



Review Article

Zona Pellucida and Reproductive Success: The Critical Role of Zona Pellucida Glycoprotein 2

Neha Rajput¹ , Jaishree Mishra¹ , Gagandeep Kaur Gahlay¹ 

¹Department of Molecular Biology and Biochemistry, Guru Nanak Dev University, Amritsar, India



***Corresponding author:**
Gagandeep Kaur Gahlay,
Department of Molecular
Biology and Biochemistry,
Guru Nanak Dev University,
Amritsar, India

gagandeepgahlay@gndu.ac.in

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ABSTRACT

Reproduction, a fundamental biological process, is vital for ensuring species continuity. Despite its complexity, sexual reproduction increases genetic diversity and allows for more efficient removal of deleterious mutations, ultimately enhancing fitness and survival. However, human fertility rates have been steadily declining, with approximately 17.5% of the global adult population affected by infertility (WHO, 2023). 30%–40% of these cases are attributed to female factors, which can be genetic, chromosomal, hormonal, physiological or epigenetic. Zona Pellucida (ZP), a glycoprotein matrix surrounding a mammalian oocyte, plays a crucial role in folliculogenesis, fertilisation, and embryo protection. Human ZP is made up of four glycoproteins (ZP1–4), out of which ZP2 is essential for species-specific sperm binding, structural integrity, and embryo development. Female knockout mice lacking ZP2 exhibit infertility, indicating its critical role in oocyte maturation and fertilisation. Post-fertilisation, ovastacin-mediated ZP2 cleavage prevents polyspermy, a critical step ensuring viable embryonic development. Hence, female infertility can be caused by mutations in the ZP2 gene, which often present phenotypically as defective ZP formation, impaired oocyte development, and compromised fertilisation in infertile female patients. In this review article, we discuss the role played by ZP2 and how mutations in this gene can cause female infertility. Understanding the effects of mutations in the ZP2 gene in females will provide valuable insights into infertility mechanisms and their possible treatment strategies.

Keywords: Female Infertility, Fertilisation, Ovastacin, Polyspermy, Zona pellucida, ZP2

INTRODUCTION

Reproduction is a fundamental process that ensures the perpetual existence of life forms. Even though sexual reproduction is lengthier and more complicated as compared to asexual reproduction, natural selection favours it, as it fosters immense genetic diversity, which ultimately provides crucial advantages to various life forms in the race for survival.^[1] The fertility rates of the human population have been steadily declining since 1950.^[2] Amongst other factors, increasing rates of infertility are significantly contributing to this decline. Globally, ~17.5% of reproductive-aged adults experience infertility at some stage in their lives.^[3]

Infertility can be defined as an inability to conceive after 12 months or more of unprotected sexual intercourse and can occur in both females and males. Depending upon the underlying causes of infertility, approximately 30%–40% of infertility cases are attributed to female factors which include ovulatory disorders (25%), endometriosis (15%), pelvic adhesions (12%), tubal blockage (11%), other tubal/uterine abnormalities (11%) and hyperprolactinaemia (7%).^[4] In many instances,

subtle genetic variations, chromosomal abnormalities and epigenetic modifications may be the possible contributors to infertility. These genetic variations can alter genes involved in key processes such as gametogenesis, fertilisation and early embryonic development, impacting the reproductive processes.

STRUCTURE OF A MAMMALIAN OOCYTE

A mature human egg, or oocyte, is a specialised cell with cytoplasm that houses vital organelles and various nutrients essential for early embryonic growth. Beneath the plasma membrane, secretory organelles called cortical granules are present that prevent polyspermy [Figure 1]. A specialised layer of granulosa cells called cumulus oophorus surrounds, supports and nourishes the oocytes. In addition to these, an oocyte is surrounded by an extracellular glycoprotein matrix that separates it from the cumulus cells and is known as the Zona Pellucida (ZP).

