Original Paper

The Expression of Markers Related to Ovarian Germline Stem Cells in the Mouse Ovarian Surface Epithelium and the Correlation with Notch Signaling Pathway

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Key Words

Ovarian germline stem cells • Notch signaling • DAPT • Mice

Abstract

Background/Aims: Ovarian germline stem cells (OGSCs) have been shown to mainly exist in the ovarian surface epithelium (OSE), but the activity changes of germline stem cells during different reproductive stages and the potential regulatory signaling pathway are still unknown. The Notch signaling pathway plays a key role in cell development, primordial follicles and stem cell proliferation. However, whether it plays a role in the proliferation of OGSCs is unknown. Here, we analyzed the activity changes of germline stem cells and the correlation between germline stem cells and the Notch signaling pathway. **Methods:** The expression of germline stem cell markers Mvh, Ooc4 and the Notch molecules Notch1, Hes1, and Hes5 were detected during 3 days (3d), and 2, 12, 20 months (2m, 12m, 20m) mouse ovarian surface epithelium samples. DAPT, a specific inhibitor of the Notch pathway, was used to observe the influence of Notch signaling in the germline stem cells. **Results:** The results showed that the levels of MVH and OCT4 decreased substantially with reproductive age in ovarian surface epithelium, and the same tendency was detected in the Notch signaling molecules Notch1, Hes1 and Hes5. Dual-IF results showed that the germline stem cell markers were co-expressed with Notch molecules in the ovarian surface epithelium. While, the expression of MVH and OCT4 were reduced when the ovaries were treated with DAPT and the levels were attenuated with increasing dose of DAPT. **Conclusion:** Taken together, our results indicate that the viability of OGSCs decreased with the age of the mouse ovaries, and the activity of OGSCs in the ovarian surface epithelium may be related to the Notch signaling pathway.

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Published by S. Karger AG, Basel

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Introduction

As a highly conserved cell signaling pathway, the Notch signaling pathway plays a crucial role in cell fate determination, cell proliferation and differentiation [1, 2]. The Notch receptor is a single pass transmembrane protein, ligand proteins binding to the extracellular domain induce proteolytic cleavage and release of the Notch intracellular domain (NICD). Finally, the NICD moves into the nucleus to regulate the expression of Notch target genes [3]. Notch signaling pathway is necessary for cell-cell communication, which involves gene regulation mechanisms that control multiple cell proliferation and differentiation processes during embryonic and adult life. Previously, Notch signaling pathway is accepted as one of key pathway in the formation and development of primordial follicles [4, 5]. Recent studies have confirmed that the Notch signaling is involved in the proliferation and differentiation of various stem cells [6-8]. Especially, these researches have shown that the Notch signaling pathway is also involved in the regulation of germline stem cells in invertebrates [9, 10]. However, it is still unknown that the role of Notch pathway in germline stems cells of mammals ovary.

Compared with males, there is a limited reproductive cycle for female mammals during the whole life. In mouse ovaries, due to a large number of reproductive follicles that rupture at birth, the limited remaining germ cells are eventually wrapped in primitive granulosa cells and to form the primordial follicle pool [11, 12]. As we known, the traditional view may suggest that the ovarian function gradually decrease with the female ages. One of the major reasons is that the consumption of the primordial follicle pool is greater than its replenishment [13]. The balance between consumption and replenishment determines ovarian function and reproductive span, so the continuous replenishment to primordial follicle pool can maintain the ovarian function, delay premature ovarian aging and restore ovarian function.

