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Impact of Retinoic Acid Receptor Alpha Conditional Knockout on Ovarian Follicle Development in the Mouse

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Impact of Retinoic Acid Receptor Alpha Conditional Knockout

on Ovarian Follicle Development in the Mouse

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Introduction

The ovaries are the primary female reproductive organs. These organs have several critical functions: secreting hormones via follicles, protecting the eggs a female is born with, and releasing eggs for possible fertilization (Urry, 2016). An ovarian follicle is composed of an oocyte (an immature egg) that is surrounded by one or more of somatic cells called granulosa cells. Outside of the granulosa cells, in larger follicles, there are a couple of layers of theca cells (Demczuk et al., 2016). Both granulosa and theca cells secrete hormones and growth factors that are essential for maturation of the egg. Vice versa, signaling from the egg is crucial for granulosa and theca cell proliferation and follicle development and maturation.

Mammalian ovarian follicle formation and development involves the establishment of the initial follicle pool, follicle selection, growth, maturation of eggs, and production and release of hormones (Kipp et al., 2011). Ovarian follicle formation and development is necessary for the propagation of species in addition to the healthy development of the female reproductive system (Kipp et al., 2011). When there is abnormal endocrine signaling in the follicles, infertility and ovarian diseases such as Polycystic Ovarian Syndrome (PCOS) (Ndefo et al., 2013), Premature Ovarian Failure (POF) (Jankowska, 2017), and different types of ovarian cancers (Budiana et al., 2019) may occur.

Mechanisms underlying the regulation of ovarian follicle development and survival are not well understood. In the past few years, studies from the Kipp lab and others have shown Retinoic Acid (RA), an active form of Vitamin A, is critical for ovarian development and fertility (Kawai et al., 2016, Kipp et al., 2011, and Demczuk et al., 2016). RA plays an important role in granulosa cell proliferation through a cell signaling cascade involving Retinoic Acid Receptors (RARs) (Demczuk et al., 2016). However, the functions of RARs (alpha, beta, and gamma) are

not well understood and how their signaling affects follicle development in the ovary has not been studied. Preliminary reports in the Kipp lab tested for mRNA expression showing out of the three isoforms, RAR alpha (RARA) had the highest expression in the ovary and granulosa cells of the mouse.

The purpose of this study is to investigate the function of RAR alpha (RARA) in mouse ovarian follicle development by characterizing a new conditional knockout mouse that the Kipp lab has created. General knockout represents deletion of a particular gene within the body, and when RARA was deleted, it led to mice mortality (Chapellier et al., 2002). To evade this occurrence, a conditional knockout mouse using the Cre-Lox system was created. The Cre-Lox system works by using the Cre recombinase enzyme (Kelmenson, 2011). This enzyme recombines and deletes target gene(s) within the Lox-p sites. This system enables us to generate tissue-specific gene knockout, giving control over the location of the specific gene deletion. A deletion of RARA in mouse granulosa cells and examining the impact of this deletion can help us understand the function of RARA in the ovarian follicles. Consequently, findings from this study will contribute to infertility treatments and give an understanding on how to prevent and treat ovarian diseases in humans.

Review of Literature

Ovarian Biology and Folliculogenesis

The female gonads are a pair of ovaries that flank the uterus. Each ovary is packed with follicles, each of which consists of an oocyte surrounded by supporting cells. Follicles nourish and protect the oocyte partially by producing hormones that control the female reproductive cycle (Urry, 2016). The process of follicle development and maturation is called folliculogenesis.

Folliculogenesis is a highly selective process with only one dominant follicle completing the cycle resulting in ovulation every menstrual cycle in the human. At birth, the human ovary contains around 400,000 primordial follicles (Pangas and Rajkovic, 2015). Primordial follicles begin to undergo morphological and hormonal changes necessary for successful ovulation to occur. This includes primordial follicles transitioning into primary, secondary, tertiary, and antral stages of follicle development (Table 1). Two hormones produced by the pituitary gland, follicle stimulating hormone (FSH) and luteinizing hormone (LH) stimulate follicle growth and development (Erickson, 2008). During each menstrual cycle, the hormones activate around 20 primordial follicles, however, normally only one follicle will mature and be selected for ovulation in mono-ovulatory animals such as humans (Erickson, 2008). The selected follicle will burst and discharge the oocyte, concluding the process of ovulation (Figure 1).

During folliculogenesis, cell differentiation occurs within the somatic cells producing granulosa and theca cells. Both cell types are critical for normal follicular development and growth. Theca cells appear in the secondary stage of folliculogenesis and are responsible for the production of androstenedione, which is used by the granulosa cells as a substrate to make estradiol (Young and McNeilly, 2010). The main function of estradiol is to mature and maintain the reproductive system. Theca cells express receptors for LH and granulosa cells express FSH receptors. Only a few follicles will complete the whole process of folliculogenesis, with 99.9% of follicles dying due to programmed cell death, leaving a woman to ovulate only around 400 times in her lifetime (Erickson, 2008).

Stage	Description
Primordial	Small primary oocyte arrested in diplotene stage of meiosis, composed of a single layer of mesodermal cells (also considered flattened granulosa cells) around the oocyte
Primary	Granulosa cells begin to take on a cuboidal cell shape.
Secondary	Composed of 2 to 10 layers of cuboidal or low columnar granulosa cells. This transition is accomplished by continuing division of granulosa cells and theca cells.
Tertiary (Antral)	A follicle characterized by an antrum containing a fluid (follicular fluid), an oocyte surrounded by granulosa cells, a basal lamina, and theca cells.

Table 1. The stages of folliculogenesis in mammalian ovary (Erickson, 2008).
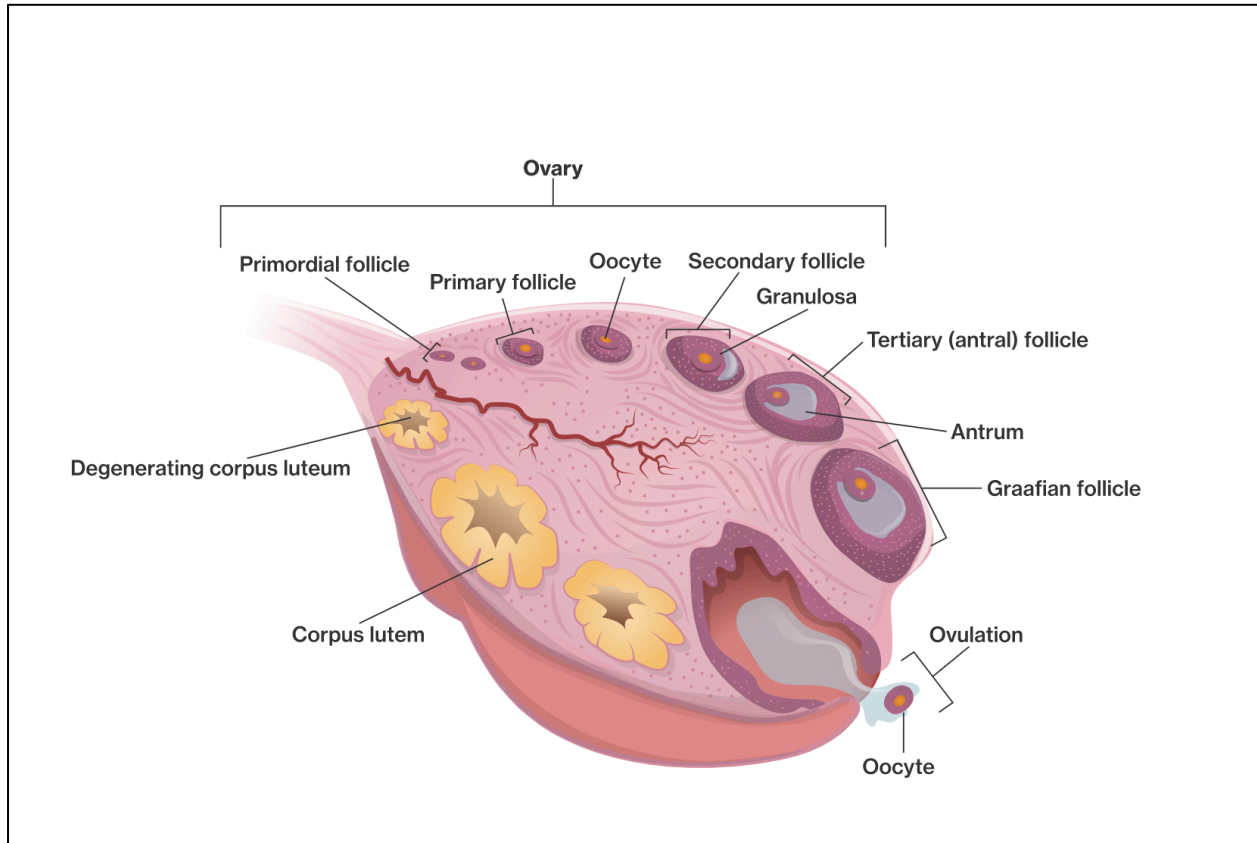
Figure 1. Diagram representing an ovarian follicle undergoing morphological and endocrinological changes during the menstrual cycle. The follicles begin to form primordial follicles that undergo various stages of development starting with primary stage of development to more advanced stage such as the tertiary (antral) stage of follicle development before the oocyte is mature enough to be released from the follicular structure. After ovulation, the follicle undergoes degradation to form corpus luteum (Image from https://www.coursehero.com/sg/anatomy-and-physiology/folliculogenesis/).

Infertility and Ovarian Diseases

Many of diseases may affect the functions of the ovary. Some of these diseases may lead to decreased ovarian stimulation resulting in an immature oocyte pool. Some are caused by an imbalanced production or supply of hormones, growth factors, and vitamins, which fail to support the process of folliculogenesis and healthy ovulation, resulting in infertility (Hart, 2016).

The three major diseases that are the leading cause of infertility and affect a significant number of women are PCOS, POF, and ovarian cancer (World Health Organization, 1992). Classically, PCOS is characterized by a combination of menstrual disturbances and symptoms of excessive androgen secretion (Ndefo et al., 2013). Clinical signs of PCOS include elevated LH secretion and muted FSH concentration, resulting in irregular signaling of follicles between the granulosa and theca cells (Ndefo et al., 2013). As a result, theca cells produce excessive amount of androgens which give rise to hirsutism (excessive body hair), acne, obesity, or androgenic alopecia (Ndefo et al., 2013). PCOS affects one in ten women of childbearing age, making it the most common endocrine abnormality among females of reproductive age in the U.S. (U.S Department of Health, 2014). PCOS is a complex condition that accounts for more than 75% of cases of anovulatory infertility (Gorry et al., 2006). Anovulation occurs when the ovaries do not release an oocyte during a menstrual cycle. Therefore, ovulation does not take place (Gorry et al., 2006). The mechanism for anovulation is uncertain but there is evidence that arrested antral follicle development is associated with the abnormal endocrine system release of LH. The etiology of PCOS remains unknown (Ndefo et al., 2013), and a significant number of female patients are left with diminutive therapeutic interventions.

POF is another ovarian disease that leads to infertility due to the cessation of ovarian function before 40 years (Jankowska, 2017). This term is in reference to the condition when the

ovaries have lost their germinative and hormonal functions because of the exhaustion of the number of ovarian follicles prior to the typical menopausal age. Most women experience menopause at the average age of 51. Menopause is a natural phase in a woman's life, typically occurring between ages 45 and 55, with an average age of around 51. It signifies the end of reproductive capacity as the ovaries gradually produce fewer hormones, particularly estrogen and progesterone, leading to the cessation of menstrual cycles (Sikon and Thacker, 2007). There are two types of POF. Type 1 is classified by a complete depletion of ovarian follicles (afollicular). Type 2 POF is classified by inflammation of ovarian follicles, very few follicles present, or ovaries with numerous primordial follicles (Jankowska, 2017).

Ovarian cancer is characterized by a cancerous growth arising from the ovary. Ovarian cancer is one of the most prevalent gynecologic cancers and is one of the leading causes of cancer-associated mortality in women (American Cancer Society, 2015). The low survival rate (~54%, 5-year survival rate) is due to the fact early detection methods for ovarian cancer have not achieved satisfying results, mostly due to the heterogenous nature of the cancer (Budiana et al., 2019). Cancer therapies include reducing the amount of hormones such as estrogen and progesterone, and, as a result, this affects follicle maturation and growth, which leads to infertility (Bedoschi et al., 2016 and Budiana et al., 2019).

There is no doubt that the ability of the ovary to produce a dominant follicle, which ovulates a fertilizable egg, is under the control of the endocrine system, most notably by the hormones FSH and LH. The diseases mentioned above interfere directly or indirectly with the standard action of the gonadotropins and lead to follicle apoptosis and infertility (Bedoschi et al., 2016, Jankowska, 2017, and Ndefo et al., 2013).

Thus, some female patients who have been diagnosed with these conditions may resort to *in vitro* fertilization (IVF). IVF is a procedure in which an egg is fertilized by the sperm outside the body with modified environments of growth media to achieve a healthy egg. Since the above diseases are prominent in having an immature oocyte pool, IVF can get relatively expensive and is not always successful. Unfortunately, the causes for these diseases are not well comprehended. If the pathway of follicle development and maturation were more understood, we could give insights into why these diseases give rise to irregular folliculogenesis.

