

# Oocyte Survival and Development During Follicle Formation and Folliculogenesis in Mice Lacking Aromatase

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## Research

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# Abstract

## Background

Assembly of oocytes into primordial follicles is essential for establishing the ovarian reserve required for female fertility. In mice, this process begins during embryonic development. Primordial germ cells form cysts by incomplete mitosis until 13.5 days post coitum (dpc). These cysts break down just before birth. Some oocytes undergo apoptosis while surviving oocytes are enclosed by granulosa cells to form primordial follicles. Cyst breakdown and primordial follicle formation were previously shown to be inhibited by estradiol and estrogenic compounds *in vitro*, suggesting that estrogen is important for regulation of this process.

## Methods

To determine the role of fetal estrogen in cyst breakdown and follicle formation these processes were quantified in aromatase deficient (ArKO) mice between 17.5 dpc and postnatal day (PND) 9. Ovaries of ArKO mice were also examined at 2-week intervals to determine if folliculogenesis is affected by lack of estrogen and the age at which the typical ArKO ovarian phenotype first appears.

## Results

Oocyte number, follicle assembly and follicle development in ArKO mice did not differ from controls between 17.5 dpc and PND9 except for a difference in the proportion of follicles at the primordial and primary stage at PND7. At 2 weeks, ArKO heterozygous and homozygous ovaries still had oocytes in cyst while all oocytes were enclosed in follicles in wildtype ovaries. From 2 to 8 weeks oocyte numbers were similar in all genotypes though there was a trend toward fewer total oocytes in ArKO homozygous females as compared to controls at 8 weeks and a significant reduction at 10 weeks. Abnormal structures such as hemorrhagic follicles and hemosiderin deposits were also observed starting at 6 weeks.

## Conclusions

These results suggest that a lack of fetal estrogen does not affect the rate of cyst breakdown or primordial follicle formation perinatally, and maternal estrogen or other signals are the chief regulators. Furthermore, the typical ArKO ovarian phenotype occurs earlier than previously reported.

## Background

Proper formation of ovarian follicles is the basis for establishing a reserve of oocytes that will serve a female for her reproductive lifespan. Correspondingly, an insufficient ovarian reserve is associated with premature ovarian failure and infertility (Nelson, 2009). In order to elucidate better methods for treating premature infertility or delaying the onset of female reproductive senescence it is critical to understand the mechanisms that contribute to the establishment and maintenance of this oocyte reserve.

The process of oocyte development and follicle formation begins in the fetus with the migration of primordial germ cells to the developing ovary (Molyneaux et al., 2001). The germ cells then undergo several rounds of mitosis, and during this time they are referred to as oogonia. Groups of oogonia, connected by intracellular bridges, are formed as the result of incomplete cytokinesis after each round of mitosis (Pepling and Spradling, 1998). These groups of oogonia are referred to as germ cell cysts and become oocytes when they enter meiosis (Bullejos and Koopman, 2004; Menke et al., 2003). Cysts first fragment into smaller cysts which then reassociate so that clusters contain some oocytes connected by intercellular bridges and other oocytes associated by aggregation (Lei and Spradling, 2013). During this time, the oocytes progress through the first stages of meiotic prophase I and become arrested at an extended diplotene stage called dictyate (Cohen et al., 2006; Dutta et al., 2016). Beginning at 17.5 days post coitum (dpc) the cells separate and individual oocytes become surrounded with pregranulosa cells (Pepling et al., 2010). This process is accompanied by apoptosis of several oocytes from each cyst (Pepling and Spradling, 2001). There is evidence that the oocytes that are lost serve to support or “nurse” the surviving oocytes (Lei and Spradling, 2016). Those that remain become enclosed by pregranulosa cells to make up the ovarian reserve consisting of diplotene arrested oocytes housed within primordial follicles (Kerr et al., 2013). Despite the significance of this process for female fertility, the precise mechanisms that regulate cyst breakdown and follicle formation in mammals remain poorly understood.

Estrogens are implicated in the regulation of cyst breakdown and primordial follicle formation. Work from our lab has demonstrated that estradiol and other estrogenic compounds inhibit cyst breakdown and primordial follicle formation in the ovaries of fetal mice (Chen et al., 2007; Karavan and Pepling, 2012). Fetal ovaries are likely exposed to estrogen from the maternal circulation. It is thought that during pregnancy high levels of estrogen in the developing ovary maintains oocytes in cysts, and when levels decrease cysts break apart and granulosa cells envelope each oocyte. One question that remains is the relative importance of fetal and maternal sources of estrogen in maintaining oocytes in cysts prior to follicle formation.

There is some evidence supporting the idea that estrogen synthesized by fetal ovaries may play a role in maintaining oocytes in cysts. First, the fetal mouse ovary is capable of synthesizing its own estrogen. Estradiol was detected in the ovaries of fetal mice along with the enzymes aromatase and 3 $\beta$ HSD, which are required for the biosynthesis of estradiol (Dutta et al., 2014). Second, when fetal mouse ovaries were grown in culture in the absence of estrogen, cyst breakdown and follicle formation were accelerated as compared to in vivo (Chen et al., 2007). In addition, if the estrogen that inhibits cyst breakdown is derived from the maternal circulation, one would expect the initiation of cyst breakdown to coincide with a decrease in circulating maternal estrogen. In mice, the initiation of cyst breakdown begins at 17.5 dpc, however a rapid decrease in maternal estrogen is not observed until the day of birth (Dutta et al., 2014). Furthermore, the capacity for fetal ovaries to secrete estradiol has been observed in other species such as cattle (Yang and Fortune, 2008). However, when fetal ovaries were cultured during the time of cyst breakdown and follicle formation in the presence of letrozole, an aromatase inhibitor, to eliminate any production of estrogen by the fetal ovary, cyst breakdown and follicle formation was not affected (Dutta

et al., 2014). These findings indicate that a source of estrogen apart from the fetal ovary regulates follicle formation.

Accurate measurement of steroid hormones such as estradiol has been problematic (Vesper et al., 2014). Instead, the enzyme aromatase can be used as a proxy of estradiol location and amount in the ovary. Aromatase is encoded by the *Cyp19* gene (Nelson et al., 1993), and is responsible for converting androgens to estrogens through a pathway originating with cholesterol (Cui et al., 2013). Aromatase has been detected in fetal and adult rat ovaries (Stocco, 2008) and in fetal through neonatal stage ovaries of mice (Dutta et al., 2014).