The OSE has been confirmed to be a source of oocytes during the embryonic or fetal period, therefore, it is also commonly taken as the germinal epithelium. OGSCs or female germline stem cells (FGSCs) are a type of germ cell that possesses the abilities of proliferation and differentiation. In recent years, these cells have been proven to mainly exist in the OSE, which can generate oocytes to replenish the primordial follicle pool [14-16]. At present, OGSCs have been successfully isolated and cultured *in vitro* via the primary culture method from neonatal and adult mouse ovaries [16-18]. In addition, the OGSCs have also been successfully isolated from other postnatal mammalian ovaries [15, 19, 20]. Especially, the OGSCs from human ovaries were primary cultured and developed to a follicle-like cells *in vitro* [21, 22], which may provide a new therapeutic approach for ovary remodeling, infertility treatment and delaying female aging in future. As a tool, OGSCs had also been successfully used to construct transgenic animals [15, 23]. The discovery of OGSCs provides enormous clinical application prospect, however, there are still some problems that need to be solved before the potential application of OGSCs can be realized. As a source of oocytes, the OSE layer may play a considerable role in OGSCs propagation and development. And the number or growth of OGSCs in OSE layer will benefit to replenish germ cell and save the ovarian function. However, the number or proliferation of OGSCs changes throughout the reproductive life cycle. Consequently, it is very meaningful that OGSCs can maintain stability for ovary function. Previous studies have indicated that the Notch pathway involves in the formation and development of primordial follicles, the regulation of OGSCs in invertebrates, as well as tumor stem cell proliferation. Hence, considering Notch signaling pathway plays a key role in stem cell proliferation and follicle development, we leaded Notch signaling pathway into the research of OGSCs in mouse. Here, the aim of this study is to reflect the changes in the number or growth of OGSCs in mouse OSE and detect the expression variation of the core components of the Notch pathway during different stages of the reproductive cycle. Further, we try to explore whether or not the number or proliferation of OGSCs in the OSE is related to the Notch signaling pathway.

Materials and Methods

Animals

Kunming (KM) female mice were obtained from the Center of Experimental Animals, NanChang University, and they were used for all experiments; the mice had free access to food and water. All procedures involving mice were approved by the animal and ethical committee of NanChang University.

OSE collection and ovary culture

The intact ovaries of mice in different phases of the reproductive cycle were isolated and cleared from the ovarian appendage as much as possible. Then, the surface epithelium of whole ovary was collected, the whole protocol was performed according to previously described methods [24]. Briefly, the surface epithelial tissue of ovaries were collected by softly brushing the OSE with a small cytology brush under stereoscopic microscope, the fragment of epithelial tissues were washed with physiological solution and collected by centrifuge. In the end, the pellet was utilized to extract total mRNA and protein.

For ovary culture *in vitro*, the intact ovaries of postnatal 5- to 7-d-old mice were cultured using the Waymouth system (Sigma, USA) and supplemented with 10% (v/v) FBS (Gibco, USA), 0.23 mM sodium pyruvate and 1% penicillin and streptomycin (P/S) (Solarbio, China) [25]. In brief, the ovaries were removed as quickly as possible under sterile conditions, washed with pre-cooled D-Hanks solution and cleaned to remove the ovarian accessory and adipose tissue as much as possible. Then, the ovaries were allocated randomly into four groups and cultured on a gelatin sponge surface for 48 h with 600 µl of Waymouth medium supplemented with different concentrations of DAPT in a 48-hole plate (Nunc, Denmark). The medium was changed every 24 h with the replacement of half fresh medium. The culture experiment was repeated at least in triplicate.

Quantitative real-time PCR

Total RNA was extracted with Tri-reagent (Life Technology, USA) from the OSE of mice of different reproductive stages. The whole protocol was performed according to previously described methods [24]. For 3-d-old or 5- to 7-d-old ovaries, more than 10 ovaries were necessary to extract enough total RNA, and for 2-, 12- and 20-m-old ovaries, two ovaries were needed to extract sufficient total RNA. The cDNA was synthesized using a PrimeScript RT reagent Kit with a gDNA Eraser (TaKaRa, Japan). Amplification was performed on an ABI7000 PCR instrument (Applied Biosystems, Foster City, CA, USA) using three-stage program parameters according to the manufacturer's instructions: 2 min at 50°C, 2 min at 95°C, and then 40 cycles of 15 s at 95°C and 60 s at 60°C, followed by a final cooling step at 4°C. The primers designed for PCR are shown in Table 1. The reaction was carried out in a 20-µl volume containing 10 µl of SYBR green Master Mix (Life Technology, USA), 0.5 µl of cDNA, 0.6 µl of 50 mM MgCl₂, 1.6 µl of 10 µM primers, and sterilized

