Mutation of rat *Zp2* causes ROS-mediated oocyte apoptosis

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Abstract

In this study, we investigated a gene-edited (*Zp2*^{MT/MT}) rat model of infertility caused by the failure to express the zona pellucida glycoprotein 2 (ZP2) due to the significant reduction of mRNA amount. We examined the defects in the zona pellucida (ZP) caused by ZP2 nullification and the influence of these defects on aspects of oocyte development, including apoptosis and fertilization ability. To investigate the cause of the influence to the oocytes' development, we evaluated the morphology of follicular transzonal projections (TZPs), known as 'bridges', which mediate the bidirectional signaling between the oocyte and surrounding granulosa cells and the level of reactive oxygen species (ROS) in ovulated eggs. Our results showed that two types of ZP defects were generated in the *Zp2*^{MT/MT} rat, that is, ZP intact but thinned and ZP cracked (or even absent). The fertilization rate of the ovulated eggs reduced in both types, while increased oocyte apoptosis was observed only in the latter type. Moreover, the increased oocyte apoptosis rate correlated closely with the reduction in follicular TZPs and increased ROS levels in ovulated egg. In conclusion, nullification of rat ZP2 destroyed the integrity of the ZP, impaired the bidirectional signaling between the oocyte and surrounding granulosa cells. Therefore, the resulting infertility likely occurs via elevation of oxidative stress and oocytes apoptosis.

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Introduction

Zona pellucida (ZP) is a unique shield-like structure enclosing the growing oocyte, ovulated egg, and preimplantation embryos (Wassarman 2008). ZP plays a vital role in oogenesis (Wassarman & Litscher 2012), fertilization (Gupta *et al.* 2012) and preimplantation embryogenesis (Suzuki *et al.* 1995, Lamas-Toranzo *et al.* 2019). In mammals, the ZP is formed by three to four zona pellucida glycoproteins (ZPGs) depending on the species, for example, four in human and rat (designated hZP1–4 and rZP1–4, respectively) and three in mouse (mZP1–3). Since we reported the first familial infertility caused by human *ZP1* mutation in 2014 (Huang *et al.* 2014), an increasing number of ZP-mutations-associated infertile cases has been reported (Chen *et al.* 2017, Liu *et al.* 2017, Dai *et al.* 2019a,b, Sun *et al.* 2019, Zhou *et al.* 2019). One of these cases, hZP2-associated infertility, is characterized by intact and thinned ZP and *in vitro* fertilization (IVF) failure, although the number of retrieved eggs (16–25 eggs per cycle) was undiminished (Dai *et al.* 2019b). However, *Zp2* null mice had unsustainable, extremely thinned ZP during folliculogenesis, producing fewer ovulated eggs

(1.4 per mouse) with no ZP (Rankin *et al.* 2001). The ZP phenotype and the damage to ovulation in humans affected by hZP2-associated infertility are much less severe than those in the *Zp2* null mouse. Furthermore, in humans, ZP4 can possibly compensate for the loss of ZP2 (Dai *et al.* 2019b). Therefore, we hypothesized that the existence of ZP4 in rat makes this a better choice as an animal model of hZP2-associated infertility.

To investigate the details of hZP2-associated infertility, we used the CRISPR/Cas9 system to create a gene-edited rat model carrying a similar *Zp2* mutation, that is, the *Zp2*^{MT/MT} rat. However, the phenotype of the *Zp2*^{MT/MT} rat was unexpected in that most antral follicular oocytes and ovulated eggs had cracked or even absent ZPs and presented apoptotic morphology. The transzonal projections (TZPs) are filamentary structures consisting of elongated cytoplasmic tips spanning the ZP to connect the oocyte and surrounding granulosa cells. These 'bridges' mediate the bidirectional signaling that is critical for the survival and development of oocytes and granulosa cells (Simon *et al.* 1997, Wang *et al.* 2019). Previously, we suggested that destruction of the integrity of the ZP could reduce the density of TZPs and decrease the quality of eggs (Wang *et al.* 2019). Several previous

studies have indicated that reduction of oocyte-granulosa cell bidirectional signaling may trigger the generation of reactive oxygen species (ROS) and that the resulting increase in oxidative stress is one of the major factors that induce oocyte apoptosis (Tiwari *et al.* 2015). Thus, we hypothesize that, in the $Zp2^{MT/MT}$ rat, destruction of the integrity of the ZP impairs the bidirectional signaling between the oocyte and surrounding granulosa cells, elevates oxidative stress, and induces oocyte apoptosis, and thus it results in female infertility. To test this hypothesis, we investigated the fertility of the $Zp2^{MT/MT}$ rat, as well as apoptosis of follicular oocytes, the morphology of TZPs in folliculogenesis, and the level of ROS in ovulated eggs. To the best of our knowledge, this is the first study to explore the mechanism of oocyte apoptosis caused by ZP gene mutations.