In order to further investigate the potential role of fetal estrogen in cyst breakdown and follicle development an aromatase deficient (ArKO) mouse strain was acquired. This strain carries a neomycin disruptive insert, which replaces 163 base pairs encoding for amino acid residues 349–403 of exon 9 of *Cyp19*, the gene encoding aromatase (Fisher et al., 1998). Adult ovaries have been characterized by Britt and colleagues (Britt et al., 2000; Britt et al., 2001; Britt et al., 2004). Their findings indicate that at 10 weeks of age, ArKO ovaries have fewer total oocytes, follicles of all stages but no corpora lutea, and hemorrhagic cysts. However, the effects of aromatase knockout on the processes of cyst breakdown and primordial follicle formation, which begin during fetal development, have not been evaluated. Furthermore, the ovarian phenotype of young animals as folliculogenesis begins has not been reported. It was noted that these animals are infertile (Britt et al., 2000; Fisher et al., 1998), however there are no data available comparing litter sizes or survival rates with wild type females. The goal of this study was to elucidate the effects of estradiol deficiency on early follicle formation, follicle development and fertility by studying the ovaries of ArKO mice between the ages of 17.5 dpc and adulthood.

## Materials And Methods

### Animals

Aromatase knockout mice on a C57BL/6J background were obtained from Dr. Orhan Oz at the University of Texas Southwestern Medical Center. They were maintained in accordance with the policies of the Syracuse University Institutional Animal Care and Use Committee. Mice were housed at a controlled photoperiod (12 h light, 12 h dark), temperature (21–22 °C), and humidity with food and water available ad libitum. Mice were kept on a phytoestrogen free diet. For timed matings, females were mated with males and checked daily for vaginal plugs. Noon on the day of vaginal plug detection was designated as 0.5 dpc. Birth usually occurred at 19.5 dpc and was designated as postnatal day (PND) 1. Juvenile and adult mice were euthanized by CO<sub>2</sub> asphyxiation. Neonatal mice were euthanized by decapitation on the appropriate day.

### Fertility Tests

Two parental pairs for each condition were set up with: two wild type parents, wild type females with heterozygous males, and ArKO females with wild type males. Pairs were left together for 3–6 months. Staff of the Department of Laboratory Animal Resources at the State University of New York Upstate Medical University recorded the number of pups born in each litter, any pups that died at birth, and any pups that died prior to weaning. Each breeding pair was sacrificed at the end of 6 months and tail snips were collected reassess parental genotype.

## Genotyping

Upon sacrifice of each mouse, a small tail snip was taken and stored in individually labeled tubes at -20°C until DNA extraction. For neonates and pups under 21 days of age this was the first and only genotyping conducted. For adults, this was the second genotyping done for reassurance of genotypes established at time of weaning. DNA was extracted from tails using the DNeasy blood and tissue kit from Qiagen. PCR reactions were completed using primer sequences listed in Table 1.

Table 1  
Primers used for PCR and genotyping of ArKO mice.

Primer Name	Sequence
9F	GTGACAGAGACATAAAGATCG
9R	GTAATTCATTGGGCTTAGGG
13	CTTGGGTGGAGAGGCTATTC
14	AGGTGAGATGACAGGAGATC

## Histological Methods

Juvenile and adult ovaries were dissected in PBS and then fixed in Bouin's solution for 2 hours at room temperature, followed by dehydration through an ethanol series. Histological processing of the ovaries was performed at the Animal Health Diagnostic Center at the College of Veterinary Medicine, Cornell University. The ovaries were serially sectioned at 5 µm and stained with hematoxylin and eosin. Follicles were counted in every 10th section at 400X magnification. To avoid bias, all ovaries were analyzed without knowledge of age or genotype. To avoid double counting of oocytes, only oocytes with a visible nucleus were counted. Oocytes were in primordial follicles if they contained an oocyte surrounded by a single layer of flattened granulosa cells. Primary follicles were characterized by an oocyte with a single layer of cuboidal granulosa cells, and the oocytes of secondary follicles were surrounded by more than one layer of cuboidal granulosa cells. Follicles with an antral space were classified as antral. Corpora lutea and hemorrhagic follicles, which were antral follicles with erythrocytes present in the antrum, were counted in each ovary. Photos were taken on an Olympus BX51 microscope with a DP74 camera.

## Statistical analysis

Data are represented as mean  $\pm$  SEM. For fetal and neonatal ovaries, statistical differences among the means were evaluated using unpaired two tailed t-tests in Microsoft Excel. Using GraphPad Prism version 6 (GraphPad Software, San Diego, CA), data from mice aged 2–14 weeks were analyzed by one-way ANOVA followed by Dunnett's post-hoc test with wild type as the control group. For all statistical analyses,  $p < 0.05$  was considered significant.

## Results

### Aromatase knockout female mice are infertile

We first confirmed that ArKO homozygous null mice were infertile by mating wildtype, heterozygous and homozygous mutant females to wildtype males. As shown in Fig. 1A, the average number of pups per litter did not differ between wild type and heterozygous females with 6.7 and 7.2 pups/litter respectively and each breeding pair produced three to four litters over six months. In contrast, there were no litters from homozygous females when paired with males for six months. Histological sections of ovaries from 10 week old wild type females contained follicles at all stages as well as corpora lutea as shown in Fig. 1B while ArKO homozygous mutant ovaries lacked corpora lutea and had abnormal hemorrhagic follicles (Fig. 1C) as previously reported (Britt et al., 2001).

### Aromatase knockout mice undergo normal cyst breakdown and primordial follicle formation

To determine if fetal estrogen played a role in regulating primordial follicle formation, we determined the percent of single oocytes, oocyte number and the percent of developing follicles between 17.5 dpc and PND 9 in ovaries from wild type, ArKO heterozygous and homozygous mice. There were no differences in the total number of oocytes counted per ovarian section between genotypes during this time period (Fig. 2A). The percent of single oocytes, a measure of cyst breakdown and primordial follicle formation, was also similar for all ages and genotypes except that at PND7, heterozygotes had a significantly greater proportion of single oocytes as compared to WT mice ( $p \leq 0.05$ ) (Fig. 2B). Differences in folliculogenesis among the three genotypes were only apparent at PND 7 (Fig. 2C-E). At this time point, a greater proportion of follicles had reached the primary and secondary stages in ArKO heterozygous and homozygous mice as compared to wild type. At PND9, there were again no apparent differences in the follicle populations of the three genotypes.