Table 1. Primer sequences used for quantitative real time PCR

Gene	Primer Sequence(5'-3')	Gene Number
<i>Notch1</i>	Forward: CAACCAGACAGACCGCACCG	NM_008714.3
	Reverse: CAGCAGCATCCACATTGTTCCACC	
<i>Hes1</i>	Forward: CCAGCCAGTGTCAACACGA	NM_008235.2
	Reverse: AATGCCGGGAGCTATCTTTCT	
<i>Hes5</i>	Forward: AGTCCCAAGGAGAAAAACCGA	NM_010419.4
	Reverse: GCTGTGTTTCAGGTAGCTGAC	
<i>Mvh</i>	Forward: GTGTATTATTGTAGACCAACTCG	NM_001145885.1
	Reverse: CACCCTTGTAATCTGTCTGAACT	
<i>Oct-4</i>	Forward: AGCTGCTGAAGCAGAAGAGG	NM_013633.3
	Reverse: GGTTCCTATTGTTGTCGGCT	
<i>Gapdh</i>	Forward: ACGTGCCGCTGGAGAAAC	NM_001289726.1
	Reverse: GTCCTCAGTGTAGCCCAAGATGC	

distilled water up to 20 μ l. All assays were independently repeated at least in triplicate, and GAPDH was used as the reference gene.

Immunohistochemistry (IHC) and immunofluorescence

Ovaries (n = 5) were individually fixed with 4% paraformaldehyde overnight. The total immunostaining protocol was performed according to previously described methods [26]. The primary antibodies used in this study are as follows: MVH (Abcam, ab27591), OCT4 (Proteintech, 11263-1-AP), NOTCH1 (Abcam, ab52627), HES1 (Abcam, ab108937), HES5 (Abcam, ab25374), and BrdU (Abcam, ab8152). All antibodies were diluted at 1:100 except Oct4, which was diluted at 1:200. All of the images were examined under a NIKON Eclipse 80i microscope.

Western blot analysis

Total protein was extracted from mouse ovaries using RIPA lysis solution (Beyotime, P0013C) according to the previous literature [27]. For 3-d-old or 5- to 7-d-old ovaries, owing to their small size, more than 100 ovaries were needed to extract sufficient total protein. For 2-, 12- and 20-m-old ovaries, more than 10 ovaries were needed to extract enough total protein. Briefly, the OSE was adequately ground using a PowerMasher (Optima INC), and the whole lysates were collected by centrifugation at 12,000 rpm for 15 m at 4°C. Then, the supernatants were mixed with the corresponding volume of 5 $\times$ loading buffer (Solarbio, China) and boiled for 5 min. The total proteins were then subjected to SDS-PAGE. After electrophoresis, the proteins were transferred onto a PVDF membrane (Millipore, USA). The membrane was blocked with 5% skim milk in TBST buffer for 20 m at room temperature and then immunoblotted overnight at 4 °C with the following primary antibodies: MVH (Abcam, ab13840, 1:500), OCT4 (Proteintech, 11263-1-AP, 1:500), NOTCH1 (Abcam, ab52627, 1:1000), HES1 (Abcam, ab108937, 1:1000), HES5 (Abcam, ab25374, 1:1000), and GAPDH (Abcam, ab37168, 1:2500). All experiments were repeated at least three times.

Statistical analysis

The quantitative data are shown as the mean $\pm$ SD. The statistical comparisons among the different age groups or DAPT groups were analyzed using either Student's t test to compare two conditions or ANOVA followed by planned comparisons of multiple conditions; $p < 0.05$ was considered to be significant, and $p < 0.01$ and $p < 0.001$ were considered extremely significant.

