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RESEARCH ARTICLE

SUMOylation of PTX3 at Lysine 203 Regulates its Subcellular Localization in Mouse Ovarian Granulosa Cells

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ABSTRACT

PTX3 is a secretary acute phase protein which is produced by cumulus cells and is critically involved in cumulus expansion, oocyte maturation and formation of cumulus extracellular matrix. However, little is known about the post-translational modifications of PTX3 and its relationship with its subcellular localization in GCs. In this study, we found out whether PTX3 could be covalently modified by SUMO-1 which is involved in its subcellular localization in ovarian GCs. Immunoblotting and immunoprecipitation analysis indicated that PTX3 was modified by SUMO-1. Mutation of PTX3 at K203R significantly reduced the modification of PTX3 by SUMO-1, suggesting that Lysine 203 is a key amino acid for SUMO-1's modification of PTX3. Further study indicated that endogenous PTX3 was mainly located in the cytoplasm of GCs and overexpressed PTX3 could also be localized in the cytoplasm of GCs, while mutation of PTX3 at K203R was only localized in the nucleus. We propose that SUMOylation of PTX3 at Lysine 203 is crucial for its cytoplasm localization and might be involved in its secretion from cytoplasmic contents into extracellular matrix, which are important in the formation of cumulus extracellular matrix.

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INTRODUCTION

The surge of ovulatory luteinizing hormone is followed by the cumulus expansion, a process started by cumulus cells, which promotes the synthesis of variety of cell surface and extracellular matrix proteins under the control of exocrine and endocrine factors (Russell and Salustri, 2006). Appropriate structure, distribution and composition of cumulus cell-oocyte complex (COC) are critical for successful ovulation and ultimately fertility (Russell and Robker, 2007). Cumulus expansion-related genes (PTX3, PTGS2, HAS2 and TNFAIP6) are prerequisite for ample cumulus expansion because null mutation in any of these genes results in defective cumulus expansion (Varani *et al*., 2002; Fülöp *et al*., 2003; Ochsner *et al*., 2003; Chang *et al*., 2015). Pentraxin 3 (PTX3) also termed as TSG-14 is composed of 381 amino acids in mice and like other members of pentraxin family, its N-terminal region couples to a 203 amino acid C-terminal domain (Garlanda *et al*., 2005). The secretions of PTX3 by cumulus cells are typically initiated by cumulus expansion from where it localizes to extracellular matrix and that assists in successful cumulus expansion (Salustri *et al*., 2004; Jang *et al*., 2015). At ovarian level, recombinant PTX3 was efficiently purified in Chinese hamster ovary cells (Rivieccio *et al*., 2007). Previous studies showed the presence of PTX3 in human cumulus cells and found the remarkable sequence similarities (82%) between mouse and human and further hypothesized that PTX3 might share the same role in human female fertility as in mice (Salustri *et al*., 2004; Zhang *et al*., 2005). PTX3 also appears to regulate apoptotic cells clearance as it binds to membrane domains of apoptotic cells (Rovere *et al*., 2000). Experimental evidence suggests that in mice GCs, growth differentiation factor 9 (GDF9) is likely to be involved for temporal and automatic restriction of PTX3 expression during the ovulatory period (Varani *et al*., 2002). $PTX3^{-/-}$ female mice are sterile because of defective cumulus matrix

formation resulting in failed ovulation and fertilization (Rivieccio *et al*., 2007).

SUMO-1 is a member of ubiquitin-related proteins, which regulates gene expression and alters multiple important physiological functions (Hochstrasser, 2001; Seeler and Dejean, 2003; Andreou and Tavernarakis, 2009; Wilkinson and Henley, 2010; Treuter and Venteclef, 2011). Although the important roles of PTX3 have been reported in cumulus expansion and ovulation, little is known about the post-translational modification and its regulation of subcellular localization in ovarian GCs.