Vitamin A and Retinoic Acid

Retinol is a scientific term for Vitamin A, which is found in numerous fruits, vegetables, and animal-sourced foods (Gilbert, 2013). Retinol is taken up by the cells and converted into Retinoic acid. Retinoic acid (RA) is the active derivative of Retinol that is required for virtually all essential physiological processes and functions because of its involvement in transcriptional regulation of over 530 different genes (Wu et al., 2016).

Retinol is mainly stored in the liver as well as the lungs and bone marrow (Vilhais-Neto and Pourquié, 2008). Retinol binds to a cellular retinol-binding protein (CRBP), and enters the circulatory system to travel to the cells that require RA. The retinol-CRBP complex enters the target cell and is metabolized in a reversible step to retinal by retinol dehydrogenase (RoDH). Retinal is then metabolized in an irreversible manner to retinoic acid by retinal dehydrogenase (RALDH). In the cytoplasm, RA is associated with the cellular retinoic acid-binding protein (CRABP) (Vilhais-Neto and Pourquié, 2008) (Figure 2).

RA acts as both paracrine and autocrine molecule. In the paracrine signaling model, RA is produced by one cell, but influences other target tissues where it is metabolized. In the autocrine signaling model, RA is produced and exerts effects in the same cell (Duester, 2008).

RA is a signaling molecule that functions as a ligand exerting its activity by binding with transcription regulatory factors, also known as RARs. These receptors are located in the nucleus and there are three isoforms of RARs present in the signaling pathway, RAR-alpha (RARA), RAR-beta (RARB) and RAR-gamma (RARG) (Duester, 2008). Immediately after RA binds to a RAR, the receptor-ligand complex binds with Retinoid X receptors (-A,-B,-G) to form a heterodimer. The heterodimer binds with a Retinoic Acid Regulatory Element (RARE) within the nuclear DNA, which activates the signaling cascade of events that result in the recruitment of transcriptional coactivators. These coactivators will either inhibit or activate the transcription of target genes that encode proteins involved in cell differentiation and development. More than 532 genes have been put forward as regulatory targets of RA (Duester, 2008) (Figure 2).
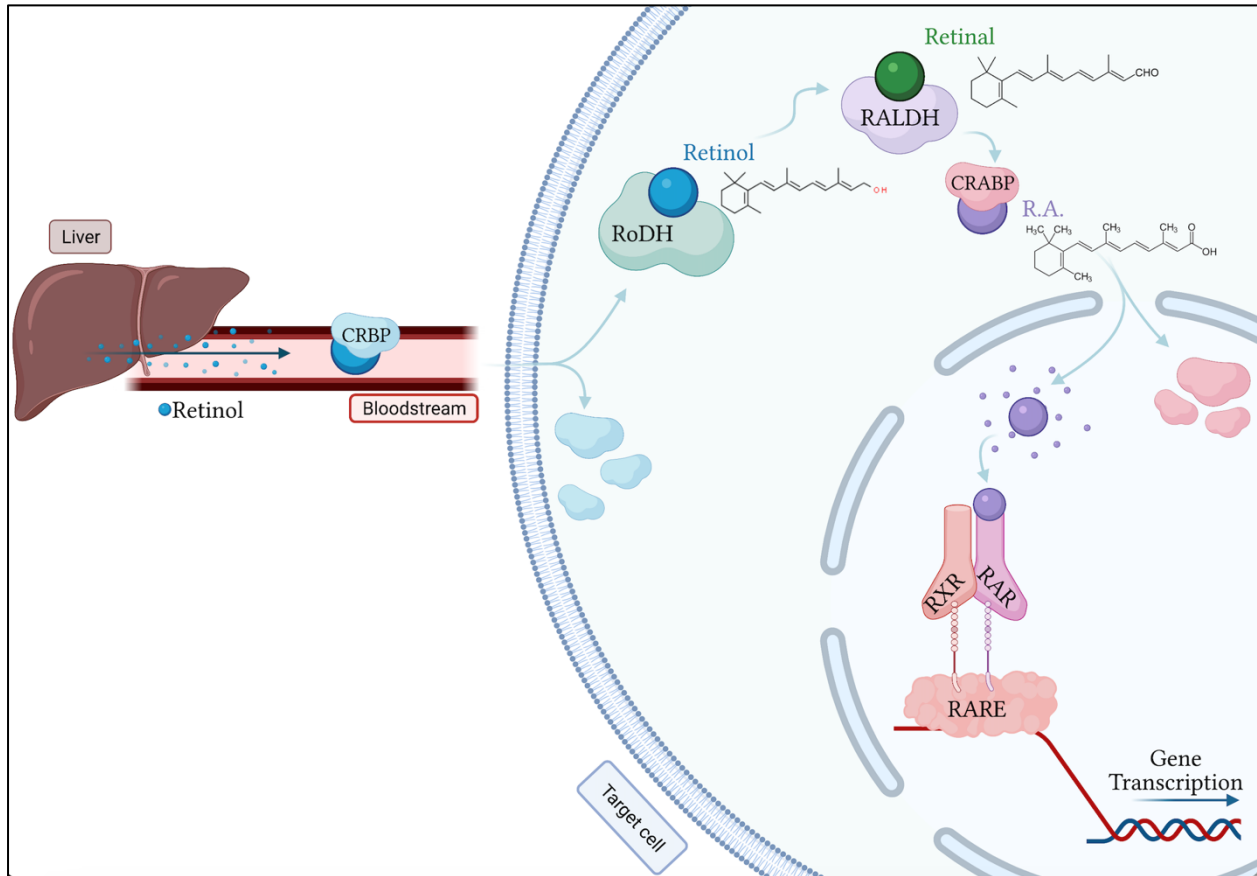
Figure 2: Retinoic Signaling Pathway. Retinol is secreted by the liver, binds to CRBP, and travels to the target cell in need of retinoic acid. Once taken up by the target cell, retinol is metabolized to retinal then oxidized to RA by dehydrogenases RoDH and RALDH. When RA is present in the cytoplasm it is bound to CRABP. Once RA enters the nucleus and binds to retinoic acid receptors RXR and RAR, the receptors heterodimerize. The binding of the two receptors activates RARE, which will recruit cofactors that will lead to transcription or inhibition of genes involved in cell growth and differentiation (Biorender).

In adults, RA plays a significant role in a number of biological processes, such as learning and memory, vision, immune function, reproduction as well as maintenance of epithelial cell function and differentiation (Vilhais-Neto and Pourquié, 2008). In addition to RA being essential for adults, it is also crucial for the development of the nervous system and notochord in embryogenesis (Blomhoff and Blomhoff, 2006). RA is used in variety of therapies from promyelocytic leukemia with 70-80% cure rate (Altucci et al., 2007) and acne vulgaris due to RA's epithelial cell development benefit (Kotori, 2015). In addition to the functions described above, there are many additional roles of RA signaling that haven't been studied such as RARA in ovarian follicle development.

Role of Retinoic Acid in the Ovary

As ovarian follicle development begins, it is regulated by multiple hormones such as FSH and LH from the pituitary gland, which are known as gonadotropins, the actions of gonadotropins are dependent on other signaling factors such as insulin-like growth factor 1 (IGF-1), transforming growth factor family-beta (TFG-β) family members (e.g., inhibin, activin, growth differentiation factor-9, and bone morphogenic proteins), and fibroblast growth factor (Richards et al., 2002). In addition to these growth factors, RA has also been found to play a role in ovarian development. By treating granulosa cells with RA, RA showed to be a potent stimulator of granulosa cell proliferation. Indicating an involvement of RAR-mediated signaling in RA- granulosa cell proliferation (Kipp et al., 2011).

Studies have shown that RA is one of the meiosis-inducing molecules, which is produced by the embryonic gonads. This was determined when mice ovaries (embryonic day 13.5) expressed RA and the receptor *Stra8,* which in turn led to the initiation of meiosis.

In mice, oocytes that progress through prophase I of meiosis will arrest in diplotene stage around birth (Dutta et al., 2016). After birth, the process of folliculogenesis begins. Recent *in vitro* studies have shown that RA can improve ovulation or oocyte maturation in several animal models including cows (Duque et al., 2002), goats (Pu et al., 2014), pigs (Alminana et al., 2008), and mice (Nasiri et al., 2011). Despite these observed effects of RA, *in vivo* studies have not been made on the requirement of RA in ovarian follicle development.

Mouse as a Model for Ovarian Follicle Developmental Studies

Humans and mice don't look alike, but both are mammals and are biologically similar. Mice share about 97.5% of genome with humans (Szuromi, 2002). Mice develop in the same way from egg and sperm, have the same kinds of organs (e.g. ovaries), as well as similar reproductive and endocrine systems. The reason our study has chosen mouse as a model organism is because mouse models currently available for genetic research include thousands of unique inbred strains and genetically engineered mutants that are beneficial for this research. Mice are also relatively easy to maintain, and they reproduce quickly. Mice are great for studying the essential principles of the ovaries and ovarian follicle development.

Cre-Lox System

The Cre-lox system is a powerful genetic recombination tool widely used in molecular biology and genetics research. It enables precise control of gene expression and manipulation of specific genes or genetic elements in various tissues or at specific developmental stages. The system consists of two main components: Cre recombinase and loxP sites. Cre recombinase is an enzyme derived from bacteriophage P1 that recognizes and binds to specific DNA sequences

called loxP sites. LoxP sites are short DNA sequences consisting of inverted repeats flanking a core region (Kelmenson, 2011).

To utilize the Cre-lox system, researchers generate a floxed allele by placing loxP sites on either side of a DNA segment of interest, such as a specific gene or genetic element. In the absence of Cre recombinase, the DNA segment remains intact. However, when Cre recombinase is introduced, it catalyzes a recombination event between the loxP sites. The outcome of the recombination depends on the orientation and positioning of the loxP sites.

If the loxP sites are in the same orientation and directly oriented, Cre-mediated recombination leads to excision or deletion of the DNA segment between the loxP sites. This results in the removal of the floxed allele. On the other hand, if the loxP sites are in opposite orientations or not directly oriented, Cre-mediated recombination can lead to inversion or rearrangement of the DNA segment between the loxP sites (Kelmenson, 2011).

We utilized the AMHR2-Cre (Anti-Müllerian hormone type 2 receptor-cre) mouse strain, which expresses Cre recombinase under the control of the AMHR2 gene promoter. AMHR2 is expressed within in granulosa cells within the ovary. By breeding these mice with mice carrying a RARA-flox allele, where the RARA gene is flanked by loxP sites, the Kipp lab was able to selectively delete or manipulate the RARA gene ovarian granulosa cells (Figure 3). This allows to investigate the role of RARA in granulosa cell function and its potential impact on ovarian development and fertility.
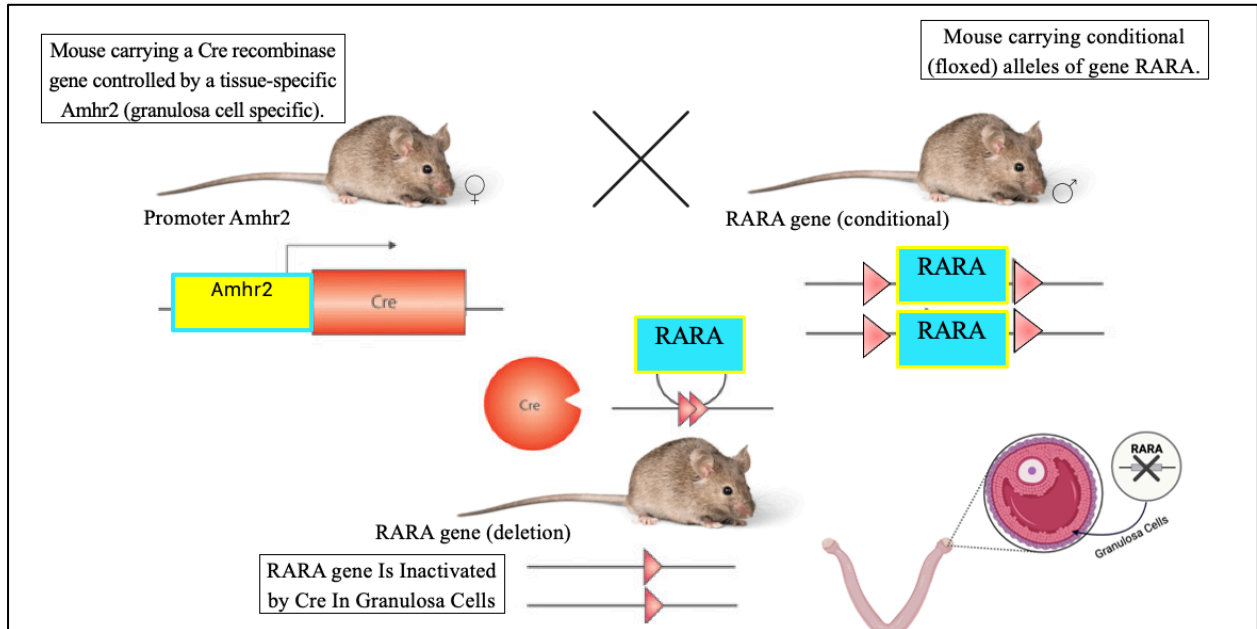
Figure 3. Diagram showing how RARA conditional knockout mice were generated using the Cre/Lox system: A mouse containing a RARA Lox sequence site was bred with a Cre gene mouse (Amhr2-Cre) targeting ovarian granulosa cells to generate a deletion of RARA in the ovarian granulosa cells.

