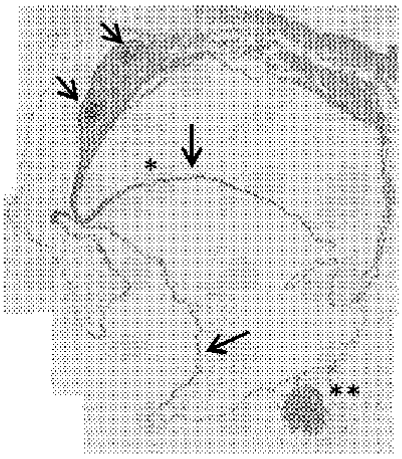


A)

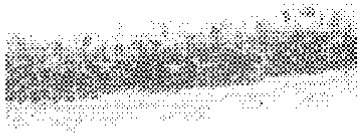
B)

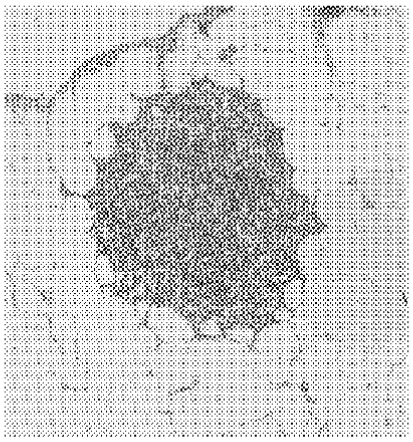


C)

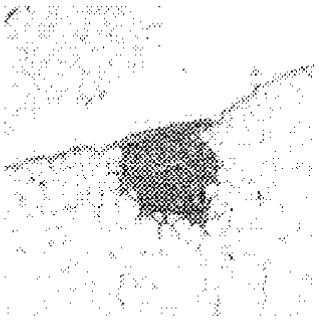


D)





E) F)



G) H)

Figure 8. H&E staining of representative ovaries from 16 week VA-mice at one year. A & B) Control with formation of a cyst present. C & D) VA-treated ovaries with several pathologies. Most noted are very large follicle and bursa cysts and hemorrhaging follicles in both sections. D) Formation of a mass of cells external and originating from the bursa membraine. E) Enlarged segment of tissues from ovary (D) surrounding the follicle cyst showing normal appearing ovarian type tissue. F) Enlarged segment of the cell mass forming outside of the bursa from ovary (D). G & H) Cell mass from from section (D) enlarged in (F) shown to originate from the bursa membrane.

Follicle Counting

R115866 Treatment Group

A count of follicle number and classification of follicle types in day 19 ovaries shows significant differences between treatment and control groups. R115866 treatment leads to a significant decrease in total follicle numbers in both treatment dose groups. ($P<0.05$; n=3-5) (*Figure 9*).

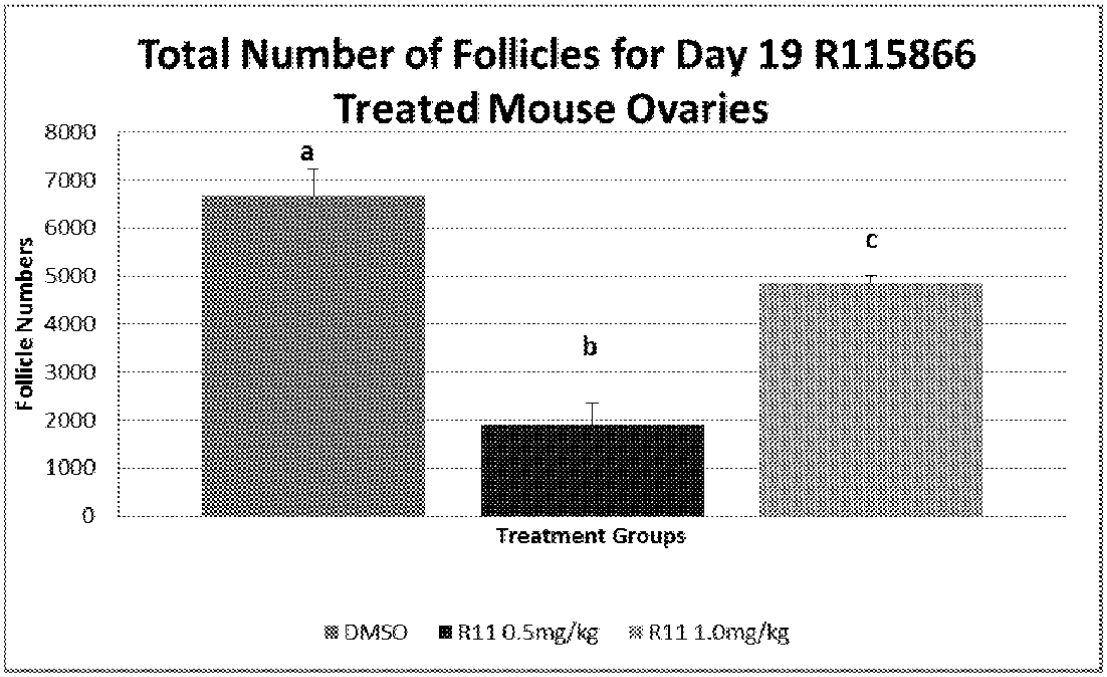


Figure 9. Total follicle numbers in ovaries harvested from day 19 control and R115866 treatment groups with different letters representing statistically significant differences. ($P<0.05$; n=3-5)

A significant difference in the numbers of primordial and BC follicles was observed in R115866 treated ovaries compared to control ovaries at day 19 for the 0.5mg/kg treatment group ($P<0.05$; n=3-5) (*Figure 10*). Although both 0.5mg/kg and 1.0mg/kg R115866 treatment groups had decreases in primordial and BC follicle counts, the higher dose did not have a statistically significant decrease in either primordial or BC follicle counts.

