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Research Paper

Ubiquitin carboxyl-terminal hydrolase L1 contributes to the oocyte selective elimination in prepubertal mouse ovaries

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Abstract: Apoptosis of abnormal oocytes is essential for defective oocyte elimination during prepubertal ovary development, and the ubiquitin system regulates the cell apoptosis via the degradation of specific proteins. Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is a component of the ubiquitin system, and the UCH-L1-dependent apoptosis is important for spermatogenesis. In the present study, the change in the number of follicles and the expression of UCH-L1 in oocytes were determined in prepubertal mouse ovaries by immunohistochemical techniques. A significant decrease in the follicular pool was found in prepubertal mouse ovaries during the period of day 21 to day 28 after birth, and accordingly, the UCH-L1 protein expression was increased, to some degree in association with Jun activation domain-binding protein 1 (Jab1) and cyclin-dependent kinase inhibitor $p27^{Kip1}$. The increased UCH-L1 protein, together with the corresponding changes of Jab1 was detected in morphologically abnormal oocytes of prepubertal ovaries. Through the immunofluorescent colocalization, UCH-L1 was shown concentrating in abnormal oocytes, and a parallel change in Jab1 was also seen. The affinity analysis confirmed the interaction between UCH-L1 and Jab1 in ovaries. These results suggest that UCH-L1 plays an important role, possibly in association with Jab1 and $p27^{Kip1}$, in selective elimination of abnormal oocytes during mouse prepubertal development.

Key words: ubiquitin carboxyl-terminal hydrolase L1; oocyte; $p27^{Kip1}$; Jab1

泛素羧基末端水解酶 L1 参与性成熟前小鼠卵母细胞的选择性剔除

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摘 要：异常卵母细胞的凋亡是性成熟前小鼠卵巢中具有缺陷的卵母细胞剔除的必要途径。泛素羧基末端水解酶 L1 (ubiquitin carboxyl-terminal hydrolase L1, UCH-L1)参与构成的泛素依赖性蛋白降解系统通过降解特定蛋白调节细胞凋亡，而 UCH-L1 依赖性的细胞凋亡对于睾丸的生精作用是必要的。本文利用免疫组化技术考察了性成熟前小鼠卵巢中不同发育阶段的卵泡数目的变化和 UCH-L1 在卵母细胞中的表达情况，结果显示卵巢中卵泡总数在出生后第 21 至 28 天显著减少；而 UCH-L1 蛋白在形态上出现异常的卵母细胞中表达显著增强，可能以某种程度与 Jab1 和 $p27^{Kip1}$ 蛋白关联。免疫荧光共定位显示 UCH-L1 在形态异常的卵母细胞中密度较高，而 Jab1 出现平行性的变化。免疫亲和分析显示卵巢中 UCH-L1 和 Jab1 发生作用。 以上结果提示，UCH-L1 在小鼠性成熟前的发育过程中异常卵母细胞的剔除方面具有重要作用，其作用可能以与 Jab1 和 $p27^{Kip1}$ 蛋白相关联的方式发生。

关键词：泛素羧基末端水解酶 L1；$p27^{Kip1}$；Jab1；卵母细胞
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Oogenesis is a key event in sexual reproduction, by which the oocytes successively acquire their intrinsic abilities critical for fertilization and the development of a healthy embryo[1, 2]. In mice, oogonia start the first meiotic division and enter the oocyte stage while developing in the fetal ovary, and shortly after birth, the meiosis is arrested in the dictyate stage of late prophase in the oocytes of developing follicles. However, the population of the oocytes that finally reach the ovulatory stage is quite small in the ovary, and the fertile lifespan of a female depends on the size of the oocyte pool at birth and the speed of the oocyte pool depletion[3, 4]. Thus, a better knowledge of this prolonged phase of oogenesis is essential for understanding the causes of oocyte pathology and optimizing the methods for oocyte culture.

While the contribution of granulosa cells to oogenesis has been studied for many years, it has recently been found that the oocyte itself plays a key role in determining its own fate besides the growth and differentiation of the follicle[5-7]. Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), formerly named as protein gene product 9.5 (PGP9.5), with the new name for its ubiquitin carboxyl-terminal hydrolase activity[8, 9], is exclusively expressed in neurons, testis, ovary, and placenta, suggesting its critical roles in spermatogenesis and placental development[10]. In our previous studies, we found that the monoclonal antibody against tUCH, the UCH-L1 homolog of toad oocytes (*Bufo bufo gargarizans*), could specifically inhibit the germinal vesicle breakdown (GVBD), indicating that tUCH is required for oocyte maturation in toad[11]. However, the functional role of UCH-L1 in oogenesis remains unknown, although it has been well demonstrated that protein degradation is essential for oogenesis and the ubiquitin-proteasome system regulates many cellular processes via the rapid degradation of specific proteins[12]. Since apoptosis controls the number of oocytes and eliminates defective oocytes during oogenesis, and the UCH-L1-dependent apoptosis has also been found to be important for normal spermatogenesis and sperm quality control during prepubertal development[13, 14], we therefore suppose that UCH-L1 might play a role in the selective elimination of abnormal oocytes during prepubertal development of ovary.