### Aromatase knockout mice have fewer oocytes starting at 8 weeks of age

It has previously been reported that ArKO homozygous mice had fewer total oocytes at 10 weeks of age but earlier time points were not examined (Britt et al., 2004). To determine when the oocyte loss first occurs, ovaries were collected from wild type, ArKO heterozygous and homozygous mice beginning at 2 weeks of age, histological sections stained with hematoxylin and eosin (Fig. 3) and the numbers of follicles in every tenth section counted. The mean number of oocytes counted per ovary in ArKO mice was

similar to wild type at 2, 4, and 6 weeks (Fig. 4). At 8 weeks, there was a trend towards fewer total oocytes per ovary in the ArKO mice as compared to wild type, although it was not statistically significant. At 10–14 weeks of age there were significantly fewer oocytes counted in the ovaries of ArKO homozygous mice as compared to wild type ( $p = 0.013$ ).

To determine if a particular stage of follicle development was affected by the lack of aromatase and estrogen, we compared the percent of oocytes at each stage among the genotypes beginning at 2 weeks of age. The percent of primordial, primary, secondary, and antral follicles were similar between the groups at the ages of 2, 4, 6, and 8 weeks (Fig. 5). There was a greater percentage of oocytes remaining in cysts in the ArKO mice as compared to wild type ( $p = 0.0124$ ) and heterozygotes ( $p = 0.0226$ ) at 2 weeks (Fig. 5A and 3B,C). This proportion was not significantly different at any other age (Fig. 5B-D). Antral follicles were first observed in wild type mice at 2 weeks old, however, they were not observed in heterozygotes or ArKO mice until 4 weeks of age.

## **Aromatase homozygous knockout mice have abnormal hemorrhagic follicles and hemosiderin deposits**

Hemorrhagic follicles were apparent upon gross examination of ovaries (data not shown) and in histological sections from ArKO homozygous mice beginning at 6 weeks old (Fig. 3I, L, Fig. 6C, E). In histological sections these structures appeared to begin as antral follicles, where the antrum filled with blood (Fig. 6E). Hemorrhagic follicles were not observed in the ovaries of any other genotype (Fig. 6A and B). Corpora lutea were observed in the ovaries of wild type and heterozygous mice beginning at 6 weeks old (Fig. 3G, H, J, K and Fig. 6A and B). They were not observed in ArKO mice at any age. Golden-brown hemosiderin deposits, which are derived from autophagy of excess iron (Theil, 2012), were found in the stroma of ovaries from ArKO mice beginning at 8 weeks (Fig. 6D). They appeared to become more prominent as the mice aged to 10–14 weeks.

## **Discussion**

The reproductive phenotype of mice lacking aromatase has been well characterized for adults. However, there is no published information on the ovaries of these animals during the critical time of cyst breakdown and follicle formation, which is important for establishing the ovarian reserve. Because *in vitro* studies have suggested that estrogen has a role in the process of cyst breakdown and follicle formation in late fetal and early neonatal life, we studied the ovaries of ArKO mice during this time period. Mice were also studied at two-week intervals to determine at what age the adult ArKO ovarian phenotype first appeared and if folliculogenesis was affected. To our knowledge, this is the first report on the ovarian phenotype of ArKO females during the period of follicle formation and early folliculogenesis.

Cyst breakdown and primordial follicle occur between 17.5 dpc and PND 5 in mice (Pepling et al., 2010), and this process is inhibited by estrogen and estrogenic compounds (Chen et al., 2009; Chen et al., 2007; Karavan and Pepling, 2012). Elimination of fetally-derived estrogen by knocking out aromatase did not affect these processes in our study. This suggests that maternal circulation is an important source of

estrogen that regulates cyst breakdown and follicle formation in mice. Our results are consistent with a previous study in which aromatase in fetal ovaries in culture was inhibited with letrozole and follicle formation was unaffected (Dutta et al., 2014). However, measurements of perinatal fetal and maternal estrogen indicates that estrogen does not decline at the time of cyst breakdown, which is expected to occur if it is the chief regulator of cyst breakdown (Dutta et al., 2014). Lack of aromatase and estrogen did not affect the total number of oocytes in the ovaries of fetal mice, suggesting that fetal-derived estrogen is not critical for oocyte survival in the developing ovary.

It is possible that a decrease in estrogen is not the initial or major initiator of cyst breakdown in mice. Progesterone also inhibits cyst breakdown in fetal mouse ovaries in culture, and the effect is additive to that of estrogen (Chen et al., 2007). Furthermore, circulating maternal progesterone rapidly declines around the time of cyst breakdown (17.5 dpc) (Dutta et al., 2014). Perhaps a drop in progesterone is actually the first stimulus for cyst breakdown in utero and the lack of estradiol after birth allows cyst breakdown to progress to completion. More information on the role of maternal vs fetal steroids in the timing of cyst breakdown and follicle formation may be gained by eliminating maternal steroid production around the time of cyst breakdown with enzyme inhibitors that block the synthesis of estrogen (i.e. letrozole) or progesterone (i.e. trilostane).

Folliculogenesis was also largely unaffected by the lack of estrogen. Follicles were classified by stage of development between PND 4 and PND 9. A difference in follicle development was observed only at PND 7. At PND 7 the ovaries of ArKO and heterozygous mice had a greater proportion of primary follicles as compared to wildtype. This suggests acceleration of primordial follicle activation in heterozygotes and ArKO mice as compared to wildtype. At this time point, the ovarian follicle populations more resemble wildtype at PND 9 than PND 7. However, by PND 9 the proportions of follicles in each stage of development in ArKO and heterozygotes again resembled those of wildtype. While this suggests an increase in primordial follicle activation at PND 7 compared to wildtype, this appears not to be contributing to the decrease in oocytes observed later in life as the ovaries have similar populations of follicles and total number of oocytes again at PND 9. They continued to have similar total number of oocytes and follicle populations through puberty and early adulthood.