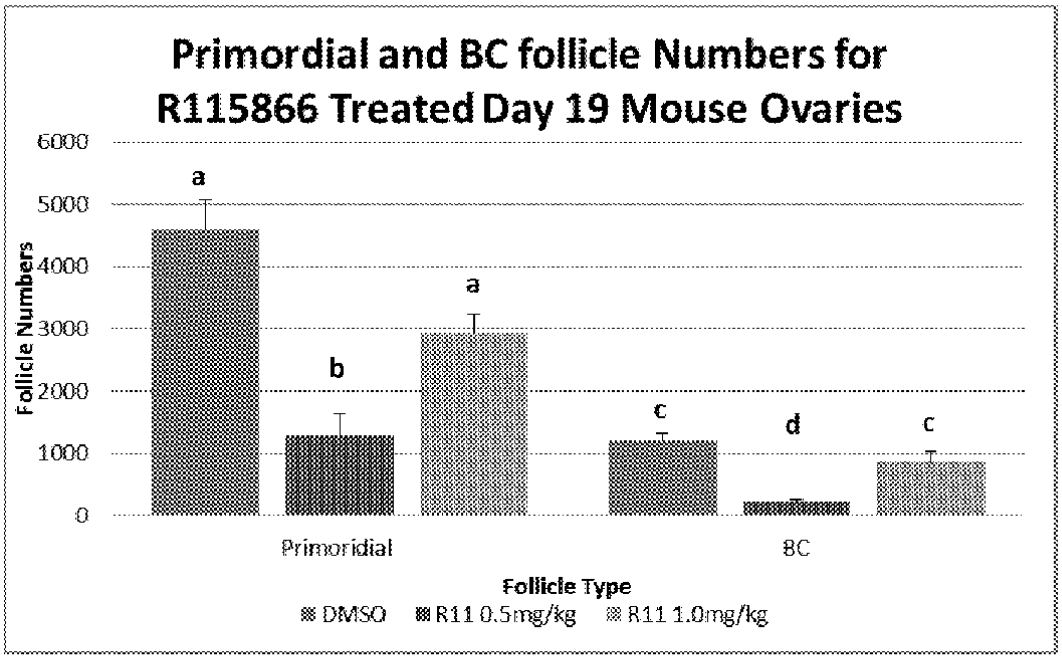


Figure 10. Primoridial and BC follicle type counts amongst ovaries harvested from day 19 control and R115866 treatment groups with different letters representing statistically significant differences. ($p<0.01$; n=3-5)

A significant decrease in primary and secondary follicles was observed in the 0.5mg/kg R115866 treatment group ($P<0.05$; n=3-5) (*Figure11*) compared to controls. Decreases in primary and secondary follicle counts for 1.0mg/kg R115866 treated ovaries were not statistically significant and for secondary follicle counts, 1.0mg/kg R115866 treated ovaries show no statistically significant difference than either control or the lower dose treatment group. Tertiary follicle counts of either the lower dose or higher dose R115866 treatment groups do not show to be statistically different compared to control, but there is a statistically significant increase in the higher dose treatment group compared to the lower dose ($P<0.05$; n=3-5). The numbers of multi-nucleoli follicles show a statistically significant increase in the higher dose R115866 treatment ($P<0.05$; n=3-5). The prevalence of other follicles types shows no significant differences.

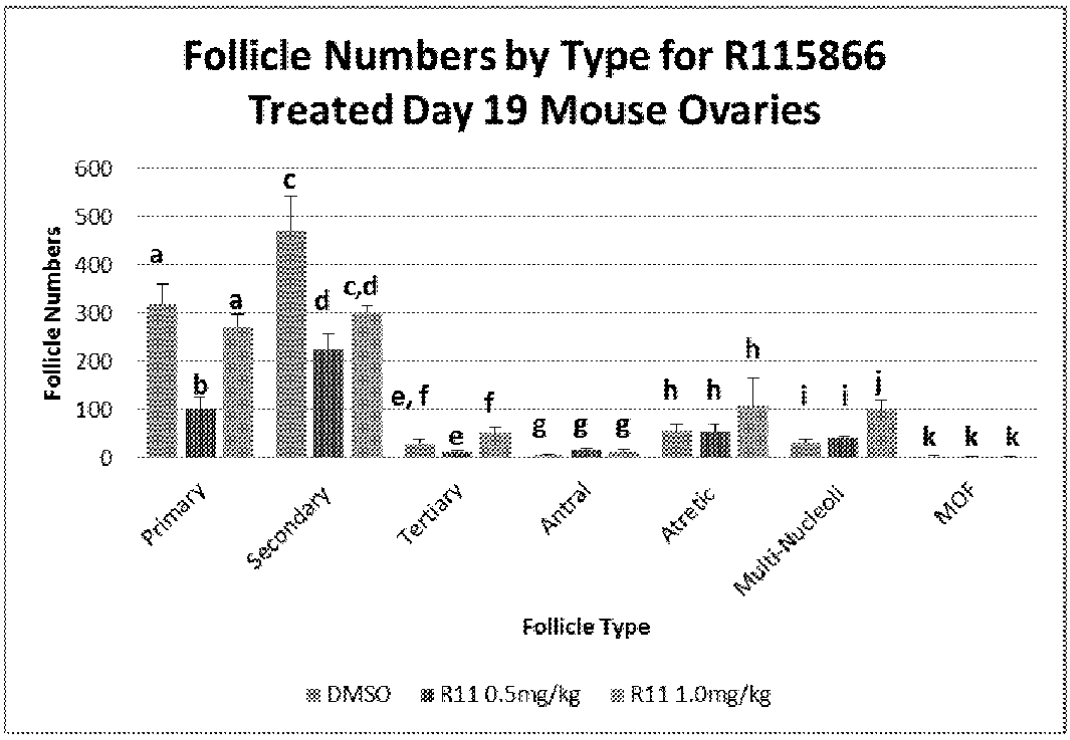


Figure 11. Primary, secondary, tertiary. antral, atretic, multi-nucleolic, and multi-oocytic follicle numbers in control and R115866 treatment groups with different letters representing statistically significant differences. ($p<0.05$; n=3-5)

VA- Follicle counts

Vitamin A deficient animals show a decrease in total follicle numbers at day 19 (*Figure 12*) (n=3), but this does not reach significance. These ovaries also show small, but not significant decreases in primordial and BC follicles (*Figure 13*). Small, but not significant differences were observed in primary, secondary, tertiary, antral, and multi-nucleolic follicles (*Figure 14*). The numbers of atretic follicles significantly increase in the VA- treated ovaries ($p=0.0238$; n=3)

