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RESEARCH ARTICLE

Small Ubiquitin-Related Modifier-1 Programs Granulosa the Cell Apoptosis in Mice Ovary

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ABSTRACT

The small ubiquitin-related modifiers (SUMO-1/2/3) have been in the limelight as crucial dictators of a spectrum of biological events including normal growth and development. SUMO-1 protein is important for post-translational modification which is required for a variety of biological processes including organ development and organelle biogenesis. However, its functional relevance in mammalian ovary still remains to be explored. Herein, we found that SUMO-1 consistently localizes in granulosa cells (GCs), oocytes and corpus luteum during mice folliculogenesis. Moreover, the SUMO-1 conjugates particularly those which molecular weight correspond to 34-, 43-, and 72 KDa were highly presented in ovaries and were dynamically influenced by PMSG and/or hCG administration, further buttressing that SUMO-1 was expressed in mouse ovary. Additionally, mutation at G96/97A (binding site) changed the localization of SUMO-1 from nucleus to both cytoplasm and nucleus. With some diffusion from nucleus into cytoplasm, and the apoptosis in GCs was significantly triggered by overexpression of both SUMO-1 and its mutant SUMO-$1^{G96/97A}$ with more significantly induced by the latter form. Over-expression of wild-type SUMO-1 and mutant SUMO-$1^{G96/97A}$ significantly induced ($P<0.001$) apoptosis in GCs, but the mutant SUMO-1 induced higher apoptotic rate ($P<0.001$) than over expressed SUMO-1. Overall, our study determines the expression of SUMO-1 in mouse ovary and pinpoints SUMO-1 as a determinant of GCs biology which is essential for mammalian ovary function.

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INTRODUCTION

SUMO is a ~12 KDa small ubiquitin-related modifier protein which conjugates to the epsilon amine group of lysine residues with substrate proteins via an enzymatic cascade analogous. To date, there are four mammalian SUMO family members: SUMO-1, SUMO-2, SUMO-3 and SUMO-4. SUMO-1 is only 18% homologues to ubiquitin but its three-dimensional structure is remarkably similar to ubiquitin (Streich and Lima, 2016). The process of SUMOylation is highly conserved, reversible and dynamic among post-translational modifications (Sarge, 2016; Wilson, 2017).

The process of SUMO conjugation can be reversed by SUMO-specific proteases (SENPs). SUMO conjugation based modifications play essential roles in the diverse cellular processes, such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, alteration of protein activity or stability, sub-cellular localization of the modified protein, cell-cycle progression, regulation of chromatin structure and antagonization of post-translational modifications (García *et al*., 2016; Zilio *et al*., 2017).

SUMO-1 mRNA is expressed in all tissues of wild-type mice including brain, spleen, lungs, and testis. SUMO-1 is mainly localized within cellular nuclei in a wide range of tissues (Zhang *et al*., 2008). Previous research has reported that SUMO-1 is predominantly localized in the head of human and mice sperm cells (Vigodner *et al*., 2013). Some studies on mouse GCs have demonstrated that SUMO-1 is expressed and regulated by gonadotropins, in particular an ovulatory hCG stimulus in mouse GCs in vivo. Furthermore, western blot analyses demonstrated that SUMO-1 was predominantly expressed

and down-regulated by hCG stimulation in the pre- and peri-ovulatory GCs nuclei (Shao *et al*., 2006).

The process of mammalian folliculogenesis is crucial for normal female reproductive functioning and is involved in follicular growth or atresia. Folliculogenesis is controlled by a series of hormones, cytokines, growth factors, paracrine and endocrine factors. The normal follicular growth includes proliferation, cell cycle control and differentiation of GCs. However, only a few follicles undergo ovulation and the majority of the follicles are lost before ovulation by atresia. This degenerative process is initiated or caused by GCs apoptosis, and the process of SUMOylation appears to be involved in apoptotic pathways in the ovary.

