Loss of ASPM Disrupts Female Folliculogenesis in Mice

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Research

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Abstract

Background: The abnormal spindle-like, microcephaly-associated (*ASPM*) gene is a causative gene of autosomal recessive primary microcephaly (MCPH) 5 in humans, which is characterized by a reduction in brain volume. It was previously reported that truncated *ASPM* proteins in transgenic mice caused major defects in the germline, a severe reduction in ovary weight and the number of follicles accompanied by reduced fertility. However, it remains unknown whether a loss of *ASPM* induces abnormal ovarian function, resulting in female infertility.

Methods: In order to assess the ovary function, we examined vaginal smear cytology from the age of 7 weeks to 100 weeks in CAG-mediated Cre-loxP conditional *Aspm*^{-/-} knockout mice and control female mice. In addition, we evaluated the ovarian size, fibrosis ratio and the number of follicles (primordial, primary, secondary, antral and atretic follicles) in mice from 15 weeks to 100 weeks old by image analyses. Mann-Whitney *U*-test was used for statistical analysis.

Results: The size of the ovary was significantly reduced in *Aspm* knockout mice at 15-20 weeks, 40-50 weeks and 70-80 weeks old. compared with the control mice. Furthermore, at all stages, we found a severe decrease in the number of developing follicles at 10-15 weeks, 40-50 weeks and 70-80 weeks old, accompanied by disrupted cyclic changes of vaginal cytology.

Conclusion: The results showing that folliculogenesis was significantly decreased and associated with abnormal vaginal cytology in *Aspm* knockout female ovaries suggested that *ASPM* might play an important role in the folliculogenesis and estrous cyclicity of the postnatal ovary.

Summary Sentence

A loss of *ASPM* protein reduced ovarian size and the number of follicles accompanied with disrupted cyclic changes in the vaginal cytology in adult female mice, suggesting that *ASPM* might play an important role in germ cell differentiation and maturation.

Introduction

The abnormal spindle-like, microcephaly-associated (*ASPM*) gene is a causative gene of autosomal recessive primary microcephaly (MCPH) 5 in humans, which is characterized by a reduction of brain volume, leading to intellectual disability and varied psychomotor disorders [1]. The pathogenetic mechanisms of microcephaly have been intensively investigated in animal models, including gyrencephalic models. The results of these studies have suggested that *ASPM* regulates cortical surface expansion by controlling the affinity of ventricular radial glial cells, an undifferentiated cell type found in the ventricular surface, thus increasing the ratio of the undifferentiated cells to more differentiated progenitors in the cortical units [2]. *ASPM* functionally acts in the symmetric division of progenitor cells by maintaining the stemness, which is essential for regulating appropriate organ size and structure.

In our previous study, we reported that a loss of ASPM induced testicular hypoplasia, composed of a decreased number of seminiferous tubules accompanied by a decrease in the number of spermatogonia, spermatocytes, spermatid and sperm, and vacuolation of Sertoli cells [3]. It has been reported that truncated ASPM proteins in transgenic mice cause major defects in the germline, not only testicular hypoplasia but also a severe reduction in ovary weight and the number of follicles, accompanied by reduced fertility [4]. However, the precise function of the ovary, including cyclic changes in the number and stages of follicles, as well as temporal changes in ovarian size and histology according to aging have not yet been examined in the absence of ASPM.

In the present study, we investigated the function and morphology of the ovary in *Aspm* knockout (KO) female mice, and found a severe decrease in the number of developing follicles at all stages in young adult mice, accompanied by disrupted cyclic changes in the vaginal cytology. These results suggested that ASPM might play an important role in germ cell differentiation and maturation during the development of the murine ovary.

Materials And Methods

1. Animals

In this study, we used CAG-mediated Cre-loxP conditional *Aspm*^{-/-} (KO) and *Aspm*^{+/+} (control: CNT) female mice, prepared as previously described [3]. Briefly, the *Aspm* KO mice retained exon 2-3 of the *Aspm* gene, encompassed by a pair of loxP signals, so that cre-recombinase activity could switch the allele from the wild-type to null zygotes. *Aspm*^{+/-} heterozygote was obtained by crossing the mice with CAG-Cre transgenic mice. *Aspm*^{-/-} (KO) offspring were also obtained as frequently as expected from the Mendelian inheritance and maintained under a 12-h light–dark cycle (on at 7 a.m.), at a controlled temperature (24–25°C) with free access to food and water. All of the animal studies were approved by the Institutional Review Board for Biomedical Research using Laboratory Animals at Kyoto Prefectural University of Medicine, and the animals were handled in accordance with the institutional guidelines and regulations.