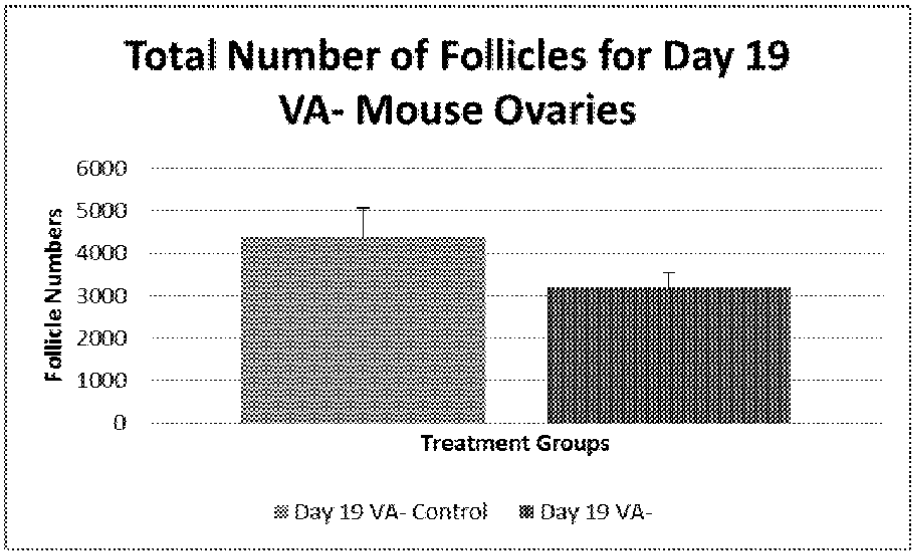


Figure 12. Total follicle numbers of ovaries harvested from day 19 control and VA- treatment groups showed no significant difference. (n=3)

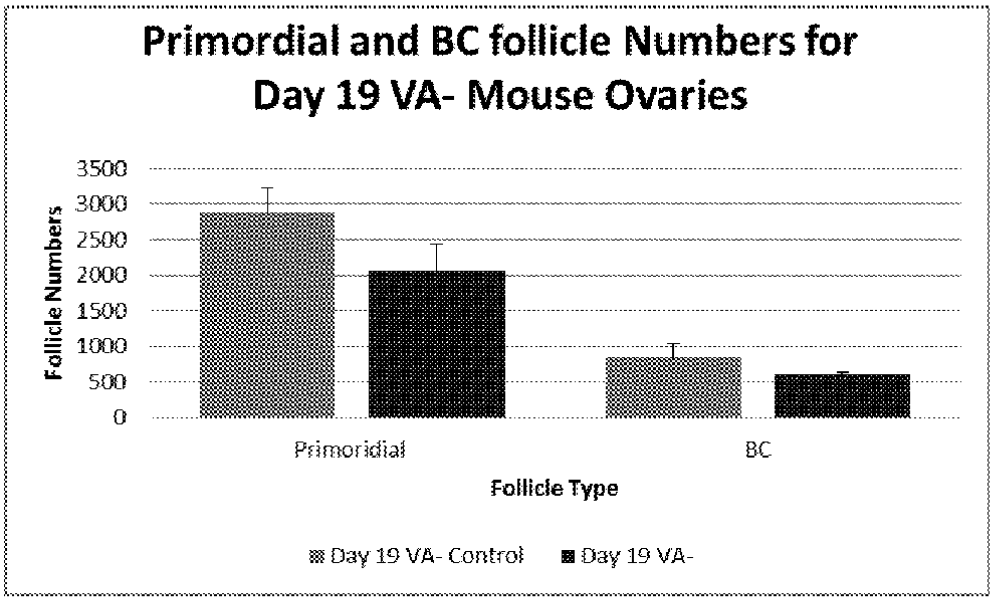


Figure 13. Primoridial and BC follicle type counts of ovaries harvested from day 19 control and VA- treatment groups showed no significant differences. (n=3)

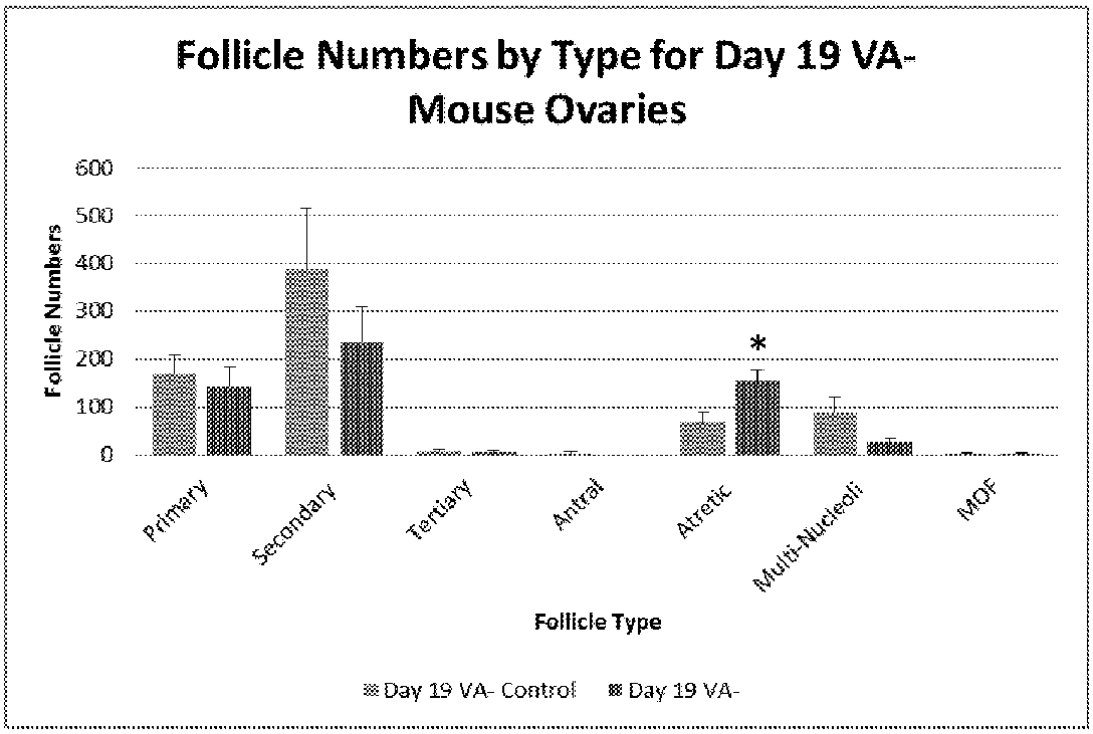


Figure 14. Primary, secondary, tertiary. antral, atretic, multi-nucleolic, and multi-oocytic follicle numbers in day 19 control and VA- treatment groups. (*$p<0.05$; n=3).

IHC

Ovarian tissue samples were stained for Cyp26b1, Ki67, and TUNEL, and images were taken using a confocal microscope. Staining for Cyp26b1 appears to be increased in R115866-treated ovaries as compared to DMSO controls (*Figure 15*). Staining for the proliferative marker Ki67 also appears to be increased in R115866-treated ovaries and shows a similar pattern seen with Cyp26b1 staining (*Figure 16*). In contrast, detection of apoptosis using a TUNEL assay shows a decrease in the appearance of apoptotic cells in R115866 treatment groups (*Figure 17*).