However, the detailed expression and localization pattern of SUMO-1 during follicular development and ovulation are still unclear. Furthermore, the roles of SUMO-1 and the effects of binding sites at G96/97 on its sub-cellular localization and apoptosis in GCs are unknown. The G96 and 97 were the last two C-terminal glycine residues of SUMO-1 that are essential for isopeptide bond formation to a lysine ε-amino group of target proteins (Zhang *et al*., 2008). Therefore, this study was undertaken to determine the expression and localization pattern of SUMO-1 in the mouse ovary and GCs during super-ovulation and luteal development. The sub-cellular localization of SUMO-1 in the GCs and its effect on apoptosis was analyzed by over-expressing wide-type SUMO-1 and mutant SUMO-1 vectors at its binding sites (G96/97A). The results indicate that SUMO-1 is highly expressed in the mouse ovary and the GCs, and its localization is seen in the nucleus of GCs. Moreover, mutation of SUMO-1 at G96/97A, which is essential for bond formation to the target proteins, alters the localization of SUMO-1 and its ability to induce apoptosis in GCs. The results in this study provide important information for SUMO-1 expression and localization profile during ovulation and the regulation of GC apoptosis.

MATERIALS AND METHODS

Ovaries collection and gonadotropin treatment: Ovaries were collected from 8, 10 and 21 days old Kunming female mice. The mice were treated with an intraperitoneal injection of 10 IU PMSG (SanSheng, Ningbo). Another group of mice was injected with 10 IU hCG (SanSheng, Ningbo) after 48 h of PMSG injection.

Plasmid construction: SUMO-1 and mutant SUMO-$1^{G96/97A}$ constructs were generated by PCR amplification of mouse SUMO-1 cDNA (NM_009460) using two-pairs of primers: SUMO-1Fw:5'-TGAATCGTCGACACCATGTCTGACCAG-3', SUMO-1Rev:5'-AAAAGAGATCTCTAAACCGTCGAGTG-3' and SUMO-1Fw:5'-TGAATCGTCGACACCATGTCTGACCAG-3', SUMO-1Rev:5'-AGAGATCTCTAAACCGTCGAGTGAGCCAC-3', respectively. The obtained sequences were subsequently cloned in the SalI-BglII sites of pCMV-N-FLAG expression vector, and later confirmed by sequencing.

Cell culture and transfection: Granulosa cells (GCs) were grown in 6-well culture dish in Dulbecco's Modified Eagle's Medium/Nutrient F-12 (DMEM/F12, Gibco) medium supplemented with 10% fetal bovine serum (FBS, Invitrogen). All cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2. The transfections were carried out by using lipofectamine LTX kit (Invitrogen) according to the manufacturer's directions.

Protein extraction and immunoblotting (Zhou *et al*., 2018): Ovarian tissues were excised by using ultrasonic cell crusher (Sonics, USA. VCX 130), and later placed into a centrifugation tube containing 200 µL of ice-cold RIPA buffer (Santa Cruz) for 10 min. Similar process was repeated for transfected GCs. Total protein was transferred to 0.45 µm PVDF membrane (Milli-pore, Bedford, MA). The membrane was placed in 5% nonfat dry milk in TBST for 1 h, and then incubated with rabbit polyclonal anti-SUMO-1 antibody (1:200, Santa Cruz) or mouse monoclonal anti-β-actin antibody (1:1000, Santa Cruz) at 4ºC overnight. It was incubated with second antibody for 1 h and incubated with the ECL chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ) for 5 min, and later exposed to X-ray film for the observation of protein bands.

Paraffin section and immunohistochemistry (Zhao *et al*., 2018): Ovarian tissues were embedded in paraffin, cut into 5 µm thickness by microtome and placed on poly-L-Lysine-coated slides. Non-specific binding sites were blocked in 10% normal donkey serum diluted in PBS for 1 h. The sections were later incubated with rabbit polyclonal anti-SUMO-1 antibody (1:100) diluted in 10% donkey serum (1:100) overnight at 4ºC. The sections were incubated with biotinylated goat anti-rabbit IgG antibody for 1 h, and then incubated with SABC solution for 30 min. After that, the sections were stained by adding fresh DAB solution for 5 min. The sections were counterstained with haematoxylin for 1 min, and then mounted with Neutral Balsam.

