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ORIGINAL INVESTIGATION

Dosage effects of ZP2 and ZP3 heterozygous mutations cause human infertility

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Abstract The zona pellucida (ZP) is an extracellular matrix universally surrounding mammalian eggs, which is essential for oogenesis, fertilization, and pre-implantation embryo development. Here, we identified two novel heritable mutations of *ZP2* and *ZP3*, both occurring in an infertile female patient with ZP-abnormal eggs. Mouse models with the same mutations were generated by CRISPR/Cas9 gene editing system, and oocytes obtained from female mice with either single heterozygous mutation showed approximately half of the normal ZP thickness compared to wild-type oocytes. Importantly, oocytes with both heterozygous mutations showed a much thinner or even missing ZP that could not avoid polyspermy fertilization, following the patient's pedigree. Further analysis confirmed that precursor proteins produced from either mutated ZP2 or ZP3 could not anchor to oocyte membranes. From these, we conclude that *ZP* mutations have dosage effects which can cause female infertility in humans. Finally, this patient was treated by intracytoplasmic sperm injection (ICSI) with an improved culture system and successfully delivered a healthy baby.

Wenqiang Liu, Kunming Li, Dandan Bai, and Jiqing Yin are contributed equally to this work.

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Introduction

All mammalian eggs are surrounded by a glycoprotein matrix termed the zona pellucida (ZP) that plays a vital role during oogenesis, fertilization, and pre-implantation development (Avella et al. 2014; Matzuk et al. 2002; Pang et al. 2011; Wassarman et al. 2001; Wassarman and Litscher 2008, 2012). During follicular development, the zona pellucida can be first observed as extracellular patches surrounding oocytes in primary follicles which then gradually increase in width around growing oocytes (Gook et al. 2008). Although the zona matrix physically separates the oocyte from external granulosa cells, intercellular interactions are closely maintained and these interactions provide an important two-way communication for oocyte growth and meiotic arrest (Fagbohun and Downs 1990; Hasegawa and Koyama 2007). During fertilization, ZP acts as a specific 'docking site' in gamete recognition, which can induce acrosome reaction and further prevents polyspermy and premature implantation (Conner et al. 2005; Gupta et al. 2007; Wassarman et al. 2004). Mammalian ZPs have conserved protein motifs, including an N-terminal signal sequence, a ZP domain followed by a C-terminal propeptide that contains a basic cleavage site (CFCS), an external hydrophobic patch (EHP), and a transmembrane domain (TMD) (Wassarman and Litscher 2012). The newly synthesized ZP proteins are transported to cell surfaces through the endomembrane system and are anchored in cell membranes by the transmembrane domain (Jimenez-Movilla and Dean 2011). After the C-terminus is cleaved from

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the CFCS by a proprotein convertase, mature proteins are released and assembled into filaments and matrices (Kiefer and Saling 2002).

The mouse ZP is composed of three sulfated glycoproteins (Zp1, Zp2, and Zp3) that are synthesized during folliculogenesis. Zp1 seems not essential for fertilization in mice; however, some mutant ZP appeared to be loosely organized and could cause ectopic accumulation of granulosa cells within the perivitelline space (Rankin et al. 1999). Although mice lacking Zp2 could develop a thin ZP composed of ZP1 and ZP3 early in oogenesis until the antral follicle stage, those ovulated eggs are ZP-free and are infertile (Rankin et al. 2001). Besides, Zp3-null mice do not form a ZP throughout oogenesis (Liu et al. 1996; Rankin et al. 1996). In addition, the absence of a ZP in both *Zp2*- or *Zp3*-null mice presents adverse influences on embryogenesis and further development (Rankin et al. 1996, 2001). Those studies also indicate that two ZP proteins are sufficient to form ZP during folliculogenesis. However, little is known about the interactions and compensations among ZPs, particularly where they are double or triple heterozygous mutants.

Humans have four genetic loci encoding *ZP1*, *ZP2*, *ZP3*, and *ZP4*, which assemble to form the insoluble ZP during oocyte development within a growing follicle (Conner et al. 2005; Gook et al. 2008; Gupta et al. 2012; Lefievre et al. 2004). In contrast to mice, human ZP1 plays a very important role in zona pellucida matrix formation. An autosomal recessive inheritance of a homozygous truncated ZP1 was reported to cause female infertility (Huang et al. 2014). Other studies indicated that sequence variations in the *ZP* genes may cause zona anomalies and infertility (Mannikko et al. 2005; Margalit et al. 2012; Pokkyla et al. 2011). ZP dysmorphology is further associated with markedly diminished oocyte fertilization, pregnancy, and implantation rates after in vitro fertilization (IVF) (Huang et al. 2014; Sauerbrun-Cutler et al. 2015). The previous studies in humans have not identified any mutations in ZP2 and ZP3 related to disorders of the zona pellucida. Thus, existence of heritable mutations in ZP2 or ZP3, and effects of such mutations on formation of zona pellucida and fertility in humans remains unknown.

In this study, we investigated two novel heritable mutations in ZP2 (NM_003460.2:c.2092C>T) and ZP3 (NM_001110354.1:c.1045_1046insT) occurring in a primary infertile female patient who produced oocytes with abnormal ZP morphologies (thin ZP or no ZP). Parallel-mutated mouse models as well as in vitro experiments indicated that these mutations have dosage effects on ZP formation. We found that these heterozygous mutations together caused very thin ZP or even missing ZP around the oocytes. Therefore, these ZP2 and ZP3 mutants had cumulative adverse effects on zona pellucida formation. Importantly, this patient was finally treated by intracytoplasmic sperm injection (ICSI) with an improved culture system and successfully delivered a healthy baby.

Results

Clinical description

The infertile patient received a diagnosis of primary infertility at 34 years of age, after 2 years of cohabitation with her partner. Her age at menarche was 12 years; her menstrual cycle was abnormal (lasting 6–7 days and occurring every 37–60 days). Infertility-related examinations revealed abnormal ovulation. The patient underwent one failed IVF attempt. At the first IVF cycle, 9 oocyte cumulus complexes (COCs) were obtained from the patient. After removing the granulosa cells for in vitro fertilization, none of the oocytes possessed ZP. Subsequently, those eggs showed polyspermy or degradation following IVF (Fig. 1a–c). At the second IVF cycle, 8 COCs were retrieved. The ZP was examined after culturing for 3 h, and COCs were digested with hyaluronidase to remove granulosa cells. Seven of those eggs possessed very thin ZP, and one showed its complete absence in contrast to a wide-type oocyte with intact ZP (Fig. 1d–f).