Materials and methods

Animals

$Zp2^{MT/MT}$ rats were generated by CRISPR-Cas9 technology-mediated DNA editing. To make the mutation in rats comparable

to those of humans affected by ZP2-associated infertility (c.1695_2A>G and c.1691_1694dup), two guide RNAs (gRNA), gRNA 1 (5'-TATCCTACCAACGGCCTTATAGG-3') and gRNA 2 (5'-GGTGCTGTGGCTGCACTAGTGGG-3'), were designed based on bioinformatic analysis to introduce site-specific double-strand breaks (DSB) in exon14 and exon18 of rat $Zp2$, respectively (Fig. 1A). Recombination of genomic DNA was completed by non-homologous end-joining (NHEJ) (Fig. 1A). The gRNAs and Cas9 mRNA were transcribed and purified *in vitro* using MEGAshortscript™ T7 Transcription Kit (AM1354, Thermo Fisher Scientific), MEGAclear™ Transcription Clean-Up Kit (AM1908, Thermo Fisher Scientific), and Cas9 plasmid (CAS9P, Sigma-Aldrich). The purified gRNAs and Cas9 mRNA were co-injected into the zygotes collected from Sprague-Dawley rats by oviduct flushing 13 h post-mating. After *in vitro* culture in the KSOM medium (MR-101-D, Sigma-Aldrich) at 37°C and 5% CO₂ overnight, two-cell embryos were obtained and transferred into the oviducts of pseudo-pregnant rats. Approximately 3 weeks later, neonates were genotyped by PCR using three pairs of primers (Fig. 1A, arrows or arrow heads) and Universal Genomic DNA Extraction Kit (9765, TaKaRa), and PCR Amplification Kit (M712B, Promega). Only the rats with the pre-designed mutation were considered as the F0 generation and were used to establish a strain. F0 rats were

Table 1 Antibodies, primers and gRNAs.

Resources	Names	Description	Manufacturer	Catalog	Other information		
					IHC	WB	Product length, bp
Primary antibodies	ZP1	Rabbit-derived	Santa-cruz, USA	134757	1:200		
	ZP2		Abcam, USA	ab2719677	1:200	1:500	
	ZP3		Santa-cruz, USA	25802	1:200		
	ZP4		Abcam, USA	ab2553903	1:200		
Secondary antibody	ZP1–4	Goat Anti-Rabbit IgG	Affinity, USA	S0001	1:200	1:2000	
Primers (5'–3')	Pair 1			BioSune, China			316
	F	AGGATGACCGTGAGATGC					
	R	AACCTATGATGACTGCTGAG					
	Pair 2			–			490
	F	TTCCAGGATGACCGTGAGATGC					
	R	CAAGCAACCCTGGTGTGTAGGAGTA					
	Pair 3						294
	F	CAGAATAGGAGAACGGGAGG					
	R	GTAAGGTCAATCCTGATTGG					
	Pair 4						$Zp2^{WT/WT}$ rats: 2108; $Zp2^{MT/MT}$ rats: 1706
	F	AGGTATCTTCCAACATGGCTAGG					
	R	GTCCAATCAGGAATTGAACCTTAC					
gRNA (PAM in bold; 5'–3')	Pair 5						189
	F	GGGCTTTGGACAAAGGAACA					
	R	AGCATGTCCCTTGGAAGTT					
	Pair 6						150
	F	CCCATCTATGAGGGTTACGC					
	R	TTTAATGTACGCACGATTTC					
	gRNA 1	TATCCTACCAACGGCCTTAT AGG					Obtained from <i>in vitro</i> transcription
	gRNA 2	GGTGCTGTGGCTGCACTAGT GGG					

F, forward; PAM, protospacer adjacent motif; R, reverse.

crossed with WT females to obtain $Zp2^{WT/MT}$ F1 rats, which were intercrossed to obtain F2 rats ($Zp2^{WT/WT}$, $Zp2^{WT/MT}$ and $Zp2^{MT/MT}$). F3 rats were obtained by intercrossing F2 rats.

In this study, the $Zp2^{WT/WT}$ and $Zp2^{MT/MT}$ rats were F2 or F3, whereas the $Zp2^{WT/MT}$ rats were F1. Only sexually mature rats (aged 13–15 weeks) were used. All rats were housed in an environmentally controlled room maintained at $23 \pm 1^\circ\text{C}$ with a 12 h light:12 h darkness cycle. Animal protocols were approved by The Animal Care and Use Committee, Central South University, China.

DNA, mRNA and protein verification

DNA verification was performed in all the F1 rats. Following the manufacturers' instructions, genomic DNA was extracted (9765, TaKaRa) from rat tail samples and *Zp2* gene was amplified (M712B, Promega) by PCR using the primers (Pair 1–3. Pair 1-forward: 5'-AGGATGACCGTGAGATGC-3', upstream of the target deletion DNA fragment, Pair 1-reverse: 5'-AACCTATGATGACTGCTGAG-3', downstream of the above fragment; Pair 2-forward: 5'-TTCCAGGATGACCGTGAG

ATGC-3', upstream of the fragment, Pair 2-reverse: 5'-CAAGCAACCCTGGTGTGTAGGAGTA-3', within the fragment; Pair 3-forward: 5'-CAGAATAGGAGAACGGGAGG-3', within the fragment, Pair 3-reverse: 5'-GTAAGGTTCAATTCCTGATTGG-3', downstream of the fragment) described in Fig. 1A and Table 1. After purification, DNA sequencing was performed. In addition, off-target analysis was carried out by examining the top ten potential off-target sites (predicted by CRISPOR, shown in Supplementary file, see section on supplementary materials given at the end of this article) of gRNA 1 and gRNA 2.

RNA was extracted from rat ovary samples according to the manufacturer's protocol of TRIzol™ Plus RNA Purification Kit (12183555, ThermoFisherScientific). RT of mRNA was performed with the use of GoScript™ RT System (A5001, Promega). PCR amplification of *Zp2* cDNA was performed with a pair of primers (forward: 5'-AGGTATCTTCCAACATGGCTAGG-3', reverse: 5'-GTCCAATCAGGAATTGAACCTTAC-3', Table 1), which covered the whole coding sequence. The lengths and sequences of PCR products were confirmed by agarose gel electrophoresis and Sanger sequencing, respectively.