CYP26B1

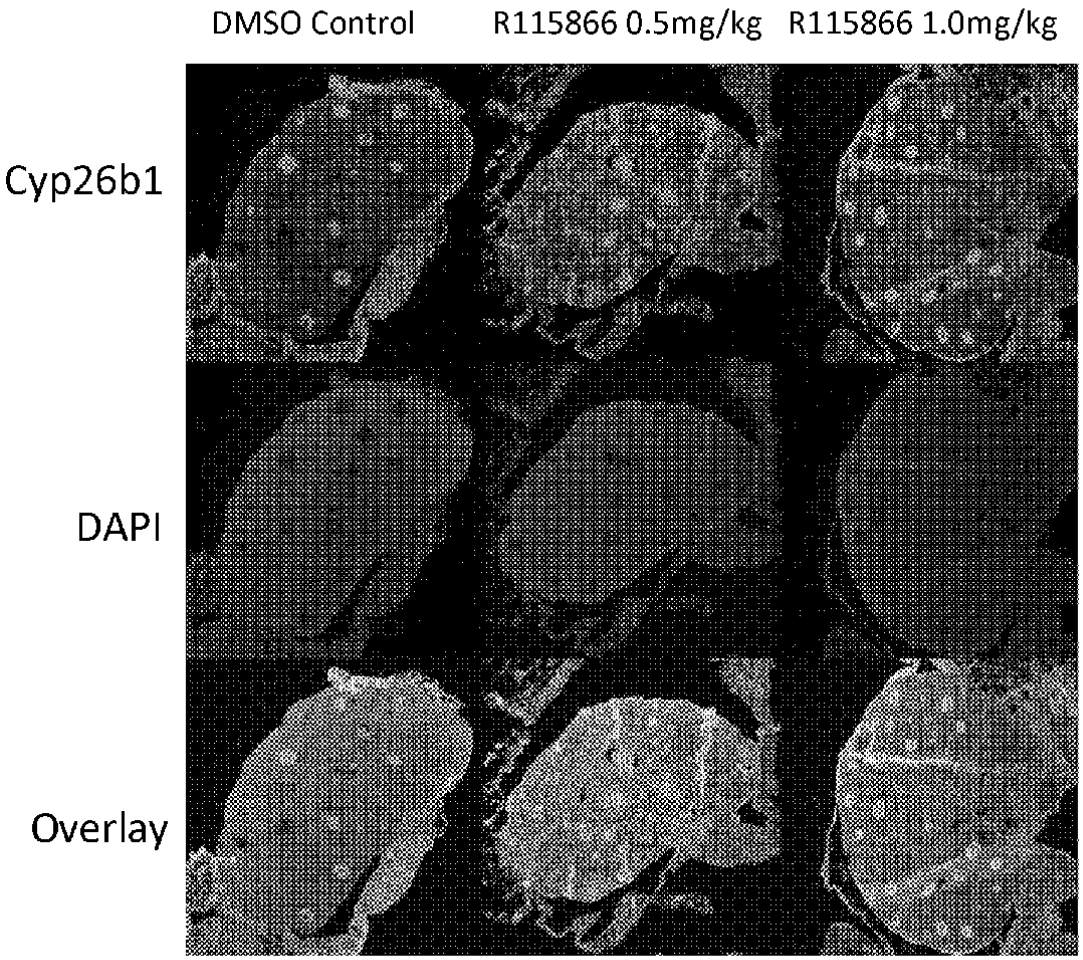


Figure 15. Immunohistochemical staining for retinoic acid degrading enzyme Cyp26b1 in control and R115866 treated day 19 ovaries.

Ki67

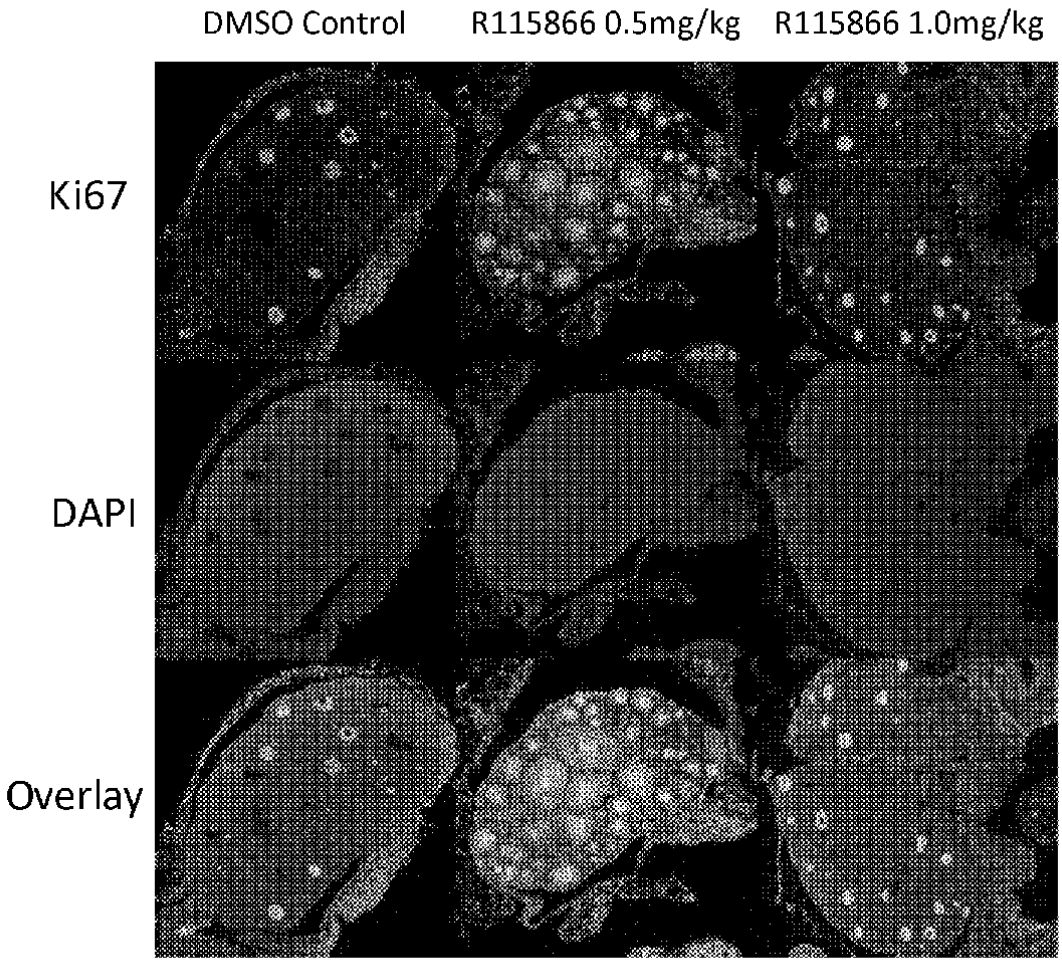


Figure 16. Immunohistochemical staining for proliferative marker Ki67 in control and R115866 treated day 19 ovaries.