Heterozygous mutations in ZP2 and ZP3

We performed Sanger sequencing of this family (Fig. 2a) on the candidate genes *ZP1*, *ZP2*, *ZP3*, and *ZP4*, showing that the patient (family member II-1) (Fig. 2a) carried a heterozygous missense mutation NM_003460.2:c.2092C>T in the coding region of *ZP2* and a heterozygous frameshift mutation NM_001110354.1:c.1045_1046insT in *ZP3* (Fig. 2b). These mutations were found to be transmitted from her parents. The father showed a heterozygous mutation in *ZP2* and *ZP3* that was in full accord with that identified in the patient. The mother carried a normal *ZP2* gene and the same heterozygous frameshift mutation in *ZP3* (Fig. 2b). Further analysis of the gene reading frame suggests that the missense mutation in *ZP2* causes a premature stop of the encoded protein, in which the 698th R amino acid is replaced by a stop codon and further results in a truncated ZP2 protein lacking the transmembrane domain (Fig. 2c). In addition, the frameshift mutation in *ZP3* also causes premature stop of its encoded protein, in which the 349th R amino acid is replaced by L amino acid followed by a stop codon and results in a truncated ZP3 protein without the whole propeptide region containing the cleavage site and the transmembrane domain (Fig. 2d).

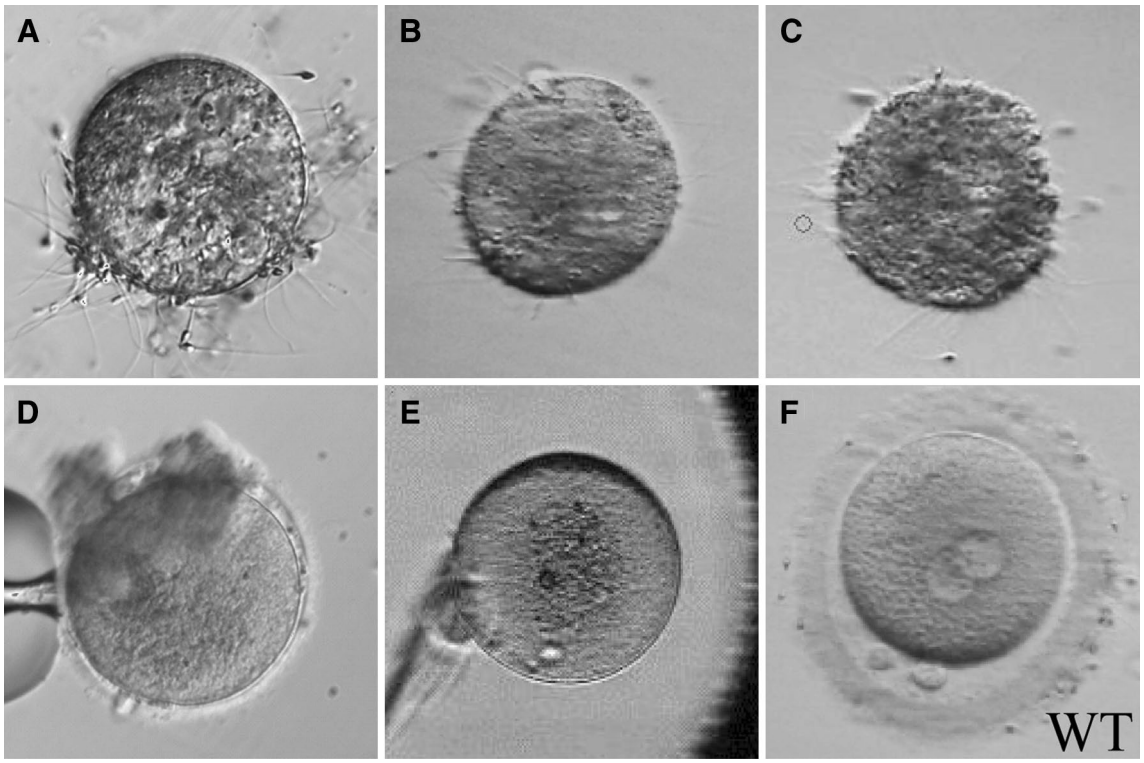

Fig. 1 Characteristics of the eggs from the patient. **a–c** At the first IVF cycle from the female patient, no ZP was observed in the patient's oocytes when granulosa cells were removed following IVF, and oocytes could not avoid polyspermy. **d** At the second IVF cycle, seven eggs possessed very thin zona pellucida and **e** one was zona pellucida-free. **f** Normal fertilized zygote of wild-type human oocyte which was treated by IVF

Characterization of ZP mutants in a mouse model

To confirm that heterozygous mutations identified in *ZP2* and *ZP3* are associated with primary infertility, we first characterized the phenotype of female mice carrying a corresponding missense mutation in *ZP2* ($Zp2^{+/mut}$) and a corresponding frameshift mutation in *ZP3* ($Zp3^{+/mut}$) (Supplementary Material Figure S1A). Genotypes of those mutant mice were confirmed using Sanger sequencing (Supplementary Material Figure S1B). In detail, in the $Zp2^{+/mut}$ (NM_011775.7:c.1999_2001delinsTAA) mouse model, the 667th S amino acid was replaced by a stop codon which resulted in a truncated mouse ZP2 protein lacking the transmembrane domain (Supplementary Material Figure S1C). In the $Zp3^{+/mut}$ (NM_011776.1:c.1049_1050delinsTGT) mice, the 350th R amino acid was replaced by L amino acid followed by a stop codon which further led to a truncated mouse ZP3 protein lacking the whole propeptide region (Supplementary Material Figure S1C). The amino acid homology of the truncated isoforms of mouse and human ZP2 and ZP3 proteins was 89.8 and 92.8%, respectively. As ZP expression was restricted to the germline of female mice, there was no overt phenotype apart from sterility and the mutant male mice were fertile as expected.

Fully grown oocytes from $Zp2^{+/mut}$ female mice were surrounded by a ZP that was approximately one-half the thickness of that in wild-type females (Fig. 3b, d). Although the $Zp2^{+/mut}$ females were as fertile as the wild-type females, oocytes from the $Zp2^{mut/mut}$ females completely lacked a ZP, which caused infertility (Fig. 3a and Supplementary Material Table S4). Moreover, eggs harvested from the $Zp2^{mut/mut}$ females showed polyspermy after in vitro fertilization, but eggs harvested from the $Zp2^{+/mut}$ females could be fertilized normally (Fig. 3e). Similar to Zp2, the oocytes from the $Zp3^{mut/mut}$ females completely lacked a ZP, which led to infertility (Fig. 3a and Supplementary Material Table S4). Oocytes from the $Zp3^{+/mut}$ females were surrounded by ZP less than half the thickness that could achieve normal in vitro fertilization and produce offspring (Fig. 3b, d, e and Supplementary Material Table S4).