Results

The expression tendency of germline stem cell markers and key Notch pathway molecules in the mouse OSE

It has been previously confirmed by other studies that OGSCs are mainly located in the OSE [14, 16]. To detect the viability of germline stem cells and the activity of the Notch signaling pathway in mouse OSE at different stages, we selected five candidate genes, including Mvh, Oct4, Notch1, Hes1 and Hes5. We chose 3-d-, 2-m-, 12-m- and 20-m-old mice to analyze the mRNA and protein expression levels of these genes in the OSE. The real-time PCR results showed that the mRNA level of Mvh and Oct4 were the highest in 3-d-old mice and were extremely down-regulated in 2-m-old mice. There was little Mvh and Oct4 expression in 12- and 20-m-old mice ($p < 0.05$) (Fig. 1A). The key components of the Notch pathway (Notch1, Hes1 and Hes5) were significantly reduced along with the increasing reproductive age. However, compared with Mvh and Oct4, expression of these Notch pathway molecules was still observed at 12-m and 20-m ($p < 0.05$) (Fig. 1B). Next, we detected the protein level of the above candidate genes. The western blotting results showed similar trends in protein expression that correspond with the mRNA expression results ($p < 0.05$) (Fig. 1C, D).

Meanwhile, we gathered 3-d-, 2-m- and 20-m postnatal mouse ovaries. The Hematoxylin-sosin (H&E) staining indicated that there are some of very small epiblast-like stem cells (VSELs) in the OSE at 3-d and 2-m OSE, a few level at 20-m, and the results of a smear of OSE cells showed the same phenomena (Fig. 2A). The IHC results showed that MVH and OCT4 expression was highest in the OSE at 3-d, a higher level was observed at 2-m, and there was less or no staining at 20-m (Fig. 2B). In addition, we detected the expression of

Fig. 1. Expression of germline stem cell markers and notch pathway components in different stage of mice OSE. (A) Quantitative real-time PCR analysis for germline stem cell markers in 3-d-, 2-m-, 12-m and 20-m mice OSE. The expression of *Mvh* and *Oct4* were relatively highest level at 3-d, and displayed at least 60% decrease at 2-m, extremely decreased at 12-m and almost cannot be detected at 20-m. (B) compared with 3-d, *Notch1* mRNA level in 20-m displayed an 80% decrease, a 70% decrease in *Hes1* and a 90% decrease in *Hes5*. (C) Western blot analysis for the protein level of MVH and OCT4 in 3-d-, 2-m-, 12-m and 20-m mice OSE. D: The protein expression of notch pathway components NOTCH1, HES1 and HES5 in 3-d-, 2-m-, 12-m and 20-m mice OSE. The results present as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

Notch signaling components, the results showed that the expression levels of NOTCH1, HES1 and HES5 were localized in the OSE in all three age stages, but compared to the results at 3-d, there was an obvious decrease in expression at 2-m (Fig. 2C). Together, in this study it has been evidenced that the germinal marker *Mvh* (expressed also in OGSCs) along with *Oct4* was indeed expressed in the OSE, and these results indicate that the expression level of germline stem cell markers and key Notch pathway molecules in the OSE of mouse decreased with ovary age.

Fig. 2. The protein expression and dynamic changes of germline stem cell markers and Notch pathway components in OSE. The scale bars 20 μ m. (A) Hematoxylin-sosin staining the OSE and a smear of OSE cells in 3-d, 2-m and 20-m mice. Some VSELs present in OSE, but there are a decrease trend along with aging. (B) Immunohistochemistry analysis the expression of MVH and OCT4 in 3-d, 2-m and 20-m OSE. The arrow represents a positive cell. MVH and OCT4 were highest level in 3-d OSE, and a higher level was observed in 2-m, there was less or no staining in 20-m. (C) Notch pathway components expression in all three age stages OSE, but there were a decrease trend along with reproductive aging.