Immunofluorescence cytochemistry (Chiu *et al*., 2018): For detection of the sub-cellular localization pattern of SUMO-1 and SUMO-$1^{G96/97A}$, GCs were cultured in a confluent monolayer on glass cover slips and transfected with pCMV-N-FLAG-SUMO-1 or pCMV-N-FLAG-SUMO-$1^{G96/97A}$ plasmids, respectively. Cells were fixed in 4% paraformaldehyde (Santa Cruz) for 30 min. GCs were permeabilized for 20 min with 0.5% Triton-X100 (Santa Cruz) and blocked in 5% bovine serum albumin (BSA, Santa Cruz). Then, the cells were incubated with anti-FLAG primary antibody (1:100 dilutions, CWBIO, Beijing) overnight at 4ºC. Then, cells were incubated with FITC-conjugated antibody (1:100 dilutions, Boster, Wuhan) for 1 h. The nucleus of the cells was stained with 10 µg/mL 4,6- diamidino-2-phenylindole (DAPI, Santa Cruz) for 5 min. After that, the cover slip was mounted onto a slide and later analyzed under a Nikon TE2000-U Fluorescence microscope.

Cell apoptosis assay (Zhou *et al*., 2018): GCs were transfected with pCMV-N-FLAG, pCMV-N-FLAG-SUMO-1 or pCMV-N-FLAG- SUMO-$1^{G96/97A}$ vectors for 48 h, respectively. Cell apoptosis was measured by using the AnnexinV kit according to the manufacturer's instructions (AntGene Co, Wuhan, China). The apoptosis

was measured by using BD FACSCalibur flow cytometric analyzer (Becton, Dickinson and Company, USA; 488 nm excitation and 530 nm emission).

RESULTS

Expression of SUMO-1 conjugates in mouse ovary and GCs: The expression of SUMO-1 conjugates in mouse ovary was studied by Western blotting. The results showed that SUMO-1 modified proteins were highly expressed in mouse ovaries after PMSG or PMSG/hCG treatment. The amount of SUMO-1 conjugates at ~72 KDa and the global SUMO-1 conjugates were significantly increased at 1 h, decreased at 12, 24, and 36 h, and later increased at 48 h after PMSG injection (Fig. 1A). Whereas, no significant different was found in ~72 KDa protein after PMSG/hCG injection. Furthermore, the amount of SUMO-1 conjugates at ~34/43 KDa was significantly increased at 9 and 11 h and was relatively decreased at 7 and 15 h after hCG injection, but the global SUMO-1 conjugates were significantly increased at 11 and 24 h (Fig. 1B). In-vitro cultured GCs also showed different expression pattern of SUMO-1 conjugates (Fig. 1C).