Interestingly, oocytes from $ZP2^{+/mut}$&$ZP3^{+/mut}$ females were surrounded by ZP that were much thinner or completely absent compared to the $Zp2^{+/mut}$ (or $Zp3^{+/mut}$) mice (Fig. 3c). Surprisingly, granulosa cells of $ZP2^{+/mut}$&$ZP3^{+/mut}$ mice could grow into perivitelline spaces owing to this ZP deformity. Moreover, in vitro

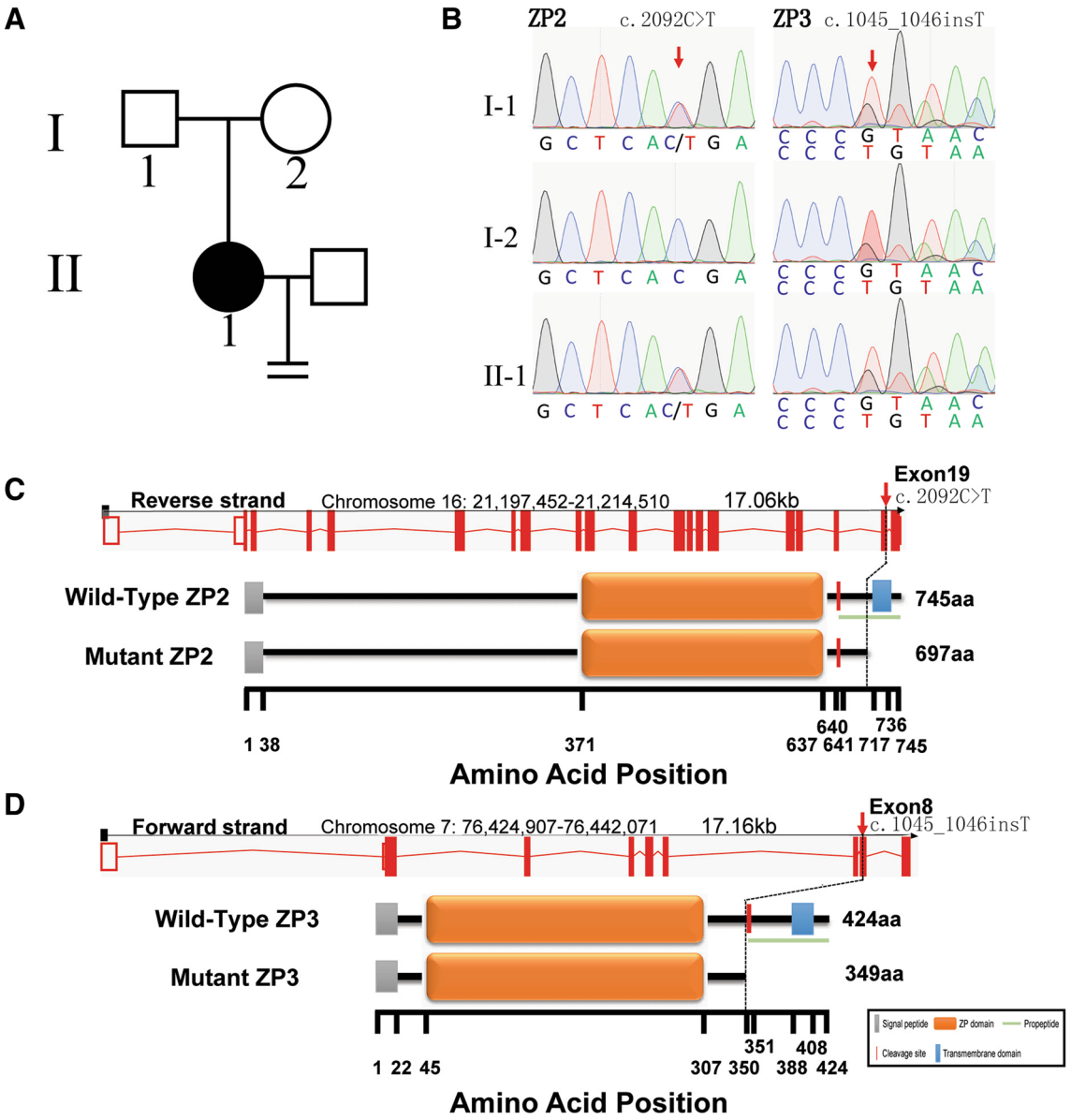

Fig. 2 Identification of ZP mutations in infertile patient. **a** Pedigree of the patient. *Square* indicates male family members, *circle* indicates female family members, and *solid stands* for affected members, equal signs denote infertility. **b** Patient (II-1), as well as her father (I-1) carried a heterozygous missense mutation NM_003460.2:c.2092C >T in the coding region of *ZP2* and a heterozygous frameshift mutation NM_001110354.1:c.1045_1046insT in *ZP3*. The patient's mother (I-2) carried a normal *ZP2* gene, but also the heterozygous mutation frameshift in *ZP3* (NM_001110354.1:c.1045_1046insT). **c** Wild-type ZP2 possesses 745 amino acids (aa); however, the missense mutation NM_003460.2:c.2092C >T appeared on the exon 19 of *ZP2* caused a premature stop codon, in which the 698th R amino acid was replaced by a stop codon, and further resulted in a truncated ZP2 protein (697aa). **d** Wild-type ZP3 possesses intact 424aa, and the frameshift mutation NM_001110354.1:c.1045_1046insT appeared in the exon 8 of *ZP3* caused a premature stop, in which the 349th R amino acid was replaced by L amino acid following produced a stop codon, and further led to a truncated ZP3 protein (349aa)

fertilization also showed that those eggs could not avoid polyspermy (Fig. 3f). Taken together, these heterozygous mutations of *ZP2* and *ZP3* in mice suggest mechanisms for the human infertility phenomenon which we observed.

ZP mutants affect the localization of ZP2 and ZP3 to the plasma membrane

To further investigate effects of these ZP mutations, wild-type and mutant expression plasmids from human or mouse encoding the $ZP2^{Venus}$ and $ZP3^{mRFP}$ fusion proteins were