Hypothesis

Based on previous studies showing that the retinoic acid is involved in ovarian granulosa cell proliferation (Kipp et al., 2011), we hypothesize that RARA conditional knockout mice will exhibit significant alterations in ovarian development and function.

Some of RA target genes are involved in differentiation and growth of ovarian follicles (Balmer and Blomhoff, 2002). Follicles play a crucial and essential role in ovulation therefore we expect that fertility studies will show a decreasing number of litters and pups.

If we knockout RARA in ovarian granulosa cells we expect to see a decrease in ovarian follicle numbers, since RA promotes granulosa cell proliferation (Kipp et al., 2011). We hypothesize that granulosa specific RARA conditional knockout in mice will have significant effects on follicular development and ovarian morphology. We hypothesize that mice with granulosa specific RARA conditional knockout will exhibit impaired follicular growth and increased abnormal ovarian morphology compared to control mice.

Materials and Methods

    Animals

    The Rara-flox/flox mice were provided by Drs. Norbert Ghyselinck and Pierre Chambon from France (MTA has been approved and signed by DePaul University).

    The Amhr2tm3(Cre)Bhr mice were acquired from the public repository Mutant Mouse Resource and Research Center (MMRRC) (Jamin, et al., 2002). Amhr2 encodes the type II receptor of anti-Müllerian hormone, which is expressed in granulosa cells in ovaries (Jorgez et al., 2004) and Cre expression alone does not cause ovarian abnormalities (Jamin et al., 2002). Amhr2-Cre mice have been used to generate several different granulosa-cell specific knockouts (Jeyasuria et al., 2004, Jorgez et al., 2004, Pangas et al., 2006).

    Mice were kept at DePaul University Animal Care Facility and were put on a 12hr light and dark cycle. Food and water were given *ad libitum* and all care of the animals followed all federal and institutional guidelines. All procedures were approved by DePaul University Institutional Animal Care and Use Committee.

    Genotyping of Mice

    For identification of RARA-flox, Amhr2-Cre (Cre-Lox transcripts) and the resulting offspring transgenic mice, genotyping was performed as directed by manufacturer protocols (Wizard® Genomic DNA purification kit for DNA purification and SYBR Master Mix from for Real-Time PCR). DNA from approximately 2-5mm biopsies of mouse tails was collected. Tails samples were lysed in a tail lysis buffer (Proteinase K buffer) (250 mM NaCl, 10 mM Tris-HCl pH 8.3; 1.5 mM $MgCl_2$; 1%SDS; 100µg/ml Proteinase K) and incubated overnight at 55°C with agitation (250rpm on a heated shaker). The following day, tail samples were brought to room

temperature and 200μL of Protein Precipitation Solution (high salt buffer provided in the kit) was added, vortexed for 20 sec, and chilled for 5min. Samples were then centrifuged for 4min at 15,000 RCF and a white pellet formed on the bottom. The supernatant was carefully removed and transferred to a 1.5 ml tube containing 650μL of isopropanol. The sample were centrifuged for 1min (15,000 RCF), washed with 70% ethanol, centrifuged again for 1min (15,000 RCF), and left to air dry for 15min. The final step of isolating DNA was to add 100μL of DNA Rehydration Solution (10mM Tris-HCl pH 7.4; 1mM EDTA pH 8.0) and resulted in stock DNA solution.

For PCR, SYBR Master Mix was used to make 20μL assay volumes in a 96 well plate. A StepOnePlus™ Real-Time PCR system was used in conjugation with the StepOne software v. 2.1 (Applied Biosystems). The thermal cycle conditions were as follows: 1x 50°C, 2 min; 95°C, 15 sec; 58°C, 45 sec; 72°C, 1 min; finished with 95°C for 15 sec. After PCR was completed, the samples were separated in a 2% agarose gel wells with added ladders and 6X loading dyes (Thermo Scientific). The gel was submerged in 1X TAE, ran at 112V/400mV for 35-40min. Gel was stained with Ethidium Bromide (EtBR).

The gels analyzed consisted of ear tag-derived DNA, each representing three distinct gene markers: RARA-floxed transcript (FR), RARA-floxed excised gene (FRe-), and Amhr2-cre (NC). These gene markers are associated with specific DNA fragments of 436bp, 357bp, and 250bp, respectively. Subsequently their sizes were separated and visualized. The gel imaging confirmed the presence or absence of the desired gene markers, allowing us to accurately identify conditional knockout alleles. Mouse alleles that resulted in negative conditional knockout alleles were used as controls in this study.

Fertility Analysis of Conditional Knockout Lines

To examine the effect of RARA allele on mice fertility a protocol provided by Dr. Varnoy from Department of Molecular Biosciences and Center for Reproductive Science at Northwestern University was used (Varnoy et al., 2014). The fertility of RARA conditional knockout mice was assessed beginning with 6-week old mice by continuously mating with a wildtype male for 6 months. As a preventative step, we rotated the male mice within the 6 set-up fertility cages every two weeks to ensure it wasn't a specific males infertility at play. The number of pups born from each female was recorded at birth. We also calculated the time between each litter, and the total number of litters born in the 6-month period. Control mice followed identical procedure to the transgenic mice.

Euthanasia and Ovary Collection

Mice were euthanized by CO_2 exposure in an enclosed chamber. Euthanasia was double confirmed by preforming cervical dislocation.

In the study, ovaries were collected from mice at different time points: week 7, week 15, and 12-15 months. Collecting ovaries from mice at different developmental time points correlated with human age, ranging from human early teen stage (week 7), mature adult (week 15), to middle-aged adulthood (12–15-month-old). These timepoints allowed us to study the progressive changes that occur in ovarian development over time. After dissection, the ovaries were dissected and cleaned of any connective tissue and stored in 10% formalin overnight. One of the cleaned ovaries, uterus, and liver from an animal were stored in a microcentrifuge tube on dry ice prior to transferring to -80°C freezer for RNA isolation. The ovary stored in formalin from the same animal was fixed for histological studies as detailed below.

Ovarian Morphology and Follicle Counting

To observe the morphology of the ovaries, one ovary form each mouse was fixed in 10%

formalin overnight. The following day the ovaries were washed 3 x 20 minutes in 50% ethanol

and then 3 x 20 minutes in 70% ethanol. Before embedding, 1-3 ovaries with the exact same

genotype were added to a plastic cassette and labeled appropriately with a pencil. The tissue

went through a series of rinses as follows: 80% ethanol for 1 hour, 95% ethanol for 1 hour, 100%

ethanol 3 times for 1.5 hours, CitraSolv (similar to xylene) 3 times for1.5 hours, 58-60°C

paraffin wax two times for 2 hours. Tissue embedding molds were placed in a paraffin oven with

clean melted paraffin (58-60°C). Air was vacuumed out of the paraffin oven to ensure no air

bubbles would be present during the wax block casting process. Third of wax is poured into the

metal mold, then ovaries were placed medially on the bottom, the rest of the wax was filled to

the top and a brand-new labeled plastic cassette was mounted to the top of the mold and left to

refrigerate at 23°C overnight. Wax blocks were pressed out of the mold and excess wax was cut

off. Ovaries were serially sectioned in 5μm sections using benchtop microtome (American

Optical 820). The resulting slides were stained by hematoxylin and eosin (H&E) using

Northwestern University Mouse Histology and Phenotyping Laboratory (Emge, 2015). Mouse

ovaries had 4-5 sections per slide.

Any abnormalities within the follicles (see examples in Table 2) were recorded. Follicles

that contain two or more oocytes that share a cytoplasm were multioocytic follicles (MOFs). An

oocyte going through atresia (programmed cell death) usually seen as a darkened and deformed

oocyte was labeled as an atretic follicle. Follicles showing presence of blood within the follicular

fluid (dark red fluid) were labeled as hemorrhagic follicle. A cyst in the follicle seen as an

abnormally large area (~20-80μm) with no oocyte present was labeled as follicular cyst, and cyst

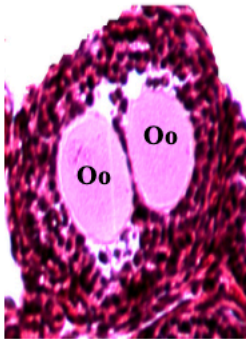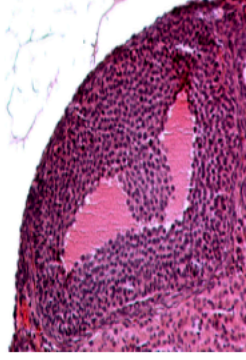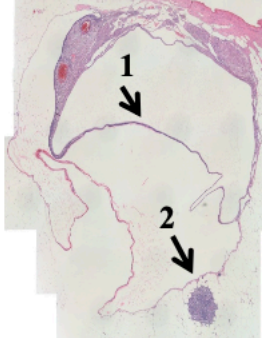present in the bursa (fluid surrounding follicle) was labeled as bursa cyst.

Abnormalities	Name
	Multioocytic follicles (MOFs)
	Atretic follicle
	Hemorrhagic follicle
	1) Follicular cyst 2) Bursa cyst

Table 2. Examples of ovarian follicle pathologies that we were looking for in the ovarian morphology experiment (images courtesy of the Kipp Lab).

Ovarian follicles were counted and identified of the H&E stained slides using the ImageJ Software. Total numbers of follicles were counted for the first and fifth ovary on each slide to prevent double counting. Only follicles containing distinct nuclei were counted. Ovarian follicle development was characterized by Table 1. Primordial cells were identified by a small primary oocyte and were composed of a single layer of mesodermal cells (also considered flattened granulosa cells) around the oocyte. Primary cells were characterized when the granulosa cells took on a cuboidal shape, secondary cells were seen to be composed of 2 to 10 layers of cuboidal or low columnar granulosa cells and theca cells were becoming present. Lastly antral follicles were classified when an appearance of a cavity containing follicular fluid was present and distinct granulosa and theca cell layers were present within the follicle. Ovaries were then multiplied by a corrective factor of 5 to calculate the total follicles of one whole ovary (Kipp et al., 2007).

Statistics

For comparison of data between two groups a two-tailed student's t-test was performed. Data is shown as Mean ± S.E. and $P \leq 0.05$ is considered significant.

Results

Genotyping of Conditional Knockout Genes

Genotyping analysis was performed on DNA samples obtained from mouse tissues to confirm the presence of specific gene markers associated with conditional knockout constructs. The genotyping was carried out using PCR amplification with primers targeting distinct DNA fragments for each gene of interest: RARA-floxed transcript (FR), RARA-floxed excised gene (FRe-), and Amhr2-cre (NC). The expected DNA fragment sizes were 436bp, 357bp, and 250bp, respectively.

Figure 1 displays representative gel electrophoresis results of PCR amplifications for each gene marker. The gel images show distinct bands corresponding to the expected DNA fragment sizes, confirming the presence of the knockout constructs.
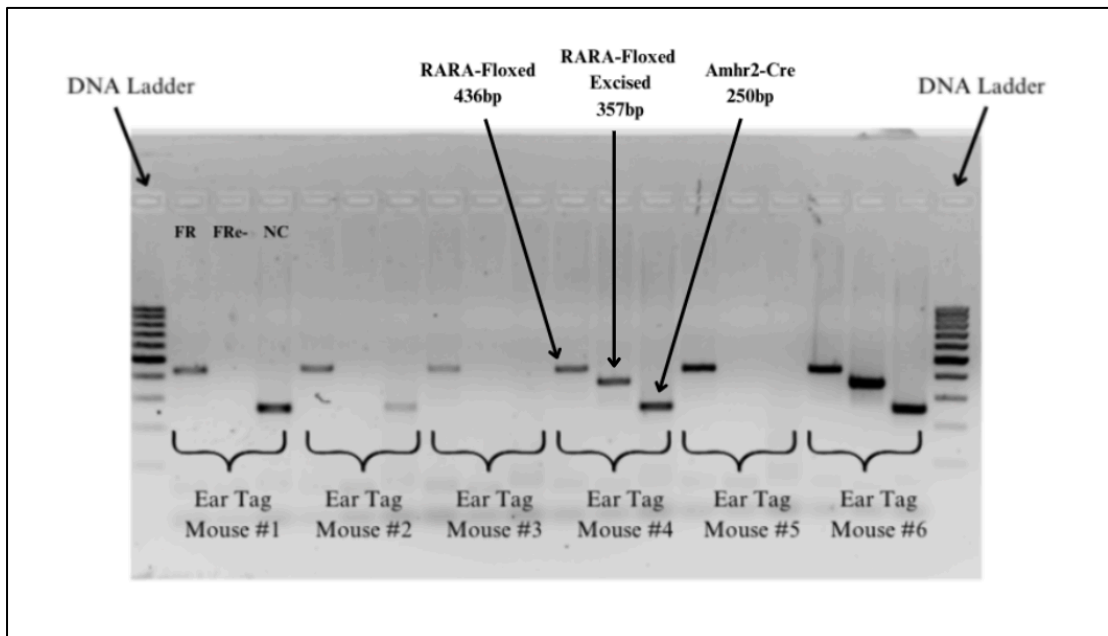

Figure 4. This gel image depicts the successful identification of conditional knockout alleles using gene marker-specific primers. Lanes 10, 11, and 12 display distinct DNA fragments at 436bp (FR), 357bp (excised RARA-floxed gene), and 250bp (Amhr2-cre), respectively. The clear bands confirm the precise genotyping of conditional knockout alleles.