There was a trend toward fewer total oocytes in the ovaries of ArKO mice as compared to wild type beginning at 8 weeks and significantly fewer by 10 to 14 weeks. The small sample size likely prevented us from detecting a statistically-significant difference at 8 weeks. There are two potential reasons for the smaller ovarian reserve in ArKO females as compared to controls. First, estrogen may influence follicle activation and its absence leads to premature primordial follicle activation. Alternatively, there may be reduced follicle survival in ArKO females. There were no differences in the proportion of primordial or primary follicles among the genotypes at any age studied, therefore it is expected that reduced follicle survival results in a decrease in ovarian reserve.

As others have suggested, the lack of estrogen likely generates a toxic environment for follicles. The lack of estrogen in ArKO mice releases negative feedback the secretion of the gonadotropins luteinizing

hormone (LH) and follicle stimulating hormone (FSH), leading to significantly elevated circulating levels of these hormones (Britt et al., 2001). Mice overexpressing LH had fewer oocytes as compared to controls, presumably because of accelerated atresia (Flaws et al., 1997), a phenotype similar to that reported here and by others for ArKO mice. The negative effect of aromatase knockout on follicle survival was first observed at 8 weeks of age.

Hemorrhagic follicles were previously observed in ArKO mice at ten weeks of age (Britt et al., 2000). They were also found in the ovaries of estrogen receptor one (*Esr1*) knockouts (3–5 weeks old) (Couse and Korach, 1999) and *Esr1* and *Esr2* double knockouts (14 weeks old) (Dupont et al., 2000). We first detected these structures at 6 weeks of age in the ArKO mice, which was earlier than expected. At this age they were apparent in gross observation of the ovary and in histological sections. In our histological sections these structures appeared to be antral follicles undergoing an abnormal atresia or death wherein the antral cavity fills with blood. Studies on mice overexpressing LH indicate that hemorrhagic follicles result from a lack of estrogen feedback and high circulating LH levels, which appears to be toxic to the larger follicles, causing the antrum to fill with blood and form a cyst-like structure (Flaws et al., 1997). Furthermore, when ArKO mice were treated with the gonadotrophin-releasing hormone (GnRH) antagonist acyline, serum LH and the number of hemorrhagic follicles were reduced (Liew et al., 2010). The appearance of hemosiderin deposits in the ovary occurred at 8 weeks in ArKO mice. These deposits are likely related to the accumulation of erythrocytes in atretic follicles.

It was previously suggested that ArKO females are infertile (Britt et al., 2001), but no data on mating outcomes have been published. In our study, all ArKO females paired for six months failed to produce litters, while heterozygotes had litter sizes no different from WT mice. Although the oocytes from ArKO females are developmentally competent (Huynh et al., 2004), our observations and those of others indicate they are anovulatory (Britt et al., 2001). Corpora lutea were not observed in the ovaries of ArKO females at any age. Furthermore, vaginal cyclicity has not been detected in ArKO females (Britt et al., 2001). A lack of estrogen is expected to eliminate the LH surge required for ovulation.

## Conclusions

To our knowledge, this is the first study on the ovarian phenotype of ArKO mice beginning during fetal development, which is when the critical process of oocyte cyst breakdown and follicle formation occur. We provide data confirming that ArKO mice are infertile as previously reported. We determined that the absence of fetal estrogen does not influence the process of cyst breakdown or follicle formation in mice during the perinatal period though results in a slight delay in completion of follicle formation at 2 weeks. Folliculogenesis was also largely unaffected by the lack of estrogen in aromatase knockout mice. Future studies will need to be done to determine the relative importance of estrogen over progesterone in the processes of cyst breakdown and follicle formation. We report that the onset of the distinct ovarian phenotype of ArKO mice occurs earlier than previously reported, first observed at 6 weeks of age with the presence of hemorrhagic follicles and a trend toward decreasing total number of oocytes starting at 8 weeks.

# Declarations

## *Ethics approval and consent to participate*

This work was approved by the Syracuse University Institutional Care and Use Committee (IACUC protocols # 14-006 and 17-003).

## *Consent for publication*

Not applicable.

## *Availability of data and materials*

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

## *Competing Interests*

The authors declare that they have no competing interests.

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## *Authors' contributions*

JMT conducted fertility tests and mutant analysis of fetal and neonatal ovaries. KR analyzed juvenile and adult ovaries and helped draft the manuscript. AV harvested and genotyped juvenile and adult ovaries and assisted with analysis. SMG assisted with fertility tests and genotyping mutants. MEP designed experiments, helped with data analysis and interpretation, obtained funding to support the project and edited the manuscript.