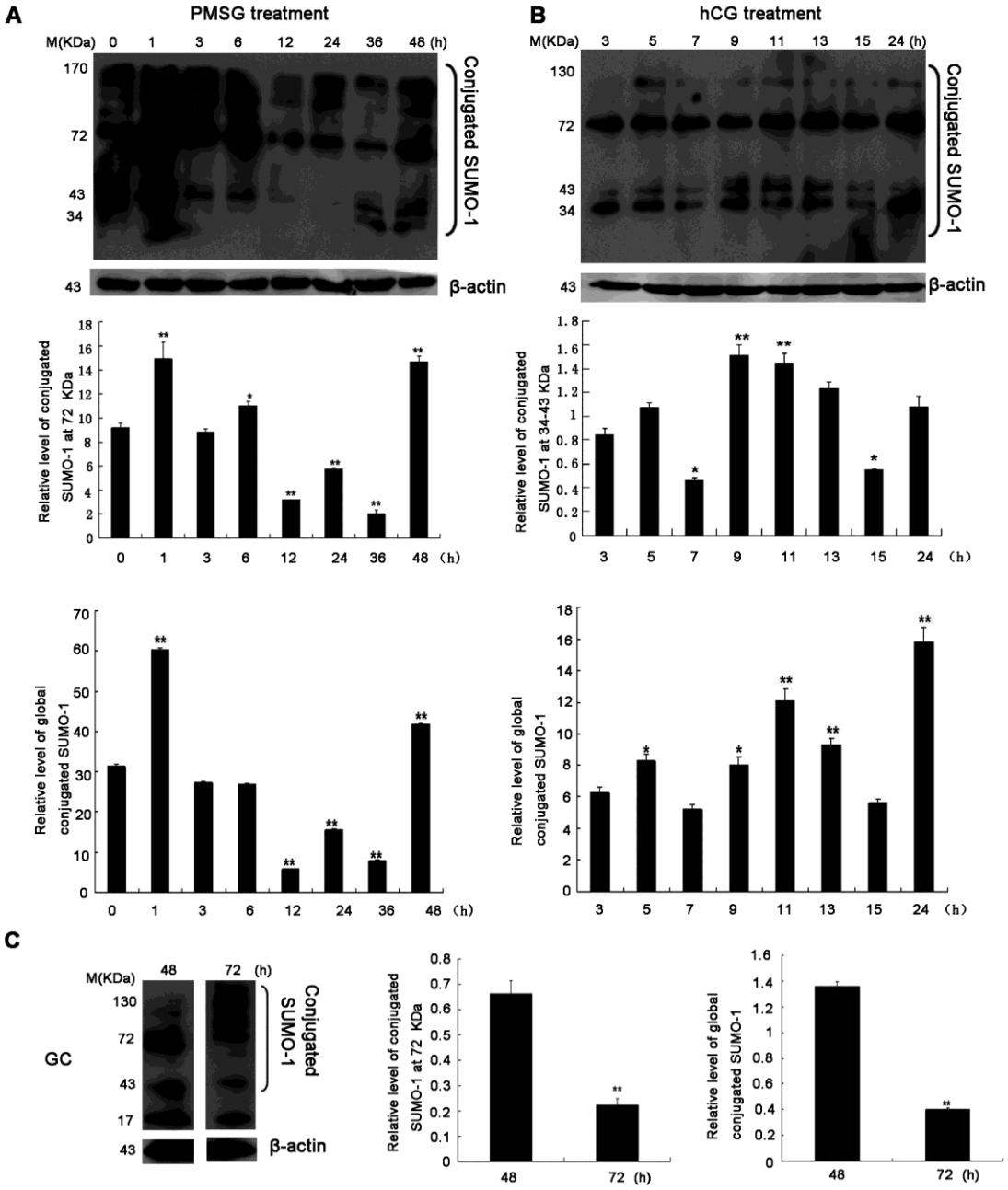

Fig. 1: The expression of conjugated SUMO-1 in ovaries during super-ovulation after PMSG and hCG treatment and in GCs after in-vitro culture. Total proteins (30 μg per lane) from ovaries was subjected to SDS–PAGE under reducing conditions and western blot analysis was performed for SUMO-1 expression. Molecular mass marker proteins are indicated on the far-left side. High-molecular-weight SUMO-1 conjugates are marked by a bracket. (A) Relative expression level of SUMO-1 conjugates at 72KDa and global conjugated of SUMO-1 were determined by densitometry after PMSG treatment. (B) Relative expression level of SUMO-1 conjugates at 34-43KDa and global conjugated of SUMO-1 were determined by densitometry after 48 h of PMSG treatment followed by hCG treatment. (C) Relative expression level of SUMO-1 conjugates at 72KDa and global conjugates of SUMO-1 were determined by densitometry in GC after in-vitro culture 48 and 72h. The total amount of β-actin present in the lower set of lanes was used to normalize the amount of SUMO-1 conjugates present on the upper set of lanes. The values are presented as means ±SEM in each treatment group (n = 3). *: P<0.05; **: P<0.001.