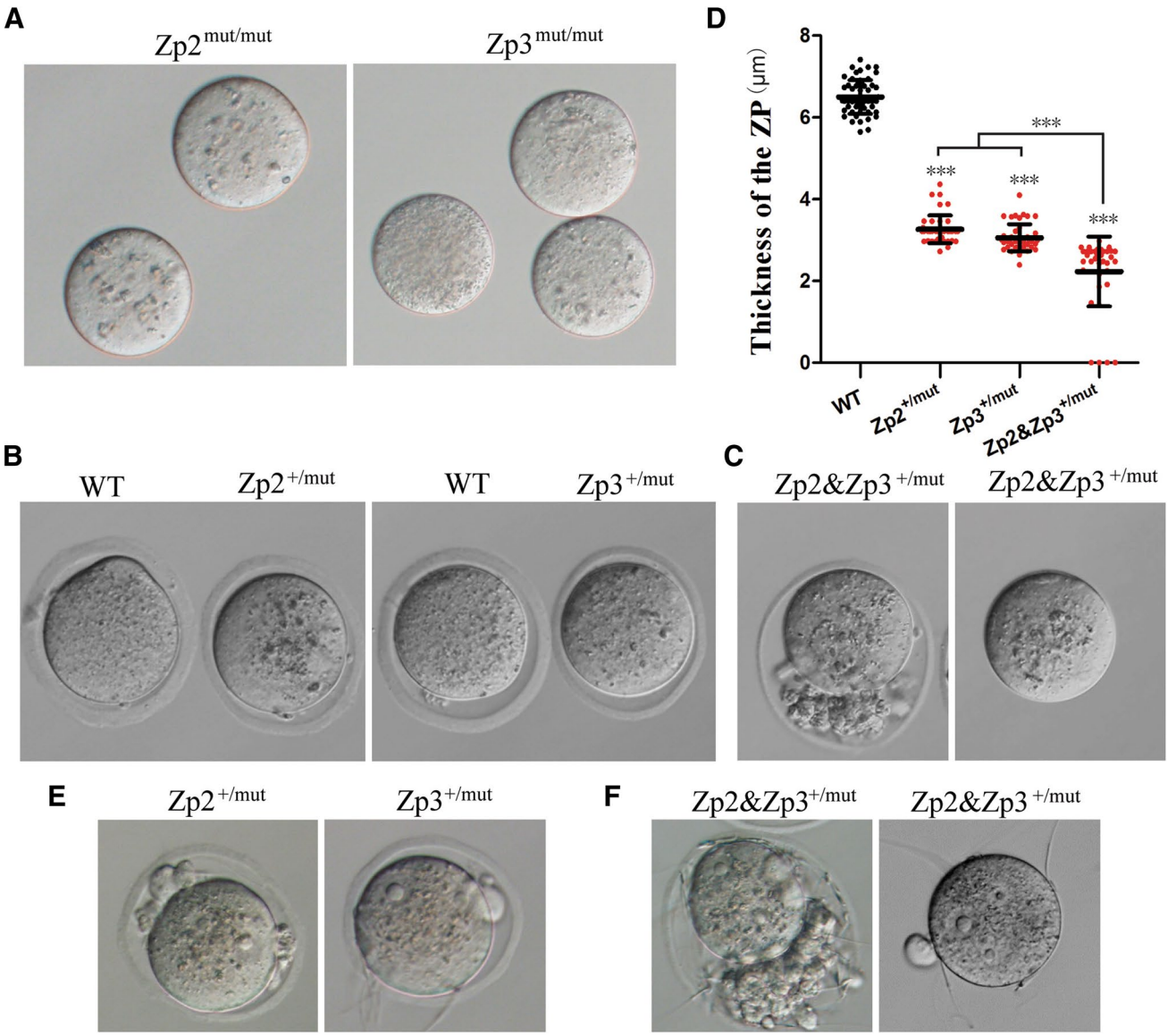

Fig. 3 Characterization of mutant ZP2 and ZP3 in mouse models**. a** The oocytes from $Zp2^{mut/mut}$ and $Zp3^{mut/mut}$ female mice completely lacked ZP which further cause infertility. **b** Both $Zp2^{+/mut}$ and $Zp3^{+/mut}$ female mice could produce fully grown oocytes, but with thinner ZP that approximately showed one-half thickness as wild-type oocytes. **c** Oocytes from $Zp2\&Zp3^{+/mut}$ females were surrounded by a ZP that was much thinner or completely absent. **d** The ZP thickness analysis of multiple mouse mutants compared with wild-type controls. Data are represented as the mean ± SD. ***$p < 0.001$ by Student's t test for comparison. **e** Oocytes from $Zp2^{+/mut}$ or $Zp3^{+/mut}$ females were surrounded by approximately one-half the thickness of wild-type ZP which yet did not influence fertilization by IVF. **f** Granule cells of $Zp2\&Zp3^{+/mut}$ could even grow into perivitelline space owing to ZP deformity. In vitro fertilization also showed that those eggs could not avoid polyspermy

transfected into Hela cells and then imaged by fluorescence microscopy. The two normal zona proteins ($hZP2^{Venus}$ and $hZP3^{mRFP}$) could localize in the endoplasmic reticulum and were concentrated in the plasma membrane. However, the mutant ZP2 and ZP3 ($DhZP2^{Venus}$ and $DhZP3^{mRFP}$) both showed diffuse staining in the cells without increased concentrations in plasma membranes (Fig. 4). We next investigated roles of mutant ZP2 and ZP3 in determining the cellular localization under more physiological conditions. Wild-type and mutant $ZP2^{Venus}$ and $ZP3^{mRFP}$ cRNA from human or mouse were in vitro transcribed and each microinjected into mouse oocytes. Wild-type $ZP2^{Venus}$ and $ZP3^{mRFP}$ from either human or mouse appeared to traffic through the cell and localize onto the plasma membrane (Fig. 5a, b). However, mutant $ZP2^{Venus}$ and $ZP3^{mRFP}$ were mostly concentrated in the endoplasmic reticulum and were