Subsequently, quantitative PCR (qPCR) was performed with two pairs of primers (Pair 5 for *Zp2* and Pair 6 for *Actb*. Pair 5-forward: 5'-GGGCTTTGGACAAAGGAACA-3', Pair 5-reverse: 5'-AGCATGTCCCCTTGGAAGTT-3'; Pair 6-forward: 5'-CCCATCTATGAGGGTTACGC-3', Pair 6-reverse: 5'-TTTAATGTCACGCACGATTTC-3'. Table 1) according to the manufacturer's instructions of MonAmp™ ChemoHS qPCR Mix (MQ00201S, Monad, China) and the products were sequenced. In addition, in each genotype, we used 3–5 normal ovulated eggs for qPCR, following the manufacturer's protocol of Fast Advanced Cells-to-CT™ qPCR Kit (A35379, Thermo Fisher Scientific).

Proteins were verified by Western blot analysis. The total proteins were extracted from ovary tissue with cell lysis kit (87787, Thermo Fisher Scientific) and separated by sodium dodecyl sulfate PAGE (SDS-PAGE). Proteins were transferred to the nitrocellulose membrane and incubated with the ZP2 primary antibody (ab2719677, abcam) at 4°C overnight. The membrane was then washed and incubated with the secondary antibody (S0001, affinity, USA.) at room temperature (RT) for 1 h. After washing, the ZP2 bands on the membrane were visualized by enhanced chemiluminescent detection reagent kit (34577, Thermo Fisher Scientific).

Isolation of ovaries and ovulated eggs

Female rats were killed by intraperitoneal injection of pentobarbital sodium with the dosage of 200 mg/kg body weight, and their ovaries were isolated directly without hormone stimulation. Immediately after isolation, the ovaries were frozen in the liquid nitrogen for mRNA sequencing, qPCR, and Western blotting, or fixed in 4% paraformaldehyde solution for periodic-acid Schiff (PAS) staining, immunohistochemistry, terminal deoxynucleotidyl transferase 2'-deoxyuridine, 5'-triphosphate nick-end labeling (TUNEL) analysis and phalloidin staining.

For super-ovulation, female rats were intraperitoneally injected with 40 IU pregnant mare serum gonadotropin (PMSG) between 5 pm and 6 pm. After 48 h, they were injected with 40 IU human chorionic gonadotropin (hCG). Fifteen hours later, these rats were killed and their oviducts were isolated and placed into the paraffin oil (pre-incubated at 37°C and 5% CO₂ for 1 h) of a culture dish containing some oil-covered droplets. These droplets were classified as M2-hyaluronidase droplets, composed of M2 medium (M7167, Sigma–Aldrich) and hyaluronidase (1 mg/mL) or M2-alone droplets, composed of M2 medium alone. Under the microscope, the expanded ampullae were torn open using two 30 G hypodermic needles and the overflowing cumulus-oocyte complexes (COCs) were drawn into the M2-hyaluronidase droplets. The COCs were gently blown with a pipette with a very fine blunt tip to disperse the granulosa cells. When the eggs were almost completely detached from the granulosa cells, they were transferred into the M2-alone droplets. After thorough washing with M2 medium, the eggs were viewed under a microscope (IX73, Olympus Corporation). For each rat, the ovulated eggs were counted and their morphology was evaluated. The eggs with normal morphology (full-grown spherical oocyte with homogeneous cytoplasm) were ready for IVF, qPCR, and ROS analysis.

Ovarian histology

After fixation in the 4% paraformaldehyde solution for 12–16 h, ovaries were dehydrated with a series of ethanol solutions with increasing concentrations (30%, 50%, 70%, 90%, and 100%). Then the ovaries were embedded in paraffin for PAS staining, immunohistochemistry, and TUNEL analysis, or in the optimal cutting temperature compound (6404, Newcomer Supply, USA) for phalloidin staining which requires frozen sections. Consecutive sections were prepared for immunohistochemistry, whereas intermittent sections were made at an interval of 200 µm for PAS staining, TUNEL analysis, and phalloidin staining. The tissue section thickness was 4 µm for PAS staining and TUNEL analysis and 8 µm for phalloidin staining. Before use, all paraffin sections were thoroughly dewaxed by dimethylbenzene, rehydrated by a series of ethanol solutions with decreasing concentrations (100%, 90%, 70%, 50%, 30%), and then washed with double distilled water and PBS solution.

PAS staining was performed according to the manufacturer's protocol of PAS Stain Kit (D004-1-2, Nanjing Jiancheng Bioengineering Institute, China). The growing follicles (GFs) were classified as follows: primary follicles (PFs), with only one intact layer of cubic granular cells; secondary follicles (SFs), with two (early secondary follicles, ESFs) or more (late secondary follicles, LSFs) intact layers of cubic granulosa cells but without any obvious antrum; and antral follicles (AFs), with an obvious antrum. The classification was based on the principle suggested by Gook *et al.* (2008), but herein some modifications were made, for example, SFs were classified into ESFs and LSFs. According to the description of Escobar *et al.* (2012), the oocytes in the GFs were considered to be morphological dead when at least one of the following conditions was met: severe deformation; fragmentation; or diffuse vacuolation. In each section, the GFs were classified and counted to calculate the constituent ratios of the total GFs in the *Zp2*^{WT/WT} and *Zp2*^{MT/MT} rats. The morphology of GFs was evaluated and the differences between *Zp2*^{WT/WT} and *Zp2*^{MT/MT} rats in terms of apoptosis were analyzed.