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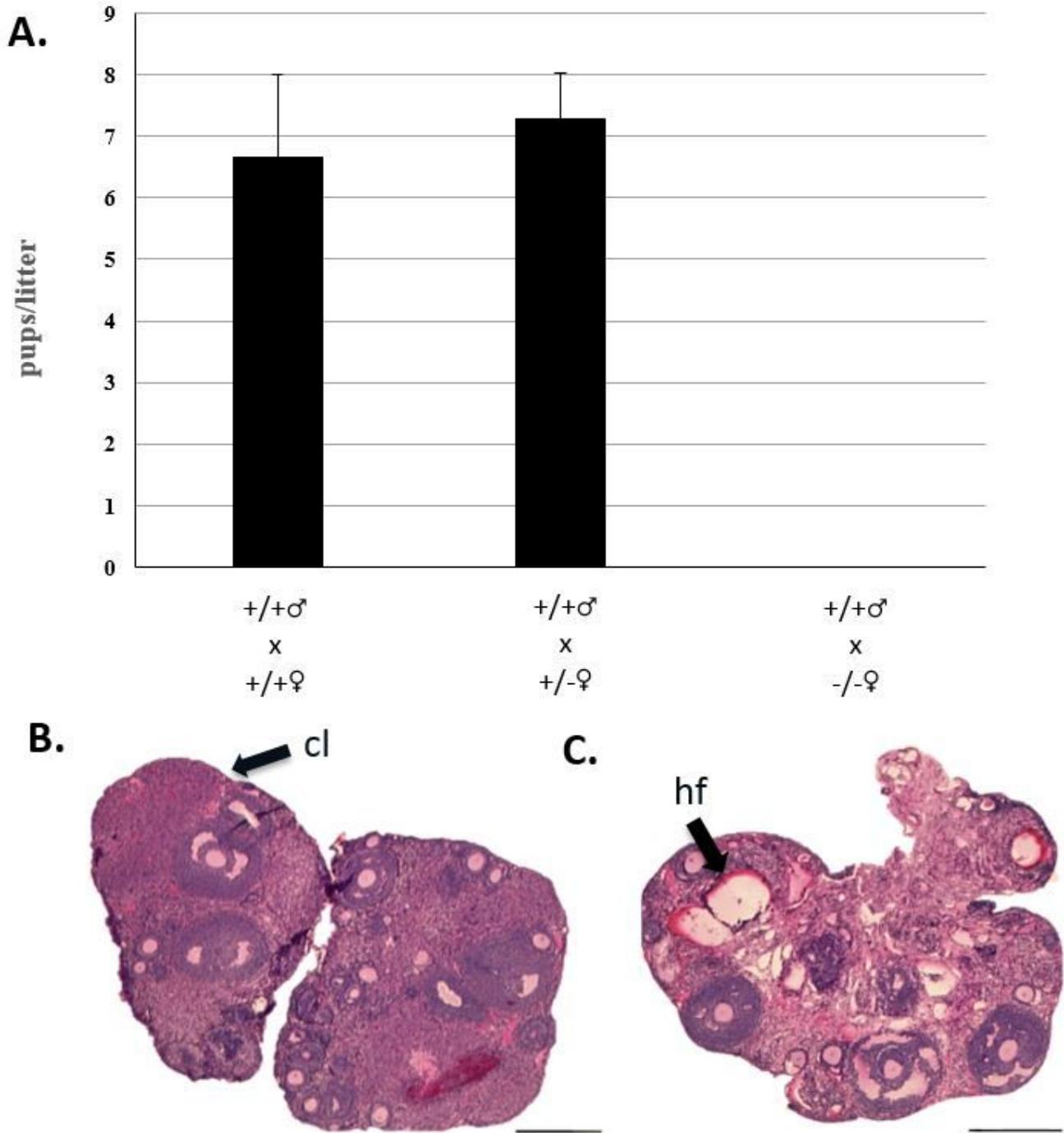
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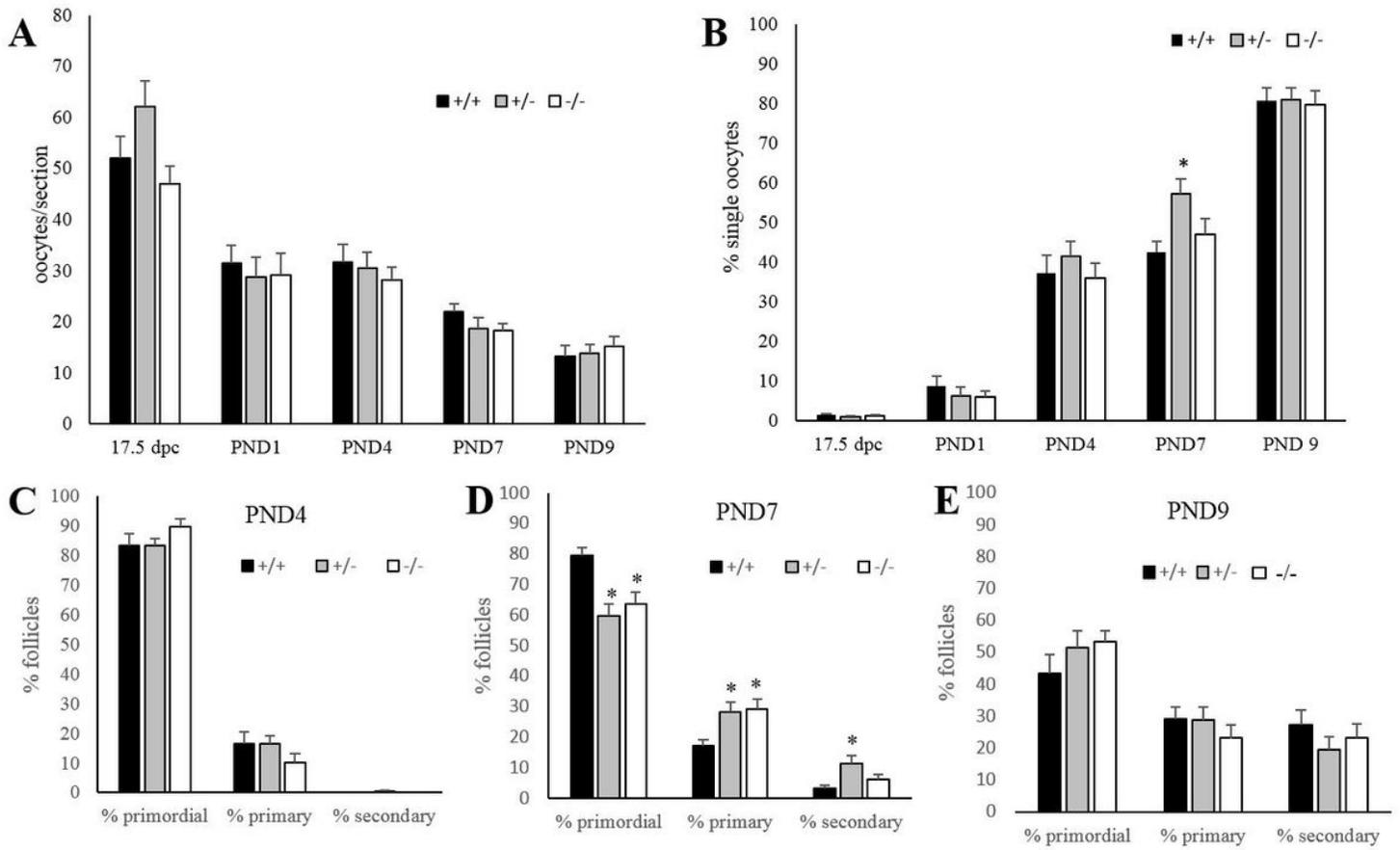
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## Figures