In the present study, we first predicted the SUMOylation sites of mouse PTX3 by using SUMO sp.2.0 software, and found there is a consensus motif (type I, ψ-K-x-E) for SUMOylation: RPMKLES. Therefore, we investigated the SUMOylation of PTX3 in GCs of early follicular phase. Because SUMOylation of PTX3 has never been investigated in mice GCs, therefore, we show that PTX3 is conjugated to SUMO-1 and is modified on lysine residue 203. In addition, we tried to detect the changes in the localization of PTX3 in GCs.

MATERIALS AND METHODS

Experimental animals: Female mice (21 days old) treatments were housed under the controlled temperature of 24ºC and 12 h light/12 h darkness with food and water ad libitum. The mice were treated with an intraperitoneal injection of 10 IU of PMSG and followed by 10 IU of hCG injection (PMSG/hCG) 48 h later, and ovaries were obtained at 6 h after hCG injection.

Plasmid construction: FLAG-SUMO-1 constructs were generated by PCR amplification of mice SUMO-1 cDNA. Then sub cloning in the SalI-BglII sites of pCMV-N-FLAG expression vector. PTX3 was amplified by PCR procedures and then sub cloned in pCMV-N-HA expression vector bearing BamHI-SalI sites. The mutant $PTX3^{K203R}$ was amplified and then sub cloned in pCMV-N-HA. The sequences of primer pairs used in this study were listed in Table 1.

Cell culture and transfection: GCs were isolated from the mice injected with PMSG/hCG. GCs were grown in 6-well culture dish in DMEM/F12 medium. Transfection was carried out using Lipofectamine LTX according to manufacturer's directions. After 48 h, GCs were collected for protein extraction, immunoprecipitation or other analysis.

Protein extraction, Immunoblotting and Co-immuno-precipitations: After transfection for 48 h, obtained protein form GCs. Protein mixed was separated by electrophoresis, and then transferred to 0.45 μm PVDF membrane. It was then incubated with polyclonal rabbit anti-PTX3 antibody (1:500 dilution), monoclonal mouse anti-HA antibody (1:1000 dilution), or monoclonal mouse anti-β-actin antibody (1:1000 dilution), at 4ºC overnight, respectively. Next day, HRP-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibody incubated. The blots were detected with the ECL chemiluminescence reagent. The primary antibodies were replaced with non-immunization rabbit or mouse IgG as a negative control.

Table 1: Sequence of primer pairs used for SUMO-1, PTX3 and $PTX3^{K203R}$ PCR amplification

Gene name		Primer sequences (5'–3')
SUMO-1	Forward	TGAATCGTCGACACCATGTCTGACCAG
	Reverse	AAAAGAGATCTCTAAACCGTCGAGTG
PTX3	Forward	GGATCCATGCACCTCCCTGC
	Reverse	CAGCTGTTAAGAAACATACTGGGCT
$PTX3^{K203R}$	Forward	CCAATGAGGCTTGAATCTTTTAG
	Reverse	CAAGCCTCATTGGTCTCACAG

Table 2: Putative consensus motif of SUMOylation sites in mammalian PTX3 predicted by SUMOsp 2.0 software

Species	Position	Peptide	Score	Cutoff	Type
Mouse	203	RPMKLES	1.422	0.13	type I:ψ-K-x-E
Pig	204	TPMKLEA	1.365	0.13	type I:ψ-K-x-E
Bovine	204	TPMKLET	1.474	0.13	type I:ψ-K-x-E
Rat	203	RPMKLES	1.422	0.13	type I:ψ-K-x-E
Goat	127	TPMKLES	1.536	0.13	type I:ψ-K-x-E
Sheep	203	TPMKLES	1.536	0.13	type I:ψ-K-x-E
Dog	166	TPMKLES	1.536	0.13	type I:ψ-K-x-E

Co-immunoprecipitation (Co-IP) of PTX3 and SUMO-1 in GCs. After co-transfection, the same amount of cell lysates was prepared in 1 mL of IP buffer. The lysate was centrifuged and supernatant was incubated with ANTI-FLAG M2 Affinity beads overnight at 4ºC with gentle rotating. Next day, eluted with 100 μL of 3×FLAG peptides and put on shaker for 30 min at 4ºC. The recovered protein was detected by Western blot using HA antibody.