TUNEL

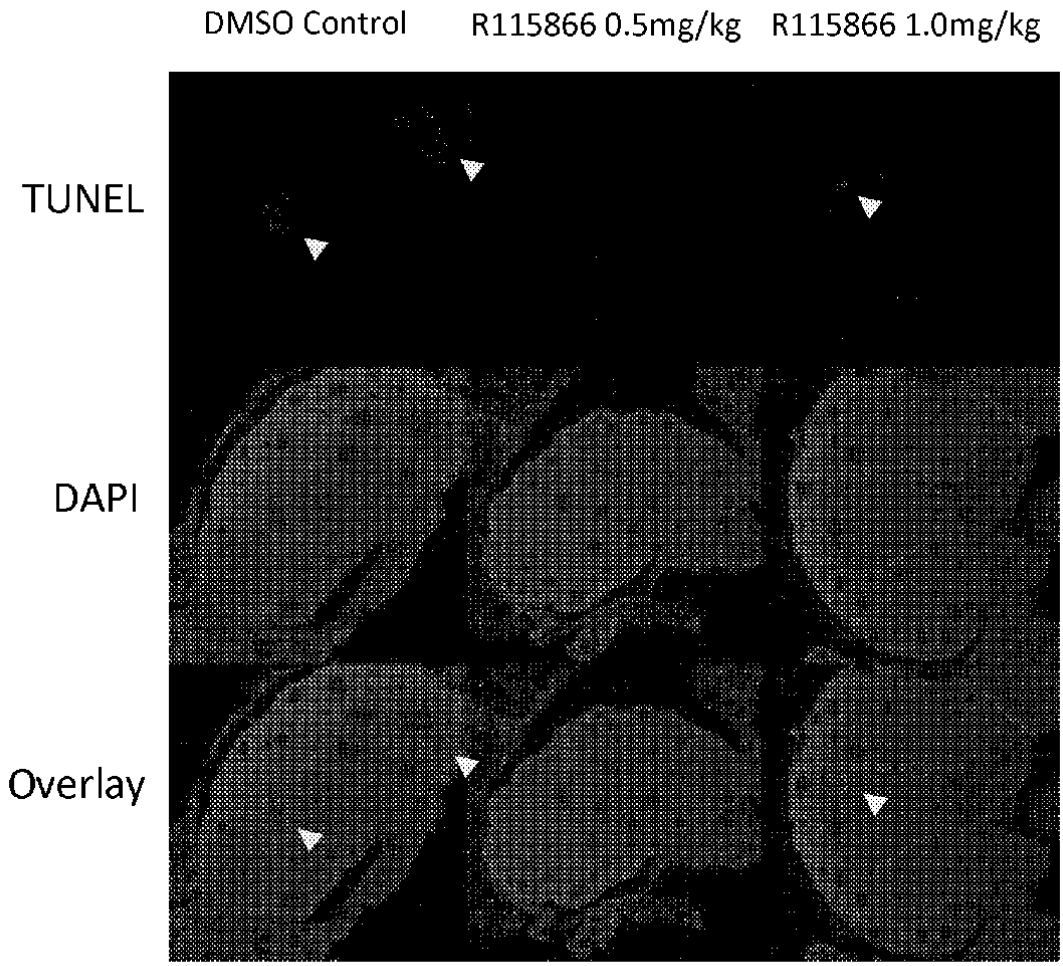


Figure 17. TUNEL staining for control and R115866 treated day 19 mouse ovaries.

Real Time PCR

To further validate and quantify the IHC results seen in day 19 R115866-treated ovaries, levels of Cyp26b1 as well as several proliferative (CyclinD1, CyclinE1, Ki67, and PCNA) and apoptotic (Apaf1, BAD, BCL121, BCL2, Caspace3, Caspace7, and Trp53) markers were assayed by rtPCR. Figure 18 shows an increase in the relative expression of Cyp26b1 in both R115866 treatment groups although this increase was not statistically significant. Interestingly, there is also an increase in proliferative markers CyclinE1, as well as PCNA which appear to be dose dependent, but these increases were also not statistically significant.

Figures 21 and 22 show the relative expression of apoptotic markers in DMSO control, 0.5mg/kg and 1.0mg/kg R115866 treated mouse ovaries. A general trend towards an increase in the relative expression of apoptotic markers is seen, with a significant increase in caspace 7 in the 1.0mg/kg R115866 treated ovaries ($P<0.05$; $n=3$-5). Increases in the relative expression of BCL121, BAD, TRP53, and BCL2 were not statisitically significant. Although TUNEL assays suggested a decrease in the presence of apoptotic cells, this suggests that it may have failed to capture the effects of the upregulation of pro-apoptotic factors, possibly due to an acceleration of the cell death process.