Germline stem cell markers and Notch components are co-expressed in the ovary cortical layer

We adopted dual-immunofluorescence histochemistry (Dual-IF) to detect the co-expression between germline stem cell markers and Notch molecules in the OSE. The Dual-IF results showed that MVH (green) and NOTCH1 (red) are co-expressed in the cortical layer of ovaries in a pattern that showed the strongest expression at 3-d, clearly decreased expression at 2-m and almost undetectable expression at 20-m (Fig. 3A). Similar co-expression patterns appeared for both MVH (green) and HES1, BrdU (red) (Fig. 3B, C). In addition, in cell level, we isolated OGSCs which showed less 20 μ m diameter and oval shape by primary culture using mice ovaries, and Dual-IF results showed that MVH and OCT4 are co-expressed in the OGSCs *in vitro* culture (Fig. 3D).

Inhibition of Notch signaling decreased the expression of germline stem cell markers

To further examine the correlations between OGSCs and the Notch pathway, 5- to 7-d-old mouse ovaries were treated with the specific inhibitor DAPT to block the Notch pathway. Mouse ovaries were divided into four groups: the DMSO group, the 10 μ M DAPT group, the 20 μ M DAPT group and the 40 μ M DAPT group. All groups except the DMSO group were treated with DAPT for 48 h. The real-time PCR results showed that compared with the DMSO group, the mRNA level of *Mvh* was slightly decreased in the 10 μ M DAPT group and decreased by nearly 50% in the 20 μ M group ($p < 0.05$). However, there was no further decrease in the 40 μ M group compared to the 20 μ M group. Meanwhile, the mRNA level of *Oct4* did not demonstrate a clear reduction in the 10 μ M group, but there was an evident decrease in the 20 μ M group ($p < 0.05$) and a much greater decrease in the 40 μ M group ($p <$

Fig. 3. The co-expression of germline stem cells marker and Notch components in ovary cortical layer. Dual-IF was performed to co-locate the MVH and Notch components in different reproductive stages OSE. The scale bars 20 μ m. (A) There was a strongest expression for MVH (green) and NOTCH1 (red) in 3-d OSE, a evidently decreased in 2-m and almost a undetectable expression in 20-m. (B and C) The similar co-expression patterns appeared both MVH, (green) and HES1, BrdU (red) in different reproductive stages. (D) Dual-immunocytochemistry results showed that MVH (green) and OCT4 (red) co-expressed in the OGSCs *in vitro* culture.

0.05) (Fig. 4A). In addition, western blot results showed that MVH protein expression was slightly decreased in the 10 μ M and 20 μ M groups, and there was an almost 50% decrease in the 40 μ M group ($p < 0.05$) (Fig. 4C). OCT4 protein levels demonstrated a successive down-regulation along with the increasing DAPT concentration (Fig. 4C). And we also measured the downregulation of Notch signaling following DAPT treatment, the results showed that the mRNA level of two key target genes Hes1 and Hes5 were a successive decrease with increasing dose of DAPT ($p < 0.05$) (Fig 4B). In general, target protein HES1 and HES5 also showed a downward trend ($p < 0.05$) (Fig 4D).

Attenuating Notch signaling decreased the activity of germline stem cells in the ovary cortical layer

Mouse ovaries were collected 5- to -7-d postnatal and treated with 20 μ M and 40 μ M DAPT for 48 h. Dual-IF was performed to detect the expression changes of MVH and OCT4 in the OSE. The results showed that compared with the DMSO group, the 20 μ M DAPT group showed a significant decrease, and a much greater decrease was observed in the 40 μ M group. The above results suggest that Notch signaling contributes to the viability of germline stem cells (Fig. 4E). Taken together, these findings suggest that the Notch pathway may relate to the viability or proliferation of germline stem cells in the OSE of mouse.

Fig. 4. Expression changes of *Mvh* and *Oct4* in OSE after treated with inhibitor DAPT. (A) Quantification real-time PCR analysis for the relative mRNA expression of *Mvh* and *Oct4* after treated ovaries with increasing dose of DAPT. Compared with DMSO group, the mRNA level of *Mvh* was decreased in 20 μ M group and 40 μ M group, and *Oct4* was an evident decrease in 20 μ M group and more decrease in 40 μ M. (B) The similar results were detected in Notch signaling pathway target gene *Hes1* and *Hes5*. (C) Western blot analysis results showed MVH protein displayed a little decrease in 10 μ M, 20 μ M groups, and an almost 50% decrease in 40 μ M group, *OCT4* protein level displayed a successive down-regulation. (D) Target protein *HES1* and *HES5* also showed a downward trend. (E) Dual-IF results showed that there was a well change in OSE after inhibited Notch pathway activity. The results present as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