Effect of RARA Conditional Knockout on Mouse Body Weight

Body weight measurements were taken for control and RARA conditional knockout mice at three different ages: week 7, week 15, and 12-15 months old. At week 7, the mean body weight of control mice was 19.901g ($\pm$) 0.272 (n = 5), while that of RARA conditional knockout mice was 17.182g ($\pm$) 0.597 (n = 3). The difference in body weight between the two groups was statistically significant ($p < 0.01$) (Figure 5). At week 15, the mean body weight of control mice was 21.900g ($\pm$) 0.563 (n = 5), whereas the mean body weight of RARA conditional knockout mice was 22.911g ($\pm$) 1.228 (n = 9). The difference in body weight between the two groups was not statistically significant (Figure 6).

At 12-15 months old, the mean body weight of control mice was 24.274g ($\pm$) 0.315 (n = 10), while that of RARA conditional knockout mice was 25.823g ($\pm$) 0.996 (n = 5). The difference in body weight between the two groups was not statistically significant (Figure 7).
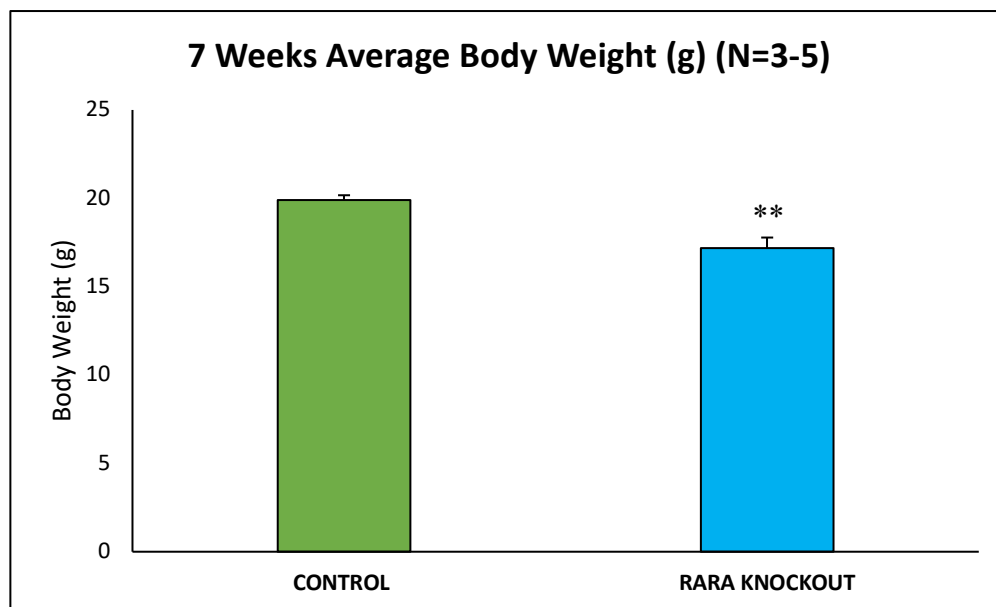

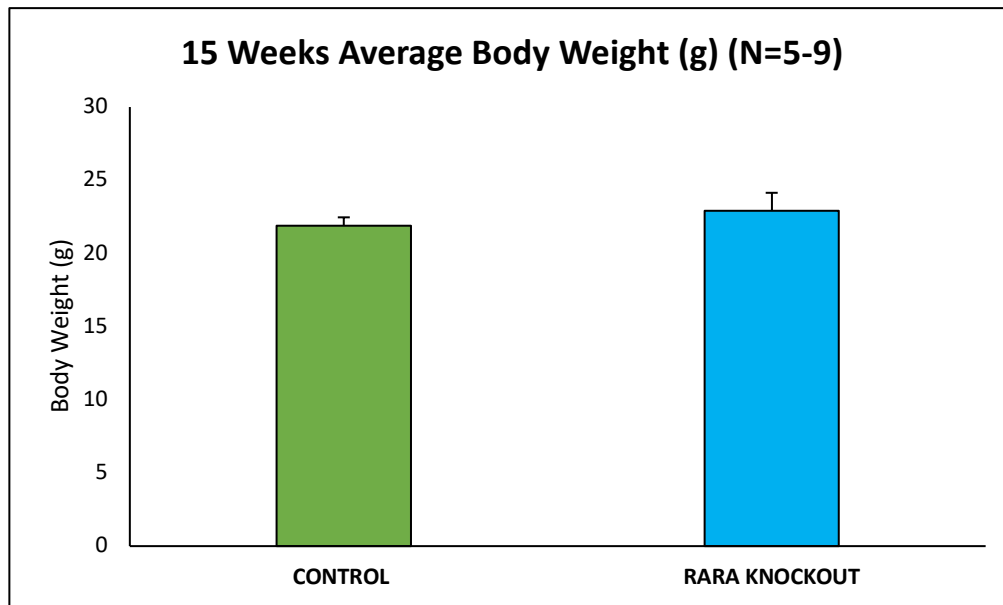**Figure 5.** Body weights of control and RARA Conditional Knockout mouse at week 7 (**p<0.01; n=3-5)

Figure 6. Average body weights of control and RARA Conditional Knockout mouse at week 15 (n=5-9).
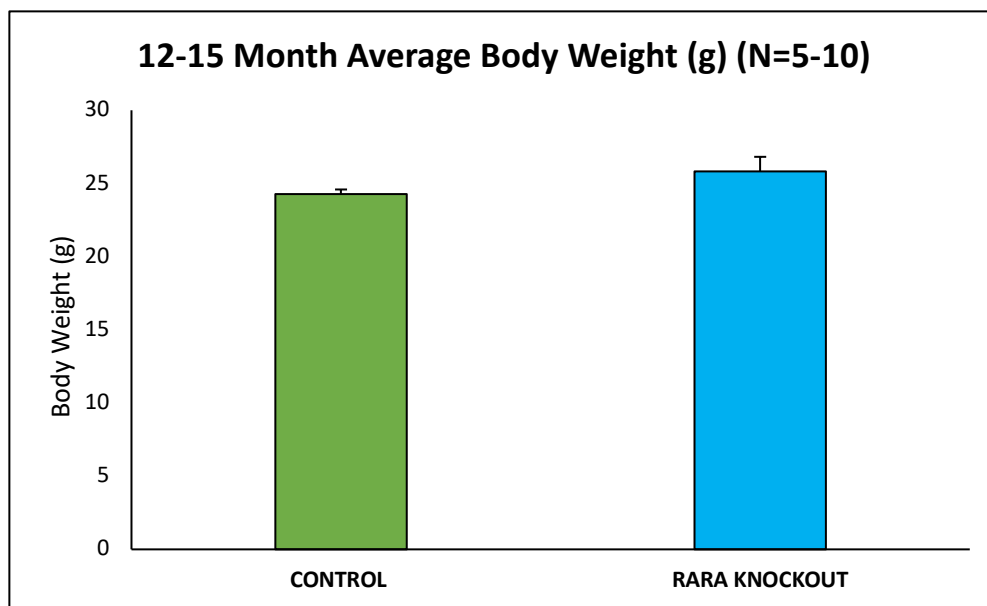

Figure 7. Average body weights of control and RARA Conditional Knockout mouse at 12-15 month (n=5-10).

Effect of RARA Conditional Knockout on Mouse Uterus Weight in Relation to Body Weight

We wanted to see the effect of conditional knockout mouse on uterus weight relative to body weight in grams. The mean uterus weight over body weight for week 7 RARA conditional knockout group was 0.002g (±) 0.00008 (n=3) while the mean uterus weight over body weight for the control group was 0.006g (±) 0.006 (n=6). The difference in uterus weight over body weight between the two groups was statistically significant (t-value=4.46) ($p<0.01$), indicating that the experimental treatment had a significant effect on uterus weight (Figure 8). Week 15 mean uterus weight over body weight for RARA conditional knockout group was 0.004g (±) 0.001 (n=5), while the mean uterus weight over body weight for the control group was 0. 0.006g (±) 0.003 (n=9) (Figure 9). 12-15 month mean uterus weight over body weight for RARA conditional knockout group was 0.022g (±) 0.002 (n=5), and the mean uterus weight for the control group was 0.024g (±) 0.007 (n=10) (Figure 10). Both mice aged week 15 and 12-15-months groups did not show significant differences.
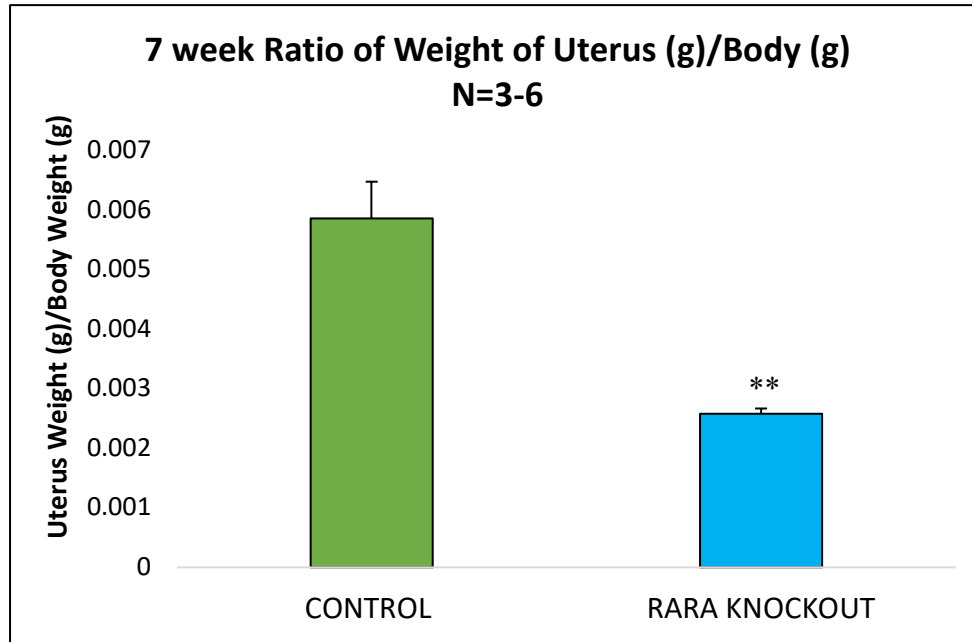
Figure 8. Average uterus weight relative to body weight of control and RARA Conditional Knockout mouse at week 7 with (**p<0.01; n=3-6).

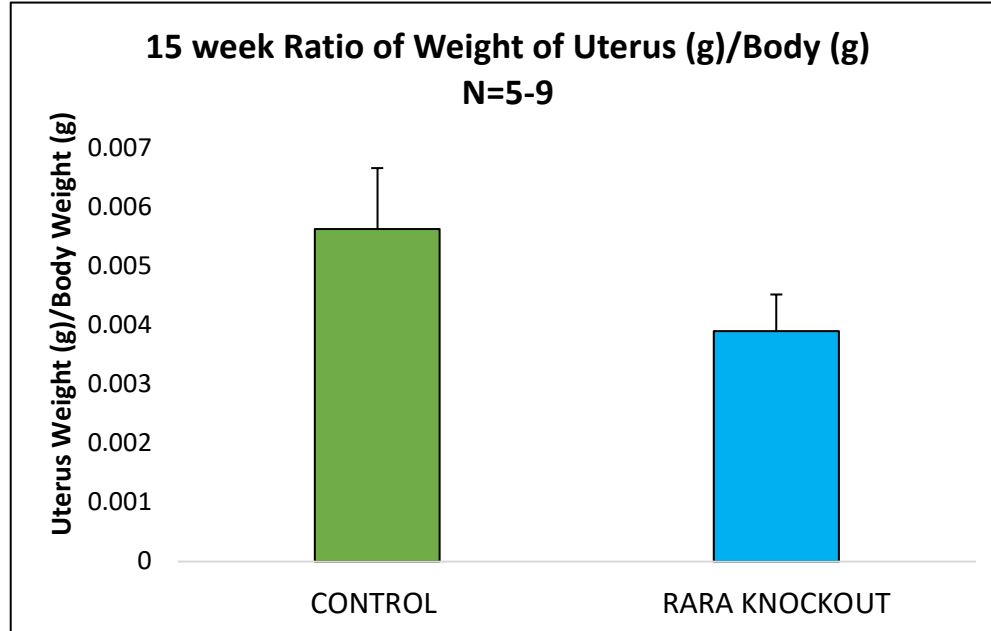

Figure 9 The bar graph displays the average uterine weight over body weight (g) for week 15 groups: RARA Conditional Knockout experimental group (n=5) and a control group (n=9).
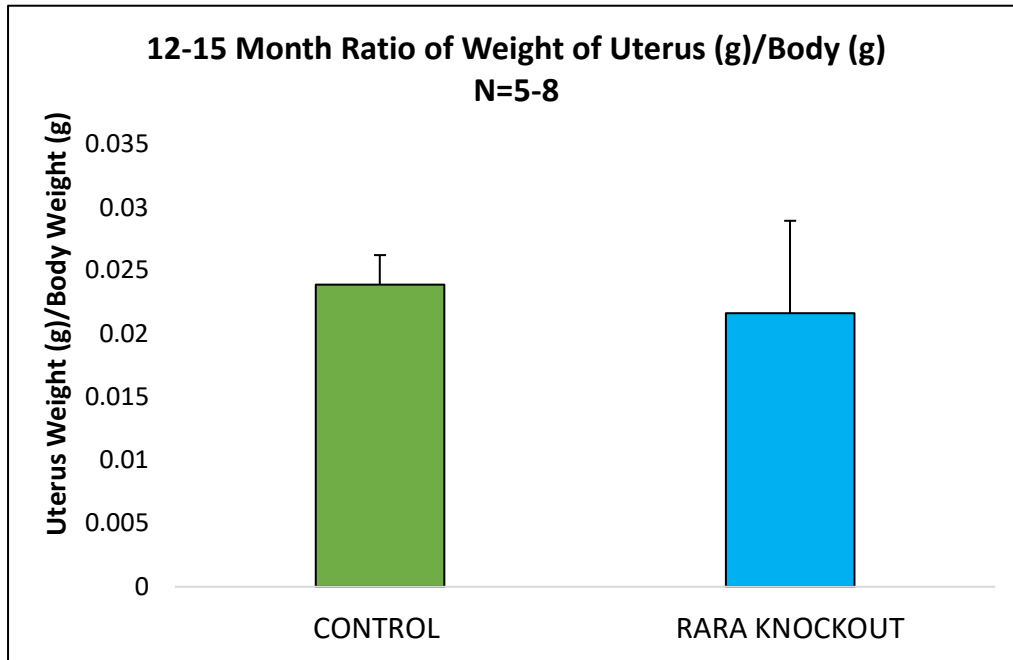
Figure 10. The bar graph presents the average uterine weight in relation of body weight in grams (g) for 12–15-month mice groups: RARA Conditional Knockout experimental group (n=5) and a control group (n=10).