For immunohistochemistry, antigen retrieval was performed using the microwave heating method as following: ovarian tissue sections were put into 1 L of boiled sodium citrate antigen retrieval solution (C1031, Solarbio, China); the solution was reboiled for 4 min and then rested for 6 min; the step was repeated three times and finally the solution cooled down naturally. After incubation with the primary antibody (ZP1: 134757, santa-cruz; ZP2: ab2719677, abcam; ZP3: 25802, santa-cruz; ZP4: ab2553903, abcam) at the dilution of 1:200 overnight, the tissue sections were washed five times with PBS and then were incubated with the secondary antibody (S0001, affinity) at 37°C for 1 h. Subsequently, sections were thoroughly washed and stained with hematoxylin. For negative controls, the same procedures were used except that the primary antibody was replaced with PBS. TUNEL assays were performed according to the manufacturer's instructions of In Situ Cell Death Detection Kit (11684795910, Roche) and phalloidin staining was performed as described by Wang *et al.* (2019). Finally, sections were stained with DAPI to indicate the cell nucleus. With the utilization of image-pro plus 6.0 software (Media Cybernetics Corporation, USA), the density

Figure 2 Follicular ZP defects and accompanied oocyte dysmorphology in *Zp2^{MT/MT}* rats. A and D indicate primary follicles; B1–3 and E1–4 indicate secondary follicles; C (C1–6) and F (F1–4) indicate antral follicles. Arrows indicate the ZP and arrow heads indicate the ectopic granulosa cells. Asterisks indicate the fragments of dead oocytes. Scale bar: 1 mm in C and F, 50 μ m in others.

of TZPs in each GFs was calculated according to the following formula:

$$\text{density of TZPs} = \frac{\text{integral optical density (IOD) within the ZP area}}{\text{the area of ZP } (\mu\text{m}^2)}$$

In vitro experiments of ovulated eggs

For IVF, male rats were killed and their epididymides were isolated. The semen was expelled into a sterile culture dish using fine tweezers. Using a glass capillary with a fine, curved and sealed tip, a small amount of semen was collected and put into the oil-covered droplets of 100 μ L human tubal fluid (HTF) solution (MR-070-D, Sigma-Aldrich), which was pre-equilibrated at 37°C under 5% CO₂ for 8–12 h. After 30 min, a certain volume of the HTF solution (containing the sperm) was transferred into a new pre-equilibrated oil-covered HTF droplet (100 μ L, not containing sperm) to create an IVF droplet with a final sperm density of 10⁶/mL. Three hours later and immediately after isolation, eggs were transferred into the IVF droplets and co-incubated with the capacitated sperm for 6 h. The eggs were then thoroughly washed with HTF solution and then cultured for 4 h. Under the relief contrast microscope (IX73, Olympus), zygotes with two pronuclei were counted and the fertilization rates were calculated.

The ROS level of eggs was assessed with the ROS assay kit (CA1410, Solarbio, China) according to the manufacturer's

instructions. Then 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was added into the M2-alone droplet containing the isolated eggs, and the final concentration of DCFH-DA was controlled at 10 μ mol/L. *In vitro* culture of eggs was continued for 30 min, during which time the eggs were gently blown with a fine pipette every 10 min. The eggs were then washed five times with M2 medium and imaged under a fluorescence microscope.

Fertility assessment

The litter size of each female was recorded after mating with a male (aged 13–30 weeks) proven to be fertile for 3 months or until a pregnancy was observed. During the 3 months of observation, females that did not become pregnant were considered infertile.

Statistical analysis

The measurement data were shown as mean $\pm$ s.d., and the differences among groups were analyzed by one-way ANOVA following the Student–Newman–Keuls test. The counting data were shown as rate $\pm$ s.e., and the differences between groups were analyzed by Chi-square test. All tests were performed with the software SPSS 19. *P* < 0.05 was considered to indicate statistical significance.

Results

The $Zp2^{MT/MT}$ rat was successfully generated, but the mutant ZP2 protein failed to be expressed due to the significant reduction of mRNA amount

A total of 97 zygotes were micro-injected and transferred into pseudo-pregnant rats, resulting in the delivery of four pups. Only one male (F0) carried the pre-designed mutations, consisting of a 2849 bp deletion between exon 14 and exon 18 and five adjacent point mutations upstream of the deletion in both *Zp2* alleles. These mutations were also confirmed in the mutant alleles of *Zp2* in all six F1 rats (two males and four females) (Fig. 1B). Off-target analysis of gRNAs for all the F1 rats showed that all the tested genomic DNA sequences (Supplementary file, site 1–10 and site 11–20 were the top 10 potential off-target sequences for gRNA1 and

gRNA2, respectively) were normal with no mutations. The four F1 females and six randomly selected F2 females (3 $Zp2^{WT/WT}$ and 3 $Zp2^{MT/MT}$) were used for mRNA and protein analyses. In addition to the five point mutations (c.1466G>T, c.1469_1571CTT>TGG, c.1475G>T) in the mutant *Zp2* mRNA, a deletion of 541 bp (c.1476_2016) was identified between exon14 and exon18, as well as retention of the complete intron 13 (Fig. 1C). This intronic retention led to a premature termination codon (PTC) in the intron 13 and resulted in a truncated protein which lacked 212 amino acids at the C-terminal. Although comparable with the mutant ZP2 protein in humans affected by ZP2-associated infertility based on bioinformatics (Fig. 1D), the mutant ZP2 protein in rats was undetectable by Western blot analysis (Fig. 1E). For each genotype, three F2 generation rats were used for qPCR analysis, with three biological replicates