Discussion

Mvh is the homolog of the *Drosophila vasa* gene, which is specifically expressed in all germ cell lineages and is known as a specific marker of reproductive cells [28, 29]. *Oct4* belongs to the POU family that plays a key role in embryonic development and stem cell pluripotency, it is specifically expressed in pluripotent stem cells [30-33]. Therefore, in general, *Mvh* and *Oct4* are taken as the markers of germ stem cells. The NOTCH1 surface receptor is a key molecule in the Notch pathway; their expression can prompt the activity of the Notch pathway [34, 35]. Hairless and enhancer of split 1 (*Hes1*) and hairy and enhancer of split 5 (*Hes5*) are the principal target genes of the Notch pathway, which regulate the proliferation and differentiation of cells [36, 37]. Hence, the expression levels of Notch1, *Hes1* and *Hes5* represent the activity of the Notch signaling pathway. Here, we selected *Mvh* and *Oct4* to represent the activity or proliferation of germline stem cells, and selected Notch1, *Hes1* and *Hes5* to represent the activity of Notch signaling pathway.

The replenishment of OGSCs and their consumption maintains the balance of the follicle pool [13]. Female mammals gradually lose reproductive ability and become infertile with ovary aging. One of the reasons for these changes in fertility is a decline of the ovarian somatic niche function. Besides, the consumption of the follicle pool is faster than the replenishment of the OGSCs, especially, a decrease in the number and proliferation ability of OGSCs in the OSE maybe accelerates the coming of menopause. Our results also show that the mRNA and protein levels of both Mvh and Oct4 decreased with ovary age. As shown in Fig. 1A, compared with the 3-d group, Mvh and Oct4 displayed decreased expression by more than half in the 2-m group, and there was very little expression in 12-m-old mice or, more dramatically, in the 20-m group. We demonstrated that the activity or proliferation of germline stem cells in the OSE will decrease as the age goes on, and our results explain one of the possible causes of infertility in old mice.

The OGSCs are mainly present in the surface epithelium, and they are characterized by their small number and low proliferation ability [21, 24]. According to ovary morphometric analysis results [13], we chose 3-d-, 2-m- and 20-m-old mouse ovaries as our experimental groups for the IHC study to represent the reproductive cycle of neonatal, adolescent and gerontal mice, respectively. The IHC results showed that both MVH and OCT4 were expressed at the highest levels in the OSE of 3-d-old mice. There was an evident decrease in 2-m-old mice, and expression was almost undetectable in 20 m old mice. The results suggested that the highest numbers and activities of OGSCs occur during the neonatal period, and these values decrease with increasing reproductive age (Fig. 2B).

The proliferation capacity of OGSCs has a direct impact on the maintenance and remodeling of ovarian function. However, at present, the proliferative mechanism of OGSCs is still unknown. Therefore, exploring the regulatory mechanism of OGSCs may help us develop methods to maintain the balance of the follicle pool and delay ovarian aging. As one of the most important cell signaling pathways, the Notch signaling pathway was detected to exist in both vertebrate and invertebrate ovaries. As a classical Notch signaling pathway, the CBF-1/RBP-Jk pathway plays a pivotal role in mammals [38]. Studies have shown that adjacent cells transfer Notch signals to each other via the binding of receptors and ligands through a signaling cascade to regulate cellular behavior, including, proliferation, development, organ formation and morphogenesis [38, 39]. In general, the ligand such as JAGGED, which can be combined with membrane receptor NOTCH1 to conduct signals, eventually activates the expression of the primary target genes Hes1 and Hes5; the latter was shown to regulate cell proliferation and differentiation [40, 41]. In fact, study indicated that Notch signaling is active in mouse ovary, the Notch components (Notch1,2 and 3) and effectors (Hes1 and Hes5) are differentially expressed during follicles and corpora lutea development throughout the oestrous cycle [42]. And some studies have also shown that Notch pathway is involved in the formation of primordial follicles and that there is an intimate connection between this pathway and mammalian ovarian function [4, 43]. As important components of notch pathway, the mRNA or protein expression levels of Notch1, Hes1 and Hes5 represent the Notch pathway activities. The real-time PCR results showed that Notch1, Hes1 and Hes5 are expressed in mouse ovaries of different phases, but they had their highest expression levels in 3-d-old ovaries and their weakest expression at 20-m, which confirmed that Notch pathway activity decreased with reproductive age. However, there is still detectable expression of Mvh or Oct4 in 12-m and 20-m-old mouse ovaries (Fig. 1B). At the protein expression level, the western blot results showed the corresponding decrease in expression with reproductive age (Fig. 1C, D). Moreover, the Notch signaling components displayed a similar trend compared with germline stem cell markers.

