Melatonin as an endogenous hormone to slow down the exhaustion of ovarian follicle reserve in mice—a novel insight into its roles in early folliculogenesis and ovarian aging

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Article

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1 **Melatonin as an endogenous hormone to slow down the exhaustion of**
2 **ovarian follicle reserve in mice – a novel insight into its roles in early**
3 **folliculogenesis and ovarian aging**

4 Running Title: Melatonin delays murine ovarian aging

5 **Abstract**

6 Previous studies have shown that long-term intake of exogenous melatonin
7 can effectively delay ovarian aging, but the mechanism has not been fully
8 elucidated. We observed that SNAT, the rate-limiting enzyme in the melatonin
9 synthetic pathway, is localized in primordial and early follicle, and that
10 granulosa cells isolated from follicle can synthesize melatonin. *In vitro* cultured
11 neonatal mice ovaries with melatonin inhibited primordial follicle activation and
12 early follicle growth. *In vivo* experiments further indicated that daily injections
13 of melatonin to neonatal mice during the primordial follicle activation phase can
14 reduce the number of activated follicles by inhibiting the PI3K-AKT-FOXO3
15 pathway; during the early follicle growth phase, injections of melatonin
16 significantly suppressed early follicle growth and atresia, and transcriptome
17 data showed that multiple pathways involved in folliculogenesis, including
18 PI3K-AKT, were suppressed. Further, *SNAT* knockout in mice resulted in a
19 significant increase in follicle activation and atresia, and eventually accelerated
20 ovarian aging. We also demonstrated that prolonged high-dose melatonin
21 intake had no obvious adverse effect on the health condition of mice. This
22 study confirms that endogenous melatonin is involved in the regulation of
23 ovarian aging, and reveals that melatonin delays ovarian aging by inhibiting
24 primordial follicle activation, early follicle growth and atresia.

25 **Keywords:** Melatonin; Ovarian aging; Primordial follicle; Early folliculogenesis;
26 Antioxidant; Follicular atresia

Introduction

Ovarian aging, which refers to the physiological process of active loss of ovarian function in females before they age, is considered as a gain-of-function mutation that can effectively avoid the physiological burden and life threatening of the maternal body brought by the elderly pregnancy¹. However, ovarian aging is not just the loss of reproductive capability, the lack of gonadal hormones caused by it can trigger many diseases including cardiovascular disease, cancer, osteoporosis, obesity and menopausal syndrome ²⁻⁵. Therefore, ovarian aging, which is called the pacemaker of female body aging, indirectly accelerates the aging of multiple organs ⁶. With the development of medical technology, the various risks brought by elderly pregnancy can be technically avoided. Therefore, the radical cure or delay of ovarian aging is becoming an urgent need for women.

The mainstream view tends to suggest that there are no germline stem cells in the ovary to replenish the continual decrease of the ovarian follicle reserve. Therefore, the exhaustion of the ovarian follicle reserve in middle age is the root cause of ovarian aging ^{7,8}. Early folliculogenesis, which begins with primordial follicle activation and ends with the formation of small antral follicle (SAF), is the directly responsible for the decrease of ovarian follicle reserve ⁹. The primordial follicle reserve established during fetal or neonatal period in mammals represents the “pre-established ovarian follicle reserve” (PreOR), which maintains the entire reproductive life of the females ¹⁰. During early folliculogenesis, a limited number of primordial follicles are progressively activated from PreOR. The activation rate of primordial follicle is strictly regulated, if too fast, it will cause premature ovarian failure ¹¹. Following primordial follicle activation, the activated follicles develop into primary, secondary, and eventually SAFs, a process also known as early follicle growth ¹². All the SAFs together constitute a “dynamic ovarian follicle reserve” (DOR), the size of which determines how many gonadotropin-dependent follicles are available to be selected for ovulation or atresia. The size of PreOR and DOR

are highly variable between individuals of similar age, but the equilibrium size of DOR in adults seems to be specific to each individual¹³.

PreOR and DOR are functionally related, and the dynamics of them are mainly controlled by two pathways, the PI3K-AKT and the BMP/AMH-SMAD signaling pathways¹³. On the one hand, DOR can be continuously replenished by the activated follicles originated from PreOR to maintain its size in balance. Hereby, before the reproductive ability declines, although the number of primordial follicles in the ovary continues to decrease, the number of SAFs in the DOR can remain stable¹⁴; on the other hand, as the ovary age, the number of primordial follicles in PreOR greatly decrease, which indirectly leads to a reduction in SAFs in DOR. Therefore, the degree of ovarian aging can be reflected by the SAFs count^{15,16}. Of note, the SAFs from the DOR will be eliminated by means of atresia if without sufficient gonadotropins. Therefore, the larger the DOR, the more SAFs will be eliminated to ensure the stability of fecundity, and accordingly, more primordial follicles will be activated from PreOR to supplement the loss of DOR. This bad circulation will eventually result in a meaningless exhaustion of ovarian follicle reserve^{13,17,18}. For these reasons, the vast majority of follicles in ovary will be eliminated before puberty, because the hypothalamic pituitary is not mature enough to produce enough gonadotropins¹⁹. In a word, inhibition of early folliculogenesis (*i.e.* primordial follicle activation, early follicle growth and atresia) can directly slow down the exhaustion of ovarian follicle reserve.

Studies have shown that the level of reactive oxygen species and inflammatory factors increase significantly in ovary during the function decline period^{20,21}. Based on this, many antioxidants such as VC, VE, N-acetyl-L-cysteine, curcumin, coenzyme Q10, proanthocyanidin, quercetin, and resveratrol have been tried to delay ovarian aging and achieved positive results²². Melatonin (MLT) is a natural antioxidant that originated 3.2-3.5 billion years ago, and has been endowed with new functions, such as rhythm regulation, sleep regulation, anti-tumor, immunoregulation, and regulation of glucose metabolism in the long evolutionary process^{23,24}. As the most

representational antioxidant, MLT is also used to delay ovarian aging ²⁵⁻³⁰. Studies have demonstrated that shortening the sunshine time can delay reproductive aging, which is considered to be related to MLT ^{31,32}; long-term supplementation of MLT in the drinking water of mice can reduce the production of ROS in the ovary, thereby increasing the number of follicles in middle age and delaying ovarian aging ^{26,28}. Supplementing middle-aged rats with MLT for 2 months during the reproductive decline period can help maintain a regular estrous cycle and normal estrogen level, and extend their reproductive age ³³; Clinical study has found that 6-month MLT treatment for perimenopausal and postmenopausal women can increase the serum LH levels and improve the pituitary and thyroid function ³⁴.