High-resolution scanning electron microscopy (SEM) on human oocytes has shown that ZP looks like a delicate meshwork of thin interconnected filaments, which are about 0.1–0.4 μm in length and 10–14 nm in thickness.^[5] It exhibits porosity, with the most porous region of ZP (outer ZP) present in contact with the cells of cumulus oophorus, whereas the more compact ZP region is present in the middle and closer to the oocyte.^[6] SEM studies also revealed that during maturation, a reorganisation of the ZP filament network in the outer ZP takes place. While the immature and atretic oocytes have a smooth-surfaced outer ZP (tightly packed filaments), the mature oocytes exhibit a spongy porous structure (loosely organised filaments). This porosity is associated with the oocyte's fertilisation potential, as the porous ZP facilitates increased sperm binding and penetration.^[5,7] In addition, the fertilised oocytes exhibit increased viscosity, stiffness and resistance to proteolytic digestion of ZP as a result of zona hardening after fertilisation.^[8] These changes in the mechanical properties of ZP after fertilisation were observed using techniques like microelectromechanical systems, microtactile sensors, quartz fibre deflection, capillary suction, etc.^[9–11]

MOLECULAR ORGANISATION OF HUMAN ZP

At the molecular level, human ZP is composed of four different glycoproteins, ZP1–4.^[12] The meshwork of ZP filaments consists of ZP2–ZP3 heterodimers, a repeated structural unit of 14 nm, with ZP1 or ZP4 incorporated as cross-linkers between the filaments [Figure 2].^[13] All ZP proteins have common structural motifs which include an N-terminal signal sequence, an N-terminal region (NTR), a ZP domain (ZPD), an internal hydrophobic patch

(IHP), a consensus furan cleavage site (CFCS), an external hydrophobic patch (EHP), a transmembrane domain (TMD) and a short cytoplasmic tail. Further, ZPD consists of a characteristic ZP-N and ZP-C sub-domain attached by a linker region, which carries the IHP. Additional extension repeats of the ZP-N sub-domain are also present in the NTR of ZP1, ZP2, and ZP4, and a trefoil domain with unknown significance is present in ZP1 and ZP4 [Figure 3].^[14] Each of these structural motifs has a specific function. The hydrophobic signal sequence guides the protein into the secretory pathway, ZPD helps in the polymerisation of ZP proteins, CFCS is the cleavage site for an endoprotease from the furin-protein-convertase family to release the mature protein into the extracellular milieu, and the IHP-EHP interactions prevent premature polymerisation of immature ZP proteins into fibrils.^[15,16]

Characterisation of glycosylation in human ZP proteins using lectins and antibodies revealed the presence of Sialyl-Lewis(a), Sialyl-Lewis(x), Neu5Ac α 2-3Gal β 1, 4GlcNAc, Gal β 1, 3GalNAc-Ser/Thr, Neu5Ac α 2,6Gal/GalNAc, fucosylated oligosaccharides, N-acetylgalactosamine residues, galactose residues and N-acetylglucosamine residues.^[17] It was observed that ZP glycoproteins have more N-linked glycosyl moieties as compared to O-linked glycosyl moieties with the Sialyl-Lewis(x) sequence (NeuAC α 2-3Gal β 1-4(Fuca1-3)GlycNAc) as the most abundant glycosyl moiety present on ZP proteins.^[18]

ROLE OF ZP DURING FOLLICULOGENESIS, OOCYTE MATURATION, FERTILISATION AND EMBRYO DEVELOPMENT

ZP plays multiple functions during various important events before and after fertilisation [Figure 4]. During folliculogenesis, ZP plays a crucial role by providing structural support to the growing oocytes and regulating communication between the oocyte and granulosa cells. A two-way communication exists between the oocyte and granulosa cells that includes direct signalling through gap junctions or through transzonal projections or by paracrine signalling via soluble oocyte secreted factors.^[19,20] This communication is essential for the completion of oocyte growth and for attaining cytoplasmic competence required for fertilisation and embryonic development.^[21–23] Disruption of this communication has a detrimental effect on folliculogenesis, as is observed in *Zp2*^{-/-} and *Zp3*^{-/-} female mice. A severe reduction in the number of antral follicles in the ovaries and fewer ovulated oocytes devoid of ZP were observed in the oviduct of these knockout females.^[24–26]

During oocyte maturation, the ZP protects the growing oocyte and prevents its premature release from the follicle, ensuring its proper development and readiness for