Is there a correlation between germline stem cells activity and the Notch signaling pathway? To address this question, we used 3-d-, 2-m- and 20-m-old mouse ovaries. The dual-IF results showed that MVH is co-expressed with NOTCH1 in the OSE, and both of them are expressed at their highest level in 3-d-old mice, followed by 2-m-old mice, and very little expression was observed in 20-m-old mice (Fig. 3A). The same results were observed for MVH and HES1 (Fig. 3B). In addition, for observing the proliferative activity in OSE, we also

detected the co-expression between MVH and BrdU, the results showed the strongest BrdU expression at 3-d, clearly decreased expression at 2-m and almost undetectable expression at 20-m (Fig. 3C). In a previous study, it was confirmed that OGSCs are mainly located in the OSE, so our results indicated that the Notch pathway may be involved in the regulation of germline stem cell proliferation in the ovarian cortical layer. The Notch signaling pathway was shown to participate in the formation of OGSCs niche and play a critical role in germline stem cell self-renewal and differentiation [10, 44]. To further confirm the link between germline stem cells and the Notch signaling pathway in mouse ovarian tissues, the 5- to 7-d-old mouse ovaries were isolated and treated with the inhibitor DAPT. As an inhibitor of γ -secretase, DAPT has been widely used to block the Notch signaling pathway [45, 46]. In our study, the mouse ovaries were continuously exposed to DAPT for 48 h. Compared with the control, all DAPT-treated groups demonstrated reduced mRNA and protein expression of Mvh and Oct4 (Fig. 4A,C). Our dual-IF results also showed that the DAPT-treated groups showed a slight attenuation of MVH and OCT4 expression in the OSE compared with the DMSO group (Fig. 4E). Therefore, our data suggested that the Notch signaling pathway may be a potential pathway involved in the regulation of germline stem cells.

In the present study, we mainly focused on OSE studies. As a next step, we will prepare to explore the correlation between the Notch pathway and OGSCs at the cell level, and cell experiments will further clarify the regulation mechanism of OGSCs. At present, we have isolated and cultured the OGSCs via the primary culture method *in vitro*, and dual-IF results showed that MVH and OCT4 are co-expressed in the OGSCs (Fig. 3D). Next, we will prepare to detect and explore the proliferation changes after activating or inhibiting the Notch signaling pathway at the cellular level.

In conclusion, our data showed that the numbers or activity of OGSCs in OSE will decrease along with the increased reproductive age. And the activity of germline stem cells in the mouse OSE is related to the Notch signaling pathway. We hope our study provides some novel evidence that the Notch signaling pathway may be a key pathway in regulating the proliferation of germline stem cells in mammals.

Acknowledgments

We thank all of the members of our lab. This work was supported by the National Natural Science Foundation of China (No. 81160081, No. 81360100, No. 81260098), the Excellence 555 Engineering of Jiangxi Province, the Natural Science Foundation of JiangXi Province (No. 20142BAB205069, No. 20142BAB205002) and the Department of Public Health of JiangXi Province (No.20121156).

Disclosure Statement

None.

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