Immunofluorescence analysis: The isolated GCs were plated and cultured on glass cover slips 48 h prior to transfection. After transfection for 48 h, GCs were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 solution, 5% BSA was added in GCs as a blocking buffer. Subsequently, cells were incubated overnight at 4ºC with monoclonal mouse anti-HA antibody (1:100). GCs were saturated with FITC-conjugated anti-mouse IgG secondary antibodies. For nuclear staining, cells were saturated in 10 mg/ml 4',6'-diamidino-2-phenylindole (DAPI), mounted on a glass slide using DABCO. For detecting the endogenous PTX3, non-transfected GCs was incubated with the polyclonal rabbit anti-PTX3 antibody (1:50) and then stained by PI to visualize the nucleus. Microscopy images were taken under a Nikon TE2000-U fluorescence microscope. The primary antibodies were replaced with non-immunization rabbit or mouse IgG as a negative control.

Data analysis: All experiments were repeated at least three times independently. The data was represented as mean±SEM and analyzed by using SPSS 17.0 software. If difference was found after One-Way ANOVA, significance was measured by using Tukey's post hoc test. A value of $P<0.05$ was considered significantly different and extremely significant when $P<0.01$.

RESULTS

Analysis and Predication of SUMOylation sites in mouse PTX3: PTX3 has been found important for assembly of cumulus extracellular matrix and cumulus expansion, little is known about the post-translational modification of PTX3. SUMOylation is an important manner for protein modification at post-translational level.

To reveal whether PTX3 has consensus motif of SUMOylation and serves as substrate for SUMO modification, we firstly used the SUMOsp 2.0 software to analyze and predict the SUMOlyation sites in mouse PTX3. Analysis of PTX3 protein revealed the presence of one conserved putative SUMOylation sites, at position of Lys203: RPMKLES, as a type I (ψ-K-x-E) SUMOylation binding site, we also found the similar consensus motif of SUMOylation site in PTX3 of other mammals (Table 2). Therefore, we predict that mouse PTX3 might be the target of SUMOylation.

Lys203 serves as a critical amino acid for covalent modification of PTX3 by SUMO-1: Although we predicted that there was consensus motif of SUMOylation sites in mouse PTX3, we had no direct evidence that PTX3 could be modified by SUMO-1. To test whether PTX3 could be a substrate of SUMO-1, and whether PTX3 lysine 203 may indeed serve as an acceptor site for SUMO-1, we used site-directed mutagenesis and constructed the mutant ($PTX3^{K203R}$) vector in which the lysine was replaced with arginine. We transfected GCs with the expression vector of PTX3 or mutant $PTX3^{K203R}$ alone, or co-transfected with FLAG-SUMO-1 expression vector, respectively. After transfection, total protein was extracted and analyzed by western blotting using PTX3 or HA antibodies. As SUMO-1 and PTX3 proteins have molecular masses of approximately 12 kDa and 43 kDa, respectively. Therefore, the predicated molecular mass of SUMO-1 modified PTX3 complex was about 55 kDa. The results showed that PTX3 at 43 kDa was detected in all samples. However, in transfection group with PTX3 or $PTX3^{K203R}$, there was also a band was detected by PTX3 antibody at 55 kDa (Fig. 1A). Interestingly, the band intensity of 55 kDa in PTX3 transfection was significantly higher than that in $PTX3^{K203R}$ transfection group ($P<0.05$). Similarly, the band intensity of 55 kDa in PTX3 and SUMO-1 co-transfection group was also significantly higher than that in $PTX3^{K203R}$ and SUMO-1 co-transfection group ($P<0.01$). We predicted that this 55 kDa protein band detected might be the SUMO-1 modified PTX3 (SUMO-1-PTX3 complexes). Moreover, by using HA antibody to detect the over-expressed PTX3, we could also observe that there was a band at 55 kDa specifically detected in transfection group, and the band intensity in PTX3 and SUMO-1 co-transfection group was significantly higher than that in $PTX3^{K203R}$ and SUMO-1 co-transfection group ($P<0.01$) (Fig. 1B). These results implied that this 55 kDa of protein band might be the SUMO-1 modified PTX3 and K203R mutation significantly reduced its SUMOylation, which suggested that Lys203 is critical for PTX3 covalently modification by SUMO-1.