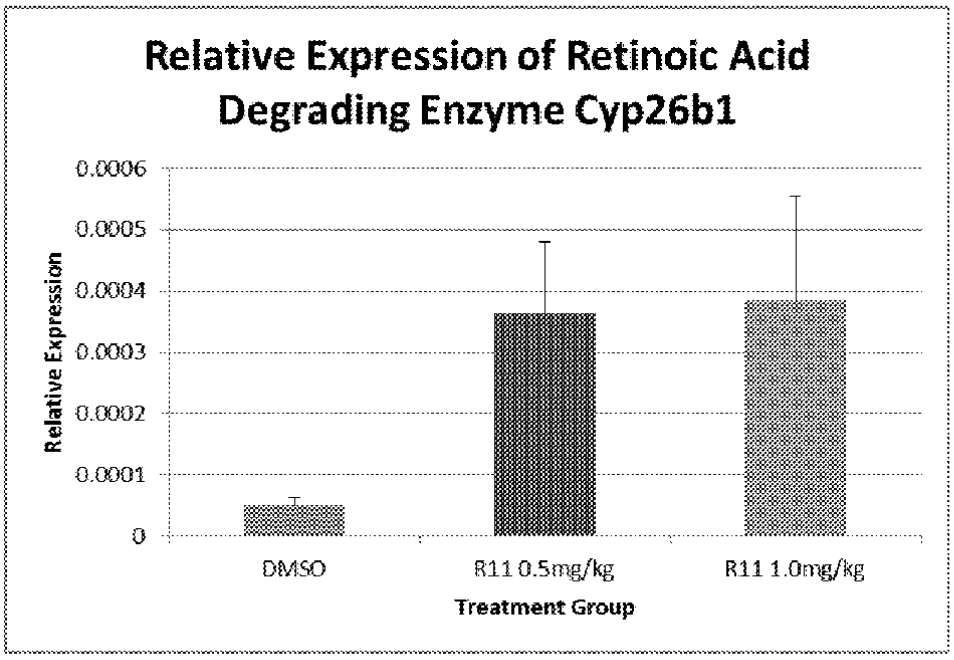


Figure 18. Relative expression of retinoic acid metabolising enzyme Cyp26b1 in day 19 control, and R115866 treated ovaries do not show a statistically significant increase in relative expression amongst R115866 treatment groups and DMSO control. (n=3-5).

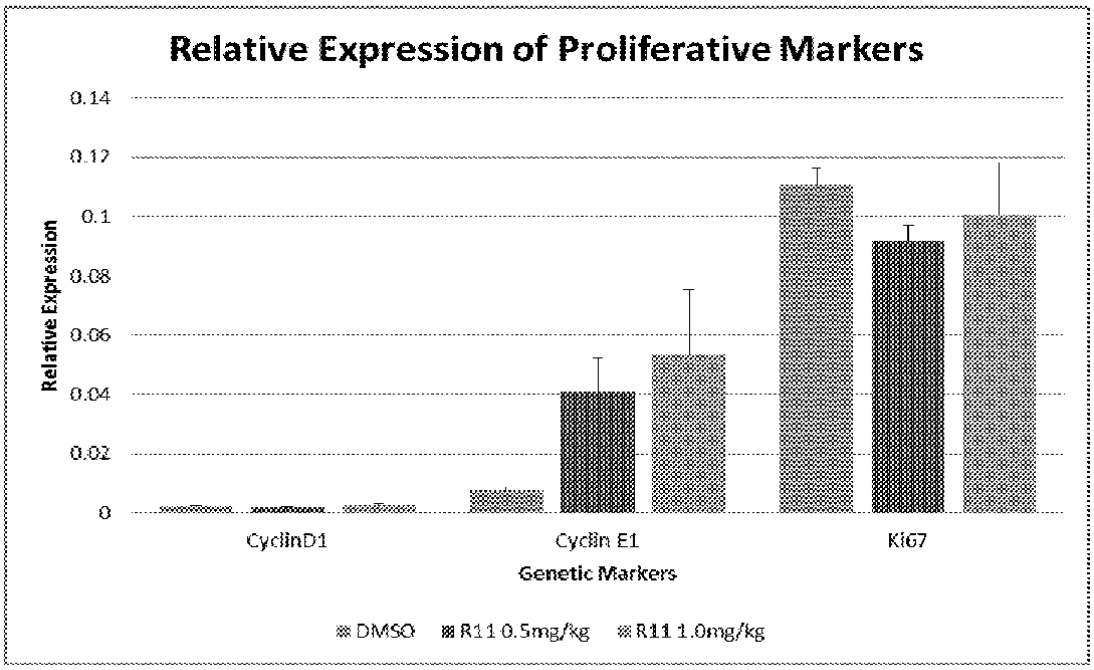


Figure 19. Relative expression of proliferative markers in day19 control and R115866 do not show statistically significant difference amongst treatment groups. (n=3-5).

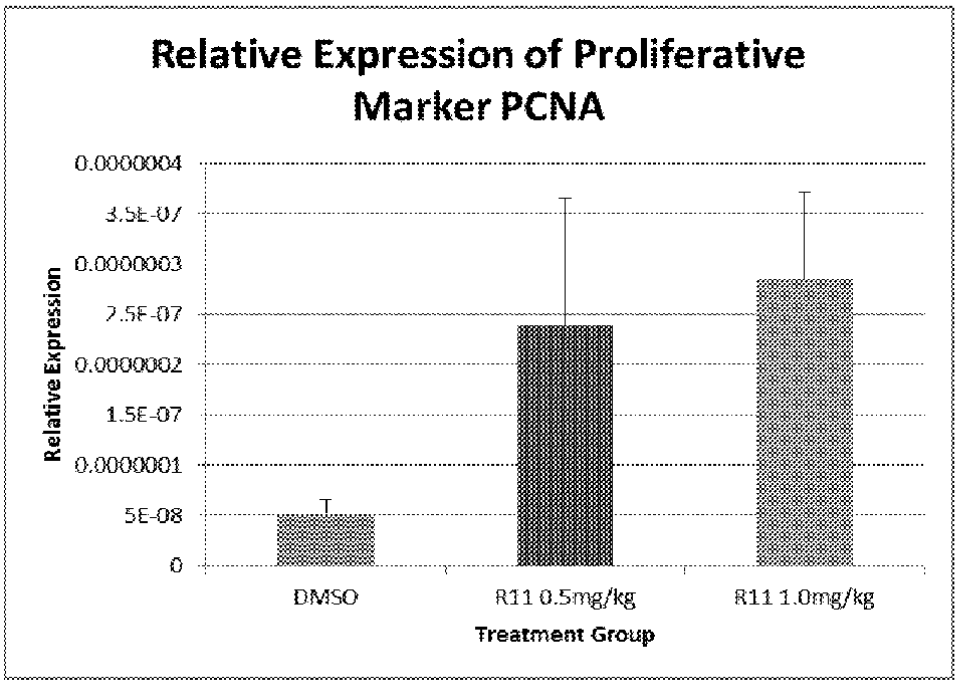


Figure 20. Relative expression of proliferative marker PCNA in day 19 control and R115866 treated ovaries do not show a statistically significant difference amongst treatment groups. (n=3-5)