2. Vaginal smear analysis

The female animals at the age of 7-11 weeks (CNT; n=5, KO; n=5), 30 weeks (CNT; n=8, KO; n=5), 40 weeks (CNT; n=9, KO; n=5) and 70-80 weeks (CNT; n=3, KO; n=3) were handled at 8:00 in the morning. Vaginal squamous epithelium was obtained by washing the vaginal cavity with 20 µL of PBS using pipette tips and fixed with 100% ethanol and 2% polyethylene glycol for 30 minutes. Post-fixed smeared cells were stained with Papanicolaou stain.

3. Tissue preparation and histopathological examination

Adult female mice were perfused transcardially with 4% paraformaldehyde in 0.1 M PBS. The mice were euthanized at the estrus stage before they were 40 weeks old. The ovaries were removed, post-fixed, and paraffin-embedded longitudinal sections were stained with hematoxylin-eosin and Masson-trichrome

stain (5 weeks: CNT; n=3, KO; n=3, 15-20 weeks: CNT; n=5, KO; n=5, 40-50 weeks: CNT; n=5, KO; n=5, 70-80 weeks: CNT; n=5, KO; n=5, 100 weeks: CNT; n=5, KO; n=5, respectively).

The ovaries were dissected, fixed overnight with 4% paraformaldehyde and embedded in paraffin. Serial coronal sections were prepared at 4 µm-thickness. Following antigen retrieval with EDTA pH 8.0, the tissue was blocked with 10% goat serum, 1% BSA, and 0.01% Triton X-100 (Sigma-Aldrich, Japan) in 0.1M PBS and incubated in primary antibody diluted in 0.1% Triton X-100 and PBS at 4°C overnight (Cleaved Caspase-3(Asp175), rabbit polyclonal, 1:200, Cell Signaling TECHNOLOGY, Danvers, MA, USA). The sections were rinsed with PBS and then incubated in the secondary antibody.

4. Image analyses

Morphometry

Four µm-thick serial paraffin-embedded sections were prepared and 10 sections at intervals of 40 µm were used for assessing the total number of follicles in the ovary. The entire area of the sections stained with hematoxylin-eosin or Masson-trichrome stain was scanned, captured on computer, and image acquisition was conducted (× 40 magnification mode) with a NanoZoomer C9600 (Hamamatsu Photonics, Tokyo, Japan). One cytologist (MM) counted the number of each type of follicle, according to the criteria as follows: Primordial follicles contain immature oocytes surrounded by flat, squamous [granulosa cells](#). In the primary follicle, the granulosa cells change from a flat to a cuboidal structure and the [zona pellucida](#) forms around the oocyte, separating it from the surrounding granulosa cells. In the secondary follicles, stroma-like theca cells encircle the follicle's outermost layer and differentiate to the [theca externa](#) and theca interna. The late-term secondary follicle is composed of a fully grown oocyte surrounded by a zona pellucida, granulosa cells layer, a basal lamina, a theca interna, a capillary net, and a theca externa. The antral follicles showed the formation of a fluid-filled cavity, which has been called the [antrum](#). In the atretic follicles, the granulosa cells undergo apoptosis, showing more than two pyknotic nuclei, an irregular arrangement and pulling away from the basement membrane.

Using the same digital HE images, the size and area of the ovary, specifically the longest diameter and the major area was measured. Quantification of the fibrosis was assessed using Masson-trichrome stained sections. For the image analysis, × 10 magnified images were exported using NDP.view 2 software (Hamamatsu Photonics, Tokyo, Japan). Fiji software was also employed (ImageJ 2.0.0-rc-61/1.5n, <http://fiji.sc>) for the computer-based image analysis. First of all, background correction was carried out to reduce any color/luminance variations in the image. The tissue area (region of interest, ROI) from each stained section was extracted based on luminance information using an auto-thresholding technique. An automatically calculated threshold was used to overcome non-uniform staining conditions [5]. Filamentous blue-stained areas of each specimen were extracted based on color information by auto-thresholding techniques. Finally, the amount of fibrosis in each tissue specimen was quantified by the following formula: fibrosis (%) = actual fibrosis area × 100 / ROI area.