To further test the SUMOylation of PTX3 and detect the critical site in PTX3, immunoprecipitation analysis was employed to detect whether PTX3 could be covalently modified by SUMO-1. We co-transfected GCs with the expression vector of HA-PTX3 and FLAG-SUMO-1 or $HA\text{-}PTX3^{K203R}$ and FLAG-SUMO-1, respectively. After transfection, the same amount of protein sample was extracted from cells and incubated with the FLAG antibody to pull down SUMO-1, and then

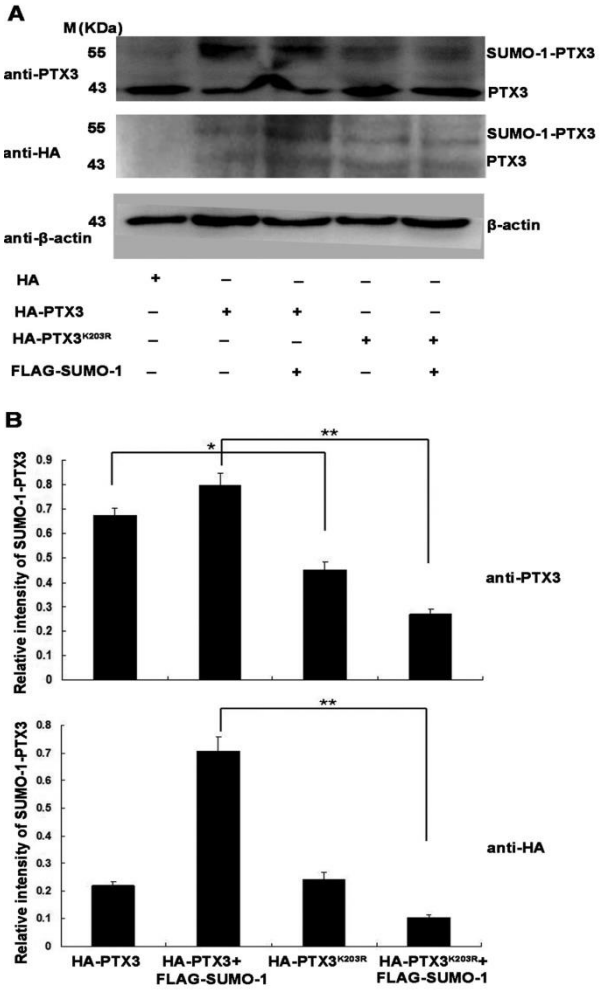

Fig. 1: Modification of PTX3 in GCs. **(A)** as detected by western blotting using PTX3 antibody (55kDa) or HA antibody. β-actin (43kDa) was served as control. (B) The relative intensity of protein bands was scanned by Gel-Pro analyzer 4.0 and normalized with β-actin. *$P<0.05$, **$P<0.01$.