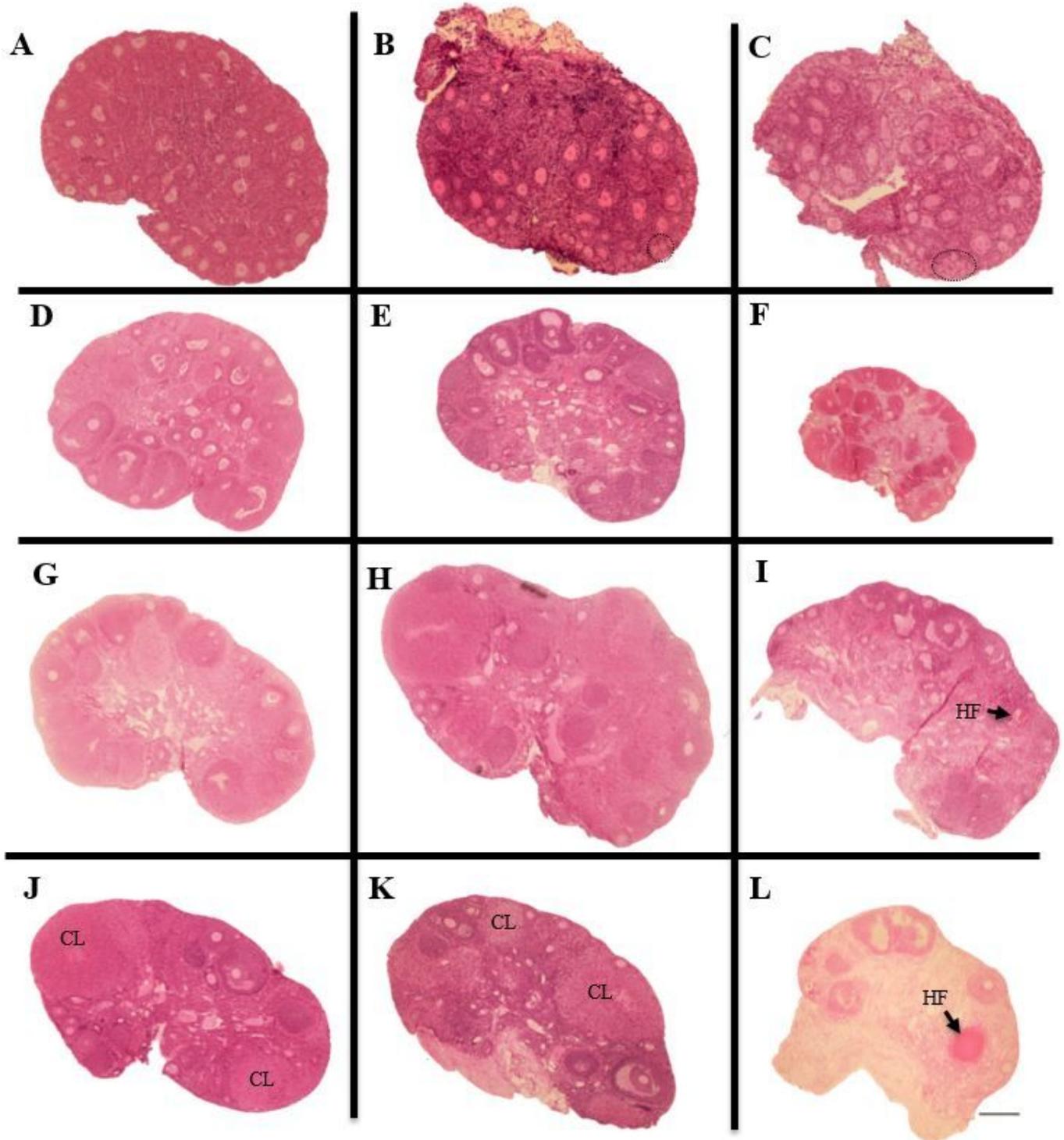
**Figure 1**

Fertility and adult ovarian morphology in wild type, ArKO heterozygous and homozygous mice. A) Fertility tests of wild type (+/+), ArKO heterozygous (+/-) and ArKO homozygous (-/-) females bred to +/+ males for 6 months. Average number of pups/litter  $\pm$  SEM. (B, C) Histological sections of ovaries from 10 week old wildtype (B) and ArKO homozygous mice (C). Scale bar = 500  $\mu$ m. cl = corpus luteum, hf = hemorrhagic follicle.