However, there are still several important issues to be elucidated with respect to the research topic of "MLT postpones ovarian aging". Firstly, previous studies have demonstrated that long-term intake of high-doses of exogenous MLT can effectively delay ovarian aging, so is endogenous MLT at physiological concentration involved in the regulation of early folliculogenesis and ovarian aging, and if so, to what extent? Secondly, in light of the free radical theory of aging, it was generally believed that the antioxidant benefit of MLT is the pivotal reason for delaying aging ³⁵. Nevertheless, unlike body aging, the root cause of ovarian aging in middle age is the exhaustion of the ovarian follicle reserve. So, in addition to the antioxidant properties, does MLT postpone ovarian aging by inhibiting the early folliculogenesis and then the loss of ovarian follicle? To address these issues, our research was conducted.

Results

Follicle-secreting MLT might play a regulatory role in early folliculogenesis

We first detected the expression and localization of SNAT in the prepubertal mice (PD5, 9, 17, 19). Immunohistochemical data indicated that SNAT was mainly distributed in the granulosa cells (GCs) of primordial follicle and early growing follicle (Fig. 1A). We next examined whether MLT can be synthesized in GCs. To this end, isolated GCs were incubated with or without 5-hydroxytryptamine (5-HT), the precursor of MLT and SNAT substrate. HPLC was then used to detect the conversion of 5-HT into MLT. In three cases, namely, no addition of GCs or 5-HT, the addition of GCs but no 5-HT, and the addition of 5-HT, no MLT was detected in the culture medium. When GCs and 5-HT were added at the same time, MLT absorption peak appeared in the chromatography. The above results indicated that the early growing follicle of mice had the capacity to synthesize MLT (Fig.1B).

To further examine the changes of ovarian MLT synthesis capacity during prepubertal folliculogenesis, we collected ovaries at PD5, 7, 9, 11, 13, 15, 17, 19, and 21. The qRT-PCR data showed that the overall expression level of *SNAT* in the ovary continued to decrease; *Fshr* and *Lhcgr* were the marker genes that reflected the process of folliculogenesis, and their expression levels gradually increased during the above process, which is opposite to the expression pattern of *SNAT* (Fig. 1C). Next, we examined the MLT level in ovarian homogenates at PD7, 9, 15, 17, and 19. The changes in MLT level, which gradually decreased with the increase of the age (Fig.1D), is similar to the expression pattern of *SNAT*. The above data suggested that MLT may be involved in the regulation of primordial follicle activation and early follicle growth.

Fig. 1 Follicle-secreting MLT might play a regulatory role in early folliculogenesis

(A) Location of SNAT in prepubertal ovaries was determined by immunohistochemistry, the scale bar = 200 μ m, and two sections from each day were used for the immunohistochemistry assay. (B) Detection of the ability of GCs to synthesize MLT. *Up*: experimental design; *Down*: representative chromatograms showing metabolites extracted from culture medium alone (#1-3), culture medium with 5-HT in the absence of GCs (#4-6), culture medium with GCs but no 5-HT (#7-9), culture medium with GCs and 5-HT (#10-12). Only GCs incubated with 5-HT show detected levels of MLT (#10-12, retention time 4.45 min). Four samples in each group were detected. (C) The expression profiles of *SNAT*, *Fshr* and *Lhcgr* in prepubertal ovaries were determined by qPCR. Three samples in each group were used for gene quantification. (D) MLT level in ovary homogenate. 4-6 samples in each group were detected. Values are mean $\pm$ S.E.M. The different superscript letters (a-b) represent a significant difference, $P < 0.05$. “*” represents significant differences, $P < 0.05$; “***” represents extremely significant differences, $P < 0.01$.

MLT supplementation inhibited early folliculogenesis in *in vitro* ovary culture system

After the establishment of PreOR of mouse, primordial follicles started to activate around PD3, which makes neonatal ovary an ideal model to study primordial follicle activation. To investigate whether MLT is involved in the regulation of primordial follicle activation in *in vitro* culture system, ovaries from mice at PD3 were cultured *in vitro* for 96h, and then the number of activated follicles with and without MLT treatment were counted (Fig. 2A). The result showed a remarkable decrease in the number of activated follicles after both 10^{-8} and 10^{-7} M MLT treatment. Additionally, 10^{-7} M MLT can also reduce the proportion of atresia follicles, but the difference was not significant (Fig. 2B). The above data indicated that MLT can inhibit the primordial follicle activation.

To further study the effect of MLT on early follicle growth, ovaries from mice at PD10 were isolated and cultured *in vitro* for 96h. Then the proportions of follicle beyond type5a-stage in the ovary with and without MLT treatment were counted respectively (Fig. 2C). It was found that 10^{-7} M MLT can reduce the proportion of follicles beyond type5a-stage, but the difference was not significant ($P = 0.092$). In addition, MLT treatment had no obvious effect on the number of atresia follicles at this phase (Fig. 2D).

Fig. 2 MLT supplementation inhibited early folliculogenesis in *in vitro* ovary culture system

(A) Experimental design and identification standards of the activated and atretic follicle. (B) The effect of MLT supplementation on follicle activation (values are mean $\pm$ S.E.M) and atresia. *Up*: the representative photographs of HE staining in each group, the scale bar = 100 μ m; *Down*: the statistical charts of activated follicles and atretic follicles. 8-12 sections in each group were counted. (C-D) The effect of MLT supplementation on early follicle growth and atresia; (C) Experimental design; (D) *Up*: the representative photographs of HE staining, the scale bar = 200 μ m, *Down*: the statistical charts of follicles beyond type5a-stage and atretic follicles. Ten sections in each group were counted. The different superscript letters (a-b) represent a significant difference, $P < 0.05$.

In vivo treatment with MLT suppressed the activation of primordial follicle though PI3K-AKT-FOXO3 pathway

To further study the effect of MLT on primordial follicle activation *in vivo*, the mice were injected with different doses of MLT starting from PD3 to PD9. The result showed that 0.1 mg/kg dose of MLT had no obvious effect on the number of activated follicles, and 1 mg/kg and 15 mg/kg doses of MLT significantly reduced the number of activated follicles ($P < 0.01$). This indicated that exogenous MLT intake can inhibit primordial follicle activation in PreOR. Unlike *in vitro* culture, the number of atretic follicles in the ovary *in vivo* was small, and MLT had no significant effect on the rate of follicular atresia (Fig. 3A). For the convenience of research, we used 15mg/kg as the optimal dose of MLT for subsequent experiments.