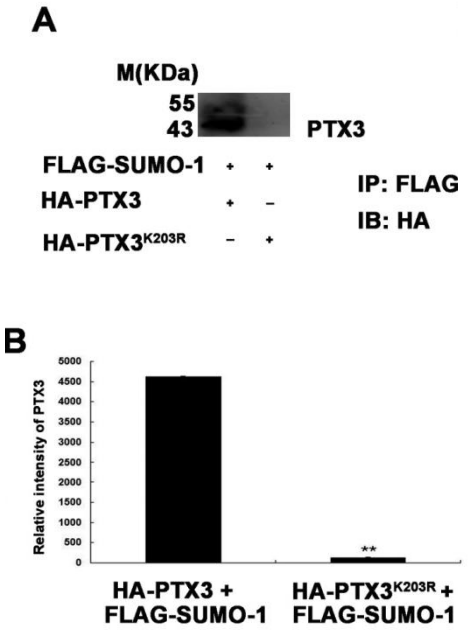

Fig. 2: Immunoprecipitation of PTX3 with SUMO-1. (A) By using a normal (HA-PTX3) and a mutant ($HA\text{-}PTX3^{K203R}$) expression vector, respectively. At 48 h it was analyzed by Western blotting using HA antibody. (B) The intensity of PTX3 or $PTX3^{K203R}$ detected by HA antibody was scanned by Gel-Pro analyzer 4.0. **P<0.01.

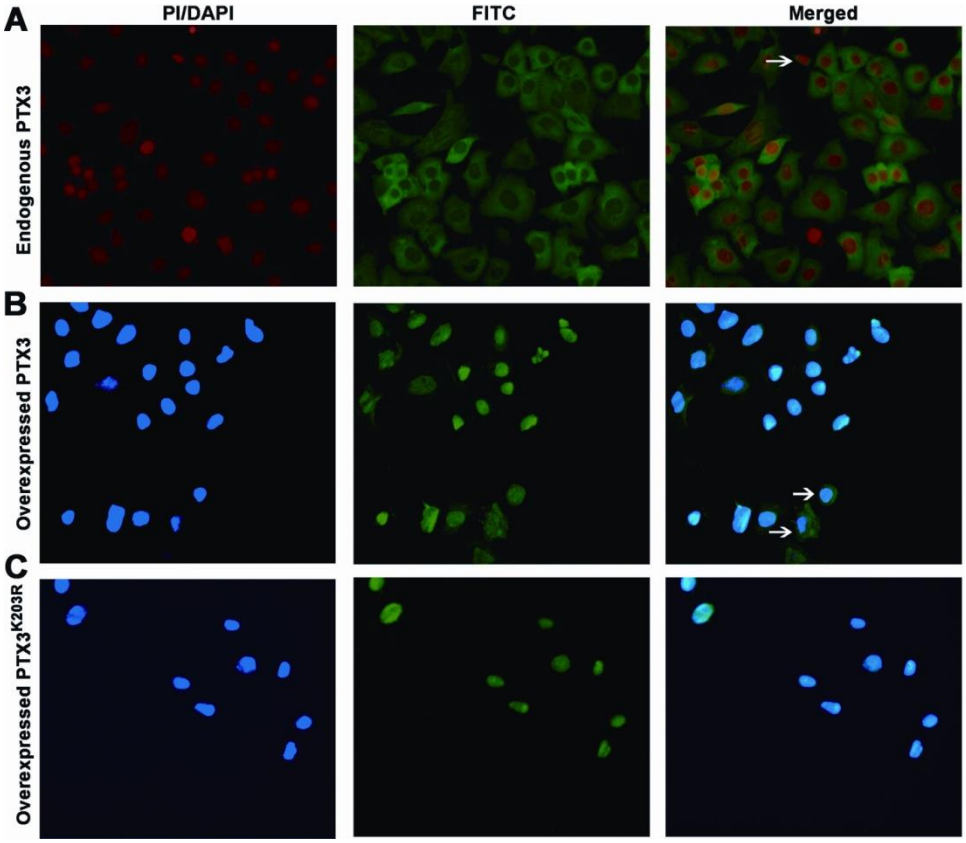

Fig. 3: Subcellular localization of PTX3 and PTX3^{K203R} in GCs. (A) Endogenous PTX3 was detected by indirect immunofluorescene using PTX3 antibody in non-transfected GCs. (B, C) Overexpressed PTX3 and PTX3^{K203R} were detected in HA-PTX3 or HA-PTX3^{K203R} transfected GCs by using HA antibody, respectively. The expressions were predominantly localized in the cytoplasm, but few were in the nucleus (arrow marked). The overexpressed PTX3 was localized in the nucleus, but few were detected in the cytoplasm of GCs (arrow marked). The mutant PTX3^{K203R} was only detected the localization in the nucleus of GCs.