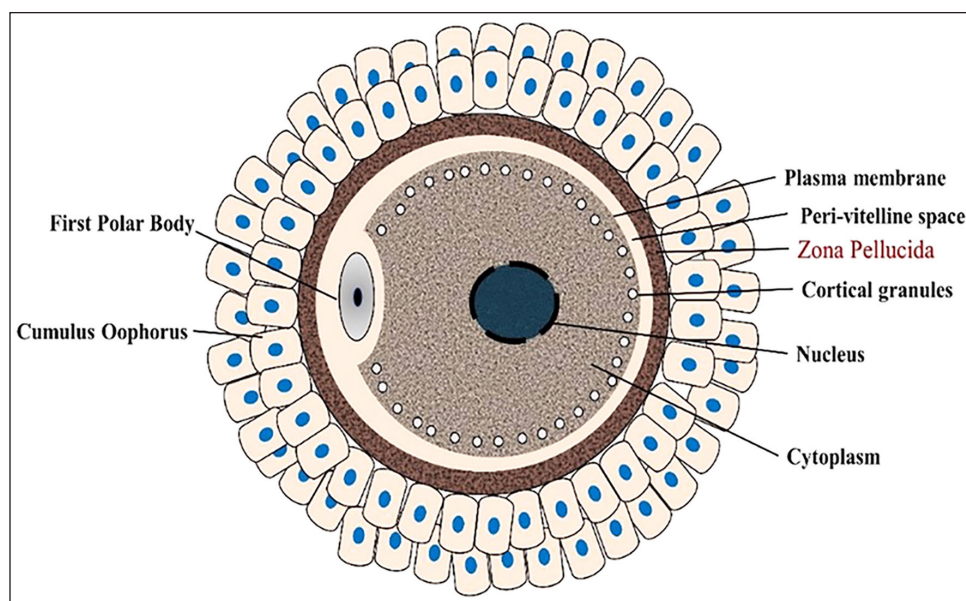


Figure 1: Structure of human oocyte.

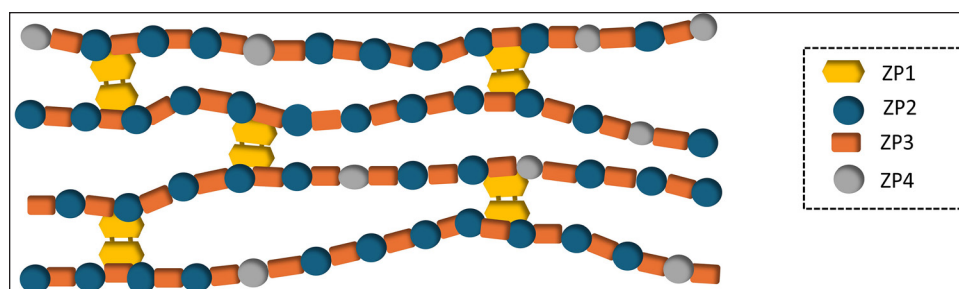


Figure 2: The structural organization of a zona pellucida (ZP) heterodimer.

fertilisation. After fertilisation, cleavage of ZP2 by ovastacin enzyme triggers oligomerisation of ZP2-N terminal fragments, leading to extensive cross-linking of ZP filaments. This causes structural reorganisation of the ZP and increases its stiffness and filament density, rendering it impenetrable to additional sperm and hence, prevents polyspermy.^[27]

ZP also plays an indispensable role during early embryonic development until implantation occurs. As the early embryo travels through the fallopian tube towards the uterus, the ZP matrix provides mechanical protection, shielding it from physical damage as well as from maternal immune cells.^[28] In addition, it prevents premature embryo adhesion to the oviductal wall and regulates embryo-maternal crosstalk during early embryonic stages.^[29-31] By acting as an interface between the growing embryo and uterine epithelium, ZP contains all the signals sent from the embryo to the mother and vice versa and thus acts as a kind of “mailbox” of embryo-maternal communication.^[32] As the blastocoele in the

growing trophoblast expands by fluid intake, an enlargement of volume by 2–3 fold occurs, which increases the internal pressure, causing stretching of the trophoblast and thinning of ZP. Ultimately, the blastocyst breaks the zona matrix and emerges out of it by a process called “zona hatching” and gets implanted into the uterine lining.^[33] Transmission electron microscopy shows that specialised trophoblastic cells called “zona breakers” help in the initial breakdown of ZP during early embryonic growth and hatching. ZP safeguards the integrity of the pre-implantation embryo during early development and aids in its transport through the oviduct.