SUMO-1 localization in mouse ovary during sexual maturation and super-ovulation after PMSG and hCG treatment: As SUMO-1 was detected in the ovary and GCs, so we examined the cellular distribution of SUMO-1 in the mouse ovary by immunohistochemistry. On day 8 and 10 after birth, we found very strong immunostaining signals of SUMO-1 in the primary and secondary follicles. The localization was mainly dominated in the oocytes and GCs (Fig. 2A, B). Treatment with PMSG had no significant effect on immunostaining signals of SUMO-1 in the oocytes and GCs of all stages of follicles (Fig. 2C-E, G-I). Strong immunostaining signals were observed in the follicles 12 h after PMSG injection (Fig. 2F). After hCG treatment, SUMO-1 was mainly accumulated in the GCs of antral follicles and pre-ovulatory follicles (Fig. 3A-E). Furthermore, the immunostaining signals of SUMO-1 were also observed in the corpus luteum after hCG treatment (>12 h) (Fig. 3F-I).

Subcellular localization of SUMO-1 in GCs: To further observed precisely the localization of SUMO-1 signals in GCs and to investigate the effect of SUMO-1 mutation at G96/97A on its subcellular localization in GCs, we transiently transfected GCs with SUMO-1 or SUMO-$1^{G96/97A}$ expression vectors, respectively. Indirect immunofluorescence showed that endogenous and over-expressed SUMO-1 was predominantly localized in the nucleus of GCs (Fig. 4A, B). However, mutant SUMO-$1^{G96/97A}$ was localized both in the nucleus and cytoplasm (Fig. 4C). These results suggested that mutation of SUMO-1 at G96/97A affected the normal localization of SUMO-1 in GCs.

Mutation of SUMO-1 at G96/97A increased its ability to induce GCs apoptosis: Mutation of SUMO-1 at G96/97A altered the sub-cellular localization of SUMO-1 in the GCs. In order to further reveal the roles of SUMO-1 on GCs apoptosis and the effect of mutation of SUMO-1 at G96/97A on GCs apoptosis, we mutated the 96th and 97th glycine residues that are critical for SUMO-1 conjugation to its substrates (Müller *et al*., 2001; Zhang *et al*., 2008). Vectors of wild-type SUMO-1 or mutant SUMO-$1^{G96/97A}$ was transfected in GCs for 48 h and then GCs were collected for apoptotic analysis by flow cytometer (Fig. 5A). The results showed that apoptosis rate was significantly increased after transfection with wild-type SUMO-1 or mutant SUMO-$1^{G96/97A}$ compared to that of the control group (Fig. 5B, $P<0.001$). Furthermore, apoptosis rate in GCs with over expressed SUMO-1 was significantly lower than that in GCs with over expressed mutant SUMO-$1^{G96/97A}$ ($P<0.001$).

DISCUSSION

Post-translational modification protein, SUMO-1 plays important role to maintain the normal cellular functions. For example, over-expression of SUMO-1 inhibits signal-induced activation of NF-kB and represses SP3 transcription activation (Ross *et al*., 2002). SUMO-1 modified caspase-7 contributes to its nuclear localization in human neuronal cells (Hayashi *et al*., 2006), while SUMO-1 enhances RbAp46 protein stabilization that helps in the suppression of NIH/3T3 cells growth (Giri *et al*., 2008).

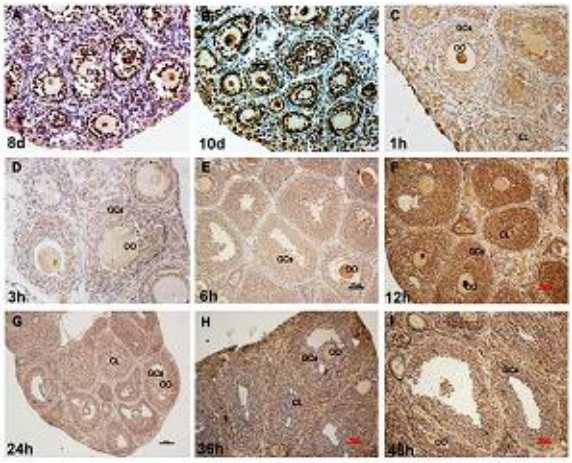

Fig. 2: Immunohistochemical localization of SUMO-1 in the mouse ovary after PMSG treatment. (A, B) In 8 and 10 days old mice, strong immunostaining signal of SUMO-1 was observed in primary and secondary follicles (bar = 20 μm). (C-F) SUMO-1 was localized in GCs and nucleus of oocyte in secondary follicles after PMSG treatment at 1, 3, 6, and 12 h (bar = 50 μm). (G-I) While, at 24, 36, and 48 h after PMSG treatment, SUMO-1 was localized in the layers of GCs in pre-antral and antral follicles (bar = 50 μm). GCs: granulose cells, OO: oocytes.