In addition, it was previously reported that, Jun activation domain-binding protein 1 (Jab1) was involved in cytoplasmic degradation of proteins[15] and related to the nucleus-cytoplasm translocation of cyclin-dependent kinase inhibitor $p27^{Kip1}$[16-18]. Jab1 binds with UCH-L1 in tumor cells[19] and interacts with ubiquitin-like molecule NEDD8 during embryogenesis[20]; Interestingly, Jab1 is also required for meiotic progression, as well as the establishment of anterior-posterior (AP) and dorsal-ventral (DV) axes of oocyte in *Drosophila* . And the primordial follicle pools of ovaries in $p27^{Kip1(-/-)}$ mouse were prematurely activated once it was endowed, and the massive follicular death occured before sexual maturity could be rescued by the loss of $p27^{Kip1}$[23], suggesting that $p27^{Kip1}$ might be an important factor for prepubertal follicular selective apoptosis. Thus, we hypothesize that UCH-L1 might participate in the regulation of prepubertal oocyte selection in association with Jab1 and $p27^{Kip1}$. The present study was undertaken to determine the expression patterns of UCH-L1, Jab1 and $p27^{Kip1}$ in oocytes of prepubertal mice with a view to preliminarily explore the role of UCH-L1 in oogenesis.

1 MATERIALS AND METHODS

1.1 *Animals and tissue preparation*

Adult ICR mice aged 6-8 weeks were obtained from the SIPPR/BK Laboratory Animal Company (Shanghai, China). All of the mice were caged at controlled temperature (approximately 22 °C) under a 14 h light:10 h dark photoperiod. All the experimentation was in full compliance with standard laboratory animal care protocols approved by the Institutional Animal Care Committee of Shanghai Institute of Planned Parenthood Research. Adult females were mated with fertile males of the same strain to achieve pregnancy. The female offsprings were sacrificed by cervical dislocation respectively on postnatal day 0, 14, 21 and 28. The whole ovarian tissues were collected, to be frozen in liquid nitrogen and stored at –80 °C for Western analysis, or to be fixed for paraffin sections.

1.2 *Follicle counting*

According to the reported methods[24], 5 ovaries were used to prepare the paraffin sections. Observed under the stereomicroscope, largest typical sections of 5 ovaries at each developmental time points were then selected for follicle counting. Follicles were counted after anti-UCH-L1 immunostaining. Primordial follicles were identified as an oocyte partially surrounded by either only squamous granulosa cells or both squamous and cuboidal granulosa cells. Primary follicles were classified to contain a small oocyte completely surrounded by a single layer of cuboidal granulosa cells, whereas secondary follicles contain a larger oocyte and more than one layer of granulosa cells, usually with the presence of theca cells. Tertiary (small antral)

follicles were similar in size to secondary follicles, but contain an antrum. Antral follicles are the largest follicles and contain large antral spaces. For only a few antral follicles were present in the ovaries up to day 28 age, all follicles with an obvious antrum were counted as the tertiary follicles. SPSS13.0 was used to process the data. Data of each stage were from 5 sections representative of ovaries for 5 mice, expressed as mean±2SE, and the difference was considered as statistically significant at the 0.05 level using one-way analysis of variance (ANOVA). If the ANOVA shows significant difference between time-points, further multiple comparisons will be taken by Bonferroni method.

1.3 *Western blot analysis*

Frozen ovarian tissues were thawed in ice-cold protein extraction buffer [EB: 50 mmol/L HEPES (pH 7.5), 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, 25 mmol/L β-glycerophosphate (Merck), 1 mmol/L Na_3VO_4, 50 mmol/L NaF, 1 mmol/L EDTA, 0.5 mmol/L EGTA; 10 mg/mL each of soybean trypsin inhibitor (Sigma), leupeptin and aprotinin (Amresco), and 1 mmol/L PMSF (Sigma)], then homogenized by the glass dounce homogenizer. Solutions were subsequently centrifuged at 14 000 *g* for 10 min at 4 °C, and the supernatant was collected as the total protein extract. Protein concentration of each total protein extract sample was determined by Bradford assay.

Following a described protocol[25], 20 μg total protein extracts of each sample and LMW SDS-PAGE Markers (Shanghai Guanyu Industry Company) were subjected to reducing 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred onto a nitrocellulose membrane (Immobilion™-NC, Millipore Corporation, Billerica, MA, USA). The membrane was incubated with blocking solution [5% skim milk powder in TBS with 0.05% Tween-20 (Sigma, St. Louis, MO, USA)] for 1 h, and subsequently, incubated respectively with the rabbit anti-UCH-L1 sera (1:400, Zymed Lab. Inc., CA, USA), or the rabbit anti-Jab1 sera (1:200, Santa Cruz Biotechnology, Inc., CA, USA), or the rabbit anti-$p27^{Kip1}$ sera (1:200 diluted in blocking solution, Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Then, the membrane was washed and incubated with goat anti-rabbit IgG-AP (1:2 000 diluted in blocking solution, Zymed Lab. Inc.) for 1 h at room temperature. After further washing, the immunoreactive complexes on the membrane were visualized by staining with BCIP/NBT kit (00-2209, Zymed Lab. Inc.) according to its user's manuals for 5 min. β-actin was applied as a sample loading control by immunoblotting detection with the rabbit polyclonal antibody against it (Abcam, Cambridge, UK).