5. Statistical analysis

All results are expressed as either mean $\pm$ SD or mean $\pm$ SEM. The Mann-Whitney *U*-test was used to analyze any differences between the two groups. Values of $P < 0.05$ were considered to be statistically significant.

Results

1. Vaginal smear analyses

The vaginal smears were classified into four categories: Proestrus, Estrus, Metaestrus, or Diestrus (Fig.1). The smears showed regular cyclic changes of E, M, D and proestrus P stage with 4-5 days intervals at the age of 30-40 weeks in control females (Fig.1). On the other hand, the vaginal smears exhibited a disrupted cycle and remarkable elongation of the estrous or diestrous stages at 30-40 weeks old in KO females (Fig.1). The frequency of the abnormal estrous cycles was shown in Fig. 2. The vaginal smears showed some individual differences and showed irregular estrous cycles at 7 to 15 weeks old, appearing in both KO and control mice (irregular estrous cycles: control (n) = 5/5; KO (n) = 5/5). The smears showed regular cyclic changes in the proestrus, estrus, metaestrus and diestrus stages with 3 to 5 days intervals at the age of 20-30 (irregular estrous cycles: control (n)=0/4), 30-40 (irregular estrous cycles: control (n) = 0/7) and 40-50 weeks old (irregular estrous cycles: control (n) = 1/9) in control females. On the other hand, the vaginal smears demonstrated disrupted cycles, remarkable elongation of the estrous, metaestrous or diestrous stages, and irregular intervals between the estrous stage in KO mice at 20-30 (irregular estrous cycles: KO (n) = 2/3), 30-40 (irregular estrous cycles: KO (n) = 3/5) and 40-50 weeks old (irregular estrous cycles: KO (n) = 5/5). Regular cycle of vaginal smear completely disappeared in control females at 70-80 weeks old (irregular estrous cycles: control (n) = 3/3). In the KO females, the disrupted estrous cycle was associated with atrophic cytology at 70-80 weeks old (irregular estrous cycles: KO (n) = 3/3).

2. Morphology of the ovaries

The ovaries were slightly smaller in size, with a reduced number of primordial, primary and secondary follicles at 5 weeks of age in the KO females. The ovaries of the KO females were much smaller in size, compared with those of the control females at both 15-20 weeks and 40-50 weeks old. The ovaries of the KO females exhibited a fibrous stromal mass composed of a very few follicles from 15 weeks to 70-80 weeks old. Although the control ovaries showed multiple cysts, atretic follicles and fibrous tissue at 70-80 weeks of age, a few primordial, primary and secondary follicles were still preserved. At 100 weeks of age, ovaries from both the control and the KO mice showed massive fibrosis. However, a few atretic follicles were found in the control mice (Fig.3).

In the control and the KO mice, respectively, the ovarian areas were 1.41 ± 0.71 (mm²), 1.18 ± 0.27 (mm²) at 5 weeks, 2.27 ± 0.14 (mm²), 1.05 ± 0.18 (mm²) at 15-20 weeks, 1.72 ± 0.42 (mm²), 0.87 ± 0.23 (mm²) at 40-50 weeks, 1.30 ± 0.28 (mm²), 1.32 ± 0.83 (mm²) at 70-80 weeks and 2.20 ± 2.33 (mm²), 2.44 ± 1.27 (mm²) at 100 weeks of age. The total ovary area was significantly smaller in the KO mice, compared with

the control mice at 15-20 weeks and 40-50 weeks of age. However, no significant differences were shown between the areas found in the KO and the control mice at 70-80 weeks and 100 weeks old. At 5 weeks old, the ovarian area showed a tendency to be reduced in the KO mice, however, the difference did not reach the significance level (Fig. 4A).

In the control and KO groups, respectively, the longest diameters of the ovary were 1.82 ± 0.57 (mm) and 1.51 ± 0.25 (mm) at 5 weeks, 2.24 ± 0.21 (mm) and 1.38 ± 0.18 (mm) at 15-20 weeks, 1.97 ± 0.24 (mm) and 1.92 ± 0.57 (mm) at 40-50 weeks, 1.69 ± 0.21 (mm) and 1.92 ± 0.94 (mm) at 70-80 weeks and 1.55 ± 0.95 (mm) and 2.20 ± 0.55 (mm) at 100 weeks of age. The longest diameters were significantly less in the KO group, compared with the control group at 15-20 weeks and 40-50 weeks of age. The diameters of the ovaries showed a trend for a decrease in the KO group. However, the difference did not approach the significance level at 5 weeks old (Fig. 4B).