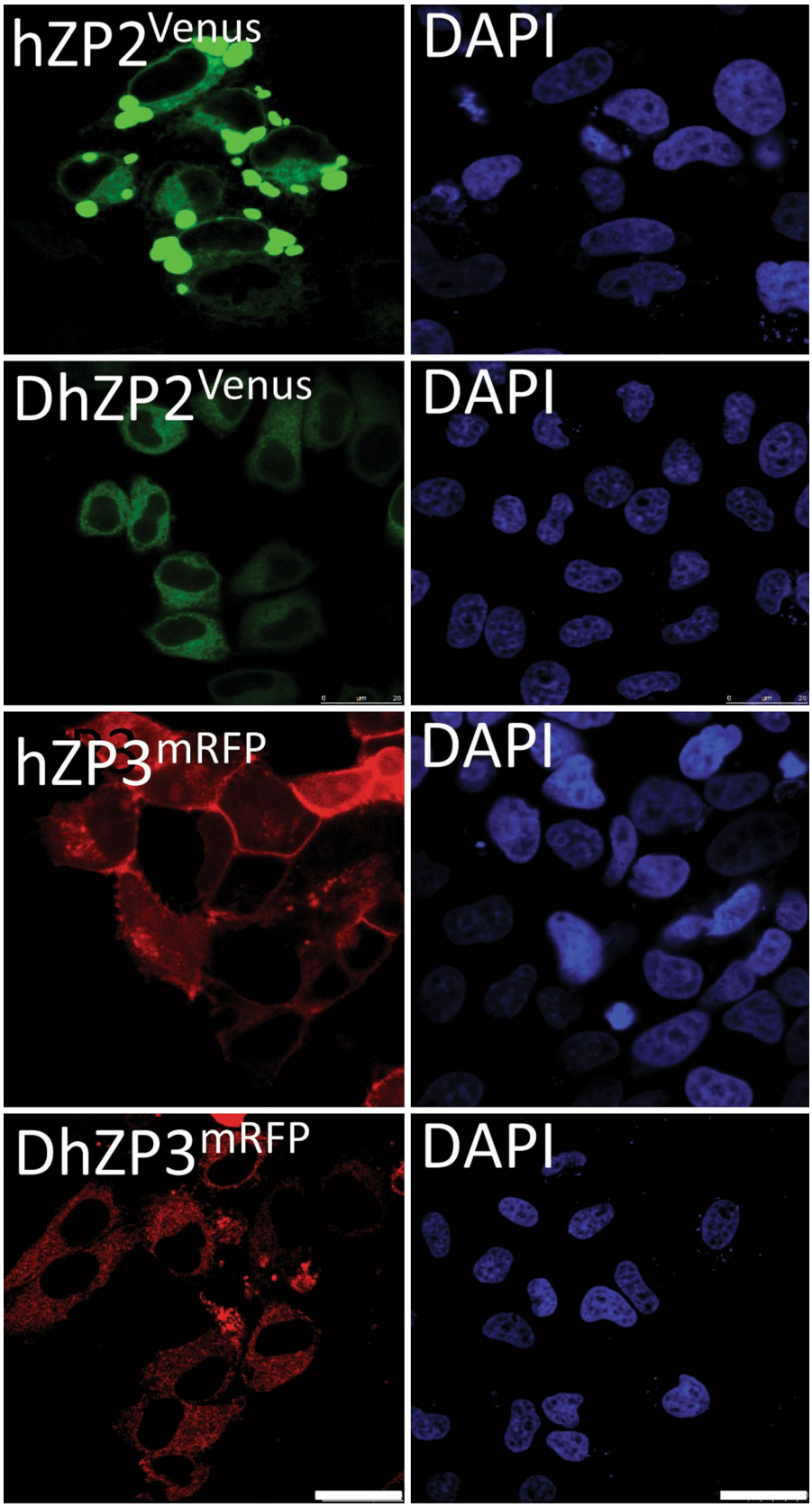

Fig. 4 Wild-type and mutant of human ZP2 and ZP3 expressed in Hela cells. Hela cells were transfected with wild-type and mutant expression plasmids from human encoding the $ZP2^{Venus}$ and $ZP3^{mRFP}$ fusion proteins, respectively. Images show that wild-type $ZP2^{Venus}$ (*green*) and wild-type $ZP3^{mRFP}$ (*red*) could be concentrated in the plasma membrane, while the mutant $ZP2^{Venus}$ (*green*) and mutant $ZP3^{mRFP}$ (*red*) could not. *Scale bar* 40 μm

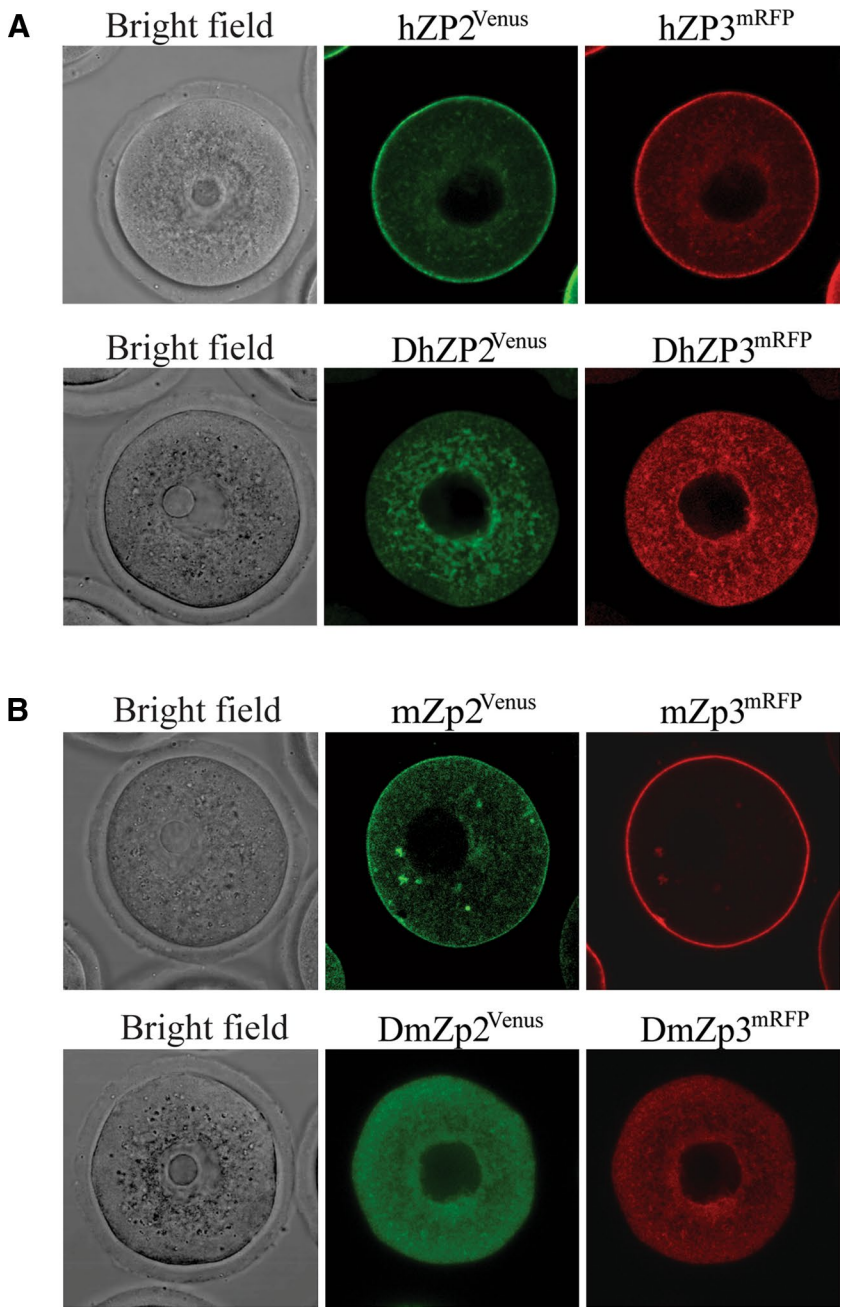

Fig. 5 ZP mutants affect the localization of ZP2 and ZP3 in plasma membrane. Wild-type and mutant expression plasmids from human or mouse encoding the $ZP2^{Venus}$ and $ZP3^{mRFP}$ fusion proteins were in vitro transcribed and separately microinjected into mouse oocytes. **a** Wild-type human $ZP2^{Venus}$ ($hZP2^{Venus}$, *green*) were assembled on oocyte plasma membrane, while mutant human $ZP2^{Venus}$ ($DhZP2^{Venus}$, *green*) were trapped in the endoplasmic reticulum. **b** Wild-type mouse $ZP3^{mRFP}$ ($mZP3^{mRFP}$, *red*) localized in the plasma membrane, and mutant mouse $ZP3^{RFP}$ ($DmZP3^{mRFP}$, *red*) were not transported and localized in the plasma membrane

not detected in plasma membranes (Fig. 5a, b). These data indicated that mutations in *ZP2* and *ZP3* resulted in altered ZP proteins that could not transport and localize in plasma membranes, thus preventing their incorporation into the zona matrix.