1.4 *The pull-down analysis for UCH-L1 binding with Jab1 in ovarian protein extracts*

Ovarian protein extract (500 μL, 1 mg total protein) from 28 days old mice was incubated for 2 h at 4 °C under constant rotation with 50 μL sepharose CL-6B (pre-washed with EB). After a centrifugation (1 000 *g*, at 4 °C for 2 min), the supernatant was removed and incubated with 20 μL glutathione Sepharose 4B beads (Pharmacia Biotech.) binding with glutathione *S*-transferase (GST), under constant rotation for another 2 h at 4 °C before obtaining the supernatant. The supernatant was incubated with glutathione Sepharose 4B beads (Pharmacia Biotech.) binding with GST-UCH-L1 fusion protein[26] for 4 h at 4 °C. The two kinds of beads were washed three times in EB and then suspended in 1×SDS-PAGE sample buffer. To determine whether Jab1 is absorbed specifically by UCH-L1, the supernatant was processed for SDS-PAGE and Western blotting immediately after boiling for 5 min and a brief centrifugation. The immunoreactive complexes, as above in Method 1.3, were detected by ECL method using PhosphaGLO™ AP Substrate kit (KPL, Gaithersburg, MD, USA).

1.5 *Immunohistochemistry*

Ovaries were directly fixed in Bouin's fixative for 24 h and then dehydrated in graded alcohol followed by embedding in paraffin (Beijing Chemical, Beijing, China). The adult mouse testes and ovaries were treated in the same method to test the specificity of our UCH-L1 antibody by comparing with reported results from other labs. Sections (5 μm) of ovarian tissues were deparaffinised and rehydrated. For antigen retrieval, the sections were boiled in a microwave oven (800 W) in 10 mmol/L citrate, pH 6.0 for 2 times of 5 min. Incubation with 3% H_2O_2 in PBS to abolish the endogenous horse radish peroxidase (HRP) activity. For immunohistochemical detection, after non-specific binding blockade for 1 h using 10% normal bovine serum in PBS containing 0.05% Tween-20 (PBST, pH 7.4), sections were incubated respectively with the rabbit anti-UCH-L1 sera (1:200, Zymed Lab. Inc.), or the rabbit anti-Jab1 sera (1:50, Santa Cruz Biotechnology, Inc.) with 10% normal bovine serum in PBS overnight at 4 °C. After washing in PBST three times, each for 5 min, sections were incubated with biotinylated bovine anti-rabbit IgG-B (1:200; Santa Cruz Biotechnology Inc., CA, USA). After washing for 3 times in PBST, the sections were incubated with biotin-conjugated bovine anti-rabbit IgG (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, followed

by strepavidin-HRP and DAB complex according to the manufacture's protocol (Liquid DAB-Plus Substrate Kit, Zymed Lab. Inc.). On some sections, before being added to the sections, the primary anti-sera were immunoneutralized respectively by recombinant GST-UCH-L1, prepared by our lab[26], and Jab1 peptide (Santa Cruz Biotechnology, Inc.) at 37 °C for 30 min as a negative control. Sections were counterstained with haematoxylin and mounted regularly. The degree of staining was assessed subjectively through the blinded examination of the slides by two investigators independently.

For indirect immunofluorescent colocalization, rabbit anti-UCH-L1 sera and goat anti-Jab1 sera (1:50, Santa Cruz Biotechnology, Inc.) were mixed together to add on the slides with the same dilution of the primary antibodies as above. The FITC conjugated bovine anti-rabbit IgG (1:200, Santa Cruz Biotechnology, Inc.) and Rhodamine conjugated donkey anti-goat IgG (1:200, Santa Cruz Biotechnology, Inc.) were mixed and used as the secondary antibody. The fluorescence labeled slides were mounted in 10% glycerol in PBS, and examined under microscope (Nikon 50i, Nikon Corporation).