The mTORC1 and PI3K-AKT-FOXO3 are the pivotal pathways that control primordial follicle activation. Generally, Foxo3 localizes to the oocyte nucleus in the dormant follicle, but exports to the cytoplasm to activate primordial follicle when phosphorylated by PI3K-AKT signaling, and mTORC1 signaling plays an important role in triggering the PI3K-AKT signaling^{36,37}. To further elucidate the mechanism of how MLT inhibits primordial follicle activation, the above pathways were determined by western blot and immunofluorescence. On PD6, MLT treatment did not affect the active state of the *mTOR* pathway but significantly suppressed the phosphorylation of AKT ($P < 0.05$). Similar to PD9, MLT treatment did not affect the active state of the *mTOR* pathway but significantly suppressed the PI3K-AKT pathway ($P < 0.05$) (Fig. 3B). Immunofluorescent staining demonstrated that MLT treatment remarkably suppressed nuclear exclusion of Foxo3 in oocyte of primordial follicle (PD6, $P=0.0221$; PD9, $P=0.0235$) (Fig. 3C). These data indicated that PI3K-AKT-FOXO3 cascade is the target of MLT action on primordial follicle activation. It was reported that mTORC1 signaling in primordial follicle plays an important role in triggering PI3K-Akt pathway. Nevertheless, in this study, MLT administration did not affect the mTORC1 signaling. Therefore, the mechanism

of how MLT inhibits the PI3K-Akt-Foxo3 cascade remains to be further elucidated.

We also studied the antioxidant capacity of MLT in mouse ovary, and observed that short-term MLT intake significantly upregulated the expression of *SOD1* in the ovary ($P = 0.0421$) (Fig. 3D), and elevated the serum levels of GSH-PX ($P < 0.01$), SOD, CAT and T-AOC ($P < 0.01$). Consistent with the change in antioxidants, the level of MDA was significantly decreased by MLT ($P < 0.01$) (Fig. 3E). These data indicated that MLT can effectively improve the antioxidant capacity of the ovary, which is consistent with previous reports.

Fig. 3 *In vivo* treatment with MLT suppressed the activation of primordial follicle through PI3K-AKT-FOXO3 pathway
(A) The effect of MLT injection on follicle activation and atresia. *Left*: experimental design

and representative photographs of *H&E* staining in each group; *Right*: the statistical charts of activated follicles (values are mean±S.E.M) and atretic follicles. 10-12 sections in each group were counted; the scale bar = 100 μm; (B) The effect of MLT injection on *mTOR* and *PI3K-Akt* pathways in ovaries at PD6 and PD9. *Left*: western blots of p-Rps6kb1, Rps6kb1, p-Eif4ebp1, Eif4ebp1, p-Akt and Akt expression in the two groups; *Right*: the densitometry of western blots, the relative intensity of phosphorylated proteins was measured and normalized by the amount of the respective total proteins (values are mean±S.E.M). (C) The effect of MLT injection on Foxo3 translocation in the oocyte at PD6 and PD9. The “red arrow” indicates cytosolic localization, the “yellow arrow” indicates nuclear localization, the scale bar = 100 μm. 5-12 sections in each group were used for immunofluorescence analysis (values are mean±S.E.M). (D) The effect of MLT injection on the expression of antioxidant genes in the ovary, 4-5 samples in each group were used for gene quantification (values are mean±S.E.M). (E) The effect of MLT injection on the serum levels of GSH-PX, SOD1, CAT, MDA and T-AOC. Five samples in each group were used for detection (values are mean±S.E.M). Significant differences are donated by **P* <0.05, and ***P* <0.01.

In vivo treatment with MLT inhibited early follicle growth and atresia

To further study the effect of MLT on early follicle growth and atresia *in vivo*, we performed continuous MLT injection on mice from PD10 and collected ovaries at PD15, 17, 19, and 21 for histological analysis (Fig. 4A). At PD15, compared with the control group, the proportion of type5b follicles and atretic follicles in the MLT treatment group was significantly reduced (*P* <0.01); at PD17, the proportion of type5b follicles and atretic follicles in the MLT treatment group was extremely significantly reduced (*P* <0.01), and the proportion of SAFs was also significantly reduced (*P* <0.05); at PD19, the proportion of type5b follicles in the MLT treatment group was significantly reduced (*P* <0.05), and the proportion of SAFs and atretic follicles was extremely significantly reduced (*P* <0.01); and at PD21, the proportion of type5b follicles and atretic follicles in the MLT treatment group was significantly reduced (*P* <0.05), and the proportion of SAFs was extremely significantly reduced (*P* <0.01); The effect of MLT intake on the growth of oocytes in follicles was also examined. It was found that the diameter of oocytes in early growing follicles did not show significant differences after MLT treatment. The expression levels of *Fshr* and *Lhcgr* gradually increased with the growth of early follicle, therefore the expression levels of the two genes can indirectly reflect the number of follicles in the

advanced development phase of the ovary. The results of qRT-PCR showed that MLT intake significantly reduced the expression levels of *Fshr* and *Lhcgr* in the ovaries at PD17 and PD19 ($P < 0.05$) (Fig. 4B), which is consistent with the histological data.

The formation of small follicular antral means that they can respond to GTH stimulation, so the number of oocytes ovulated directly reflects the number of SAFs in the ovary. Therefore, to obtain further phenotypic evidence that MLT inhibits early follicle growth, superovulation was performed. The result was partially consistent with the histological data. At PD15, the mice could not yet respond to superovulation stimulation; and at PD17, the mice began to respond to the stimulation. There was no significant difference in the number of oocytes ovulated between the control group and the MLT treatment group; at PD19, the number of oocytes ovulated in the MLT treatment group was significantly lower than that in the control group ($P = 0.0431$) (Fig. 4C). In addition, the decrease in the number of SAFs means the decrease in the number of follicles involved in periodic recruitment, which indirectly leads to the decrease in the number of implantation embryos after breeding. Therefore, to further demonstrate the inhibitory effect of MLT on early follicle growth, the continuous MLT treatment was performed on PD10-21 mice, breeding was conducted after their vagina opened (a sign of puberty onset), and the quantity of implantation embryos was counted (Fig. 4D). The results showed that the number of implantation embryos in the MLT intake group was significantly smaller than the control group ($P = 0.0354$), which is also consistent with the section data, and the embryo size was bigger than the control group ($P < 0.01$). Of note, the embryos in the MLT intake group showed unequal distribution on both uterine horns, and the cause is still unclear.