Discussion

Although ZP formation has extensively been investigated in mice (Hoodbhoy et al. 2006; Liu et al. 1996; Rankin et al. 1996; Rankin et al. 1999, 2001), where any individual knockout of ZP2 or ZP3 will lead to the MII oocyte being ZP-free and infertile, genetic causes of human ZP dysmorphology and interactions among human ZPs are largely unknown. In the clinic, ZP dysmorphology markedly diminishes oocyte fertilization, pregnancy, and implantation rates (Mannikko et al. 2005; Margalit et al. 2012; Sauerbrun-Cutler et al. 2015). Here, for the first time, we identified an additive-effect pattern of inheritance for both *ZP2* and *ZP3*. The patient carried a heterozygous missense mutation in *ZP2* (NM_003460.2:c.2092C>T) as well as a heterozygous frameshift mutation in *ZP3* (NM_001110354.1:c.1045_1046insT). Our results showed that those

heterozygous mutations together, but not homozygous mutations or separate gene knockouts cause thin or absent ZP around oocytes in humans (and also mice), leading to abnormal fertilization and accounting for this patient's infertility. The patient was finally treated by intracytoplasmic sperm injection (ICSI) with an improved culture system and successfully delivered a healthy baby.

ZP polypeptides from different mammals show high degrees of conservation (Kiefer and Saling 2002; Wassarman 2008). Both human and mouse ZP precursor polypeptides contain an N-terminal signal sequence, a zona domain, a C-terminal propeptide with a consensus furin cleavage site, a transmembrane domain, and a short cytoplasmic tail (Gupta et al. 2007). A previous report indicated that Zp2 and Zp3 are sufficient to form the insoluble zona matrix in the mouse (Rankin et al. 1999). In addition, disrupted N-termini of Zp2 or Zp3 individually or together that abolished their expression could result in zona-free eggs and further lead to infertility in the mouse model (Rankin et al. 1996, 2001). However, little is known about C-terminal truncations of ZP proteins and the interactions between them in heterozygous cases are still unclear. These may be more common than homozygous mutations in humans. In detail, transmembrane domains and cytoplasmic tails localized in the C-terminal of ZP2 and ZP3 are necessary to traffic and co-localize in the plasma membrane before being released by hydrolase and incorporated into the extracellular matrix (Jimenez-Movilla and Dean 2011). The *ZP2* and *ZP3* mutants identified in this study were missing transmembrane domains and cytoplasmic tails of the C-terminal propeptide, which were expected to abrogate the prevention of intracellular polymerization as well as trafficking those proteins in both intracellular and extracellular domains. Our in vitro experiments showed that a truncated proteins of ZP2 and ZP3 (both human and mouse) could not anchor on cell membranes and were mostly accumulated in the endomembrane system, which is similar to that observed previously (Hoodbhoy et al. 2006; Jovine et al. 2002; Qi et al. 2002). Moreover, we demonstrated that ZP surrounding $ZP2^{+/mut}$&$ZP3^{+/mut}$ double heterozygous mutant female eggs were much thinner or completely absent (Fig. 3c), an observation similar to the patient (Fig. 1d, e). Interestingly, we also noticed that some ovulated eggs had ectopic granulosa cells in the perivitelline space between the zona pellucida and oocyte plasma membrane (Fig. 3c), which was previously reported only in the Zp1-null mouse model. Together, these results demonstrated that transmembrane domains and cytoplasmic tails of ZP proteins are essential for functional ZP formation in both human and mouse, and such joint heterozygous mutations could further lead to polyspermy and infertility.

It is interesting to find the same ZP3 mutation in both parents. However, whether it is a genetic hot spot remains unclear as no other cases are reported in human until now and all other members in this family pedigree are fertile. The ZP thickness of human oocytes is important for IVF; a thick ZP will cause unsuccessful fertilization, while a thin ZP leads to a higher polyspermy fertilization rate (Bertrand et al. 1996). The previous mouse studies have shown that oocytes from $mZP2^{+/-}$ or $mZP3^{+/-}$ females are surrounded by one-half the thickness of normal ZP, although these oocytes can be fertilized normally with IVF and those mice are fertile (Gupta et al. 2012; Lefievre et al. 2004; Wassarman et al. 1997). In a human IVF clinic, we found that patients with two-thirds of normal ZP thickness showed approximately 50% polyspermy fertilization rates (Supplementary Material Table S5). Thus, we suggest that human oocytes are more sensitive than mouse oocytes regarding ZP thickness during IVF and natural fertilization.

Finally, our study for the first time demonstrates that such *ZP* mutants together can cause dosage effects on ZP formation and fertility which were in accordance with both the patient's pedigree and mouse models. As these heterozygous mutations occurred in *ZP2* and *ZP3* at the same time, oocytes showed much thinner ZP and polyspermy after IVF (Fig. 3f). The mother of the patient carrying the *ZP3* mutant was fertile, while the patient carrying both the *ZP2* and *ZP3* mutants showed complete fertilization failure. Therefore, both human and mouse models effectively show that *ZP2* and *ZP3* mutants have cumulative effects. Thus, understanding the association between the genetic variants in *ZP* genes and abnormal fertilization will facilitate overcoming fertility problems.

Materials and methods

Genetic analysis

All genomic DNA from patient and patient's family members was extracted from peripheral blood with TIANamp Genomic DNA Kit (Tiangen, Beijing, China). All 53 exons of *ZP1*, *ZP2*, *ZP3,* and *ZP4* genes were examined by Sanger sequencing according to specific primers in all 35 segments (Supplementary Material Table S1). PCR amplification was performed using Q5 High-Fidelity Polymerase (5x, NEB, MA, USA). The PCR products were collected through agarose gel electrophoresis and extracted with Universal DNA Purification Kit (Tiangen, Beijing, China). The purified products were sequenced for the initial screening by GENEWIZ Biological Technology Limited Corporation. The purified PCR products with suspected mutated exons were cloned into *p*LB vectors using Lethal Based Fast Cloning Kit (Tiangen, Beijing, China); more than five individual clones of each suspected mutated exon were sequenced de novo.