2 RESULTS

2.1 The relatively quantitative difference of follicles in ovarian tissues of prepubertal mice

The ANOVA analysis results indicated that the summed data of various kinds of follicles dropped from day 14 to day 28 postnatal, and the change from day 21 to day 28 time-point reached the statistically significant degree ($P<0.05$). The morphologically abnormal follicles mainly occurred during day 21 to day 28 postnatal. As for specific types of follicles, the primordial follicles decreased throughout the period studied ($P<0.05$). The number of total follicles in the ovaries of 14 and 21 days old mice was significantly greater ($P<0.05$), suggesting the total number of follicles during the prepuberty dropped obviously at the transition from day 21 to day 28 (Fig.1*A*). Consistently, the number of primordial follicles also decreased significantly from day 14 to day 21, and from day 21 to day 28 postnatal ($P<0.05$), and the number of primary follicles was only obtained significant change during the prepubertal period from day 21 to day 28 ($P<0.05$), though secondary and tertiary follicles were shown to increase to a less degree from day 21 to day 28 (Fig.1*B*). Despite that total number of abnormal follicles only took a small proportion, it could be found more primary follicles included than other follicular types.

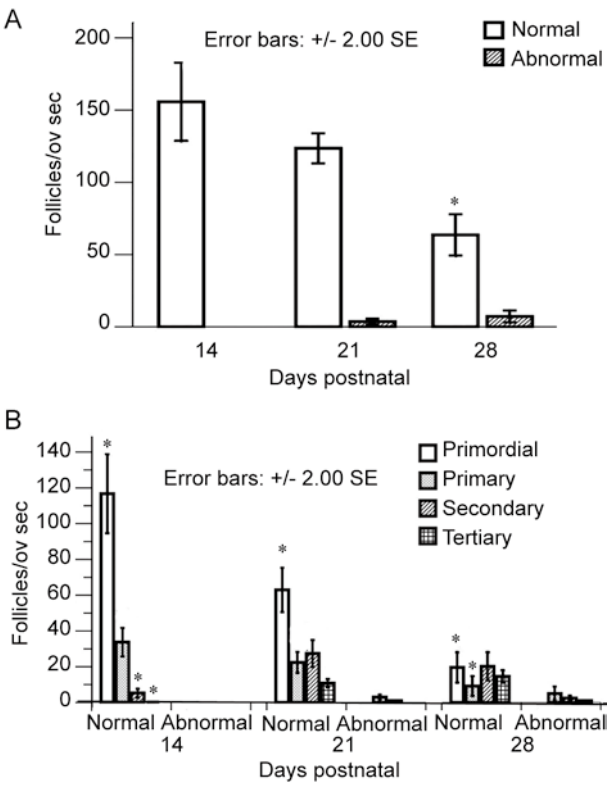

Fig. 1. The follicular pool in mouse ovaries changed with time during prepuberty. *A*: The sum of all kinds of follicles with normal and abnormal shapes is graphed on time. The bars express the mean ± 2SE of follicles from 5 ovarian sections. The asterisk at the top of the bar indicates significant differences between time-points in comparison by Bonferroni multiple method in ANOVA ($P<0.05$). *B*: The changes of various kinds of follicles in mouse ovaries with time are described separately. The amounts of primordial, primary, secondary and tertiary follicles in the ovaries of 14, 21 or 28 days old mice were individually analyzed by ANOVA, the corresponded asterisks at the top of the bars indicate significant differences ($P<0.05$) between time-points for a follicular type, and the data were expressed by mean ± 2SE to show the change of each follicular type from day 14 to day 28 postnatal. The Follicles/ov sec in the ordinate shows the numbers of follicles in one ovarian section.

2.2 Western blotting analysis of UCH-L1, Jab1 and $p27^{Kip1}$ proteins in various tissues

The total protein extracts isolated from various tissues of mice were used to determine the tissue distribution of UCH-L1, Jab1 and $p27^{Kip1}$ proteins by Western blot analysis (Fig. 2*A*). The results showed that, UCH-L1 protein was detected by rabbit anti-UCH-L1 antibody in samples extracted from mouse brain, testis and ovary (Fig. 2*A*, top bar) specifically, whereas the bands of Jab1 (Fig. 2*A*, medium bar) and $p27^{Kip1}$ (Fig. 2*A*, bottom bar) proteins were