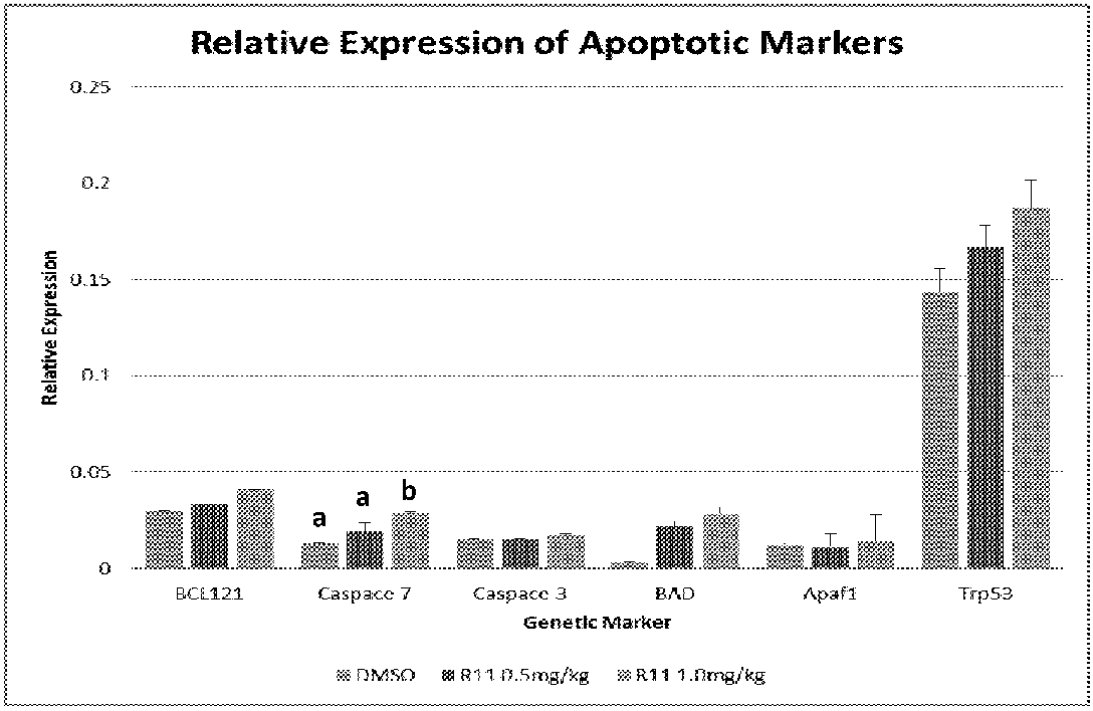


Figure 21. Relative expression of apoptotic markers in day 19 control, 0.5 and 1.0mg/kg R115866 treated ovaries with different letters representing statistically significant differences. ($P<0.05$; n=3-5).

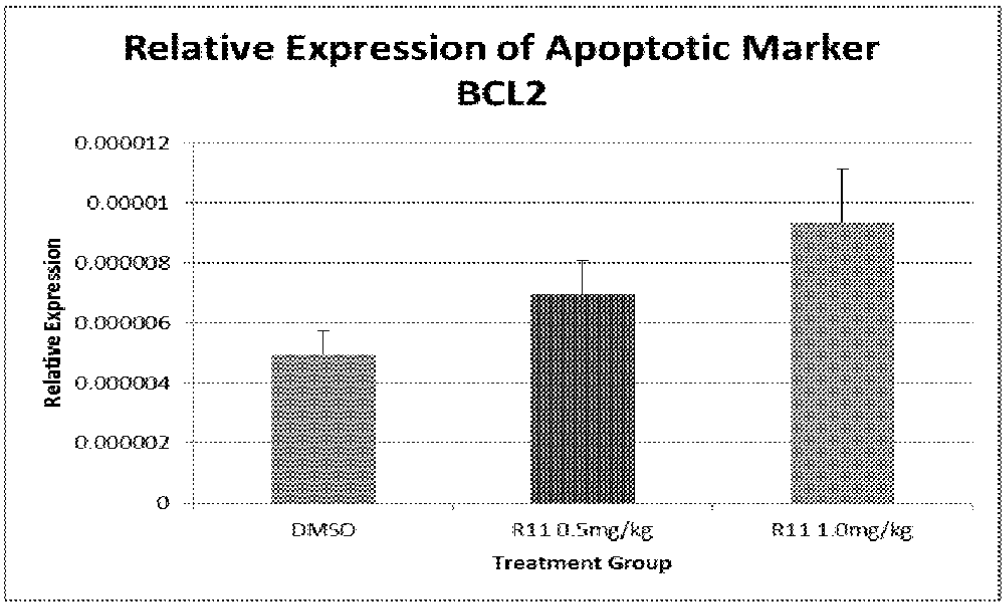


Figure 22. Relative expression of apoptotic marker BCL2 in day 19 control and R115866 treated ovaries show no statistically significant differences amongst treatment groups. (n=3-5).

Discussion

The results of this study demonstrate that imbalances in retinoic acid levels lead to improper development of the mouse ovary. Histology show pathologies in the morphology of all treatment groups. Day-19 VA-deficient and R115866 groups show differences in development; demonstrated by follicle counting and day-19 R115866 treated group show a significant difference in development assayed by IHC and quantified by real time PCR.

Injection Procedure

Previous treatment of mouse pups with retinoic acid resulted in an unacceptably high mortality rate. Due to the highly toxic effect of direct retinoic acid treatment, we instead based our strategy on the inhibition of RA degradation by R115866 treatment. Such pharmacological inhibition of RA degrading enzymes is shown to effectively increase endogenous RA levels. We still observed a high mortality rate in mouse pups injected with 2.5mg/kg R115866; it is unknown whether this was due to RA toxicity, the toxicity of the drug itself, or other unknown side effects of the drug. A lower mortality rate was seen in mice treated with 1.0mg/kg R115866, and no lethal effects were observed in mice treated with 0.5mg/kg R115866. Due to these effects, the number of individuals in this group is higher than other groups.

Subcutaneous injection of one day old mouse pups proved to be difficult, since the skin of these young pups was very thin and taut. Practice showed the best spot was the hind quarter, where a skin fold could most easily be made. As the mouse pups grew, the skin thickened, and injections became easier by day 3. The large injection volume (20ul) also caused problems with seepage from the injection site. Despite this, we maintained this volume to limit the concentration of DMSO and R115866 injected to favorable levels. This may have resulted in variability of dose within each treatment group. It is uncertain how much R115866 was effectively administered by each injection. Precaution was taken by z-tracking injections (a method of shifting the skin before injection and releasing it after injecting as the needle is retracted to shift the injection site away from the injected

liquid), injecting the solution slowly, and waiting a few seconds before retracting the needle.