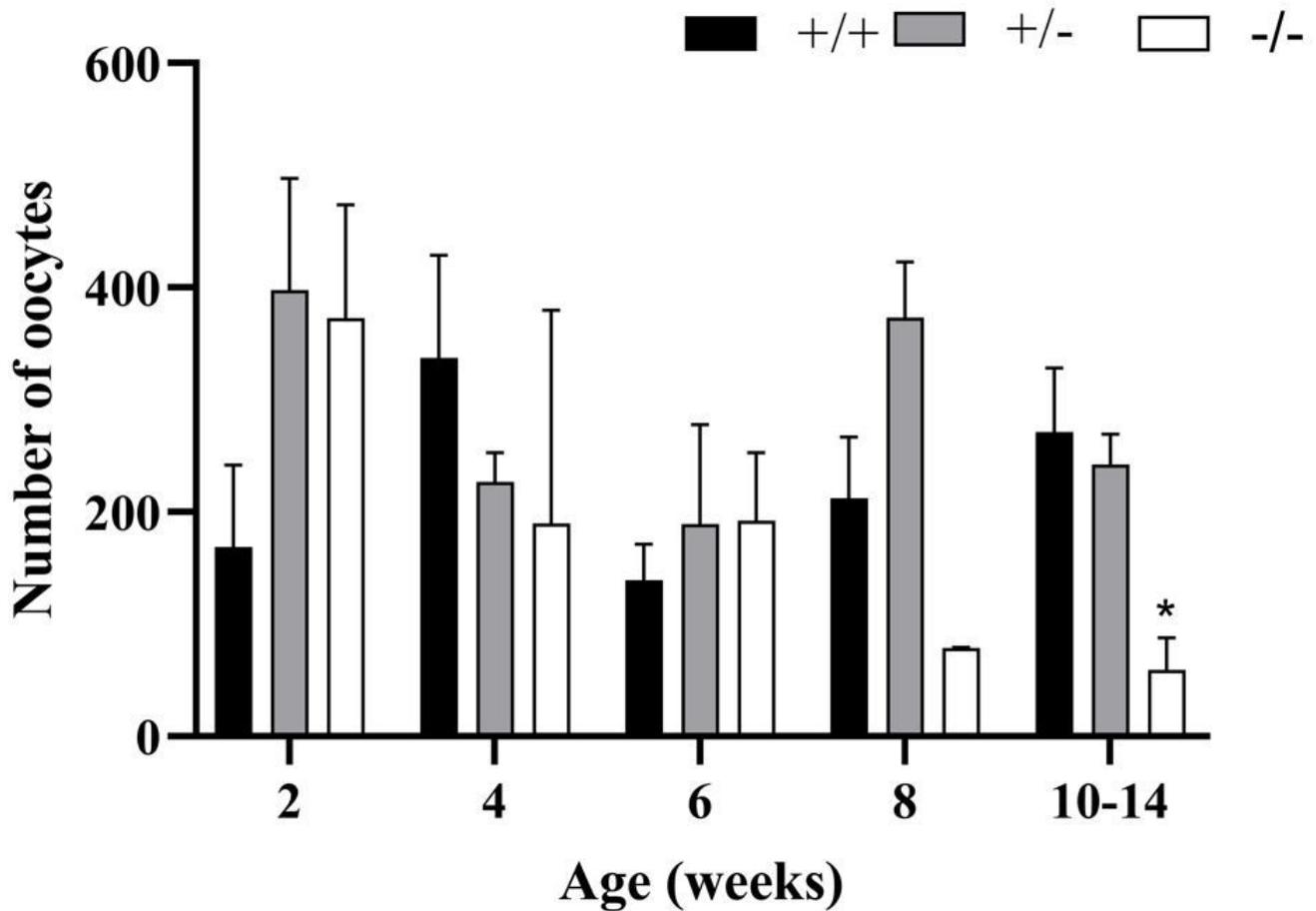
**Figure 2**

Oocyte number, cyst breakdown and follicle development in wild type, ArKO heterozygous and homozygous developing mice. The percentage of single oocytes (A) and the number of oocytes (B) in ovaries from wildtype, heterozygous and homozygous mutant mice at 17.5 dpc and PND 1, 4, 7 and 9. (C-E) The percentage of primordial, primary and secondary follicles relative to the total number of follicles from wildtype, heterozygous and homozygous mutant ovaries at PND 4 (C), PND 7 (D) and PND 9 (E). Data are presented as the mean  $\pm$  SEM. \*indicates a significant difference between control and treated ovaries (unpaired two-tailed t-tests  $p < 0.05$ ).