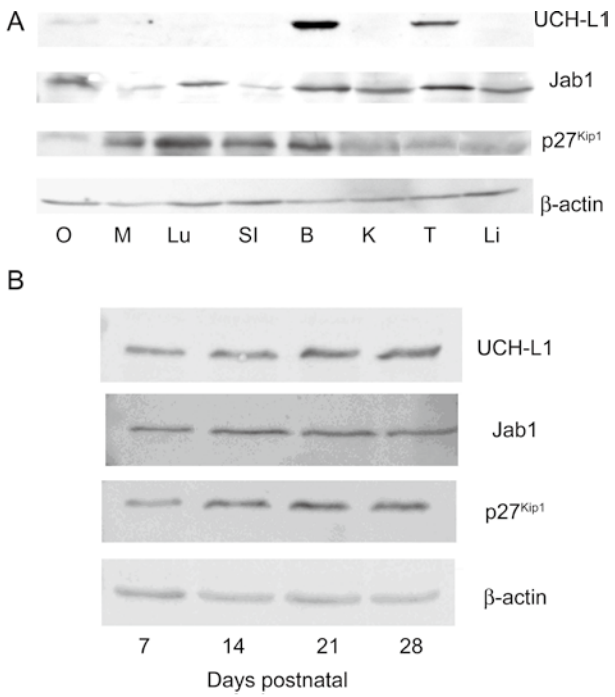

Fig. 2. Western blot analysis of UCH-L1, Jab1 and $p27^{Kip1}$ proteins in the mouse tissues. *A*: The tissue distribution of UCH-L1, Jab1 and $p27^{Kip1}$ proteins. The total proteins were isolated from ovary (O), muscle (M), lung (Lu), small intestine (SI), brain (B), kidney (K), testis (T) and liver (Li) of the 8 weeks old ICR mice. Total protein extract (20 μg) of each sample was loaded into each lane. The top panel shows the ~28 kDa signal detected by rabbit anti-UCH-L1 antibody, whereas the medium and bottom panels respectively show the ~38 kDa and ~27 kDa bands recognized respectively by rabbit anti-Jab1 and anti-$p27^{Kip1}$ antibodies. *B*: The protein levels of UCH-L1, Jab1 and $p27^{Kip1}$ in mouse ovaries during the prepuberty. Relative to β-actin, the contents of UCH-L1, Jab1 and $p27^{Kip1}$ in ovaries were shown, to different degrees, changing with time after birth.

observed in all tested tissues, with β-actin as a control, though higher degrees were detected in brain and gonads for Jab1, in parallel to UCH-L1.

2.3 The protein levels of UCH-L1, Jab1 and $p27^{Kip1}$ in prepubertal ovaries

The results of Western blotting analysis (Fig. 2*B*) indicated that UCH-L1 protein in ovaries was shown to increase with time during prepuberty, though more obviously from day 14 to day 21, relatively to β-actin. Jab1 maintained at similar level; $p27^{Kip1}$ only at day 7 was shown lower. Though no evident paralleled change was found for Jab1 to justify its relation with UCH-L1, and no corresponded decrease of $p27^{Kip1}$ owing to UCH-L1 increase, their abundance at the prepubertal stage enhances the possibility of their association as a complex.

2.4 The UCH-L1 antibody recognized native and recombinant antigens specifically

To demonstrate the specificity of UCH-L1 signals detected by rabbit anti-UCH-L1 antibody, the GST-UCH-L1, recombinantly expressed GST fusion protein of mouse UCH-L1, was used to neutralize the UCH-L1 antibody, which had been shown to react specifically by immunoblotting results (unpublished). The immunohistochemical results from 8 weeks old mice were in consistent with the reported data[14]. UCH-L1 protein staining was mainly localized in spermatogonia and Sertoli cells of testis (Fig. 3*A*). UCH-L1 protein signal was also detected in oocytes of 8 weeks ovary (Fig. 3*C*). The positive signals were diminished effectively by neutralizing with GST-UCH-L1 in the controls (Fig. 3 *B*, *D*).

2.5 Expression of UCH-L1 protein in oocytes of developing follicles by immunohistochemistry

In negative control, positive signal was completely eliminated by neutralizing with recombinant GST-UCH-L1 (Fig. 4*A*), assuring the immunohistochemical specificity of UCH-L1 staining. In ovarian tissues of prepubertal mouse aged 0-28 days, UCH-L1 immunostaining was predominantly localized in the cytoplasm of all oocytes contained in follicles at different stages (Fig. 4*B-F*). Among most of the stained oocytes, the intensities of positive signal were almost equal. However, more intense staining was discriminated in a few of oocytes with abnormal shapes (indicated by blue arrows in Fig. 4*B-D*), suggesting that UCH-L1 protein was more abundant in these abnormal oocytes. Meanwhile, in day 28 postnatal ovaries, three kinds of abnormal secondary follicles were observed: (1) with a fragmental or abnormally shaped oocyte (Fig. 4*D*, indicated by blue arrow); (2) two or more oocytes crowded in one follicle (Fig. 4*E*, indicated by blue arrow); (3) their granular cells dissociated from oocytes (Fig. 4 *F*, indicated by blue arrow). By repeated and careful comparisons, it was found that the UCH-L1 protein level was unusually high in oocytes of those morphologically abnormal follicles.

2.6 The binding of UCH-L1 and Jab1 in the protein extract of day 28 mouse ovaries *in vitro*

The UCH-L1 affinity chromatography, or pull-down analysis, confirmed the interaction between UCH-L1 and Jab1 in prepubertal ovaries (Fig. 5), as in the tumor cells[19]. The GST-UCH-L1 pulled out the Jab1 from protein extracts of day 28 mouse ovaries, and GST did not. So we can deduce that the Jab1 was bound specifically and pulled out by UCH-L1. But we found the Jab1 has two bands in