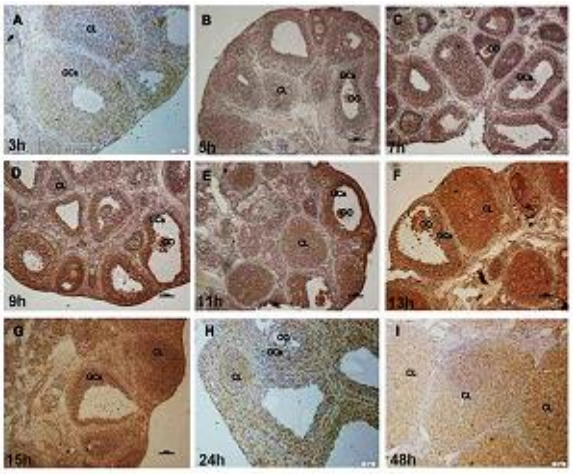

Fig. 3: Immunohistochemical localization of SUMO-1 in the mouse ovary after 48 h of PMSG treatment followed by hCG treatment. (A) At 3 h after treatment of hCG, SUMO-1 was detected in small antral follicles and GCs (bar = 50 μm). (B-G) At 5, 7, 9, 11, 13, and 15 h, SUMO-1 was appeared on GCs of antral follicles (bar=100μm). (H-I) At 24 and 48 h, SUMO-1 was clearly observed in the luteal tissues (bar=50μm). GCs: granulosa cells, CL: corpus luteum.

In this study, we demonstrated that over-expression of SUMO-1 significantly increased GCs apoptosis, which is in agreement with the previous findings that SUMO-1 protein was associated with pre- and peri-ovulatory follicular development (Shao *et al*., 2006). In mammalian ovary, SUMO-1 is involved in the regulation of GCs growth, differentiation and cell death. Several investigators have shown that SUMO-1 recognizes and interacts with target proteins that are related to the apoptotic process. For instance, Caspase-8 can be sumoylated at lysine 156 (Besnault-Mascard *et al*., 2005). Bax/Bak-dependent stimulation of SUMO-1 conjugation to DRP1 during the apoptotic cell death (Wasiak *et al*., 2007). SUMO-1 increases apoptotic responses by enhancing p53 transcriptional activities (Liu *et al*., 2013).

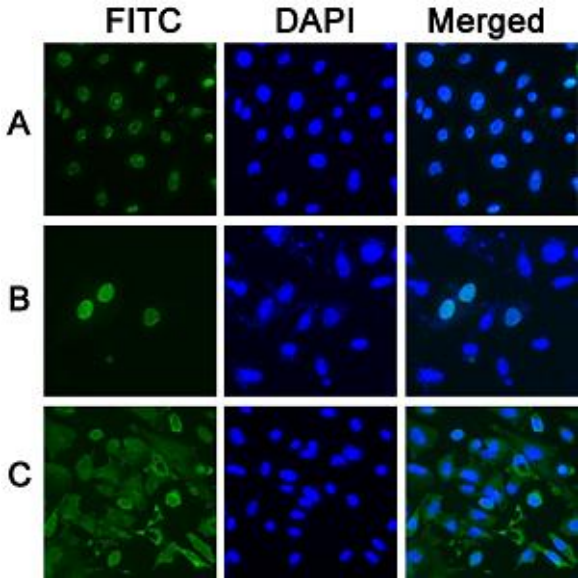

Fig. 4: Sub-cellular localization of SUMO-1 and its mutant (G96/97A) in GCs by immunocytochemistry. In non-transfected GCs, endogenous SUMO-1 detected by SUMO-1 antibody was localized in the nucleus of GCs (green). The nucleus was stained by DAPI (blue). (A) After transfection with SUMO-1 expression vector or the mutant SUMO-1$^{G96/97A}$ expression vector for 48 h, the subcellular localization of Flag-SUMO-1 or Flag-SUMO-1 $^{G96/97A}$ was studied by FLAG antibody. (B) Immunostaining signals of SUMO-1 were observed in the nucleus of GCs. (C) The signals of SUMO-1$^{G96/97A}$ were observed both in the nucleus and cytoplasm of GCs (green). Original magnification is 40x.