Among the four ZP proteins, ZP1 and ZP4 function as structural cross-linkers of ZP filaments. The absence of *Zp1* in mice results in a loosely organised ZP leading to subfertility, while oocytes lacking *Zp4* have a morphologically normal zona matrix with no overt fertility defects.^[34,35] In contrast, the absence of either *Zp2* or *Zp3* leads to complete infertility, underscoring their essential roles in fertilisation.^[24,25]

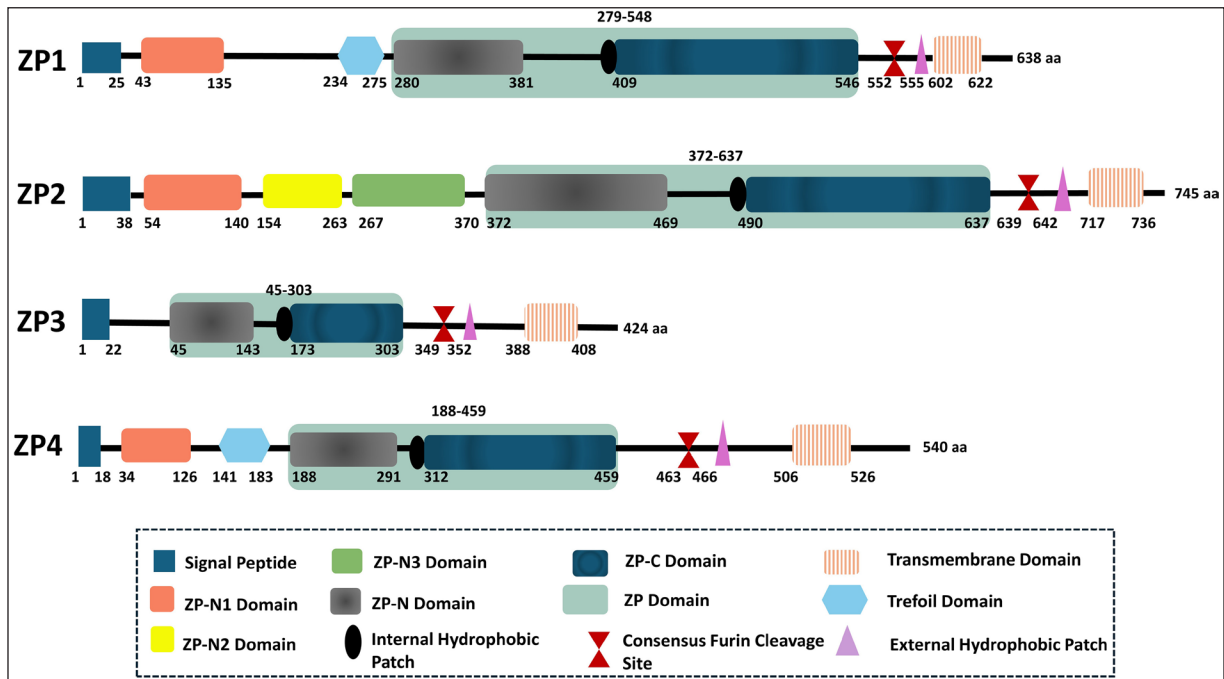


Figure 3: Domain structure of the four human zona pellucida (ZP) proteins.