Effect of RARA Conditional Knockout on Ovarian Cyst Development

During the dissections of control and RARA conditional knockout mice at week 7, week 15, and 12-15 months old, we observed the presence of cysts in the ovaries of some mice. A mouse ovarian cyst is a fluid-filled sac or growth that develops within the ovary of a mouse. The cyst may range in size from a few millimeters to several centimeters and can be either unilocular (having one chamber) or multilocular (having multiple chambers) (Figure11).

The percentage of ovaries that have cysts was first calculated for each animal, e.g. 0%, 50% or 100%. The resulting data were then averaged for all animals to obtain Mean ± S.E.

Week 7 mice had an average percentage of mice with cysts for RARA conditional knockout mice of 16.67% (±0.129), while average control mice had ovarian cysts was 25% (± 0.272) (Figure 12). At week 15, average RARA conditional knockout mice ovaries that had cysts was 30% (± 0.153), while control mice had an average of 0% of ovarian cysts (Figure 13). At 12-15 months, average RARA conditional knockout mice ovaries had cysts was 10% (± 0.100), while the average of control mice that had ovarian cysts was 19.23% (± 0.070) (Figure 14).

A student's t-test was performed to determine if the difference in cyst incidence between the control and RARA conditional knockout groups was significant. The results showed a statistically significant difference at week 15 ($p < 0.05$). No significant differences were observed at week 7 or 12-15 months old.
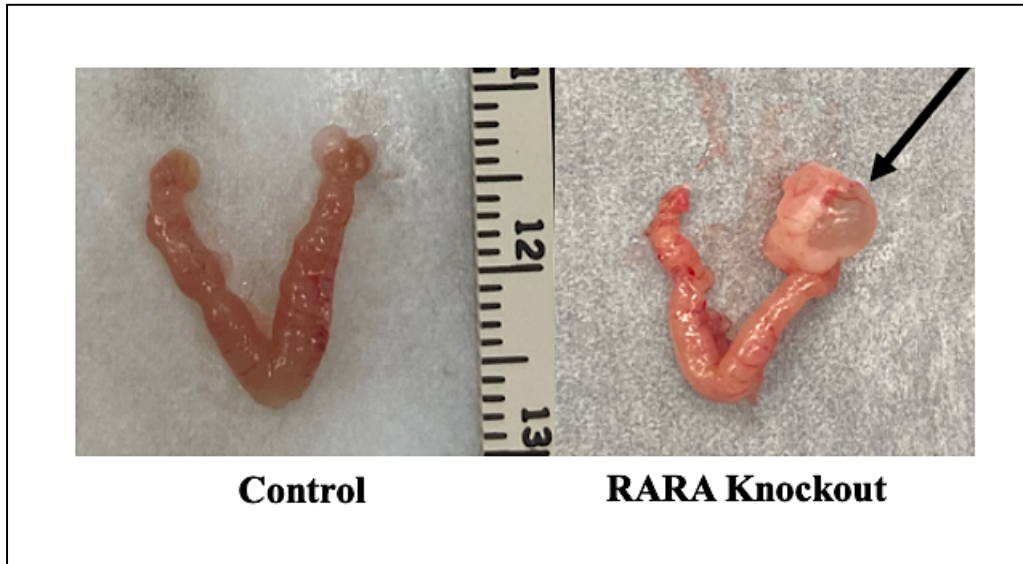
Figure 11. Photograph of dissected mouse ovaries comparing an ovary with a control ovary without cysts (left) and an ovary with a cyst (right). The mouse ovary with a cyst (right) shows a round, fluid-filled sac (yellow arrow) that is approximately 5 mm in diameter. The control ovary (right) appears normal and does not show any visible cysts or abnormalities.

Figure 12. The graph shows the percentage of cysts in ovaries of control and RARA conditional knockout mice at week 7. The data were obtained by examining the ovaries of mice in each group (n=6-10). Statistical analysis did not reveal a significant difference between the control and RARA conditional knockout groups at week 7.
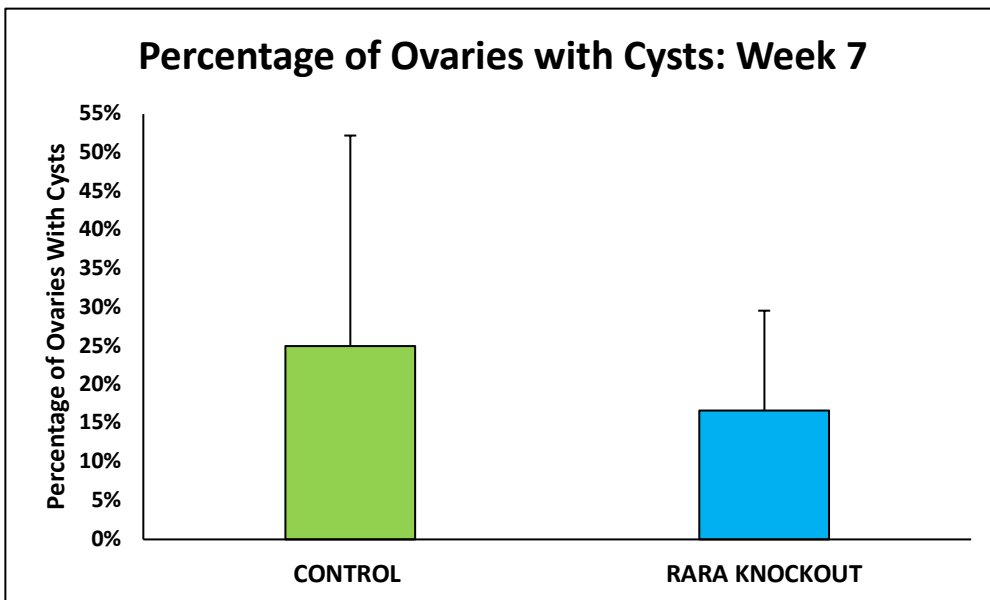
Percentage of Ovaries with Cysts: Week 15
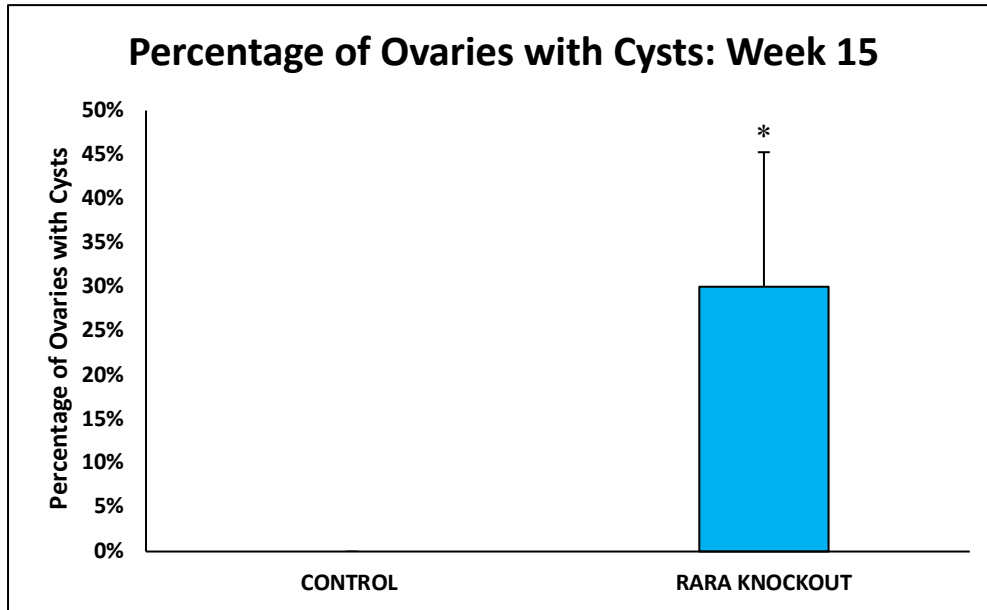
Figure 13. The graph shows the percentage of cysts in ovaries of control and RARA conditional knockout mice at week 15. The data were obtained by examining the ovaries of mice in each group (n=5-9). Statistical analysis revealed a significant difference between the control and RARA conditional knockout groups at week 15.

Percentage of Ovaries with Cysts: 12-15 Months

Figure 14. The graph shows the percentage of cysts in ovaries of control and RARA conditional knockout mice at 12-15 Months. The data were obtained by examining the ovaries of mice in each group (n=5-11). Statistical analysis did not reveal a significant difference between the control and RARA conditional knockout groups at 12-15
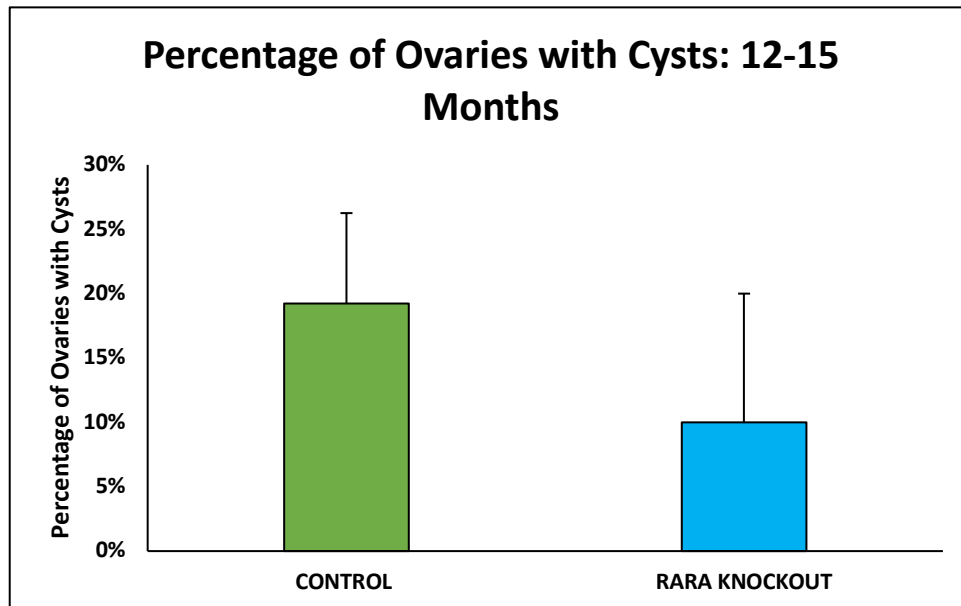
Fertility Analysis

In this 6-month study, we compared the fertility of control and RARA conditional knockout mice by analyzing the number of litters produced, the number of pups per litter, and the overall fertility rate. Over the course of the study, the control group produced an average of 6.4285 ($\pm$) 0.060 litters, while the RARA conditional knockout group produced an average of 2.3846 ($\pm$) 0.104 litters. This difference was statistically significant ($p<0.05$) (Figure 15).

We also compared the number of pups per litter in each group. The control group had an average of 5.9555 ($\pm$) 0.622 pups per litter, while the RARA conditional knockout group had an average of 5.9231 ($\pm$) 1.385 pups per litter. This difference was not statistically significant (Figure 16).

Finally, the experiment aimed to investigate the pup birth rate over a six-month period, with a focus on monthly variations. A total of 282 pups were born during the study period, with the number of pups born each month ranging from 0 to 46.

The total number of litters recorded during the six-month study period was analyzed for both control and RARA conditional knockout mice. The results showed that the control mice produced a total of 35 litters, while the RARA conditional knockout mice produced a total of 10 litters.