Mouse Weights

Mice injected with R115866 showed a significant decrease in weight at day 19 compared to DMSO control. By week 7, we observed a more dramatic effect on weight that appears to be dose-dependent. This difference disappears as the mice age, and by week 15, there was no difference in weight between the treatment groups and DMSO control. This would suggest that there is a delay in development through puberty, but that this is resolved by adulthood. Mice treated with R115866 or VA- diets also show a different pattern of adipose remodeling. Vitamin A has been shown to be involved in adipose remodeling, and in the liver this effect may be mediated through PPAR expression [65, 66]. Since PPAR activity is assosiated with metabolic processes, an increase in PPAR activity could explain the decrease in weight seen in these mice. This effect may resolve as PPAR expression levels normalize as the mice age.

Ovary Harvesting and Follicle Counts

Ovaries harvested from R115866 treated mice appear smaller than control ovaries; however, control ovaries appeared larger than expected relative to untreated ovaries. We currently have no explanation for this, as increased ovary size due to DMSO treatment has not been previously reported. Total follicle numbers show a significant decrease in all R115866 treated groups (*Figure 9*). The 0.5mg/kg treated group shows a much more

dramatic decrease in total follicle number than the higher dose (1.0mg/kg) group. Without a direct measure of the effect of R115866 on Cyp26b1 expression or the subsequent levels of endogenous RA, it is difficult to say why lower does R115866 has a more dramatic effect. RT-PCR data shows that the observed increase in *Cyp26b1* levels appears to be dose dependant, but a statistically significant increase was not seen in either R115866 treatment groups, possibly due to a larger standard deviations in these treatment groups. However, the relative expression levels of *Cyp26b1* appear to correlate positively with a higher R115866 dose. In spite of this, endogenous RA levels were not measured due to the lack of appropriate assays. Thus, we cannot explain why lower total follicle numbers were seen with the lower dose R115866 treatment.

We expected that R115866 would cause an increase in RA levels based on *in vitro* studies in the Kipp lab showing increased proliferation of granulosa cells in response to both R115866 and RA treatment. Such stimulation of granulosa cell growth would cause follicles to prematurely progress to later stages of development. The R115866 1.0mg/kg treatment group did show a significant increase in the number of multi-nucleolar follicles. This may indicate an acceleration of cellular processes that correlate with increases in transcription; an increase in nucleolar size is further consistent with precancerous/cancerous cells **[67, 68]**. This suggests that oocytes may be directly stimulated by R115866, causing an increase in overall levels of transcription. Both CyclinE1 and PCNA proliferation markers showed an increase mRNA relative expression level; however, Ki67 did not. Apoptotic markers are also elevated, which complicates our interpretation. However, there is precendence for uncontrolled proliferation itself being an apoptotic trigger [69, 70].

We did not observe a significant difference in the number of atretic follicles in R115866 treated groups compared to control; however, there appears to be a small increase in the 1.0mg/kg R115866 treatment group. It is possible that higher dose injections cause a subsequent increase in RA to toxic levels, which may cause follicle maturation to fail. Vitamin A-deficient ovaries likewise show a significant increase in atretic follicles, which might be caused by failure of RA stimulation of follicle progression.

IHC and RT-PCR

IHC analysis showed that there is an increase in the proliferative marker Ki67 and retinoic acid metabolising enzyme Cyp26b1 upon R115866 treatment relative to control ovaries (*Figures 15,16*). It also appears that the same areas of each section exhibit increased signals for both Ki67 and Cyp26b1. This may suggest that there is an interaction between proliferation and retinoic acid degradation. In line with this, TUNEL assays done on sections show decreased apoptotic signals in these same areas (*Figure 17*). However, RT-PCR analysis of apoptotic markers shows somewhat contrary results to IHC data. Of 7 apoptotic markers, 5 show an increased trend with increased dose of R115866. Of those 5 markers, Caspase 7 showed a statistically significant increase in the 1.0mg/kg R115866 treatment group (*Figures 21, 22*). Other RT-PCR data was consistent with the IHC results. Of 4 proliferative markers, none show statistically significant increases in the R115866 treatment group, However, CyclinE1 and PCNA show an increase realtive expression with increased dose of (*Figures 19, 20*). Cyp26b1 also showed increases in the R115866 treatment group that are not statistically significant (*Figure 20*). This increase in Cyp26b1 may be as an effect of inhibition of the enzyme

causing increases in RA levels and thereby signaling in a feedback loop for higher expression of Cyp26b1.

The increases in apoptotic markers shown in the RT-PCR data, although statisitcally insignificant, would suggest increased apoposis was present, which was not detectable by the IHC methods used. This may be because of the particular sections of the ovaries chosen for staining, or apoptosis might be accelerated or interrupted by retinoic acid signaling. Based on the decrease in ovary size seen in the R115866 treatment group, we expected to detect increased apoptosis and decreased proliferation. However, the increase in some of the proliferative markers is in line with *in vitro* studies that showed an increase in granulosa cell proliferation in response to RA treatment. It is possible that the inappropriately high levels of proliferation we observed early in follicle development itself drives increased apoptosis of primoridal ovarian cells.

Overall, much of this data shows trends amongst the different treatment groups that were not statistically significant. These trends still provide insight for some of the phyotypes observed in our studies and may have not been statistically significant due to low sample size in each of the treatment groups.

Long Term Effects of Early VA- diet

One mouse placed on VA- diet from gestation to 16 weeks of age was alowed to recover for a year on regular diet to see the long term effects of early VA- diet. Morphology revealed several pathologies including bilateral follicle and bursa cysts as well as atretic and hemorrhaging follicles. In contrast, control ovaries showed an overall normal

appearance with a unilateral small follicle (*Figure 8*). It therefore appears that the role of VA in early development is vital through adulthood, and that an attempt to rescue mice kept on a VA-diet after 16 weeks was not successful. Furthermore, a growth of cells appears forming on the bursa of VA- treated ovaries (*Figure 8 F, G, H*), which may suggest the formation of carcinoma but is uncertain at this time.

Conclusion:

We hypothesized that retinoic acid plays a critical role in the development of the ovary. Treatment with R115866 causes an increase in the endogenous RA levels and we were able to observe the impact of the increase in levels of RA on ovarian morphology. Subsequently, we also treated mice with a VA- diet and observed the effect of decreased levels of RA on the ovary. We saw pathological defects in both groups, with decreased numbers of follicles, cyst formation, smaller ovaries, and atretic follicles as compared to controls. These findings suggest that RA plays a significant role in the development of the mouse ovary, and that levels of RA must be tightly regulated for healthy development. In addition, we observed that the effects of RA early in ovarian development and the pathologies involved in the improper balance of RA levels are long lasting and irreversible. This study demonstrates that RA signaling during early stages of mouse development may play an important role in establishing normal ovarian morphology and in follicle maturation.

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