The above data collectively indicate that exogenous MLT intake restricts the size of DOR and inhibits its atresia. To further explore the possible molecular mechanism, we performed RNA-Seq. The results showed that 598 genes in ovaries at PD17 exhibited differential expression (Fig. 4E,F). GO

enrichment analysis showed that the differentially expressed genes were mainly clustered into “Negative regulation of cell proliferation and differentiation”, “Regulation of cellular response to growth factor stimulus”, and “Blood vessel development”, (Fig. 4G). We further validated the accuracy of GO analysis. The proliferation activity of GCs was suppressed by MLT, as PCNA, the proliferation marker, was down-regulated in MLT-treated group ($P=0.0291$) (Fig. 4G). The KEGG heatmap showed that the differentially expressed genes were mainly enriched in the typical pathways associated with folliculogenesis, including “PI3K-AKT signaling”, “MAPK signaling”, “VEGF signaling”, “Pathways in cancer” and “TGF-beta signaling pathway” (Fig. 4H). It was suggested that, in “Pathways in cancer”, 17 genes were significantly induced by MLT, and all demonstrated a downward trend; 11 genes in “PI3K-Akt signaling” were significantly induced, and nine of them demonstrated a downward trend; and 11 genes in “MAPK signaling” were significantly induced, and nine of them demonstrated a downward trend. We further validated KEGG analysis by using a western blot, and the result demonstrates that “PI3K-Akt” signaling was suppressed by MLT, as the phosphorylation of Akt was decreased in the MLT-treated group ($P=0.0048$).

Fig. 4 *In vivo* treatment with MLT inhibited early follicle growth and atresia

(A) The effect of MLT injection on early follicle growth and atresia, and oocyte growth. *Left:* experimental design and representative photographs of H&E staining in each group. The scale bar = 200 μ m, 33-38 sections in each group were used for this analysis. *Right:* the

statistical charts of the proportion of early growing follicles at different stage and atretic follicles, and oocyte diameter (values are mean $\pm$ S.E.M.). (B) The effect of MLT injection on the expression of *Fshr* and *Lhcgr*. 3-4 samples in each group were used for gene quantification (values are mean $\pm$ S.E.M.); (C) The effect of MLT injection on the quantity of ovulation after superovulation treatment. *Up*: The representative photographs of reproductive organs in each group after superovulation; *Down*: The statistical chart of the ovulation quantity (values are mean $\pm$ S.E.M.). (D) The effect of MLT injection on the quantity of implanted embryos after natural mating. *Up*: the representative photographs of implanted embryos in each group; *Down*: the statistical charts of the number, size and distribution of implanted embryos. Con: N = 21, MLT: N = 25. (E) The heatmap of all the differentially expressed genes after MLT injection. (F) Volcano map; (G) GO enrichment analysis of differentially expressed genes, and validating the effect of MLT injection on PCNA by immunohistochemistry and qPCR (values are mean $\pm$ S.E.M.), respectively. The typical biological process (BP) associated with folliculogenesis is marked in red, the scale bar = 200 μ m. Three sections in each group were used for immunohistochemistry assay, and four samples were used for gene quantification. (H) KEGG enrichment analysis of differentially expressed genes, and validating the effect of MLT injection on PI3K-AKT pathway by western blot. *Left*: KEGG analysis of differentially expressed genes, the typical pathways associated with folliculogenesis are marked in red; *Middle*: interaction and overlapping of associated molecules among significant pathways, the “blue circle” represents downregulation and “red circle” represents upregulation; *Right*: validating the effect of MLT injection on PI3K-AKT pathway by western blot (values are mean $\pm$ S.E.M.). Significant differences are donated by * P <0.05, and ** P <0.01.

SNAT-knockout in mice accelerated the exhaustion of ovarian follicle reserve and age-related fertility decline

To evaluate the effect of endogenous MLT on follicle activation, atresia, and ovarian aging, we prepared *SNAT* knockout mice, in which a total of 1440 bp genomic sequences, including all exons 3 and most of exons 2 and 4 in *SNAT*, were deleted (Fig. 5A). We then counted the effect of *SNAT* knockout on follicle activation and atresia (Fig. 5B). It was found that the number of activated follicles could be slightly increased after *SNAT* knockout, and the difference was significant (P <0.05). After MLT supplementation, the increase in the number of activated follicles caused by *SNAT* knockout can be completely reversed (P <0.01). TUNEL staining validated that obvious apoptosis could be observed in the ovary of both wild type and *SNAT* knockout mice, but after MLT supplementation, apoptosis was reduced. Consistent with the TUNEL results, the number of atretic follicles in the *SNAT*-KO ovary

increased with respect to the wild type, but the difference was not significant. After MLT supplementation, the number of atretic follicles in *SNAT* knockout mice decreased significantly ($P < 0.05$). We further examined the effect of *SNAT* knockout on the distribution of FOXO3 in oocytes. It was also observed that *SNAT* knockout significantly enhanced the transport of Foxo3 to the ooplasm ($P < 0.05$). By contrast, MLT administration effectively alleviated the influence of *SNAT* depletion on Foxo3 transportation ($P < 0.05$) (Fig. 5C). The above data further suggested that endogenous MLT can reduce the activation of primordial follicle, and inhibit follicular atresia by reducing apoptosis.

Since MLT can affect the activation, growth and atresia of follicles, will it affect ovarian aging? To this end, we first analyzed the changes of litter size in mice with age after *SNAT* knockout (Fig. 5D). At the age of 2-3 months, *SNAT* knockout had no significant effect on the litter size; as the age increased to 5-6 months, the litter size in *SNAT* knockout mice was significantly smaller than that in the wild type ($P = 0.0398$); and at 8-9 months the litter size in *SNAT* knockout mice was extremely significantly smaller than that in the wild type ($P = 0.0028$). The degree of ovarian aging can be reflected by the number of growing follicles in ovary, so we counted the number of growing follicles in the ovary of 11-month-old mice. Compared with the wild type, the *SNAT* knockout mice showed a significant decrease in the number of follicles in the ovary ($P = 0.0018$) (Fig. 5E). *Gdf9*, *Nobox*, *Figla*, *Ddx4* and *Zp3*, which solely expressed by oocyte, were the marker genes that reflected the total number of primordial follicles in ovary. Among them, the expression levels of *Gdf9*, *Nobox*, *Figla* and *Zp3* in *SNAT* knockout ovary were group was significantly lower than that in the wild type ($P < 0.05$) (Fig. 5F), which suggested that the number of primordial follicles in *SNAT* knockout ovary was lower than wild type. The above phenotypic, histological and molecular data collectively indicated that MLT deficiency accelerated ovarian aging by speeding up the age-related exhaustion in ovarian follicle reserve.