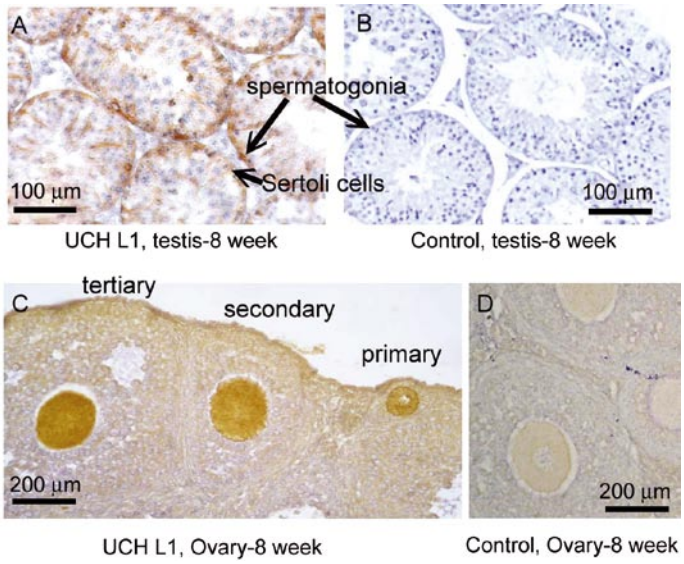

Fig. 3. Immunostaining for UCH-L1 in seminiferous tubules (*A*) and ovary (*C*) of adult mice (aged 8 weeks). *B* and *D* are the negative controls respectively for *A* and *C*, in which the rabbit anti-UCH-L1 antibody, prior to the incubation with sections, was neutralized by recombinant GST-UCH-L1 (16 μg/μg primary antibody). Scale bar in *A*, *B*: 100 μm. Scale bar in *C*, *D*: 200 μm.

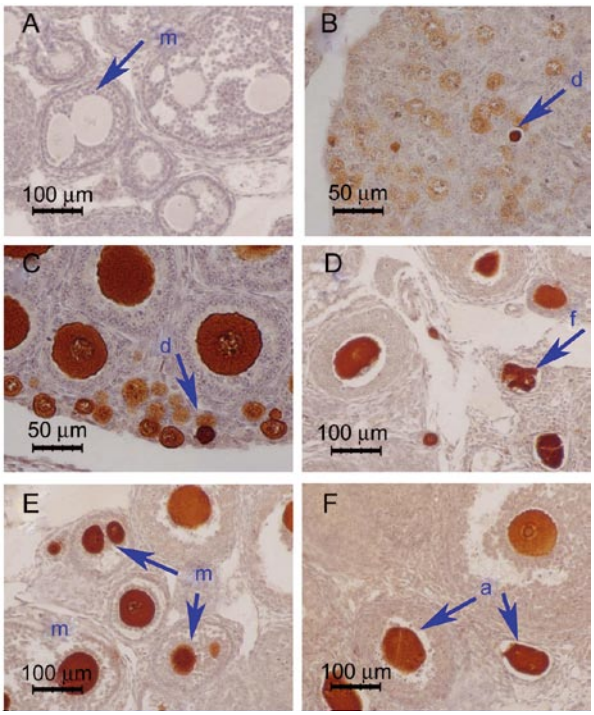

Fig. 4. Immunohistochemical detection of UCH-L1 protein in mouse oocytes at different postnatal stages. The specific signals of UCH-L1 protein were detected in newborn (*B*), 14 days old (*C*), and 28 days old (*D-F*) oocytes; oocytes in morphologically abnormal follicles were indicated by blue arrows. Picture *A* is the negative control, in which the rabbit anti-UCH-L1 antibody, prior to the incubation with sections, was neutralized by recombinant GST-UCH-L1. d = degenerate primary oocyte, a = oocytes with abnormal shape, f = fragmented oocytes, m = multiple oocytes in one follicle. The scale bar is indicated in the individual pictures.

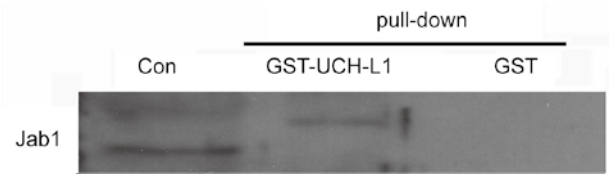

Fig. 5. UCH-L1 binds to Jab1 in the protein extract of 28 days old mouse ovaries. The protein extract of 28 days old mouse was pulled-down by glutathione Sepharose 4B beads incorporate with GST and GST-UCH-L1, and the bound proteins were detected by Western blotting for Jab1. The 20 μg total protein extract was used as control (Con), presented before the results for GST-UCH-L1 and GST as indicated at the top.

the Western blotting analysis of the ovarian protein extracts (control), and only the upper one was absorbed by UCH-L1. Moreover, the migration of the band detected in the UCH-L1 pull-down was not identical to that in control since the absorption process could possibly affect the phosphorylation of Jab1.