The fibrosis ratio was analyzed at 15-20, 40-50 and 70-80 weeks old. The ratio was significantly increased in the KO group, compared with the control mice (Two-way ANOVA). A slight tendency was shown for an increase in the fibrosis ratio in the KO mice, and a decrease in the control mice during aging, but those differences did not reach the level of significance (Fig. 4C).

At each follicle stage, the average number of the primordial, primary, secondary, antral and atretic follicles per ovary decreased with aging in the control mice (Table 1). The number of primordial, primary and secondary follicles showed a tendency for a reduction in the KO females, however the difference did not reach the significance level at 5 weeks old (Fig. 5A). The number of follicles at each stage, specifically, primordial, primary, secondary, antral and atretic follicles, was significantly reduced in KO mice at 15-20 (Fig. 5B), 40-50 (Fig. 5C) and 70-80 weeks old (Fig. 5D), compared with the control mice. At 100 weeks of age, the number of atretic follicles only showed a significant decrease in the KO mice, compared with the control mice (Fig. 5E).

The cleaved caspase-3-immunoreactivity of the ovaries showed no significant difference between the *Aspm* KO and the control mice at 5 weeks old (Supplement Fig. 1), suggesting that apoptosis of the granulosa cells was not involved in the histological ovarian changes observed along with the loss of *Aspm*.

Discussion

In this study, we evaluated the function and morphology of murine ovaries lacking ASPM and found a significant decrease in the number of follicles and altered follicular maturation accompanied with a loss of estrous cyclicity in vaginal smears.

The abnormal spindle-like, microcephaly-associated (*Aspm*) gene is the mouse ortholog of human *ASPM* [3]. *Aspm* mRNA is widely expressed in organs during fetal development, including the liver, heart, lung, kidney, spleen, stomach, colon, bladder, skin, and skeletal muscle, in addition to the brain, testis and ovary [6]. The mutation of *Aspm* induces infertility in males and females. However, only a few studies

have been conducted on infertility related with *Aspm* function [3,4]. It was reported that truncated ASPM proteins in transgenic mice caused major defects in the germline, a severe reduction in ovary weight and follicle numbers, accompanied by reduced fertility [4]. It has been suggested that the underlying mechanism of reduced fertility is related to the primary reduction of follicles caused by a massive loss of germ cells [4]. The *Aspm* gene encodes a centrosomal molecule, which is involved in spindle organization and orientation during cytokinesis [7]. ASPM is functionally active in the symmetric division of progenitor cells, which might be essential for ovarian development.

The cytological assessment of vaginal smears, composed of samples obtained in the proestrus, estrus, metaestrus and diestrus stages, showed that the ovarian function was regulated by secretory hormones such as estrogen and progesterone [23,24]. The cyclicity was relatively irregular at 7-11 weeks old in both control and *Aspm* KO mice, which might be due to sexual immaturity, individual differences, and no exposure to male animals. A regular cyclicity was established in the control group at 30-40 weeks of age when female mice were sexually matured for reproduction. On the contrary, the cyclicity was absolutely disrupted at 30-40 weeks of age in *Aspm* KO mice. Furthermore, an histological analysis confirmed that the size of the ovaries and the number of follicles were significantly decreased, and furthermore, that maturation of the follicles was altered even at 15-20 weeks old in *Aspm* KO female mice. It is suggested, therefore, that ASPM is an important molecule involved in the formation of an appropriate number of primordial follicles and in the cyclic regulation and maturation of the primordial follicles in murine ovaries. The initial decrease in the number of primary follicles was induced by a loss of ASPM, suggesting that ASPM is required for normal germ cell proliferation and differentiation during fetal ovarian development. In addition, the finding that an altered maturation of follicles was observed in *Aspm* KO females suggested that normal maturation of the primordial follicles might partly be regulated by ASPM after birth. As shown by cleaved caspase 3 immunoreactivity, the apoptotic cells in the granulosa cells of the antral follicles, showed no significant differences in *Aspm* KO, compared with the control females, suggesting that accelerating early cell death of granular cells might not be induced by a loss of ASPM. However, it is speculated that an increase in cell death of germ cells or any excessive loss of primordial follicles early in postnatal life might have contributed to the decrease in the number of primordial follicles shown at 5 weeks and 15-20 weeks of age in *Aspm* KO mice. This appears consistent with an observation in the cortical development of the present *Aspm* KO mice, showing increased apoptosis of stem/progenitor cells in the fetal brain (our unpublished data). Ovarian development in fetal and early postnatal stages should be further evaluated to show that loss of germ cells might be involved in the underlying mechanisms related to the decreased number of primordial follicles demonstrated in the present *Aspm* KO mice.