Fig. 5 *SNAT*-knockout in mice accelerated the exhaustion of ovarian follicle reserve and age-related fertility decline

(A) Schematic diagram of *SNAT* knockout in the mouse genome. (B) The effect of *SNAT* knockout and MLT therapy on follicle activation and atresia. *Upper left*: experimental design and representative photographs of *HE* staining in each group, the scale bar = 50 μ m, 25 sections in each group were counted; *Upper right*: the statistical chart of activated follicles (values are mean $\pm$ S.E.M); *Left bottom*: TUNEL staining of ovary samples in each group, the “green dot” represents apoptosis-positive cell. The scale bar = 100 μ m, five sections in each group were counted; *Lower right*: the statistical chart of atretic follicles. (C) The effect of *SNAT* knockout and MLT therapy on Foxo3 translocation in the oocyte. The “red arrow” indicates cytosolic localization, 9-10 sections in each group were used for immunofluorescence analysis (values are mean $\pm$ S.E.M). The scale bar = 100 μ m. (D) The

effect of *SNAT* knockout on litter size (values are mean±S.E.M). (E) Morphology of ovaries from *SNAT* knockout and wild type group was analyzed by *H&E* staining at 11 months of age. *Left*: the representative photographs of *H&E* staining in each group; *Right*: the statistical chart of early growing follicle (values are mean±S.E.M). The scale bar = 200 μm, 15-20 sections in each group were counted. (F) Real-time PCR of oocyte marker genes in *SNAT* knockout and wild type groups. Three samples in each group were used for gene quantification (values are mean±S.E.M). The different superscript letters (a-c) represent a significant difference. “*” represents significant differences, $P < 0.05$; “**” represents extremely significant differences, $P < 0.01$.

Long-term ingestion of excessive MLT did not disturb the reproductive rhythm, growth and health condition of mice

In the present study, both 1.0 mg/kg and 15 mg/kg MLT were effective in inhibiting follicle activation. However, these doses are far above the physiological dose of MLT in the plasma³⁸, and this is a problem that cannot be ignored. Therefore, the safety of high-dose MLT was assessed in this section. We firstly studied the effect of MLT on mice physical growth. It was observed that long-term intake of 15mg/kg MLT neither affected body development nor visceral index (Fig. 6A, B). Subsequently, we evaluated the effect of MLT on reproductive rhythm, the length of the estrous cycle, mating timings, length of pregnancy, labor timings. The results showed that all above indexes were not affected by long-term administration of 15mg/kg MLT (Fig. 6C-F). To evaluate the effect of MLT on the health condition of mice, the rectal temperature and blood biochemical indexes were measured. The rectal temperature was not affected by 15mg/kg MLT (Fig. 6G); RBC, MCV, HGB, MCHC and MPV, the indexes that reflect the status of red blood cells and platelets, were not affected by 15mg/kg MLT. By contrast, W-LCC and W-MCC, the indexes that reflect the level of immune cells, were significantly decreased ($P < 0.05$) (Fig. 6H). All the results above indicated that there is no obvious safety risk associated with long-term ingestion of excessive MLT in adult animal, but note that high-dose MLT may affect the immunity of target animals.

Fig. 6 Long-term ingestion of excessive MLT did not disturb the reproductive rhythm, growth and health condition of mice

(A) The effect of MLT intake on the daily weight gain, eight mice in each group were used for this analysis. (B) The effect of MLT intake on the visceral indexes, 9-10 mice in each group were used for this analysis. (C) The effect of MLT intake on the estrous cycle, ten mice in each group were used for this analysis. (D) The effect of MLT intake on the mating timings of female mice, Con: N=10, MLT: N=11. (E) The effect of MLT intake on the pregnancy length. Con: N=9, MLT: N=10. (F) The effect of MLT intake on labor timing, ten mice in each group were used for this analysis. (G) The effect of MLT intake on the body temperature. Con: N=9, MLT: N=8. (H) The effect of MLT on plasma biochemical indexes, five mice in each group were used for this analysis. Values are mean $\pm$ S.E.M. “*” represents significant differences, $P < 0.05$.

Discussion

This study presented a novel insight into the roles of MLT in early folliculogenesis and ovarian aging

Cisplatin is a commonly used chemotherapeutic drug that excessively activates the PI3K-AKT pathway in the follicles during use, resulting in accelerated loss of PreOR. Studies have found that MLT can effectively inhibit the excessive activation of PI3K-AKT pathway caused by cisplatin, thereby protecting and stabilizing PreOR³⁹; in addition, MLT can also effectively relieve the interference of nicotine and DEHP on the establishment of PreOR in neonatal mice⁴⁰; and in this study, we also found that *SNAT* was expressed in both primordial follicle and early growing follicle, and demonstrated that GCs had the capacity to synthesize MLT (Fig. 1). Based on the above data, we speculate that MLT may directly delay ovarian aging by regulating the activation of follicles in PreOR, and the growth and atresia of follicles in DOR. Combined with *in vitro* and *in vivo* experiments and gene knockout models, this study demonstrated for the first time that MLT reduced the activation of primordial follicles, and reduced the growth and atresia of early follicle by inhibiting the PI3K-AKT pathway. These findings offer a novel insight into the roles of MLT in early folliculogenesis and ovarian aging, that is, MLT not only has the antioxidant properties, but also acts as a signaling molecule to postpone ovarian aging by slowing down the exhaustion of ovarian follicle reserve. In particular, we found that type 1 receptor of MLT was extremely lowly expressed in prepubertal mice ovaries, while type 2 receptor was not expressed (data not shown). Therefore, the specific way of MLT inhibiting PI3K-AKT signal in follicles is still unclear. In fact, MLT not only has two transmembrane receptors (MT1/2), but can also transmit signals through intracellular receptors GPR50, NQO2 and RZR/ROR α ⁴¹. Recent studies have found that MLT can also play a physiological role by binding to the vitamin D3 receptor VDR⁴². Therefore, further study is needed on how MLT inhibits PI3K-AKT signaling in follicles.