2.7 The colocalization of UCH-L1 and Jab1 in abnormal oocytes of 28 days old mouse ovaries

As for the colocalization of UCH-L1 and Jab1, in the negative controls, the positive signal was obviously diminished by neutralizing the primary antibody in the immunofluorescent analysis (Fig. 6*A*, *B*), indicating the antigen-specificity of the positive staining. In consistent with the described data, strong positive signal for UCH-L1 protein was detected in all oocytes of 28 days old mouse ovaries

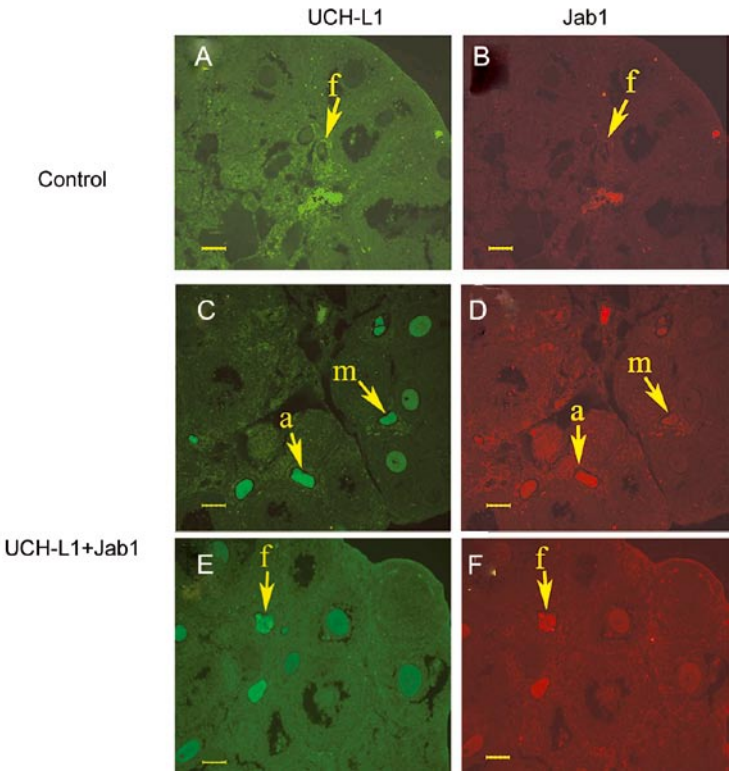

Fig. 6. The expression of UCH-L1 and Jab1 in abnormal follicles at 28 days postnatal mouse ovaries detected by indirect immunofluorescence. UCH-L1 was stained into green by FITC labeled bovine anti-rabbit IgG, while Jab1 was dyed into red by Rhodamine labeled donkey anti-goat IgG. *A*, *B*: the primary antibodies were neutralized respectively by recombinant GST-UCH-L1 and Jab1 peptide as negative controls; *C*-*F*: the colocalization of UCH-L1 (*C*, *E*) and Jab1 (*D*, *F*) in oocytes of abnormal follicles (AO). a = oocytes with abnormal shape, f = fragmented oocytes, m = multiple oocytes in one follicle. Scale bar, 100 μm.

(Fig. 6*C* and *E*), and the staining was obviously more intense in the abnormal oocyte (Fig. 6*C* and *E*, indicated by yellow arrow). Just like UCH-L1, more intense staining for Jab1 in abnormal oocytes was also observed (Fig. 6*D* and *F*, indicated by yellow arrows). So the results of indirect immunofluorescent analysis showed that both UCH-L1 and Jab1 proteins were detected in oocytes with abnormal shapes (Fig. 6*C*-*F*) to a higher degree.

3 DISCUSSION

The oocyte pool of mice changed to great degree during prepuberty, especially from day 21 to day 28, both primordial and primary follicles diminished significantly, without comparable compensation by the increase of secondary and tertiary follicles, leading to the total pool reduced. We found a certain number of morphologically abnormal oocytes or follicles, although it seemed very small relative to the loss of the pool. The oocytes or follicles with abnormal shapes mainly occurred from day 21 to day 28, and primary follicles ranked predominant. We suppose that some oocytes should have been eliminated at primordial or even earlier stages, but they are neglected in our observation. In all we can infer that oocytes have been lost to an evident degree from day 21 to day 28, and these oocytes showed some abnormal changes including the appearances of abnormal shapes.

The current paper found that the UCH-L1 specifically distributes in the brain and the gonads, and it is widespread in the cytoplasm of oocytes, but another paper thought it mainly close to the cell membrane in adult mouse oocytes[12]. The Jab1 and $p27^{Kip1}$ were found extensively expressed in various tissues. The immunohistochemical results on adult supplied the control for prepubertal mouse ovaries in our experimental operation, and in testes the distribution of UCH-L1 coincides with other reports[13, 27], so the inconsistent results above could be assured not to result from our using prepubertal mice and the cross-reaction of the UCH-L1 antibody.

This study demonstrates an abundant expression of UCH-L1 in the cytoplasm of oocytes with abnormal shapes in the developing follicles of prepubertal mice, as well as the

parallel up-regulated ovarian expression of Jab 1 and $p27^{Kip1}$ in 28 days old mice, suggesting a potential interaction among UCH-L1, Jab 1 and $p27^{Kip1}$ in the selective elimination of abnormal oocytes during the process of oogenesis.