Primary ovarian insufficiency can be caused by a reduction in the primordial follicles pool, accelerated follicle atresia, or an abnormal maturation of primordial follicles. Many genetic, as well as environmental, factors have been reported that contribute to the disruption of oocytes dynamics [14,15]. Genetic defects associated with primary ovarian insufficiency, including X chromosome defects, rare autosomal defects such as *GALT*, *FOXL2*, *AIRE*, *POLG*, *GNAS*, etc. and isolated diseases, such as FSH/LH resistance, variants

of *INHA*, *GDF9*, *FOXO3*, *NOBOX*, *FIGLA*, *CDC42*, *ADAMTS19*, and DNA replication/meiosis and DNA repair gene variants such as *DMC1*, *FANCA*, etc., but not *ASPM*, have been reported previously [16-22].

Although the size of the ovaries decreased along with normal aging, no significant difference was shown in the ovarian diameters between the control and the *Aspm* KO mice at ages older than 40-50 weeks, and likewise, no significant difference was shown in the ovarian areas at ages older than 70-80 weeks. The ovarian areas from *Aspm* KO mice were significantly smaller than those from control mice at the age of 15-20 weeks, and at the age of 40-50 weeks. Regarding fibrosis of the ovaries, we did not find any significant differences between the control and the *Aspm* KO mice. Since it is argued that the ovaries of *Aspm* KO mice demonstrate hypoplasia as well as accelerating aging, the KO mice can be used as a model animal for primary ovarian insufficiency in order to develop therapeutic strategies. In *Aspm* KO mice, the hypothalamus pituitary axis might be affected in association with abnormal brain development. Unfortunately, we did not analyze the hypothalamus or the pituitary of the *Aspm* KO mice. However, they showed no problems in growth, metabolism, immune system and autonomic functions, which are regulated by growth hormone, thyroid hormone and steroid hormones through the hypothalamus pituitary axis. Although we analyzed gonadotropins including lutenizing hormone and follicle stimulating hormone in serum, we found no significant differences between the control and the *Aspm* KO mice.

In this study, we demonstrated altered function and histology of murine ovaries associated with a loss of *Aspm*, suggesting that *Aspm* might play an important role in ovary development and ovulation homeostasis, and thus, it could be a target molecule for the treatment of primary ovarian insufficiency.

Conclusions

A loss of ASPM protein was associated with a reduction in the ovarian size and the number of follicles, accompanied with disrupted cyclic changes in the vaginal cytology in adult female mice, suggesting that ASPM might play an important role in germ cell differentiation and maturation.

Declarations

Ethics approval:

All of the animal studies were approved by the Institutional Review Board for Biomedical Research using Laboratory Animals at Kyoto Prefectural University of Medicine, and the animals were handled in accordance with the institutional guidelines and regulations.

Consent to participate: not applicable

Consent for publication: not applicable

Availability of data and material:

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

Competing interest:

The authors hereby declare that there are no conflicts of interest related to this study.

All of the authors agree to the publication. All of the authors agree to the order of authors.

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Authors' contributions:

MM conceived the present study and performed experiments. ST and HO performed experiments and analyzed data. AF generated the knockout mice. KI contributed to the conception of the present study and wrote the draft.