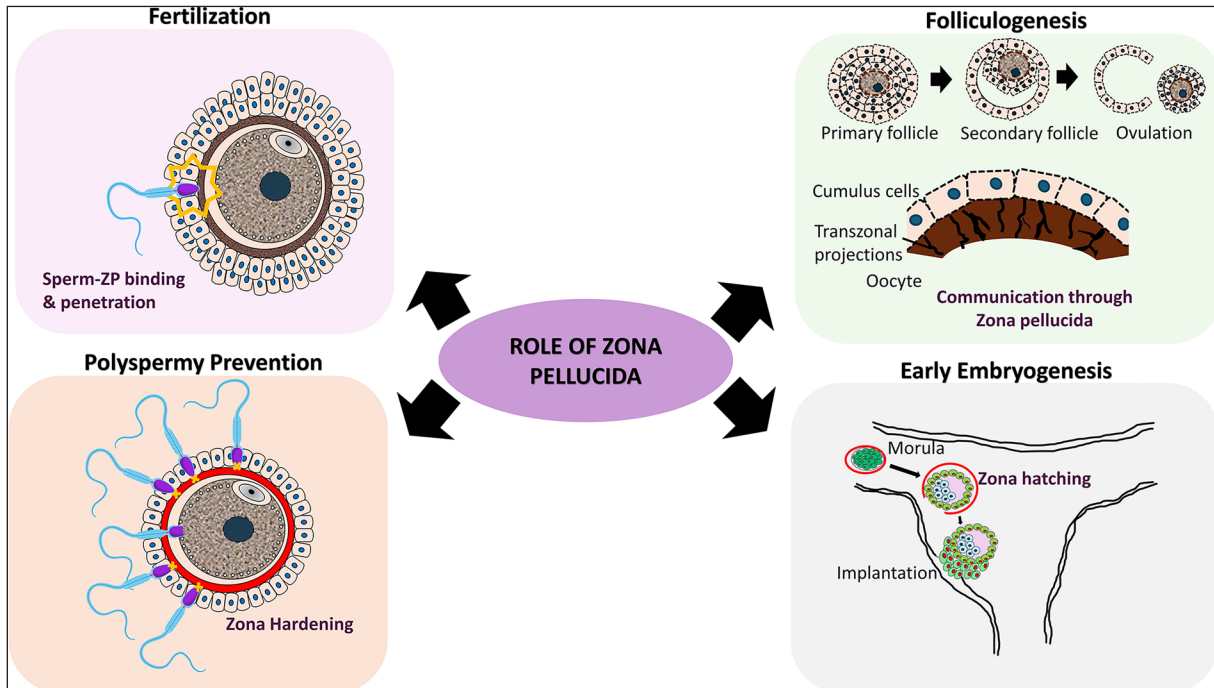


Figure 4: The various roles of zona pellucida (ZP).

According to the previous fertilisation model, also known as the glycan model, ZP3 acts as a primary sperm receptor, recognised by a sperm protein, after which the sperm undergoes the acrosome reaction. The acrosome-reacted

sperm continues to bind to the ZP through the secondary sperm receptor, the ZP2.^[36] The receptor activity of ZP3 in mice was determined to be due to the O-glycans on ZP3, more specifically Ser³³² and Ser³³⁴. Post-fertilisation, an

unknown glycosidase released from the cortical granules removes these O-glycans, as a result of which no more sperm can bind and hence polyspermy is prevented.^[37-39] However, this view has since been challenged. Mass spectrophotometric studies using mouse oocytes found only 5 O-glycan sites with glycosylation out of the 58 sites present on the mature protein. Moreover, no O-glycosylation was observed on Ser³³² and Ser³³⁴.^[40] Subsequently, using biochemical experiments and transgenic mice, it was shown that when Ser residues were mutated, neither fertilisation nor the post-fertilisation block to polyspermy was affected.^[41-44]

Although ZP3 was shown to induce acrosome reaction, a significant proportion of sperm undergo acrosome reaction before encountering ZP, suggesting that ZP3 may not be the sole factor.^[45,46] More recently, transgenic, biochemical and structural studies have provided evidence that ZP2 is the receptor for sperm's interaction and is also essential for maintaining the post-fertilisation block to polyspermy.^[27,44]

How the Absence of ZP2 Affects Folliculogenesis, Fertilisation and Embryonic Development