To further understand the effects of RARA conditional knockout on fertility, over a span of 6 months, we assessed the fertility outcomes of conditional knockout mice and control mice to investigate potential changes in breeding productivity. The following table represents the total number of litters born per month in each group (Table 3).

Statistical analysis revealed significant differences in the average number of litters born per month between control and RARA conditional knockout mice from the second to the sixth month of the study (Figure 17).

Figure 15. The bar graph shows the average number of litters produced by the control and RARA conditional knockout groups over a 6-month period. Error bars represent the standard error of the mean (SEM). The difference in the average number of litters produced between the two groups was statistically significant ($p > 0.05$), as indicated by an asterisk above the RARA knockout bar.

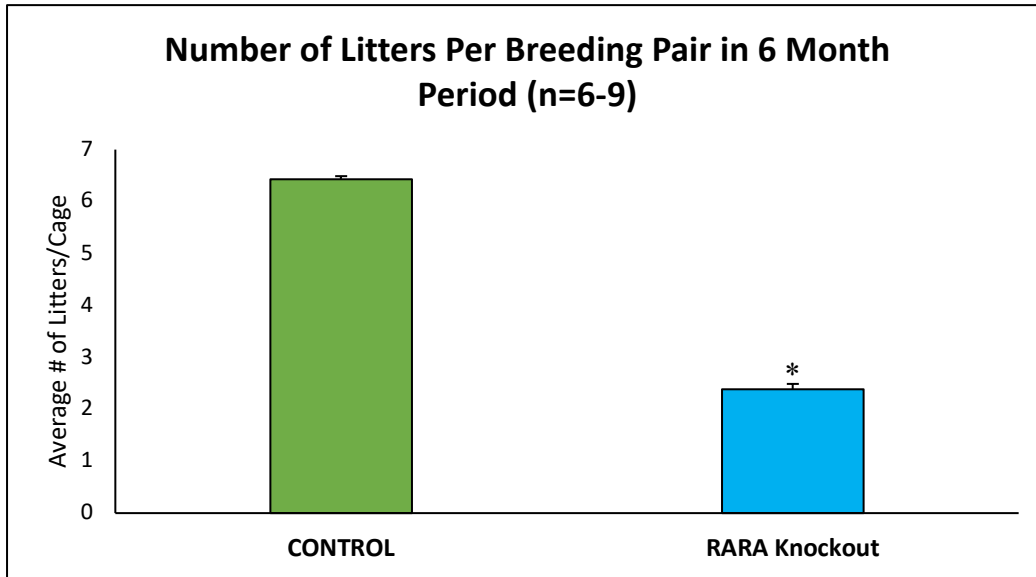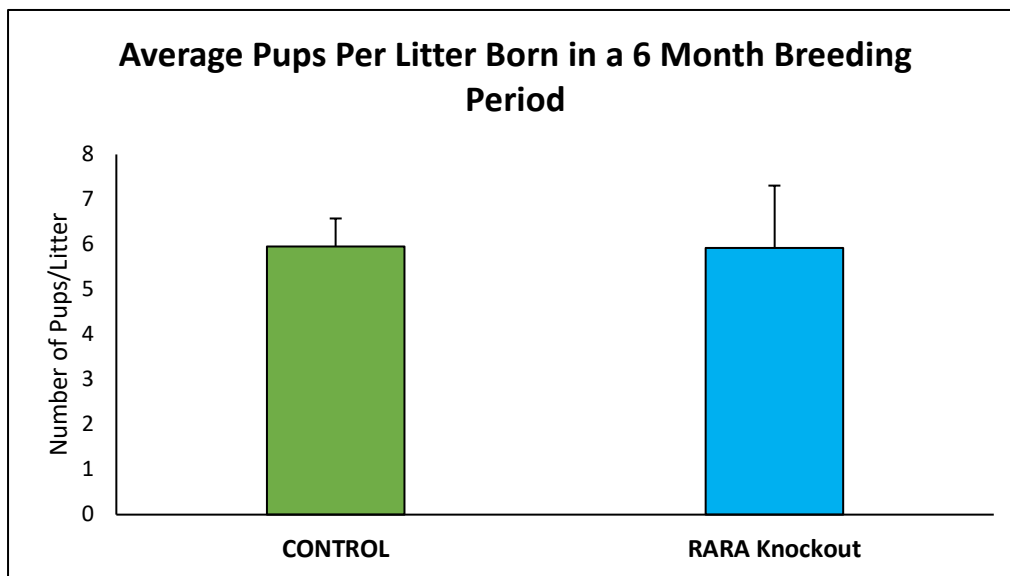**Figure 16.** The bar graph shows the average number of pups per litter for the control and RARA conditional knockout groups over a 6-month breeding period. Error bars represent the standard error of the mean (SEM).

Month	Control	RARA KO
1	6	5
2	7	2
3	8	3
4	5	0
5	6	0
6	3	0

Table 3. Table represents the monthly distribution of total litters born for control and RARA conditional knockout mice over a six-month period (n=6-9 breeding pairs).

Figure 17. Bar graph illustrates a visual comparison of the average number of litters born per month for control and RARA conditional knockout mice. The x-axis represents the months of the study period, while the y-axis represents the count of litters (n=6-9 breeding pairs; ***p<0.001)

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Effect of RARA Conditional Knockout on Ovarian Morphology

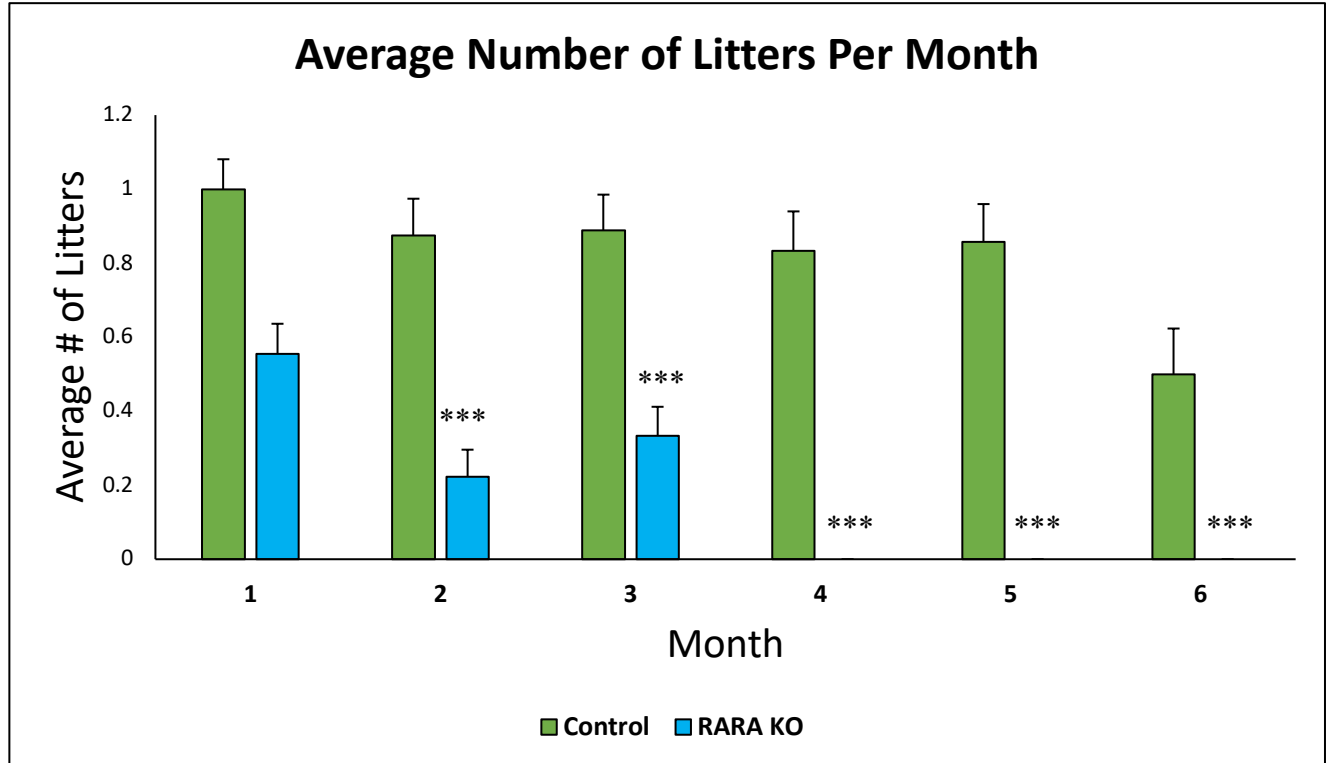H&E staining for sections of both control and RARA CKO groups show pathologies at all time points analyzed. Figure 18 shows representational pictures of week 7 control ovary (18A), and RARA CKO ovaries (18B). RARA CKO week ovaries show increase in atretic follicles (average count of 76) (18D), which was common among other samples in the group. Additionally, week 7 RARA CKO mice showed a follicle with multi oocyte follicle (18E). Along with these pathologies we observed a bilateral decrease in primordial follicles in 12 months (Figure 20) and prominent cysts in week 15 (figure 19).

Hemosiderin is an iron-containing pigment that is derived from the breakdown of hemoglobin, which is the protein in red blood cells that carries oxygen. When there is an excess of iron in the body or when there is bleeding in a tissue, the body may deposit hemosiderin as a way of storing the excess iron.

We observed the presence of hemosiderin (Figure 18C), which were identified by their characteristic morphology. In the context of the ovaries, the presence of hemosiderin may be indicative of a variety of conditions, including endometriosis, ovarian cysts, or ovarian tumors, all of which can cause bleeding or inflammation in the ovarian tissue.

Overall, the presence of hemosiderin in the week 7 mouse ovaries suggests that there may have been some degree of bleeding or inflammation in the tissue at that time point.
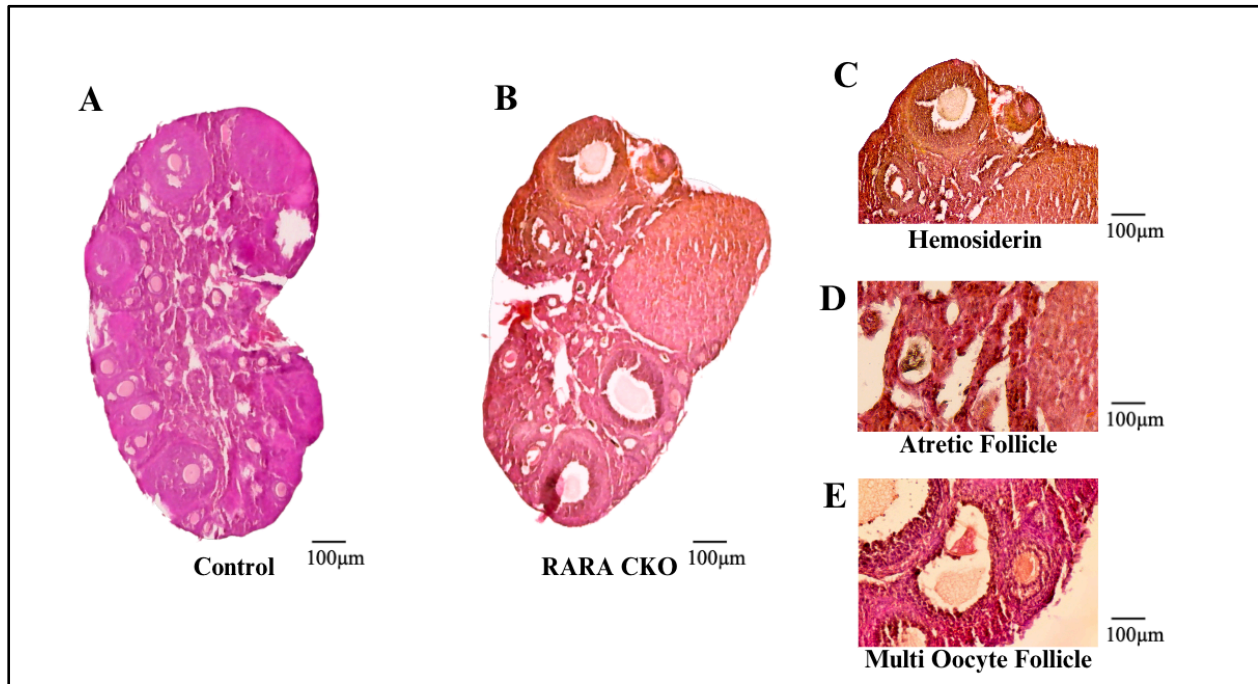
Figure 18. Histology of week 7 ovaries. A) week 7 control, B) week 7 RARA CKO, C) magnified image representing hemosiderin, D) magnified image showing atretic follicle, and E) magnified image showing multi oocyte follicle, present in week 7 mice. Size bar; 100µm.
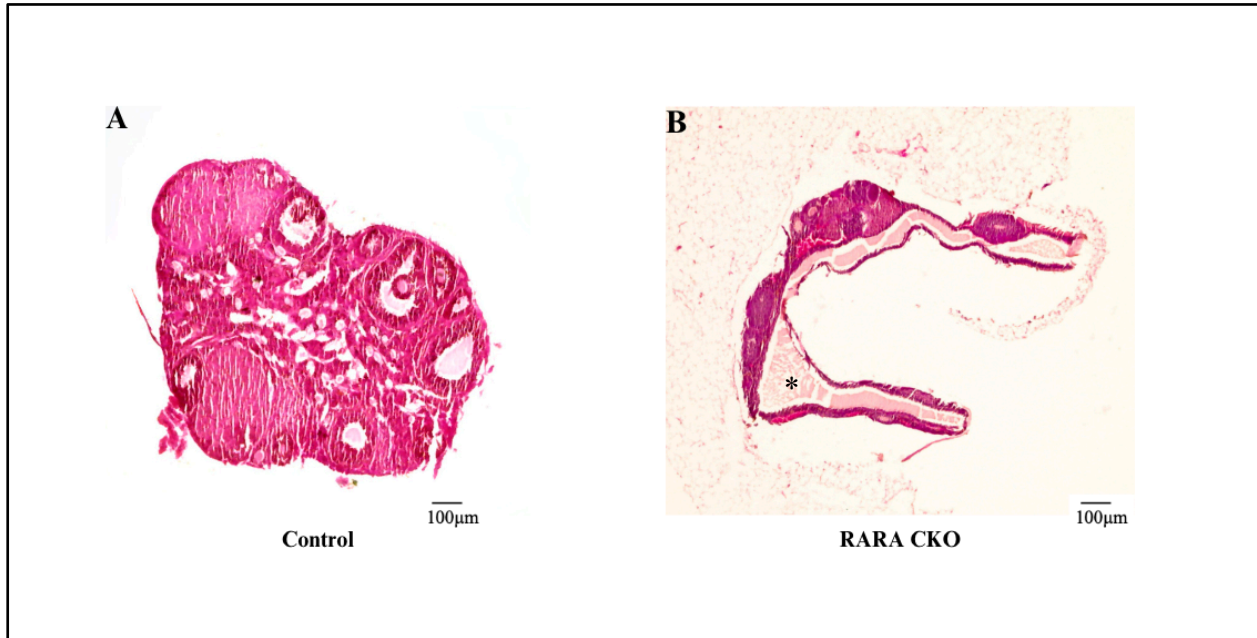
Figure 19. H&E staining of representational 15-week ovaries A) control ovary B) RARA KO mouse ovary with * representing cyst that prevented the ovary to development properly. The "*" represents the presence of the cyst. Size bar; 100µm.