This study revealed that endogenous MLT is involved in the regulation of ovarian aging

Cell culture and gene knockout data indicated that follicular cells had the ability to secrete MLT (Fig. 1B), the loss of follicular reserve in MLT-deficient mice was faster than that of the wild type, and the phenotype of ovarian aging was more obvious in middle age (Fig. 5). These results collectively indicated that endogenous MLT was indeed involved in the regulation of ovarian aging. In particular, we found that MLT-deficient mice did not exhibit premature ovarian failure or other serious abnormal phenotypes, and the lack of MLT did not significantly affect the fecundity of young mice. This suggested that MLT was not necessary for folliculogenesis and ovarian aging, that means, the above process can still be carried out without MLT. We speculate that because MLT has many physiological functions, each of which cannot reach the level of completely interrupting the related physiological process. MLT is more like a lubricant for life activities: with it, the activities run more smoothly; without it, the activities can still be carried out, and it may take a long time to show the adverse impact. However, it should also be noted that the knockout mice used in this study are of the C57BL/6 strain, which had a natural mutation in MLT synthesis gene, resulting in a comparatively lower body MLT level than other strains⁴³. Therefore, the possibility that MLT deficiency has a more significant effect on reproductive activity in other strains cannot be excluded.

This study indicated that the ovary is another organ that secretes MLT

Studies have found that preovulatory follicular fluid contains high levels of MLT⁴⁴; oocytes synthesize MLT through mitochondria to resist free radical⁴⁵; *MT1* and *SNAT* in follicles are significantly induced before ovulation, and the MLT synthesized by cumulus cells is involved in the regulation of luteinization⁴⁶; *SNAT* and *MT2* are expressed in high abundance in human CL tissue. After MLT treatment, the balance of luteotrophic and luteolytic factors can be effectively maintained, thereby improving the CL function⁴⁷. Our study further demonstrated that follicular GCs can synthesize MLT (Fig. 1B). Therefore, even though the MLT that regulates follicle activation, growth, and atresia in

this study is unlikely to be entirely derived from GCs, we can still reasonably speculate that the ovary is another MLT secreting organ based on the above research progress.

This study provides a theoretical basis for using MLT to delay ovarian aging

With the further study of follicle activation, some substances (including rapamycin, bpV, ZCL278, Metformin and 2-DG) have been listed as candidate drugs to delay ovarian aging or treat premature ovarian failure⁴⁸⁻⁵². For example, rapamycin can strongly inhibit the mTOR signaling, so it is used in cancer chemotherapy. Recently, researchers have significantly prolonged the reproductive age of mice by short-term injection of rapamycin⁵¹; as PTEN-PI3K-AKT signaling controls follicle activation, some doctors have activated the primordial follicles *in vitro* by adding the PTEN inhibitor bpV, which successfully allowed women who have premature ovarian failure gave birth to healthy children^{49,50}. But the above methods also have limitations. For example, during the administration of rapamycin, experimental animals had estrous cycle disruption, sudden weight loss, *etc.* because of its serious side effects; the activity of the PTEN-PI3K-AKT pathway is high in actively dividing tumor cells and stem cells, so both artificial suppression and activation will bring unpredictable risks to the body. In contrast, MLT has an advantage in terms of safety, as there are already commercial MLT health products on the market. This study also demonstrated that high-dose MLT intake that lasted for one month had no significant effect on the estrous cycle, reproductive rhythm, and physical development of experimental animals (Fig. 6). Nevertheless, the question of whether MLT can be used as a human ovarian health product needs further investigation. In this study, the minimum dose of MLT to inhibit follicle activation is 1 mg/kg, if converted to a human dose, it is about 50 mg per day, but the current recommended maximum intake of MLT products on the market is 10 mg/day. Therefore, the dosage intake of mice cannot be applied directly to humans. In addition, the average effective reproductive life of humans is 40 years, while the average of mice is 1.5 years. Therefore, it is

technically difficult to determine whether MLT can delay ovarian aging in humans. Even if MLT can delay ovarian aging in humans, the using method, optimal dose and potential side effects at that dose should also be reassessed.

CONCLUSIONS

In summary, this study demonstrates for the first time that endogenous melatonin is involved in the regulation of ovarian aging, and presents a novel insight into the roles of melatonin in early folliculogenesis and ovarian aging, that is, in addition to its well-known antioxidant properties, melatonin as an endogenous hormone slows down the exhaustion of ovarian follicle reserve and ovarian aging by directly inhibiting primordial follicle activation, early follicle growth and atresia (Fig. 7). This discovery helps us to understand the functions of melatonin in reproductive regulation comprehensively. It will be of great importance in future studies to ascertain whether MLT has the similar effects in humans and whether it can be used as a health product for delaying ovarian aging.

Fig. 7 Schematic illustration of a proposed model to explain MLT delaying ovarian aging

Methods

Animals

KM-strain mice were purchased from the Center for Animal Testing of Huazhong Agricultural University. *SNAT*-knockout (*SNAT-KO*) mice with C57BL6 background were obtained from Cyagen Biosciences (Guangzhou, China). The genotyping method of indentifying *SNAT-KO* mice is as follows: Primers F1 (5'-GATGAACGCCAGACTCTCCCTC-3') / R1 (5'-AGCACAGTAAACAGAGCA GGCAG-3') were used for first PCR screening. The target allele has amplicon of 627 bp. Primers F2 (5'-AGCACAGTAAACAGAGCAGGCAG-3') / R1 (5'-AGCACAGTAAACAG AGCAGGCAG-3') were used for PCR reconfirmation. The heterozygote mice have two amplicons, while the homozygote mice have only one amplicon. Mice were housed in a temperature controlled facility ($24 \pm 2^{\circ}\text{C}$) with constant 12-h light–dark cycles. Animals were allowed access to food and water *ad libitum*. All experiments and animal handling were conducted in accordance with the institutional guidelines for animal experimentation after obtaining prior approval from the Institutional Animal Ethics Committee of Huazhong Agricultural University (HZAUMO-2017-035).