HA antibody was used to detect the PTX3 or PTX3^{K203R}. The results showed that PTX3, but not PTX3^{K203R}, could be co-immunoprecipitated in the sample of SUMO-1 pull down (Fig. 2A, B). This result provided the direct evidence that PTX3, but not PTX3^{K203R}, could be covalently modified by SUMO-1, and suggested Lys203 is a critical amino acid for PTX3's modification by SUMO-1 in mouse GCs.

Mutation of PTX3 at K203R affected its subcellular localization in GCs: PTX3 is a secreted acute phase protein that is critically involved in cumulus expansion, oocyte maturation and plays a non-redundant role in the formation of cumulus extracellular matrix. In this study, we confirmed that PTX3 could be covalently modified by SUMO-1 at Lys203, and mutation of this amino acid significantly reduced its modification by SUMO-1. Furthermore, SUMOylation was reported affect the localization of target protein in cells. Therefore, we used immunofluorescent staining method to detect the subcellular localization of PTX3 and PTX3^{K203R} after transfection in GCs.

The results showed that the endogenous PTX3 protein was predominantly localized in the cytoplasm and few was localized in the nucleus (Fig. 3A, arrow). The overexpressed PTX3 was also detected the localization in both cytoplasm and nucleus in GCs (Fig. 3B, arrow). However, the overexpressed mutant PTX3^{K203R} was only localized in the nucleus (Fig. 3C). Because K203 serves as consensus motif for PTX3's modification by SUMO-1, we predicted that SUMOylation of PTX3 at K203 is critical for its cytoplasmic localization in GCs, and mutation of PTX3 at K203R disrupted the cytoplasm localization of PTX3 and might affect its secretion from cytoplasm into extracellular matrix.

DISCUSSION

SUMOylation has come out as a crucial post-translational modification that plays vital regulatory roles in various cellular pathways, involved in regulation of protein-protein interactions, the subcellular localization and stability of target proteins (Meulmeester and Melchior, 2008), thus functions in many kinds of biological processes and disease control (Kotaja *et al*., 2002; Liu *et al*., 2013; Yun *et al*., 2013). In human cumulus cells, PTX3 may be indicative of the quality of their enclosed oocyte (Zhang *et al*., 2005). In addition, PTX3 mainly played a role in fertilization process because it acts as a nodal point for the assembly of the cumulus oophorus hyaluronan-rich extracellular matrix (Garlanda *et al*., 2011; Huang *et al*., 2013). In addition, PTX3 plays a key role in the assembly of extracellular matrix, which is essential for COC matrix formation, ovulation and in vivo fertilization (Chang *et al*., 2015). The present study revealed that PTX3 is a target of SUMO-1 modification and its covalently binding of Lysine 203 in the consensus sequence ψ-K-x-E with SUMO-1 was involved in its subcellular localization in GCs. Mutation of Lysine 203 significantly reduced its

modification by SUMO-1 and inhibited its localization in cytoplasm of GCs, thus might affect its secretion from cytoplasm into cumulus extracellular matrix and function of cumulus expansion.