Figure 20. H&E staining of representational 12-month ovaries A) control ovary B) RARA KO mouse ovary showing absence of primordial follicles. White small circles in control ovary represent primordial follicles (*). RARA conditional knockout show no primordial follicles. Size bar; 100μm.

Follicle Counting

In this study, we analyzed the follicle count of ovaries from female mice at different ages: week 7, week 15, and 12 months. The ovaries were categorized into six different follicle types: p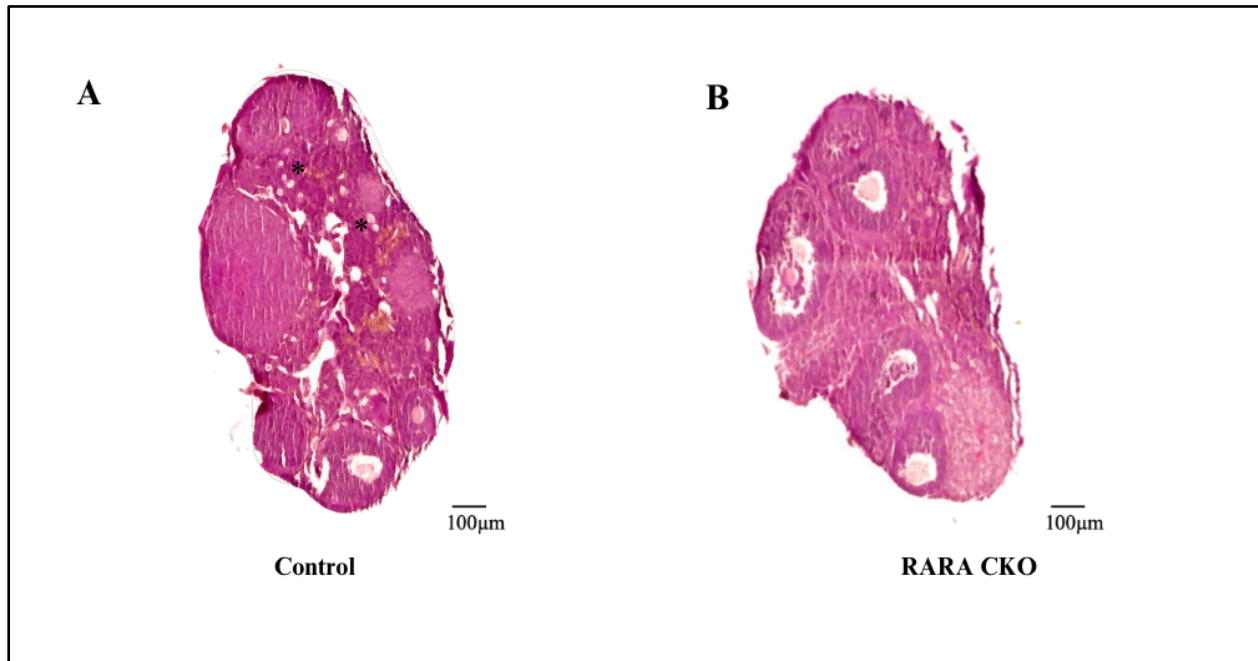rimordial, primary, secondary, preantral, antral, and atretic. Additionally, we also evaluated the presence of multi oocyte follicles (MOFs) in the ovarian tissues.

No significant differences were seen between control and RARA KO groups (n=3) in week 7 mice (Figure 21). We observed an increased number of primordial follicles in RARA conditional knockout mice compared to control at week 15 ($p<0.05$), and in decreased number of RARA conditional knockout mice compared to control at antral follicles at week 15 ($p<0.05$). RARA conditional knockout 12-month mice showed a significant decrease in primordial follicles compared to control ($p<0.05$). Specifically, the RARA KO group had a higher number of primordial follicles at week 15 (average count of 361) compared to the control group (average count of 205) (Figure 22). At 12 months the RARA KO group had a lower number of primordial follicles (average count of 48) compared to the control group (average count of 197.5) (figure 23). The RARA KO group also had a lower number of antral follicles at week 15 (average count of 13) compared to the control group (average count of 35). No other significant differences were observed between the two groups at any other age or for any other follicle type.

Figure 21. Primordial, primary, secondary, pre-antral, antral, atretic, and MOF follicle numbers in control and RARA KO with no significant differences in week 7 mice (n=3).

Figure 22. Primordial, primary, secondary, pre-antral, antral, atretic, and MOF follicle numbers in control and RARA KO with significant differences are indicated by asterisks: *p<0.05 and **p<0.01; n=3.
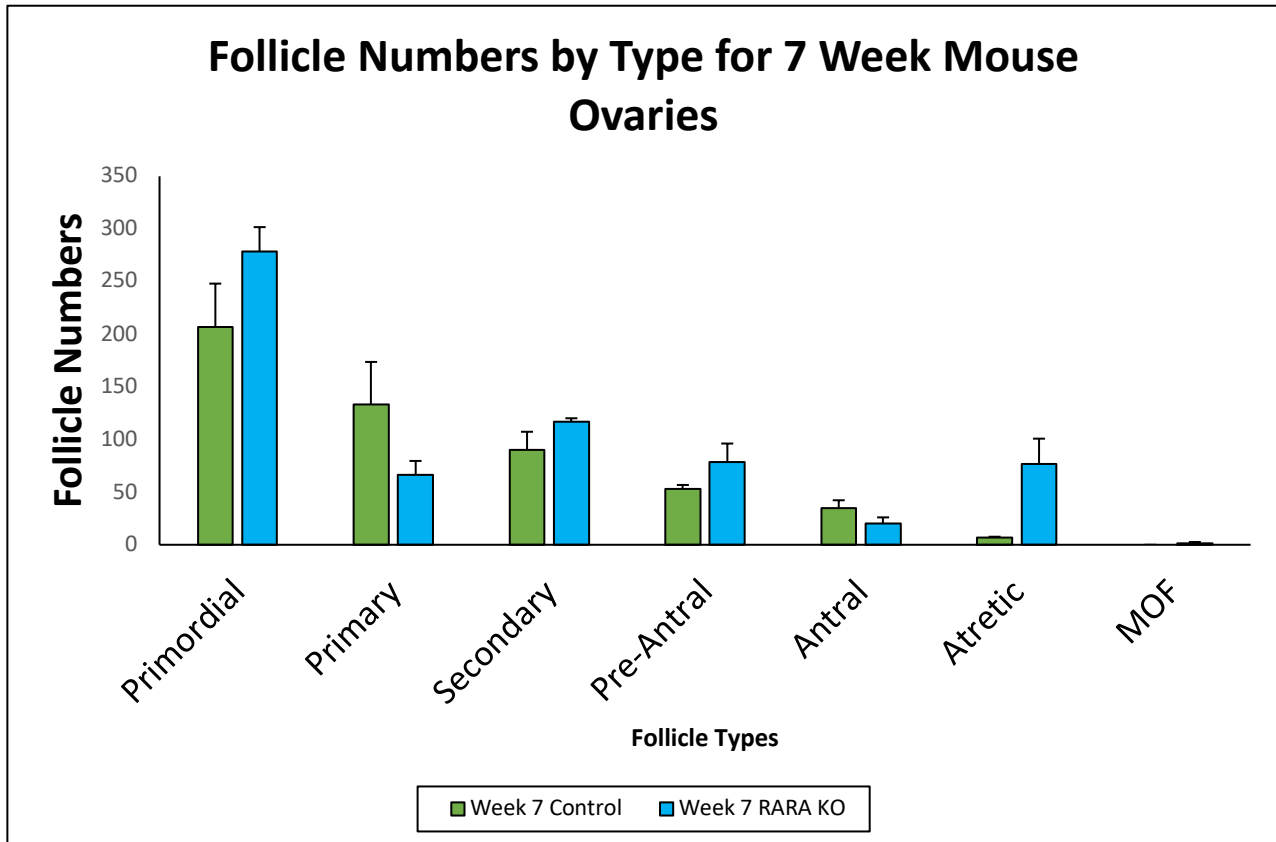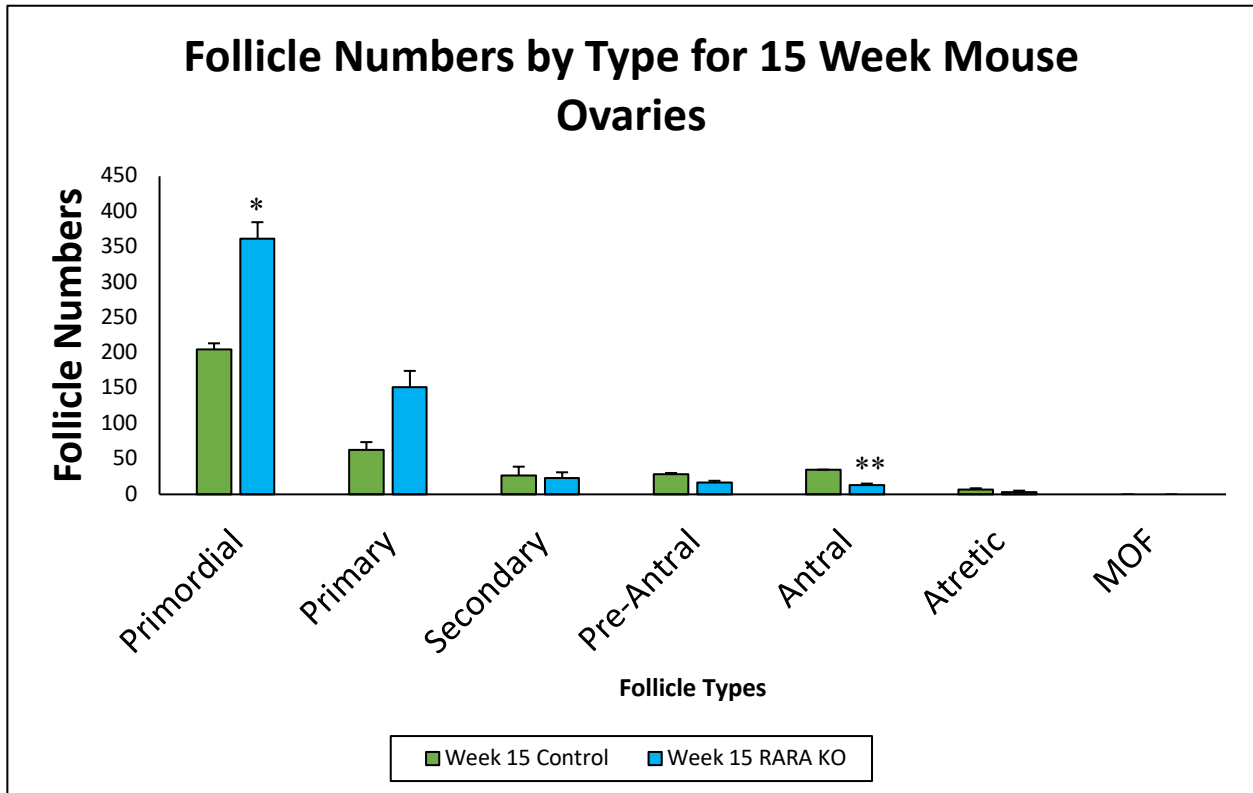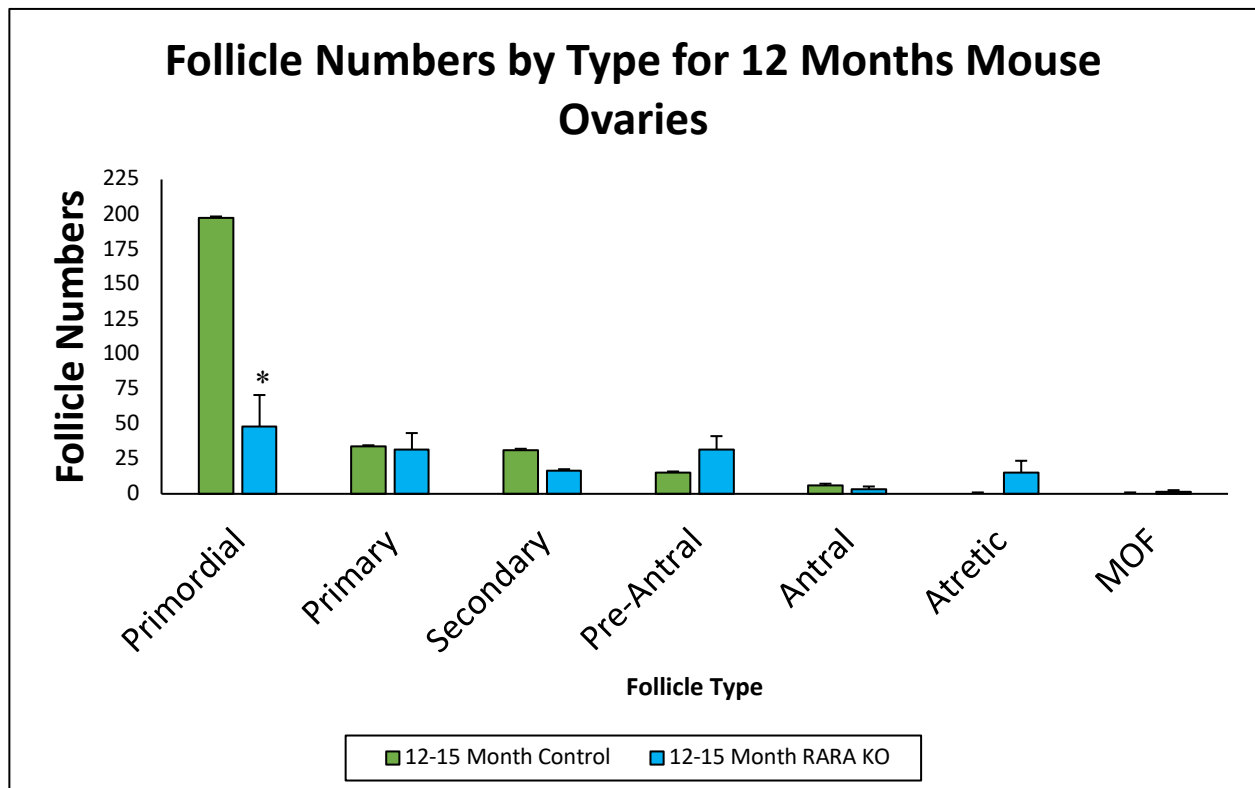
Figure 23. Primordial, primary, secondary, pre-antral, antral, atretic, and MOF follicle numbers in control and RARA KO with significant differences are indicated by asterisks: *p<0.05; n=3.