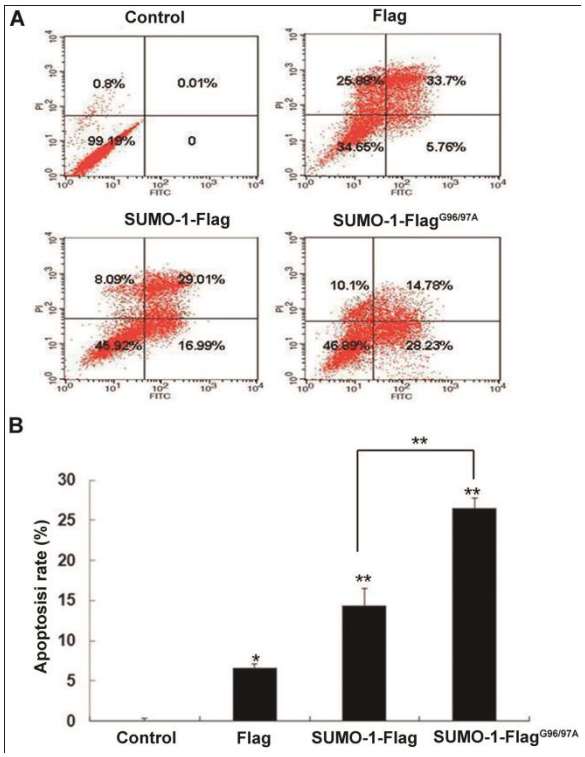

Fig. 5: SUMO-1 induced apoptosis in the mice GCs. Wild type SUMO-1 or mutant SUMO-1 $^{G96/97A}$ vectors were transfected in GCs for 48 h, while non-transfected cells and empty vector transfected group were served as control. (A) Representative flow cytometric analysis of apoptotic cells stained with Annexin V-FITC and PI, the lower right quadrant contains apoptotic cells. (B) Flow cytometric analysis was used to measure the apoptosis rate. The value expressed by each bar represents the mean ±SEM (n=4). *: P<0.05; **: P<0.001.

Previous study has found that the 96th and 97th glycine residues in SUMO-1 are important for SUMO conjugation to its substrate (Müller *et al*., 2001). The SF-1 did not SUMOylate by SUMO-1 when of 96th and 97th glycine residues were mutated (Chen *et al*., 2004). We initially predicted that mutation of SUMO-1 at these two sites might inhibit or decrease GCs apoptosis. However, in this study, we found that over-expression of mutant SUMO-1 at glycine 96 and 97 induced apoptosis rate was significantly higher than that by over-expression of wildtype SUMO-1. This result implied that normal function of SUMOylation mediated by SUMO-1 is also crucial for the living of GCs.

We predict that, firstly, the SUMO-1 modification of target proteins is dynamically regulated by SUMO ylation/de-SUMOylation, which dynamic equilibrium is important for the normal function of cells, increased or decreased SUMOylation might disrupt the equilibrium and result in the cell dysfunction, and eventually cell death. Secondly, SUMO-1 could covalently bind to many kinds of target proteins and involved in many important cellular functions in living cells, mutation of SUMO-1 could disrupt these cellular processes and might result in cell death. However, further study to uncover the target proteins of SUMOylation is necessary to understand the regulation of apoptosis by SUMOylation in GCs.