In the previous study, SUMO-1 was localized in the nucleus of different cells (Rodriguez *et al*., 2001; Giri *et al*., 2008), which implied that the SUMOylation of PTX3 is a nuclear process and its SUMOylation facilitated the translocation from nucleus into cytoplasm and secretion from cytoplasm into extracellular matrix. In this study, we found that overexpressed PTX3 and wide type of PTX3 could be detected in the cytoplasm of GCs, but the mutant $PTX3^{K203R}$ was only detected in the nucleus of GCs. We predicted that after PTX3 translation in the cytoplasm, the protein was transported into the nucleus for SUMOylation, which was necessary for its re-translocation to the cytoplasm and secretion from cytoplasm into extracellular matrix to perform its biological function in cumulus matrix. Previous studies also suggested the presence of specific nuclear localization signal for successful SUMOylation (Rodriguez *et al*., 2001). Due to only a fraction of certain protein was SUMOylated, we predict that endogenous PTX3 localization in the cytoplasm was caused by most of PTX3 proteins was not SUMO-1 modified, or has already been SUMOylated and transported into the cytoplasm. Most of the overexpressed PTX3 localized in the nucleus, but just a few detected in the cytoplasm of GCs, the possible explanation for that is: the over-expressed PTX3 was transported into the nucleus waiting for SUMOylation, but inside of the cells, the level of PTX3 or SUMOylated PTX3 has been enough for its functioning, therefore, the efficiency for SUMOylation of PTX3 was not as it used to be, which resulted in only a few of cells showed the overexpressed PTX3 signal in the cytoplasm, but most of PTX3 localized in the nucleus. However, further studies are required for its validation.

The level of PTX3 and SUMO-1 co-expression was higher than that SUMO-1 and PTX^{K203R}. The results of Co-IP indicated that PTX3 could be SUMO-1 modified Lys203. A possible explanation is that $PTX3^{K203R}$ competes with the endogenous wild-type PTX3 for SUMO-1, and this leads to reduced levels of PTX3 and SUMO-1. Previous studies confirmed the lower expression of PTX3 after 1 h hCG treatment in cumulus and mural GCs. The expression level of PTX3 was increased in the surrounding GCs of follicular antrum after 5 h of hCG exposure. Similarly, PTX3 mRNA was detected in cumulus GCs after the LH surge. Further experiments confirmed its expression in the lining of the preovulatory follicles and documented its peak expression 8 h after the exogenous LH stimulation (Varani *et al*., 2002). Some researchers demonstrated that PTX3 is likely to impact on cumulus matrix formation and conferring PTX3 as a stably nodal molecule in the cumulus matrix (levoli *et al*., 2011). Some results indicated that miR-224 may affect ovulation and subsequent embryo development by targeting PTX3 (Yao *et al*., 2014). mRNA expression levels of leptin receptor isoforms were correlated with expression of PTX3 (Van *et al*., 2010). It has been shown that PTX3 multimeric organization is essential to the protein activity in cumulus matrix assembly and stabilization, and previous studies showed that PTX3-deficient mice were unable to organize the glycosaminoglycan HA in a stable cumuli matrix and exogenously recombinant PTX3 could rescue defective cumulus expansion in vivo (Bottazzi *et al*., 1997; Scarchilli *et al*., 2007). PTX3 binds to HCs of IαI and TSG-6, which are essential for cumulus matrix formation and able to interact with hyaluronan. Cys/Ser mutants of recombinant N-terminal region of PTX3 is unable to rescue cumulus matrix organization, and that the tetrameric assembly of the protein is the minimal oligomeric state required for accomplishing this function (levoli *et al*., 2011). In this study, we found that PTX3 could be SUMO-1 modified at Lys203 amino acid residue. We predicted that SUMOylation of PTX3 might also be involved in its normal functioning in cumulus matrix and interaction with other molecules in the extracellular matrix, thus important for the assembly of cumulus matrix and cumulus expansion.

Conclusions: This study demonstrated that PTX3 could be covalently SUMOylated by SUMO-1 at Lysine 203 in mice GCs, Mutation in K203R significantly reduced its SUMOylation and altered its subcellular localization. The SUMOylation of PTX3 might be involved in its translocation from cytoplasm into cumulus matrix or its normal functioning in the assembly of cumulus matrix and interaction with other molecules in the extracellular matrix. This study also reported the regulation of PTX3 at the post-translational manner by SUMOylation, which provided new insights for the regulation of PTX3 function during follicular development and ovulation.

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