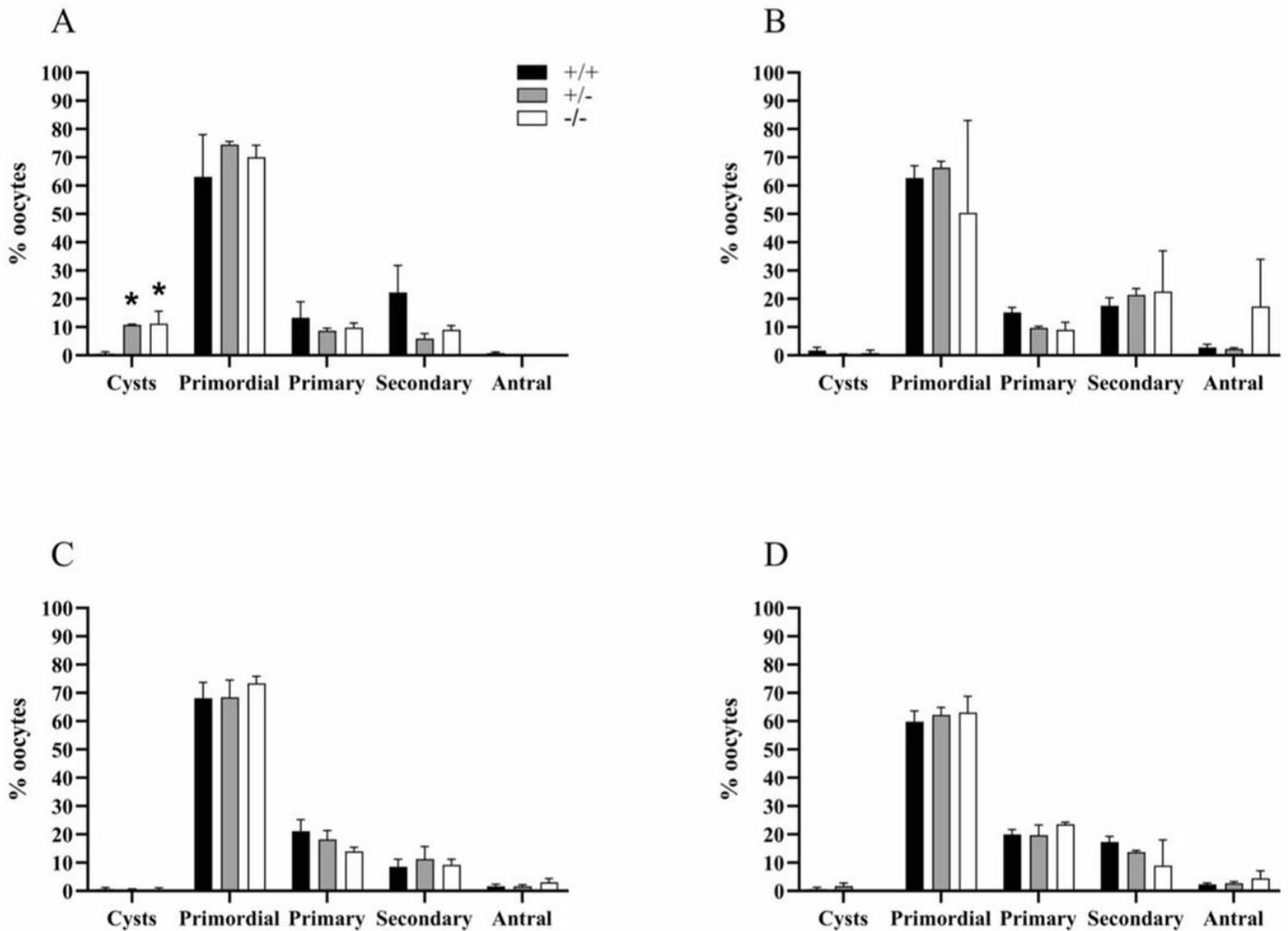
**Figure 3**

Representative mid-section images of ovaries from wild type and ArKO homozygous juvenile mice. Images of hematoxylin and eosin stained ovaries from 2 (A-C), 4 (D-F), 6 (G-I) and 8 (K-L) week-old wild type (A, D, G, J), ArKO heterozygous (B, E, H, K) and homozygous (C, F, I, L) females. Scale bar = 500  $\mu$ m. Corpus luteum = CL, Hemorrhagic follicle = HF.



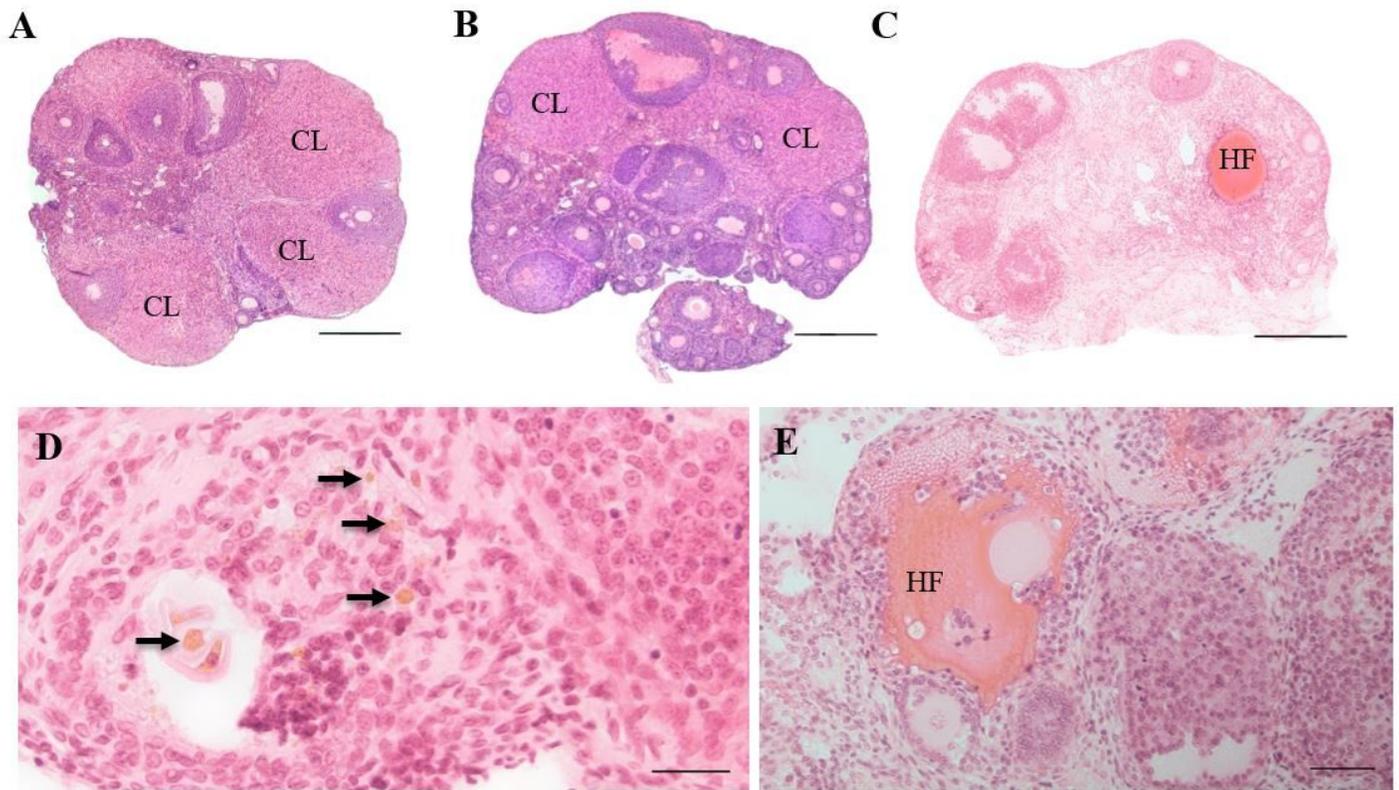
**Figure 4**

Number of oocytes in ovaries from juvenile and adult wild type, ArKO heterozygous and homozygous mice. Mean ( $\pm$  SEM) number of oocytes counted in every tenth histological section of ovaries of wild type, ArKO heterozygous and homozygous juvenile and adult female mice at two-week intervals ( $n = 2-3$  females per group). Asterisk denotes significant difference from wild type ( $p < 0.05$ ).



**Figure 5**

Percent of follicles at each stage of development in ovaries from juvenile and adult wild type, ArKO heterozygous and homozygous mice. Percent of oocytes at each stage of development counted in every 10th histological section of ovaries from wild type, ArKO heterozygous and homozygous mice. Ovaries were collected and follicles counted at two-week intervals (A: 2 weeks old, B: 4 weeks old, C: 6 weeks old, and D: 8 weeks old). Sample sizes were 2-4 females per group. Asterisks indicate significant differences from wild type ( $p < 0.05$ ).



**Figure 6**

Representative mid-section images of ovaries from 8 week old wild type, heterozygous and homozygous ArKO mice. (A-C) Images of hematoxylin and eosin stained ovaries from 8-week-old wild type (A), heterozygous (B) and ArKO homozygous (C) mice. Scale bar = 500  $\mu\text{m}$ . (D, E) Higher magnification images of 8-week-old ArKO homozygous ovaries showing Hemosiderin (D, arrows) and hemorrhagic follicles (E, HF). Scale bar = 50  $\mu\text{m}$ . Corpus luteum = CL.