Figure 3 Normal constituent ratios and increased oocyte apoptosis of growing follicles in $Zp2^{MT/MT}$ rats. (A) Constituent ratios of growing follicles. (B) Constituent ratios of secondary (left) and antral (right) follicles. (C) Oocyte death rates of secondary and antral follicles based on morphological evaluation. * $P < 0.05$; *** $P < 0.001$.

Figure 4 Absence of ZP2 expression while other ZPGs are normally expressed in $Zp2^{MT/MT}$ rats. The absence of a ZP2 signal in the ZP or cytoplasm is indicated by the arrows or arrow head, respectively. Scale bar: 50 μ m.

Table 2 Number of ovulated eggs and live births.

	Ovulated eggs			
	Dead	Live		Live births
		With intact ZP	With cracked or absent ZP	
$Zp2^{WT/WT}$	$0.38 \pm 0.74(8)^*$	21.75 ± 5.34	0.00 ± 0.00	$11.43 \pm 2.23(7)^{\#}$
$Zp2^{MT/MT}$	$8.50 \pm 2.42(10)$	2.00 ± 0.67	1.60 ± 1.07	$0.00 \pm 0.00(10)$

*Mean $\pm$ s.d. (number of animals); $^{\#}$ Mean $\pm$ s.d. (number of litters).

for each rat. The results of qPCR analysis suggested an obvious decline in $Zp2$ mRNA levels in the $Zp2^{WT/MT}$ and $Zp2^{MT/MT}$ rats, regardless of the samples, that is, ovaries (Fig. 1F) or ovulated eggs (Fig. 1G). Notably, the decline in $Zp2^{MT/MT}$ rats was for more dramatic than that in the $Zp2^{WT/MT}$ rats (Fig. 1F and G). Taken together, these findings suggested the reduction of mRNA amount resulting in the failure of mutant ZP2 protein expression.

Two types of oocytes were observed in the GFs of $Zp2^{MT/MT}$ rats, one with intact but thinned ZP, another with cracked or no ZP

A total of 24 ovaries from 12 rats (six $Zp2^{WT/WT}$ and six $Zp2^{MT/MT}$) were isolated. For each ovary, four intermittent ovarian tissue sections were stained with PAS. The ZP was stained red in GFs (Fig. 2, arrows). In the $Zp2^{WT/WT}$ rats, most PFs contained a distinguishable and intact ZP (Fig. 2A), which was thickened and much more conspicuous in the ESFs (Fig. 2B1) and LSFs (Fig. 2B2). However, in the $Zp2^{MT/MT}$ rats, the ZP was absent in all the PFs (Fig. 2D) and the majority of ESFs (Fig. 2E1), although an intact ZP was present in each LSF. This observation indicated a delay in ZP formation in the $Zp2^{MT/MT}$ rats. Two types of growing oocytes were observed in the LSFs of $Zp2^{MT/MT}$ rats: one with intact ZP, the other with cracked ZP (Fig. 2E2 and E3). The severity of ZP cracking varied greatly. In some LSFs, the cracking of the ZP was very slight and only a few granulosa cells were ectopic in the perivitelline space (PVS), some of which contacted the oocytes directly instead of via the TZPs (Fig. 2, the arrow head in E2). In other LSFs, the ZP cracking was severe and multiple granulosa cells were ectopic (Fig. 2E3). In the $Zp2^{WT/WT}$ rats, the ZP in AFs was intact and thick (Fig. 2C1–5). In the $Zp2^{MT/MT}$ rats, there were two types of oocytes in the AFs: one with intact but extremely thinned ZP (Fig. 2F1), the other with cracked (Fig. 2F2 and F4) or even absent (Fig. 2F3) ZP. Similar to the LSFs, the cracking of the ZP in the AFs of $Zp2^{MT/MT}$ rats was always accompanied by the ectopia of the granulosa cells (Fig. 2, arrow heads in F2 and F4). In both $Zp2^{WT/WT}$ and $Zp2^{MT/MT}$ rats, no morphological characteristics of death were observed in oocytes in the PFs, but in some SFs (Fig. 2B3 and E4) and AFs (Fig. 2C6 and F3–4), the oocytes presented obvious morphological characteristics of death.

In the $Zp2^{MT/MT}$ rats, morphological characteristics of death of oocytes were significantly increased in SFs and particularly in AFs

In total, 96 ovarian tissue sections were analyzed for folliculogenesis (48 $Zp2^{WT/WT}$ and 48 $Zp2^{MT/MT}$). The total number of GFs in $Zp2^{MT/MT}$ rats was 628, which was comparable with that in the $Zp2^{WT/WT}$ rats ($n=681$). Moreover, there was no significant difference in the constituent ratios of GFs between the $Zp2^{WT/WT}$ and $Zp2^{MT/MT}$ rats (Fig. 3A; $P=0.097$). This result indicated that folliculogenesis of $Zp2^{MT/MT}$ rats was not affected quantitatively. However, in terms of quality, oocytes showing morphological characteristics of death accounted for the largest proportion, whereas ZP-intact oocytes accounted for the smallest proportion in both the SFs and AFs of $Zp2^{MT/MT}$ rats (Fig. 3B). Notably, the opposite results were observed in the $Zp2^{WT/WT}$ rats (Fig. 3B). Furthermore, in the $Zp2^{MT/MT}$ rats, the proportions of oocytes showing morphological characteristics of death were significantly increased in both SFs and particularly in AFs, in which the oocytes showing morphological characteristics of death (Fig. 2C6 and F3–4) accounted for 65% in $Zp2^{MT/MT}$ rats in contrast to only 17% in the $Zp2^{WT/WT}$ rats (Fig. 3C).