Experimental design and animal treatment

To assess the effect of MLT on primordial follicle activation *in vitro*, the ovaries from mice at postnatal day (PD) 3 were cultured with MLT (Sigma, St. Louis, MO, USA) for 96h, and then sectioned for histological analysis; To assess the effect of MLT on early follicle growth *in vitro*, the ovaries from mice at PD10 were cultured with MLT for 96h, and then sectioned for histological analysis; To assess the effect of MLT on primordial follicle activation *in vivo*, MLT were injected into mice from PD3. The ovaries at PD9 were sectioned for follicle count, the blood samples at PD9 were collected for the antioxidant assay, and the ovaries at PD6 and PD9 were collected for western blot and immunofluorescence assays; To assess the effect of MLT on early follicle

growth and atresia, MLT was injected into mice from PD10. The ovaries at PD15, 17, 19 and 21 were sectioned for histological analysis. The ovaries were collected for RNA-seq and western blot at PD17; To explore the effect of *SNAT* knockout on mice fecundity, the activated and atretic follicles were evaluated in juvenile mice. Adult mice were mated with wild-type males at the ages of 2-3 months, 5-6 months and 8-9 months respectively, and then the litter size was recorded. The ovaries from mice at the age of 11 months were collected for *H&E* staining and qRT-PCR assay.

The safety of MLT administration was evaluated. To study the effect of MLT on physical growth and health condition, 15 mg/kg MLT was administered to mice for 30 days. The rectal temperature was recorded for 4 days, and the body weight was recorded every day. On the last day, the weight of organs (heart, liver, spleen, and kidney) was measured and then divided by body weight to calculate visceral index. Additionally, the blood samples were collected for biochemical index assay; To examine the effect of MLT on reproductive rhythm, 15 mg/kg MLT was administered every day, and then the estrous phase was recorded through vaginal smear and the mating timings was also recorded. To examine the effect of MLT on pregnant rhythm, 15 mg/kg MLT was administered to mice when gestational age was over 12 days, and then the length of pregnancy and labor timings were recorded.

Histomorphological analysis

Paraffin-embedded ovaries were serial sectioned (5- μ m-thick paraffin sections); every fifth section was chosen for hematoxylin and eosin (*H&E*) staining. Thereafter, morphometric measurements were performed using digitalized images obtained directly from the microscope (Olympus BX53, Tokyo, Japan) through a video camera. Then, images were saved as *TIFF* graphic files. The morphometric parameters were measured using Image Pro-Plus software (Image Pro-Plus 6.0; Media Cybernetics, Silver Spring, MD, USA). Early growing follicles at different stages of development, including

primary (type 3), secondary (type 4, 5a, 5b), small antral follicles, and atretic follicles were counted in all sections based on the well-accepted standards established by Pedersen and Peters's criterion⁵³.

Ovary culture

Mouse ovaries were collected on the designated time by microdissection in preheating phosphate buffered saline (PBS). The formulas of ovarian culture medium were based on references^{54,55} and have been modified slightly. Briefly, the ovaries from mice at PD3 were cultured on an insert (PICM0RG50, Millipore, USA) in 6-well culture dishes in 1500 μ L Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (DMEM/F12) (GIBCO, Life Technologies, USA) containing 0.1% BSA (Sigma, St. Louis, MO, USA), ITS (1:100, Sigma, St. Louis, MO, USA) and Penicillin-Streptomycin Solution at an incubator (5% CO₂, 37°C and saturated humidity); The ovaries from mice at PD10 were cultured in the above medium supplemented with FSH (30ng/ml, Sigma, St. Louis, MO, USA). Ovaries were cultured for 96h in either medium alone or medium supplemented with 10⁻⁸M and 10⁻⁷M MLT.

MLT assay with high performance liquid chromatography (HPLC)

1ml syringe was used to puncture the follicles on the ovary surface to release the GCs and then washed to remove impurities. Isolated GCs were incubated with 10⁻⁵M hydroxytryptamine (5-HT) (Sigma, St. Louis, MO, USA) or culture medium alone (DMEM/F12 containing 0.1% BSA, 1.0% ITS) for 6h to test their MLT-biosynthetic capacity. Then mediums were dried down under nitrogen and were reconstituted in acetonitrile/ultrapure water (4/6) mixture. Ovaries were collected from mice at PD7, 9, 15, 17 and 19, respectively, and then homogenized immediately in 1mL pre-cooling methylalcohol (Sigma, St. Louis, MO, USA) and centrifuged at 12000 rpm for 10 min at 4°C. The supernatants were dried down under nitrogen and were reconstituted in acetonitrile/ultrapure water (4/6) mixture. The fragments of ovarian tissue were lysated with RIPA lysis buffer. Total proteins in fragments were measured with BCA Protein

Assay Kit (CWBiotech, Beijing, China) to normalize MLT concentration.

Chromatographic separation of MLT was performed on Agilent 1260 Infinity II HPLC system (Agilent, America) equipped with a variable wavelength detector. The analysis was carried out on InertSustain C18 column (250mm×4.6 mm, 5μm, GL Sciences Inc. Japan) with acetonitrile/ultrapure water (4/6) mixture flowing at a rate of 1mL/min. Sample temperature, injection volume and detection wavelength were 25 °C, 20 μL and 222 nm. The MLT standard was purchased from Sigma, and was subsequently diluted to different concentrations for testing the standard curve. The substances of interest were identified by their peak retention times compared to those of standards and were quantified based on peak height.

Measurements of antioxidant activity and lipid peroxidation

Blood samples of each group were collected according to the above design. After clotting for 30 min, the serum was obtained by centrifugation at 3000 rpm for 10 min and stored at -20°C. The levels of glutathione peroxidase (GSH-PX), catalase (CAT), superoxide dismutase (SOD1), malondialdehyde (MDA) and total antioxidant capacity (T-AOC) were measured as detected by radioimmunoassays (RIAs). The detection work was entrusted to Beijing North Institute of Biological Technology (<http://www.bnibt.com/>). The detection kits were all purchased from Jiancheng Bioengineering Institute (Nanjing, China).

Superovulation, mating and embryos statistic

Mice were injected with 5 I.U. PMSG (Ningbo Hormone Products Co., Ltd. Zhejiang, China) to stimulate follicle growth. A dose of 5 I.U. hCG (Ningbo Hormone Products Co., Ltd. Zhejiang, China) was then injected to trigger ovulation after 44 hr. Subsequently, mice were sacrificed, the reproductive organs were collected for visual observation, and the MII-stage oocytes were collected by puncturing the oviduct for quantitative analysis. Female mice were caged with adult males after vaginal opening. The day of the mating date was recorded as the first day of gestation. Female mice were sacrificed at day 8.5

of gestation. The uterus was collected for photographic documentation, and the size and distribution of implanted embryos were measured.