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Tables

Table 1

	CNT					KO				
Follicle type	5W	10-20W	40-50W	70-80W	100W	5W	10-20W	40-50W	70-80W	100W
Primordial follicle	79.0 ± 28.0	66.0 ± 25.0	10.0 ± 3.7	2.0 ± 2.8	0.0 ± 0.0	7.7 ± 7.4	1.0 ± 1.7	0.4 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
Primary follicle	23.7 ± 7.4	30.0 ± 7.8	12.8 ± 3.3	2.2 ± 1.8	0.2 ± 0.4	9.7 ± 2.9	1.2 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Secondary follicle	43.0 ± 18.2	33.4 ± 8.9	16.2 ± 6.9	4.8 ± 2.7	0.6 ± 1.3	19.3 ± 4.0	7.6 ± 3.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Antral follicle	15.3 ± 2.5	42.4 ± 17.4	17.6 ± 5.3	7.0 ± 6.0	1.0 ± 1.0	14.0 ± 1.7	11.2 ± 5.0	0.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
Atretic follicle	44.0 ± 19.0	41.0 ± 31.0	20.2 ± 9.3	8.6 ± 7.8	2.8 ± 1.3	23.3 ± 31.8	4.2 ± 4.7	0.4 ± 0.5	1.8 ± 1.6	0.6 ± 1.3

Figures

Figure 1

Vaginal smear analyses in mice at 30 weeks and 40 weeks of age. Proestrus (P) stage, showing mixed cells from superficial, middle and parabasal layers without neutrophils; Estrus (E), showing anuclear superficial cells predominant; Metaestrus (M), composed of mixed cells from superficial, middle and parabasal layers with neutrophils; Diestrus (D), with a few cells from middle and parabasal layers are mixed with a large amount of neutrophils. The smear showed regular cyclic changes in the E, M, D and proestrus P stage with 4-5 day intervals at the age of 30-40 weeks in control females. The vaginal smear exhibited a disrupted cycle, showing a loss or a prolonged E stage, elongation of the metaestrous/diestrous stages.at 30-40 weeks old in Aspm KO females. CNT: control, KO: Aspm knockout

Figure 2

Vaginal smear cycle in control and Aspm KO females (A) This figure shows the frequency of the abnormal estrus cycle. Individual differences and irregular cycle were noted at 7 to 11 weeks old, both in KO and the controls. The vaginal smear exhibited a regular estrous cycle in control females at 30-50 weeks old, whereas 60-100% of the KO females showed a disrupted cycle at the same age. The regular vaginal smear cycle completely disappeared both in the KO and the control mice at 70-80 weeks old. (B) Regular estrous cycles in control and irregular ones in Aspm KO are shown. CNT: control, KO: Aspm knockout E: Estrus, M: Metaestrus, D: Diestrus, P: Proestrus

Figure 3

Morphology of the ovaries in the control and KO females. This figure shows the ovaries at 5, 15-20, 40-50, 70-80 and 100 weeks of age in control (CNT) and *Aspm* knockout (KO) mice. The ovaries at 5 weeks appeared slightly smaller in the KO females. The ovaries of the KO females were much smaller in size, compared with those of the control mice at both 15-20 weeks and 40-50 weeks old. The ovaries of the KO mice exhibited a fibrous stromal mass composed of only a very few follicles and a degenerative cyst from 70-80 weeks old. At 100 weeks of age, ovaries from both the control and the KO mice showed massive fibrosis. However, a few atretic follicles were found in the control mice. (A) HE: Hematoxylin-eosin stain, (B) MT stain: Masson and Trichrome stain CNT: control, KO: *Aspm* knockout

Figure 4

Morphometrical analyses of the ovaries (A) Ovarian area, showing a significantly smaller size in Aspm KO (KO), compared with the control (CNT) at 15-20 and 40-50 weeks old. (B) Ovarian longest diameter, showing a significantly smaller diameter in Aspm KO (KO), compared with the control (CNT) at 15-20 weeks old. (C) Analyzed by two-way ANOVA, the fibrosis ratio showed a significant difference (genotype). CNT: control, KO: Aspm knockout

Figure 5

Morphometrical analyses of each follicle in the ovary The number of follicles at each stage, including primordial, primary, secondary, antral and atretic follicles, was significantly reduced in the *Aspm* KO mice at 15-20 weeks (B), 40-50 weeks (C) and 70-80 weeks old (D), compared with the control females, at each corresponding age. At 5 weeks of age (A), each stage of the follicles showed a tendency to decrease in the *Aspm* KO mice, but the differences were not significant. At 100 weeks of age (E), only the number of atretic follicles showed a significant decrease in the KO mice, compared with the control mice. CNT: control, KO: *Aspm* knockout

Supplementary Files

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- [Supplementlegend.docx](#)
- [SupplementFig1.tif](#)