The ZP of $Zp2^{MT/MT}$ rats was composed of only ZP1, ZP3, and ZP4, while the mutant ZP2 protein was absent

Each immunohistochemistry analysis was performed three times. In the $Zp2^{WT/WT}$ rats, all the four ZPGs (ZP1–4) were successfully detected in the ZP (Fig. 4A, D, G and J). However, in the $Zp2^{MT/MT}$ rats, only three ZPGs (ZP1, ZP3, and ZP4) were successfully detected in the ZP (Fig. 4B, H and K). Notably, the mutant ZP2 was completely undetectable, indicating its absence in both the ZP (Fig. 4, arrows in E) and the cytoplasm of oocytes (Fig. 4, arrow head in E). This result indicated the absence of the expression of the mutant ZP2 protein in the $Zp2^{MT/MT}$ rats, which was consistent with the results of Western blot analysis.

In the $Zp2^{MT/MT}$ rats, most ovulated eggs exhibited morphological characteristics of death, and the rest presented two types: one with intact ZP, the other with cracked or no ZP

In total, 298 ovulated eggs were collected (177 from 8 $Zp2^{WT/WT}$ rats and 121 from 10 $Zp2^{MT/MT}$ rats)

(Table 2). In the $Zp2^{WT/WT}$ rats, most eggs (98.3%) were morphologically normal and only three eggs were fragmented and considered to be dead (Fig. 5A, B, left and Table 2). In contrast, most eggs (70.3%) from the $Zp2^{MT/MT}$ rats showed morphological characteristics of death (Fig. 5A, B, right and Table 2) including flattened, coarse, and deformed structure and even severe shrinkage. The severity of shrinkage differed among the eggs, with some eggs showing slight shrinkage (Fig. 5A, crossed arrow I), whereas others were drastically reduced in size (Fig. 5A, crossed arrow II) or even completely unobservable (Fig. 5A, crossed arrow III). The ZP phenotype of these morphologically dead eggs also differed greatly, with some greatly expanded and thinned (Fig. 5A, arrow 1), while others were cracked with obviously severed tips and normal in size (Fig. 5A, arrow 2) or absent (Fig. 5A, arrow 3). In the PVS of the dead eggs, ectopia of granulosa cells was consistently observed (Fig. 5A, arrow heads). These results indicated that all the dead eggs in the $Zp2^{MT/MT}$ rats originated from GFs with cracked or absent ZP. Although the number was small, some eggs in the $Zp2^{MT/MT}$ rats survived (Fig. 5B, right and Table 2), showing normal cellular morphology (Fig. 5A, asterisks). These eggs were classified into two types: one with intact but thinned ZP (Fig. 5A, arrow 4) and another with cracked or absent ZP (Fig. 5A, arrow 5). All these live eggs from the $Zp2^{MT/MT}$ rats, as well as 129 live eggs from $Zp2^{WT/WT}$ rats, were fertilized *in vitro*. The fertilization rates of both types of eggs from $Zp2^{MT/MT}$ rats were much lower than that of the eggs from $Zp2^{WT/WT}$ rats (Fig. 5C and D).

In the $Zp2^{MT/MT}$ rats, only the eggs with a cracked or no ZP showed significantly increased apoptosis, which was closely correlated to the reduction in TZPs and increased ROS levels

A total of 45 ovary tissue sections (15 from 3 $Zp2^{WT/WT}$ rats and 30 from 6 $Zp2^{MT/MT}$ rats) were analyzed by the TUNEL assay. Only the oocytes in AFs with a clear germinal vesicle were analyzed (Fig. 6A). There were 17 such oocytes in the $Zp2^{WT/WT}$ rats and 29 in the $Zp2^{MT/MT}$ rats (7 with intact ZP and 22 with cracked or absent ZP). There was no significant difference in the apoptosis rates of the oocytes in $Zp2^{WT/WT}$ rats and the ZP-intact oocytes in the $Zp2^{MT/MT}$ rats ($P=1.000$), whereas the apoptosis rate of oocytes with cracked or absent ZP was much higher ($P=0.003$) (Fig. 6B). For the TZPs analysis, five ovary tissue sections from three rats of each genotype were subjected to phalloidin staining. In the GFs of the $Zp2^{WT/WT}$ rats, as well as in the ZP-intact GFs of $Zp2^{MT/MT}$ rats, the TZPs were abundant and usually laid perpendicularly to the oocyte surface (Fig. 6A, white arrow heads). However, in some ZP-cracked GFs of $Zp2^{MT/MT}$ rats, the TZPs were very sparse and laid in a disorderly manner (Fig. 6A, white arrow heads). The GFs