Discussion

Role of RARA on Body Weight and Uterus Weight

The results of this study demonstrate that RARA has significant effect on fertility and ovarian development; demonstrated by 6-month fertility analysis, follicle counting, and histological morphologies.

Control mice and RARA KO mice showed dramatic difference in weight at week 7 but that effect did not continue at week 15 and 12 months on conditional knockout mice. The reason why the effects of RARA conditional knockout on mouse weight may be different at different time points (week 7, week 15, and 12 months) is likely due to differences in the physiological and metabolic state of the mice at these different ages.

At week 7, the mice are still growing and developing, and their metabolism is likely more active than at later time points. Therefore, any disruption in ovarian function caused by RARA conditional knockout may have a more pronounced effect on body weight at this age. We believe the effect could have been cause by an indirect increase of testosterone levels. Conditional knockout of RARA in granulosa cells that are responsible for synthesis of estrogen from androgens could have increased testosterones level in the week 7 mouse. Increased testosterone has been linked to weight loss and muscle gain, at week 7 the mice having new hormones beginning to synthesize during puberty could have had a greater effect on body loss.

At week 15, the mice have reached sexual maturity and are no longer growing as rapidly, but their metabolic rate may still be relatively high. By this time, compensatory mechanisms may have also kicked in to buffer the effects of RARA conditional knockout on body weight, such as changes in food intake or energy expenditure.

By 12 months, the mice are considered mature adults and may have settled into a relatively stable metabolic state. Any effects of RARA conditional knockout on body weight at this age may be more subtle or no longer present, as the mice may have adapted to the absence of this gene and/or compensated for its loss through other mechanisms.

It's possible that the absence of RARA in the granulosa cells could have indirectly affected estradiol levels on the uterus at week 7, which could explain why the weight of the uterus was significantly less at this time point compared to week 15- and 12-months groups. For females, uterine weight can be used as an indicator of estrogens in non-pregnant mice. Studies show the higher the weight, the higher the estrogen levels (McCarthy, 2020). In this study the low weight could be linked to low estrogen levels because of the decrease in uterus weight. Overall, the exact reasons for these differences would need to be explored further through with additional experiments and analyses, specifically testing estrogen levels using hormone analyses to see if CKO RARA mice hindered the synthesis of estradiol in the mouse.

Effect of RARA Conditional Knockout on Cyst Development

We saw no statistical significance in ovarian cyst development in week 7- or 12–15-month-old mice. Week 15 RARA KO had a significant higher rate of cysts developed rather than in control mouse. The reasoning behind seeing it in week 15 mouse could be at week 15, the mice have reached sexual maturity, and their ovaries are fully developed and functional. At this stage, the hormonal and physiological changes associated with reproductive function may make the ovaries more susceptible to disruption by RARA conditional knockout, potentially leading to changes in cyst development.

Hormonal assays of estrogen and progesterone could provide important information about the potential mechanism of cyst development in mice with RARA conditional knockout in the ovaries. Estrogen and progesterone are key hormones involved in regulating the menstrual cycle and ovarian function, and changes in their levels can indicate disruptions in these processes. Estrogen and Luteinizing hormone are responsible for ovulation in the mouse. CKO RARA hindering the production of estrogen in the granulosa layer can point to decrease of ovulation and if the egg does not ovulate it arrests in the ovary causing a cyst to form.

Serum samples were collected at each timepoint and by analyzing the estrogen and progesterone levels in the blood samples, this could potentially determine whether changes in these hormones are associated with the development of ovarian cysts in mice with RARA conditional knockout. We predict that estrogen levels would be lower than average due to CKO RARA mice hindering the granulosa cell layer and effecting the production of estrogen. Due to estrogen synthesis being affected testosterone levels would increase in the theca cells affecting the total body weight of the mouse.

RARA's Role in Fertility

RARA conditional knockout had a significant effect on fertility. Not only were there less litters born in the 6-month period, but 4 out of the 9 cages were completely infertile and never gave birth to offspring. RARA plays a significant role in mouse fertility. One potential mechanism underlying this effect could be related to disruptions in the development and function of the ovarian follicles. RARA has been shown to play a role in the development and maturation of ovarian follicles, as well as in the regulation of the estrous cycle (Damdimopoulou et al., 2019).

The results of our study have provided evidence that a potential sign of premature ovarian failure in the RARA conditional knockout mice. Premature ovarian failure (POF), also known as primary ovarian insufficiency, refers to the loss of normal ovarian function and depletion of the follicle pool before the age of 40. It is characterized by menstrual irregularities, hormonal imbalances, and decreased fertility (Jankowska, 2017).

In our fertility analysis, we observed a significantly lower number of litters born per month in the RARA conditional knockout group compared to the control group. The reduced reproductive performance observed in the RARA conditional knockout mice suggests a disruption in ovarian function, possibly resembling a phenotype associated with premature ovarian failure.

The consistent and significant differences in litter counts between the control and RARA conditional knockout groups from the 2nd to the 6th month of the study further support the notion of compromised ovarian function in the RARA conditional knockout mice. These findings show that the absence of RARA may lead to an accelerated decline in ovarian reserve and premature depletion of the follicle pool, mimicking the characteristics of premature ovarian failure.

In future studies, confirming premature ovarian failure (POF) in mice can be achieved through various methods, including the examination of vaginal smears, and monitoring the estrous cycle for the six months. These techniques can provide valuable insights into the reproductive status of the mouse and help determine if POF has occurred (Na and Kim, 2020). Another approach to confirm POF in mice is by assessing hormonal changes associated with ovarian function. Hormone analysis, such as measuring serum levels of estradiol (an estrogen

hormone) and progesterone, can provide valuable information about the functionality of the ovaries.

RARA's Contribution to Follicle Count and Development

Total follicle count numbers being increased in primordial follicles in week 15 but decreasing by the time the follicle was ready to mature and ovulate in the antral follicle stage. A possible explanation for the observed increase in primordial follicles in RARA conditional knockout mice at week 15 is that the absence of RARA directly affecting the maturation of folliculogenesis. However, without RARA to regulate follicular development, these follicles may have decreased ability to progress to the antral stage, leading to a decrease in antral follicles at later time points. This is why we saw a decreased level of primordial cells by the time age progressed to 12-month mouse.

The follicles in week 7 mice showed several pathological changes. We identified several atretic follicles as well as multi oocyte follicles. When imaging the ovaries, the color of the of the RARA mice ovaries were browner in color. Upon further research we the brown in color could be the cause of several factors but we are concluding this could be hemosiderin.

Hemosiderin is a brownish-yellow pigment that accumulates in tissues because of iron overload or local bleeding. In the case of the ovaries, hemosiderin deposition may occur due to a variety of factors, including inflammation, hemorrhage, or hormonal imbalances (Salomao, 2021).

When tissues containing hemosiderin are stained with H&E, the pigment appears as granular, golden-brown deposits within the tissue. The hemosiderin deposits may be surrounded by macrophages, which phagocytose the pigment and help to remove it from the tissue.

While hemosiderin deposition in the ovaries is not necessarily indicative of a specific pathology, it may be associated with conditions such as endometriosis, pelvic inflammatory disease, or ovarian tumors (Salomao, 2021).

Control ovaries had a pinkish-red coloration due to their high vascularity and the presence of numerous follicles containing developing oocytes. However, week 7 RARA conditional knockout mice showed phenotypically the presence of hemosiderin.

Limitations of the Study

The present study is not without its limitations, which must be considered when interpreting the results. The most prominent limitation is the small group size of n=3 for the Week 7 CKO RARA mice. This limitation stemmed from unexpected breeding issues that constrained the availability of suitable mice for the experimental group. Consequently, the limited number of animals in this group could potentially compromise the statistical power of the analysis and may limit the generalizability of the findings to a larger population. To mitigate this limitation, future studies should strive to increase the group size to enhance the reliability and robustness of the results.

Another limitation pertains to the absence of confirmed RARA expression using immunohistochemistry in the granulosa cells of the mice. Without this crucial verification, uncertainties arise regarding the presence and distribution of RARA in the targeted cell population. Thus, incorporating immunohistochemical analyses in future research would be necessary to accurately ascertain the expression of RARA in the granulosa cells and validate its potential role in the observed phenotypes.

Furthermore, the lack of macrophage markers in the current study limits our ability to confirm the presence of hemosiderin in the granulosa cells, which could provide important

insights into macrophage activity. Incorporating macrophage markers in subsequent investigations would shed light on the involvement of macrophages in the context of RARA depletion.

In conclusion, while the present study has contributed valuable insights into the role of RARA in granulosa cells, it is crucial to acknowledge these limitations to avoid potential misinterpretations. Addressing these limitations in future research endeavors will strengthen the validity and impact of the findings, enabling a more comprehensive understanding of the underlying mechanisms and implications of RARA depletion in granulosa cells.

Conclusion

We hypothesized that RARA would play a critical role in mice fertility and ovarian development. This study has characterized a new conditional knockout strain, and showed how CKO RARA has a significant effect on birth rate itself showing phenotypically signs of POF. Through our investigation into the conditional knockout of RARA in granulosa cells of the ovary, we have unraveled a previously unrecognized role for this alpha receptor in guiding the intricate journey of follicular development. The maturation of ovarian follicles is a tightly orchestrated process critical for successful ovulation and subsequent fertility. Our findings underscore the essential contribution of RARA to this process, suggesting that its presence within granulosa cells orchestrates molecular events pivotal for follicle maturation and ultimately the fertility of the female reproductive system. We saw pathological effects in RARA group, with decreased number of antral follicles, cyst formation, increase in atretic follicles, and presence of hemosiderin. Using RA treatments could benefit patients who are suffering with POF and could help them with the regulation of ovarian development. Consequently, findings from

this study will contribute to infertility treatments and give an understanding on how to prevent

and treat ovarian diseases in humans.

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