Strict control of cell proliferation and cell loss is essential for oogenesis. Most oocytes die during the fetal period or shortly after birth[3]. It has been proposed that the ovary eliminates excess follicles containing oocytes of poor quality prior to puberty to achieve an optimal number of oocytes present at puberty[24]. Otherwise, the abnormal oocytes would be eliminated through follicle atresia during puberty, for if an oocyte has genetic defects, its follicle may be handicapped concomitantly[28-30]. In the present study, it was observed that in prepuberal mice with no treatment, the total number of follicles at different stages in ovaries decreased sharply at the transition from day 21 to day 28 postnatal, and most of lost follicles were primordial and primary follicles (Fig. 1). From day 21 to day 28 postnatal, some follicles showed abnormal appearances, but no abnormal follicle was found appeared in the adult mouse ovaries, we therefore deduced that those abnormal oocytes might be gradually eliminated during prepuberty through appropriate apoptosis partly regulated by the ubiquitin-proteasome system.

The ubiquitin-mediated protein degradation pathway exerts a wide spectrum of effects and modulates a variety of biological processes including cell cycle progression, apoptosis (or programmed cell death), oncogenesis and DNA repair[31]. UCH-L1, a component of the ubiquitin system, is associated with germ cell apoptosis[13] and involved in the regulation of cell morphology[32]. Furthermore, it was previously reported that, in mice, the abnormal ovarian differentiation caused by the treatment of genistein could result in the production of multioocyte follicles[33], and fragmented-oocyte could lead to the oocyte elimination in ovary[34]. Interestingly, multioocyte and fragmented-oocyte follicles were also observed in prepubertal ovaries in the present study, and UCH-L1 protein was more abundant in oocytes of follicles with abnormal shapes, suggesting that UCH-L1 is possibly involved in the elimination of abnormal oocytes and the preservation of the normal follicular morphology during oogenesis.

A number of specific ubiquitin-binding domains have been found to mediate most of the effects of protein ubiquitination, and Jab1 has one of these known domains[15]. Jab1 is abundantly expressed in a variety of adult tissues as well as in mouse embryos, and its protein was localized in both the nucleus and cytoplasm[35]. As the cyclin-dependent kinase inhibitor, $p27^{Kip1}$ represents a protein of central activity for the control of cell proliferation, differentiation and malignant transformation[36]. The expression of $p27^{Kip1}$ was observed in the cytoplasm in growing oocytes, but was not found in the cytoplasm of fully grown oocytes[37]. Furthermore, it was well demonstrated that the expression of Jab1 led to down-regulation of the negative cell cycle regulator $p27^{Kip1}$ in carcinogenesis[38, 39]. Lack of Jab1 could lead to the high expression of $p27^{Kip1}$, resulting in impaired proliferation and accelerated apoptosis of mouse embryonic cells [17]. However, in the present study, it was observed that both $p27^{Kip1}$ and Jab1 were more abundant, in parallel to UCH-L1, in the abnormal oocytes of prepubertal ovaries. Since $p27^{Kip1}$ controls oocyte growth and activates follicle apoptosis[23], and the morphological changes of oocyte is accompanied with follicular apoptosis [28-30], we therefore suppose that, UCH-L1 might play a critical role in the elimination of abnormal oocytes during mouse prepubertal development in association with Jab1 and $p27^{Kip1}$. The high expression of UCH-L1 in morphologically abnormal oocytes could be deduced to result in the apoptosis of these oocytes, and the up-regulated Jab1 protein might be a responsive reaction to maintain its own homeostasis. The UCH-L1 affinity analysis confirmed that UCH-L1 binds to Jab1 as in tumor cells[19], but the precise interactions among UCH-L1, Jab1 and $p27^{Kip1}$ during the process of prepubertal development of mouse ovaries still need direct evidence because we did not found any $p27^{Kip1}$ decrease in response to UCH-L1 or Jab1 up-regulation. Since two Jab1 bands were detected in Western blotting analysis, we deduced that the Jab1 can be phosphorylated, and only the phosphorylated form was bound by UCH-L1, but the real situation needs further study.

In summary, it was found in mice that the sum of all kinds of follicles diminished significantly during the period of day 21 to day 28 postnatal; accordingly, there was an increasing trend in the level of UCH-L1 proteins in the ovaries. The morphologically abnormal oocytes mainly appeared in prepubertal ovaries from day 21 to day 28, and the up-regulation of UCH-L1 and Jab1 protein contents in these oocytes suggests that UCH-L1 may play an important role in association with Jab1 and $p27^{Kip1}$ in selective elimination of abnormal oocytes during prepubertal development. Affinity analysis results further showed that the interactions of UCH-L1 and Jab1 in prepubertal oocytes as in the tumor cells may lead to a better knowledge of the molecular mechanisms underlying the oogenesis, and the development of contraceptive or/and assisted

reproduction technologies.

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