with abnormal TZPs were in the majority, particularly in the ZP-cracked GFs with multiple ectopic granulosa cells in the PVS (Fig. 6A, black arrow heads). These changes in TZPs were pervasive, rather than confined to the region of ectopic granulosa cells (Fig. 6A). For statistical analysis, 13 GFs from $Zp2^{WT/WT}$ rats and 21 GFs from $Zp2^{MT/MT}$ rats (including 11 ZP-intact GFs and 10 ZP-cracked GFs) were included for the calculation of TZPs' density. In the ZP-intact GFs, the density of TZPs was comparable ($P>0.05$) with that in the GFs of $Zp2^{WT/WT}$ rats, but in the ZP-cracked GFs, the density of TZPs was significantly reduced ($P<0.001$) (Fig. 6C). For ROS analysis, 45 eggs from five $Zp2^{WT/WT}$ rats and 24 eggs from six $Zp2^{MT/MT}$ rats (14 ZP-intact eggs and ten eggs with cracked or absent ZP) were evaluated. There was no significant difference in the IOD of ZP-intact eggs in the $Zp2^{MT/MT}$ rats and that of ovulated eggs in the $Zp2^{WT/WT}$ rats ($P=0.493$) (Fig. 6D). However, the average IOD of the eggs with cracked or no ZP in the $Zp2^{MT/MT}$ rats was significantly higher than that of eggs in the $Zp2^{WT/WT}$ rats ($P<0.001$) (Fig. 6D). This indicated a significant increase in ROS levels in the eggs with cracked or no ZP, but not in the eggs with intact ZP, in the $Zp2^{MT/MT}$ rats.

The $Zp2^{MT/MT}$ female rats were infertile

The litter sizes of seven $Zp2^{WT/WT}$ females and ten $Zp2^{MT/MT}$ females were recorded (Table 2). Every $Zp2^{WT/WT}$ female produced a litter with the average litter size of 11.43 (Table 2). However, none of the $Zp2^{MT/MT}$ females became pregnant and no pups were produced (Table 2).

Discussion

In this study, we described a ZP2-null rat strain characterized by extreme vulnerability of the ZP, severe apoptosis of the oocytes, and absolute loss of fertility. Most importantly, our results indicate that the increased ROS levels associated with the destruction of ZP integrity and reduction in TZPs may contribute to the oocyte death. This result indicates a detrimental, even lethal consequence in the metabolism of the oocyte caused by the perforation or absence of the ZP due to mutations in *Zp2* and the resulting protein nullification. It is still unknown that by which means *Zp2* mutation caused ZP2 nullification. However, considering that *Zp2* mutation results in drastic mRNA reduction, the nonsense mediated mRNA decay, a RNA surveillance system that ensures accelerated degradation of PTC-containing mRNA to avoid the synthesis of truncated proteins with potentially deleterious effects for cells (Lambert et al. 2020) may be a plausible explanation. These findings highlight the importance of ZPGs coding genes on the survival of oocytes during folliculogenesis and provide novel insights in the potential mechanism, especially from the aspect of ZP2 gene and its functions.

Figure 5 Abnormalities in the morphology and fertilizing ability of ovulated eggs in *Zp2*^{MT/MT} rats. (A) Morphology of ovulated eggs. Arrows indicate the ZPs: arrow 1, the cracked, extremely expanded and thinned ZP; arrow 2, the cracked ZP with obviously severed tips, but unexpanded; arrow 3, the absence of the ZP in a morphologically dead oocyte; arrow 4, the intact but thinned ZP; arrow 5, the absence of the ZP in a morphologically normal oocyte. Crossed arrows indicate the shrinkage of oocytes: crossed arrow I, slight shrinkage; crossed arrow II, extreme shrinkage; crossed arrow III, the oocyte was not visible. Arrow heads indicate the ectopic granulosa cells, and asterisks indicate the morphologically normal oocytes. Scale bar: 100 μ m. (B) Constituent ratios of ovulated eggs based on morphological evaluation. (C) Fertilization rates of ovulated eggs *in vitro*. *** P < 0.001; NS: not significant. (D) Representative images for fertilized eggs. Arrow heads indicate the pronucleus. Scale bar: 100 μ m.

Figure 6 Abnormalities in the oocytes' apoptosis and ROS level as well as the TZPs' density in $Zp2^{MT/MT}$ rats. (A) Representative images of TUNEL assay, phalloidin staining, and ROS level analysis. The degree of TUNEL assay positivity is shown in yellow-green. White arrows indicate the locations of ZPs, white arrow heads indicate the filamentary TZPs, and black arrow heads indicate the ectopic granulosa cells. Scale bar: 10 μm . (B) Apoptosis rates of oocytes in antral follicles based on the TUNEL assay. $**P < 0.01$; NS: not significant. (C) Densities of TZPs in growing follicles. $***P < 0.001$; NS: not significant. (D) ROS levels of ovulated eggs based on integral optical density analysis. $***P < 0.001$; NS: not significant.