Production of Cas9 mRNA, sgRNA, and ssODNs

T7 promoter was added into Cas9 coding sequence by PCR amplification using PX330 vector and the primer T7-Cas9 (F and R). T7-Cas9 PCR products were collected through agarose gel electrophoresis and purified by QIAquick Gel Extraction Kit (Qiagen, USA). The acquired products were used as templates (500 ng) for in vitro transcription using mMESSAGE mMACHINE T7 ultra transcription kit (Ambion, Thermo Fisher Scientific, USA). Both T7 promoter and targeting sgRNA sequences were added into sgRNA backbone template by PCR amplification using primers T7-sgZp2 (F and R) and T7-sgZp3 (F and R), respectively. Each T7-sgRNA PCR product was purified on gels using QIAquick Gel Extraction Kit (Qiagen, USA) and then used as templates (250 ng) for in vitro transcription using MEGA shortscript T7 kit (Ambion, Thermo Fisher Scientific, USA). Both Cas9 mRNA and specific sgRNA were purified according to the standard protocol by phenol:chloroform extraction and ethanol precipitation, and then dissolved in DNase/RNase-free water (Life Technologies). Primer sequences are listed in Supplementary Material Table S2. Gene-specific single-stranded donor oligonucleotides (ssODNs) for HDR (homology directed repair) are listed in Supplementary Material Table S2.

Generation of knock-in mice

C57BL/6n female mice (7–8 weeks old) were used as embryo donors. C57BL/6n female mice were superovulated by intraperitoneally injecting with PMSG and hCG, and then mated to C57BL/6n male mice. Fertilized embryos (zygotes) were collected from their oviducts. Cas9 mRNA (100 ng/μL), sgRNA (Zp2 or Zp3) (50 ng/μL), and targeting ssODNs (10–20 ng/μL) were mixed and injected into the cytoplasm of fertilized eggs with both pronuclei visible in CZB (Chatot–Ziomek–Bavister) medium. The injected zygotes were then cultured in Quinn's Advantage cleavage medium (In-Vitro Fertilization, Inc.) containing SCR7 (50 μm, TOCRIS) for about 24 h, and 18–20 2-cell stage embryos were transferred into the oviduct of a pseudo-pregnant ICR female mouse at 0.5 dpc.

Mouse identification and maintenance

$Zp2^{+/mut}$ mice and $Zp^{3+/mut}$ mice were generated by CRISPR/Cas9 system with targeting homologous single-stranded donor oligonucleotides (ssODNs). $Zp2\&Zp3^{+/mut}$ double mutant female mice were produced by crossing $Zp2^{+/mut}$ mice with $Zp3^{+/mut}$ mice. All mice had access to food and water. All experiments were performed in accordance with the University of Health Guide for the Care and Use of Laboratory Animals and were approved by the Biological Research Ethics Committee of Tongji University. Genotyping of $Zp2^{+/mut}$ and $Zp3^{+/mut}$ was performed by PCR of mice tail-tip genomic DNA and then analyzed by Sanger sequencing. Primer sequences are listed in Supplementary Material Table S2.

Expression constructs of wild-type and mutant ZP2, ZP3 with fluorescent proteins

For observing mutant ZP2, ZP3 protein localization in vitro, full-length and C-terminal truncated ZP2, and ZP3 cDNA original from human and mouse were constructed and then recombined with eukaryotic expression vector *p*cDNA3.1 containing yellow (Venus) or red (mRFP1) fluorescent proteins. The fluorescent tag was fused in C-terminal of wild-type or mutant ZP2, ZP3. Human and mouse ZP2/ZP3 cDNA sequences' information was acquired from NCBI (www.ncbi.nlm.nih.gov), and the primers including mutant Zp2, Zp3 genes were designed according to mutant site of patient and restriction site of vectors by Soft Primer Premier 5 (Supplementary Material Table S3). All cDNA was produced by PCR amplification with Q5 High-Fidelity Polymerase (5x, NEB, MA, USA). The purified PCR products were cloned into *p*LB vector using Lethal Based Fast Cloning Kit (Tiangen, Beijing, China), and sequenced by GENEWIZ Biological Technology Limited Corporation. The correct clones were amplified through *E. coli* and the plasmids were extracted using TIANprep Rapid Mini Plasmid Kit (Tiangen, Beijing, China). The intended cDNA fragments were cut from plasmids by restriction enzymes and linked with fluorescent vectors using T4 Ligase (NEB, MA, USA). The products were transformed into competent *E. coli* cells, and the plasmids were extracted with EndoFree Plasmid Midi Kit (cwbiotech, Beijing, China).

Wild-type and mutant ZP2, ZP3 expression in Hela cells

Hela cells were cultured in fresh DMEM medium (Merck, Millipore) supplemented with 10% fetal bovine serum (Hyclone, Thermo Scientific Corporation, North America), penicillin/streptomycin (100×, Merk Millipore), at 37 °C/5% CO_2, which was renewed every day. Full-length and C-terminal truncated two types of ZP2 and ZP3 reconstructed vector with fluorescent protein were transfected into Hela cells, separately. VigoFect (Vigorous Biotechnology, Beijing, China) was used as the transfection reagent. Fluorescence in transfected cells was observed 24 h post-transfection; then, the cells were fixed and labeled with DAPI (Sigma) at 48 h for imaging using a Confocal Microscope (Leica TCS SP5 II). At least 20 fields contain transfected cells were captured from each sample.

Oocytes microinjection

GV oocytes isolated 48 h after PMSG injection from 4-week-old female mice were incubated in MEM medium (Chemicon-Millipore, Billerica, MA) supplemented with IBMX (3-isobutyl-1-methylxanthine) at 37 °C before microinjection. Wild-type and mutant ZP2, ZP3 cRNA were synthesized with the mMESSAGE T7 Ultra Kit (Ambion, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The storage concentration of each cRNA was optimized to 1000 ng/μL. Oocytes were injected with approximately 10 picoL of cRNA using a Piezo-driven micromanipulator. Surviving oocytes were cultured (37 °C/5% CO_2) for 36–48 h in MEM medium containing IBMX. Oocytes were fixed with 4 % paraformaldehyde and images were obtained by confocal microscopy.

In vitro fertilization (IVF) of wild-type and mutant mouse oocytes

Female BDF1 mice (8–9 weeks old) were super-ovulated by injection with 5 IU of pregnant mare serum gonadotropin (PMSG), followed by injection of 5 IU of human chorionic gonadotropin (hCG) (San-Sheng Pharmaceutical Co. Ltd.) 48 h later. At 14 h, after hCG injection, the cumulus oocyte complexes were released from the oviducts. Sperm were obtained from the caudal epididymis of 10-week-old BDF1 males. Cauda were cut several times with clippers and transferred into HTF medium under 5% CO_2 in air. Sperm were passively released into the culture medium through slits in the bases of cauda. Sperm were capacitated for 0.5 h before IVF. Oocytes were placed in sperm suspension for 4–6 h and then washed and cultured in CZB medium.

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Compliance with ethical standards

Conflict of interests On behalf of all authors, the corresponding author states that there is no conflict of interests.