Measurement of biochemical indexes

The whole blood samples in each group were collected in anti-coagulation tubes. Thereafter, the biochemical indexes were analyzed by use of a blood cell analyzer (pochH-100iV Diff, Sysmex, Japan). The detection work was entrusted to the Veterinary Hospital of Huazhong Agricultural University. The test indexes included red blood cell count (RBC), corpuscular volume (MCV), hemoglobin (HGB) concentration, mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), mean platelet volume (MPV), white blood cell count (WBC), total lymphocyte count (W-SCC), granulocytic amount (W-LCC), intermediate cell amount (W-MCC), neutrophilic granulocyte percentage (W-LCR) and intermediate cell percentage (W-MCR).

RNA-Seq and data analysis

The sequencing and data annotation were completed by Novogene (<http://www.novogene.com/>). Briefly, total RNAs were extracted using the Trizol reagent. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA). To select cDNA fragments of preferentially 250-300 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Then, the library quality was assessed on the Agilent Bioanalyzer 2100 system. Gene expression was normalized as the fragments per kilobase of exon per million fragments mapped (FPKM). FPKM values were calculated in each RefSeq gene for differential expression analysis. The $P < 0.01$ obtained with the Audic Claverie test was considered to be statistically significant. Gene ontology (GO; <http://www.geneontology.org>) was used to annotate biological terms, and the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/>) was used to find the associated pathways.

Immunohistochemistry and immunofluorescence

Paraffin-embedded ovaries were subjected to routine 5- μ m thickness sectioning for immunohistochemistry. Briefly, for immunohistochemistry, the sections were incubated with rabbit polyclonal antibodies (SNAT: 1:100; ab3505, Abcam; PCNA: 1:100, GT2253, Genetech, Shanghai, China) overnight at 4°C; then, the sections incubated with secondary antibody (goat anti rabbit IgG: 1:200, G1213, Servicebio Technology, Wuhan, China) for 1 hr at 37°C. After washing, sections were incubated with streptavidin–HRP (CWBioTech Inc., Beijing, China) for 2 hr. Target protein was visualized using DAB (Sigma-Aldrich, Shanghai, China) as the chromogen. For immunofluorescence, the sections were incubated with rabbit monoclonal antibodies (Foxo3a: 1:200, 2497S, CST) overnight at 4°C. Afterward, the sections were incubated with fluorescent secondary antibody (goat anti rabbit IgG: 1:200, GB21303, Servicebio Technology, Wuhan, China) for 50 min, subsequently stained with DAPI for 5 min. The images obtained directly from ortho-fluorescence microscopy (Olympus Co., Japan).

Western blotting

Total protein was extracted with RIPA lysis buffer (Servicebiotech technology, Wuhan, China). Thereafter, proteins were separated via SDS–PAGE and then were subsequently transferred to polyvinylidene fluoride (PVDF) membranes. Subsequently, the membranes were incubated with primary antibodies for the following proteins: AKT (1:1000; 9272S, CST), p-AKT (Ser473; 1:1500; 4060S, CST), Rps6kb1 (1:1000; 9202S, CST), p-Rps6kb1 (Thr-389; 1:1000; 9205S, CST), Eif4ebp1 (1:1000; 9452S, CST), p-Eif4ebp1 (Ser-65; 1:1000; 9451S, CST), GAPDH (1:2000; CW0100, CWBiotech, Beijing, China), Actin (1:2000; CW0096, CWBiotech, Beijing, China). The membranes were then washed incubated with the corresponding secondary antibody (goat anti rabbit IgG: 1:4000; BF03008, Biodragon-immunotech, Beijing, China; goat anti mouse IgG: 1:4000, BF03001, Biodragon-immunotech, Beijing, China) for 2 hr. Finally,

the immunoblots were visualized with an ECL kit (CWBioTech, Beijing, China). The relative intensity of phosphorylated proteins (p-AKT, p-Rps6kb1, and p-Eif4ebp1) was measured by ImageJ software program and normalized by the amount of the respective total proteins.

Real-Time quantitative PCR analysis

Total RNA was extracted using the Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA). Reverse transcription was carried out by using the PrimeScript™ RT reagent kit with genome DNA Eraser (Takara Bio Inc., Dalian, China). Real-time qPCRs were run using a QuantiFast SYBR Green PCR Kit on a BioRad CFX Manager Machine (Bio-Rad, Hercules, CA, USA). Normalization was performed using the housekeeping gene *β-actin* as a control. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in supplementary *Table-S1*.

TUNEL Staining

TUNEL staining was utilized to detect cellular apoptosis in the ovaries from wild-type, *SNAT-KO* and *SNAT-KO+MLT* groups according to the manufacturer's protocol (*In Situ* Cell Death Detection Kit, Roche, Germany). Briefly, paraffin-embedded ovaries were subjected to routine 5-μm thickness sectioning. All sections were incubated in *TUNEL* reaction medium for 2h at 37°C in the dark. After the reaction was stopped, the sections were washed and stained with DAPI for 5 min. The *TUNEL*-positive cells in ovarian tissues from all of the groups were observed and analyzed using a fluorescence microscope (Olympus Co., Japan).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey post hoc test and unpaired two-tailed t-test was used to analyze the statistical significance among multiple groups and between two groups, respectively. The comparison between the percentages uses the chi-square test. *P*-value <0.05

749 was considered statistically significant, and P -value <0.01 was considered
750 statistically highly significant.

751 **Additional information**

752 Additional supporting information may be found online in the Supplementary
753 Material section at the end of the article.

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Figures

Figure 1

Follicle secreting MLT might play a regulatory role in early folliculogenesis

Figure 2

MLT supplementation inhibited early folliculogenesis in in vitro ovary culture system

Figure 3

In vivo treatment with MLT suppressed the activation of primordial follicle through PI3K AKT FOXO3 pathway

In vivo treatment with MLT inhibited early follicle growth and atresia

Figure 5

SNAT knockout in mice accelerated the exhaustion of ovarian follicle reserve and age related fertility decline

Figure 6

Long term ingestion of excessive MLT did not disturb the reproductive rhythm, growth and health condition of mice

Figure 7

Schematic illustration of a proposed model to explain MLT delaying ovarian aging

Supplementary Files

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