Several studies have demonstrated that SUMO-1 gene is highly conserved in humans and mice, and its mRNA is abundantly expressed in the ovaries (Su and Li, 2002). Similarly, higher expression of specific proteases, which are crucial for maturation and deconjugation of SUMO-1, is reported in the human ovary (Kim *et al*., 2000). In this study, immunoblot analysis revealed the expression profiles of SUMO-1 conjugates in the mouse ovary and GCs. Previously, our group has also successfully identified SUMO-1 modified proteins including P53, PTX3 and CEBPβ in the ovaries of different species (Liu *et al*., 2013; Zhen *et al*., 2014; Yang *et al*., 2017). In this study, the SUMO-1 conjugates were dynamic during super-ovulation after PMSG/hCG treatment. Previous reports have confirmed that RanGAP1, as the first identified SUMOylation target, was expressed in many tissues, with a molecular weight of 76 KDa (Evdokimov *et al*., 2008). We predict that the SUMO-1 conjugates at ~72 KDa might be the SUMO-1-RanGAP1 complexes. Furthermore, the SUMO-1 conjugates at 34 KDa might be the SUMO-1-UBC9 complexes, which showed 34 KDa band in other cells (Su *et al*., 2012). Furthermore, the expression of SUMO-1 conjugates was dynamic in ovaries after PMSG or hCG injection, which indicates that SUMO-1 mediated SUMOylation might be dynamically regulated during super-ovulation and folliculogenesis. The SUMO-1 is covalently conjugated to multiple protein substrates via SUMOylation pathway, acting as a specific effect on gene regulation and protein–protein interaction. In our previous study, we observed that the SUMO-1 protein is co-expressed with p53, PTX3 and CEBPβ in mouse GCs (Liu *et al*., 2013; Zhen *et al*., 2014; Yang *et al*., 2017), this provide the evidence that SUMO-1 may conjugate to its various substrates and can further modify their actions in GCs. This study also revealed that the higher molecular conjugates of SUMO-1 were abundantly expressed in GCs at 72 h in an in-vitro culture. Further studies are needed to explore the targets of SUMO-1, its regulation and roles of SUMOylation in GCs.

Immunohistochemical analysis showed that SUMO-1 exhibited highest expression on the GCs in the primary and secondary follicles, but lower signals were detected in antral follicles after gonadotrophin treatment. This observation is consistent with a previous report that the expression of SUMO-1 protein was greatly reduced in GCs of pre-ovulatory follicles but not in the GCs of early stages of follicular development (Shao *et al*., 2006). This is indicative of LH-mediated regulation of SUMO-1 expression in the mouse ovary. In rodents, PMSG-induced LH surge occurs at 56 h followed by ovulation 12-14 h later (Shao *et al*., 2006). Disruption of endogenous LH surge blocked the SUMO-1 inhibition in GCs. These results suggest that the activation of LH receptor is important for inhibition of SUMO-1. It is also possible that the SUMOylation of different targets may increase or inhibit SUMO-1 expression after gonadotropin treatment. However, further studies will be required to address this issue.

The immunostaining signal of SUMO-1 was found in the nucleus of GCs. However, after mutation of SUMO-1 at glycine 96 and 97 to alanine, the localization of mutated SUMO-1 was present both in the nucleus and the cytoplasm. In a previous study, p53-SUMO-1 was normally localized to PML bodies, however, site mutation in the SUMO-1 changed its localization to the nuclear envelope with some cytoplasmic staining (Carter *et al*., 2008). These findings implied that amino acids of SUMO-1 at G96/97 are important for the sub-cellular localization and functioning of SUMO-1 in mice GCs. The nuclear localization of SUMO-1 in GCs might regulate the sub-cellular localization of target proteins and may involve in the regulation of apoptosis.

Conclusions: Our results demonstrated the expression and localization pattern of SUMO-1 conjugates in the mouse ovary after PMSG/hCG injection and in the GCs during in vitro culture. We further revealed that over-expression of SUMO-1 significantly induced the GCs apoptosis, and amino acids at G96/97A site of SUMO-1 are crucial for the sub-cellular localization of SUMO-1 and its regulation of apoptosis.

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Authors contribution: FFY and LJH participated in its design, conceived of this study and coordination. HR and JJX participated in verification of data and helped modification the manuscript. All authors critically reviewed and edited the manuscript.

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