Although loss of ZP2 function always leads to infertility, the severity of the ZP defect (evaluation based on the existence and integrity of eggs' ZP: ZP-free is severest, ZP-cracked is intermediate, and ZP-intact is slightest) varies in different species, with the ZP2-null rat (some eggs are ZP-free, the others are ZP-cracked or ZP-intact) exhibiting intermediate severity compared with the ZP2-null mouse (all the eggs are ZP-free) and the ZP2-truncated human (all the eggs are ZP-intact). Avella *et al.* reported that the thickness and robustness of the ZP in ZP2-null mouse were restored by the expression of hZP4, suggesting that hZP4 can compensate for the absence of mZP2 (Avella *et al.* 2014). According to amino acid sequences alignments, hZP4 and rZP4 show similar rates of coverage on their respective ZP2 (49% and 54%, respectively), but hZP4 has a higher identity (to ZP2) rate (40.1%) than rZP4 (33.8%), supporting a stronger putative compensatory ability of hZP4 compared with that of rZP4. In addition, although both containing ZP4, the human ZP is twice as thick as that in the rat. This observation may suggest that, compared with rZP4, hZP4 plays a much more important role in increasing the ZP thickness. Taken together, these findings indicate that the ZP defect in ZP2-truncated humans is less severe than that in ZP2-null rats because hZP4 can better compensate for hZP2 and that the ZP2-null mouse, which lacks ZP4, has the most severe ZP defect.

Previous studies have clarified a view that only the ZP-intact eggs can contribute to pups production and that the ZP-cracked and ZP-free eggs are eliminated during preimplantation development *in vivo* because of the poor or no embryo protection (Rankin *et al.* 1999, 2001). The major reason for the infertility of the ZP2-null rat and mouse is the drastic reduction in the number of ovulated eggs, especially the ZP-intact eggs. However, the number of ovulated eggs in ZP2-truncated humans is not reduced. The ZP phenotypes indicate that the integrity of the ZP is the key factor for preventing the impairment of oogenesis. This phenomenon is also supported by studies in ZP3-null mice or ZP4-null rabbits (Liu *et al.* 1996, Wassarman *et al.* 1997, Lamas-Toranzo *et al.* 2019). In mice lacking a single *Zp3* allele, or rabbits lacking both ZP4 alleles, the ZP is thinned but still intact and the number of ovulated eggs is not reduced (Wassarman *et al.* 1997, Lamas-Toranzo *et al.* 2019). In mice lacking both *Zp3* alleles, the ZP is absent throughout folliculogenesis and the numbers of antral follicles and ovulated eggs are significantly reduced (Liu *et al.* 1996). Additionally, in the ZP1-null mouse, a few of the growing follicles are ZP-cracked, which leads to the reduction in the numbers of AF and ovulated eggs, although these effects are not as severe as those in the ZP3-null mouse (Rankin *et al.* 1999). Similarly, the number of eggs (without degeneration) retrieved from all the 11 human beings affected by reported infertilities

associated with cracked or absent ZP due to mutations in ZPGs coding genes are universally lessened (averagely 0–8.5 eggs per cycle depending on the patients, 1.4 eggs per cycle as a whole) (Huang *et al.* 2014, Chen *et al.* 2017, Liu *et al.* 2017, Dai *et al.* 2019a, Sun *et al.* 2019).

Cracks in ZP are always accompanied by ectopia of the granulosa cells in the perivitelline space, which leads to direct contact of the oocyte and surrounding granulosa cells rather than through the TZPs. Previous studies have implicated granulosa cell ectopia as the initiator of the developmental impairment of the oocyte mediated by changes in its metabolism, leading to alterations in the density and distribution of TZPs, which may be exacerbated by a form of vicious circle by further changing the oocyte's metabolism (Wang *et al.* 2019). This putative positive feedback explains the pervasive nature of the decline in follicular TZPs in the *Zp2^{MT/MT}* rat, rather than an effect that is confined to the area of ectopic granulosa cells. Although the metabolic changes may be complicated, the results are clearly detrimental and even often lethal to the oocytes. Oocyte degeneration or apoptosis associated with the destruction of the integrity of the ZP has been reported in cases of human infertility caused by ZPGs coding genes, although the mechanism remains unclear (Chen *et al.* 2017, Sun *et al.* 2019). ROS may play a critical role, and although the mechanism underlying the accumulation of ROS remains to be elucidated, some clues have been provided by previous studies. Pandey *et al.* suggested that decreased levels of cyclic 3', 5'-AMP (cAMP), an important signaling molecule that is transferred from the granulosa cells to oocytes through TZPs, are closely related to the increased ROS levels (Pandey & Chaube 2014). However, it is not practicable to detect the egg's cAMP level in the *Zp2^{MT/MT}* rat because of the drastic decreased number of survived eggs. The increased ROS may cause oocyte damage by contributing to activation of the mitochondria-mediated apoptotic pathway (Tiwari *et al.* 2015).

It can be speculated that the altered metabolic changes described previously also contribute to the reduced fertilization rate of the *Zp2^{MT/MT}* rat eggs with cracked or absent ZP. However, the *Zp2^{MT/MT}* rats' ZP-intact eggs also show a reduced fertilization rate which cannot be attributed to the metabolic change only. Notably, because of the failure of sperm binding, the eggs of ZP2-truncated humans cannot be fertilized unless intracytoplasmic sperm injection (ICSI) is performed. This indicates a vital role of hZP2 in sperm recognition and binding. Strikingly, the ZP-intact eggs of *Zp2^{MT/MT}* rats recognize and bind the sperm effectively, indicating a much less important role of rZP2 than hZP2 in fertilization. This might make the ZP-intact eggs of *Zp2^{MT/MT}* rat fertilizable, although the fertilization ability remains impaired.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0037>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H X designed the research. Y W, M Z, and H H performed the experiments. Y W and R-P Q analyzed the data. J-T Y, D G, and Y S contributed to discussion. Y W wrote the paper. H X and H D reviewed and revised the paper. H D contributed to finalization of the paper.

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