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References

Avella MA, Baibakov B, Dean J (2014) A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans. J Cell Biol 205:801–809. doi:10.1083/jcb.201404025

Bertrand E, Van den Bergh M, Englert Y (1996) Clinical parameters influencing human zona pellucida thickness. Fertil Steril 66:408–411

Conner SJ, Lefievre L, Hughes DC, Barratt CL (2005) Cracking the egg: increased complexity in the zona pellucida. Hum Reprod 20:1148–1152. doi:10.1093/humrep/deh835

Fagbohun CF, Downs SM (1990) Maturation of the mouse oocyte-cumulus cell complex: stimulation by lectins. Biol Reprod 42:413–423

Gook DA, Edgar DH, Borg J, Martic M (2008) Detection of zona pellucida proteins during human folliculogenesis. Hum Reprod 23:394–402. doi:10.1093/humrep/dem373

Gupta SK, Bhandari B, Shrestha A, Biswal BK, Palaniappan C, Malhotra SS, Gupta N (2012) Mammalian zona pellucida glycoproteins: structure and function during fertilization. Cell Tissue Res 349:665–678. doi:10.1007/s00441-011-1319-y

Gupta SK, Chakravarty S, Suraj K, Bansal P, Ganguly A, Jain MK, Bhandari B (2007) Structural and functional attributes of zona pellucida glycoproteins. Soc Reprod Fertil Suppl 63:203–216

Hasegawa A, Koyama K (2007) Contribution of zona proteins to oocyte growth. Soc Reprod Fertil Suppl 63:229–235

Hoodbhoy T, Aviles M, Baibakov B, Epifano O, Jimenez-Movilla M, Gauthier L, Dean J (2006) ZP2 and ZP3 traffic independently within oocytes prior to assembly into the extracellular zona pellucida. Mol Cell Biol 26:7991–7998. doi:10.1128/MCB.00904-06

Huang HL, Lv C, Zhao YC, Li W, He XM, Li P, Sha AG, Tian X, Papasian CJ, Deng HW, Lu GX, Xiao HM (2014) Mutant ZP1 in familial infertility. N Engl J Med 370:1220–1226. doi:10.1056/NEJMoa1308851

Jimenez-Movilla M, Dean J (2011) ZP2 and ZP3 cytoplasmic tails prevent premature interactions and ensure incorporation into the zona pellucida. J Cell Sci 124:940–950. doi:10.1242/jcs.079988

Jovine L, Qi H, Williams Z, Litscher E, Wassarman PM (2002) The ZP domain is a conserved module for polymerization of extracellular proteins. Nat Cell Biol 4:457–461. doi:10.1038/ncb802

Kiefer SM, Saling P (2002) Proteolytic processing of human zona pellucida proteins. Biol Reprod 66:407–414

Lefievre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, Pavlovic B, Lenton W, Afnan M, Brewis IA, Monk M, Hughes DC, Barratt CL (2004) Four zona pellucida glycoproteins are expressed in the human. Hum Reprod 19:1580–1586. doi:10.1093/humrep/deh301

Liu C, Litscher ES, Mortillo S, Sakai Y, Kinloch RA, Stewart CL, Wassarman PM (1996) Targeted disruption of the mZP3 gene results in production of eggs lacking a zona pellucida and infertility in female mice. Proc Natl Acad Sci USA 93:5431–5436

Mannikko M, Tormala RM, Tuuri T, Haltia A, Martikainen H, Ala-Kokko L, Tapanainen JS, Lakkakorpi JT (2005) Association between sequence variations in genes encoding human zona pellucida glycoproteins and fertilization failure in IVF. Hum Reprod 20:1578–1585. doi:10.1093/humrep/deh837

Margalit M, Paz G, Yavetz H, Yogev L, Amit A, Hevlin-Schwartz T, Gupta SK, Kleiman SE (2012) Genetic and physiological study of morphologically abnormal human zona pellucida.

Eur J Obstet Gynecol Reprod Biol 165:70–76. doi:10.1016/j.ejogrb.2012.07.022

Matzuk MM, Burns KH, Viveiros MM, Eppig JJ (2002) Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296:2178–2180. doi:10.1126/science.1071965

Pang PC, Chiu PC, Lee CL, Chang LY, Panico M, Morris HR, Haslam SM, Khoo KH, Clark GF, Yeung WS, Dell A (2011) Human sperm binding is mediated by the sialyl-Lewis(x) oligosaccharide on the zona pellucida. Science 333:1761–1764. doi:10.1126/science.1207438

Pokkyla RM, Lakkakorpi JT, Nuojua-Huttunen SH, Tapanainen JS (2011) Sequence variations in human ZP genes as potential modifiers of zona pellucida architecture. Fertil Steril 95:2669–2672. doi:10.1016/j.fertnstert.2011.01.168

Qi H, Williams Z, Wassarman PM (2002) Secretion and assembly of zona pellucida glycoproteins by growing mouse oocytes microinjected with epitope-tagged cDNAs for mZP2 and mZP3. Mol Biol Cell 13:530–541. doi:10.1091/mbc.01-09-0440

Rankin T, Familari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, Dean J (1996) Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. Development 122:2903–2910

Rankin T, Talbot P, Lee E, Dean J (1999) Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss. Development 126:3847–3855

Rankin TL, O'Brien M, Lee E, Wigglesworth K, Eppig J, Dean J (2001) Defective zonae pellucidae in Zp2-null mice disrupt folliculogenesis, fertility and development. Development 128:1119–1126

Sauerbrun-Cutler MT, Vega M, Breborowicz A, Gonzales E, Stein D, Lederman M, Keltz M (2015) Oocyte zona pellucida dysmorphology is associated with diminished in vitro fertilization success. J Ovar Res 8:5. doi:10.1186/s13048-014-0111-5

Wassarman PM (2008) Zona pellucida glycoproteins. J Biol Chem 283:24285–24289. doi:10.1074/jbc.R800027200

Wassarman PM, Litscher ES (2012) Influence of the zona pellucida of the mouse egg on folliculogenesis and fertility. Int J Dev Biol 56:833–839. doi:10.1387/ijdb.120136pw

Wassarman PM, Litscher ES (2008) Mammalian fertilization: the egg's multifunctional zona pellucida. Int J Dev Biol 52:665–676. doi:10.1387/ijdb.072524pw

Wassarman PM, Jovine L, Litscher ES (2001) A profile of fertilization in mammals. Nat Cell Biol 3:E59–E64. doi:10.1038/35055178

Wassarman PM, Jovine L, Litscher ES (2004) Mouse zona pellucida genes and glycoproteins. Cytogenet Genome Res 105:228–234. doi:10.1159/000078193

Wassarman PM, Qi H, Litscher ES (1997) Mutant female mice carrying a single mZP3 allele produce eggs with a thin zona pellucida, but reproduce normally. Proc Biol Sci 264:323–328. doi:10.1098/rspb.1997.0046