As discussed before, the *Zp2*^{-/-} female mice are infertile. During the early follicular stage, a thin zona matrix composed of ZP1 and ZP3 was formed around the oocytes from these knockout mice, which failed to persist in the antral follicular stage, and thus, the ovulated oocytes lacked ZP matrix. Upon hormonal stimulation, only half of the *Zp2* null females ovulated and produced significantly fewer eggs than normal females, pointing towards the significant decrease in the number of surviving follicles in the absence of the ZP matrix.^[26] The association between oocytes and granulosa cells was less intense, and the corona radiata cells were unorderedly arranged around the oocytes of these knockout mice compared to those of the wild type. It was proposed that ZP may help in stabilising the gap junctions between the oocytes and granulosa cells, and in its absence, fewer gap junctions are formed, leading to impaired transfer of nutrients, nucleotides, metabolites, ions, etc., to the oocyte, which ultimately compromises its growth.^[26] On mating with normal males, no two-cell embryos were found in the oviducts of these females, rendering them unable to conceive or produce offspring. Thus, infertility in *Zp2* null females can be attributed to the scarcity of oocytes and their rapid reabsorption into the oviduct's epithelial lining. However, *in vitro* fertilisation (IVF) of zona-free oocytes retrieved from *Zp2* null females was successful, and the resulting zygote grew to the blastocyst stage, but their developmental potential beyond this stage was compromised, and after transfer to foster mothers, live births were not observed.^[25]

ZP2 Mediates Species-Specific Sperm-ZP Interaction in Humans Through Its N-Terminal Domain

Human sperm-ZP binding occurs in a species-specific manner, which means human sperm does not bind with the ZP of mice or any other non-hominoid.^[47] When transgenic mice were engineered to express human-mouse chimeric ZP, i.e., human ZP1 along with mouse ZP2 and ZP3 (huZP1 rescue mice); human ZP2 along with mouse ZP1 and ZP3 (huZP2 rescue mice); human ZP3 along with mouse ZP1 and ZP2 (huZP3 rescue mice); and human ZP4 along with mouse ZP1-3 (huZP4 transgenic mice), human sperm could bind and penetrate the ZP of oocytes from huZP2 rescue mice but not with those from other rescue mice [Figure 5] suggesting the species-specific binding nature of ZP2.^[48] The sperm binding site was localised to the NTR of ZP2 between amino acids 51 and 149 using baculovirus-expressed recombinant protein as well as rescue mice expressing chimeric ZP2, where mouse ZP2 had its N-terminus replaced with human ZP2 N-terminus (hu/moZp2 rescue mice) or human ZP2 had its N-terminus replaced with mouse ZP2 N-terminus (mo/huZP2 rescue mice).^[48,49] As expected, human sperm bound to only rescue mice in which the mouse ZP2 had its N-terminus replaced with the human ZP2 N-terminus. This confirmed the role of the NTR of ZP2 in human sperm-ZP binding.

Although we have delineated the sperm binding region on ZP2, the corresponding receptor(s) on the sperm remain elusive. Several sperm candidate proteins like β 4GALTI, ADAM3, SP56, sFUT5, ACROSIN, etc., have been implicated in ZP binding.^[50] However, transgenic males in which these proteins are absent are fertile, probably because most of these were identified based on the classical model involving O-glycan-mediated interaction with ZP3 and hence were misidentified. To date, no specific receptor responsible for binding to ZP2/ZP has been identified.^[50]

ZP2 Cleavage Prevents Polyspermy

Fertilisation triggers a cascade of intracellular signalling pathways, leading to cortical granule exocytosis that results in the release of its contents, including enzymes, into the perivitelline space (PVS). One of the enzymes released is ovastacin, a zinc metalloprotease critical for post-fertilisation remodelling of ZP to prevent polyspermy.^[51] Polyspermy is a condition where an egg is fertilised by more than one sperm, and is lethal in most mammals, including humans.^[52] Time-lapse imaging of human fertilisation shows that once a leading sperm penetrates the ZP matrix and attaches to the oocyte's membrane, subsequent sperm are halted from further penetration into the ZP matrix within 10 seconds.^[53]

Ovastacin cleaves ZP2 in its NTR at a specific site,^[71] LA↓DD^[74]. This site is highly conserved among mammals, and cleavage at this position inactivates sperm binding and

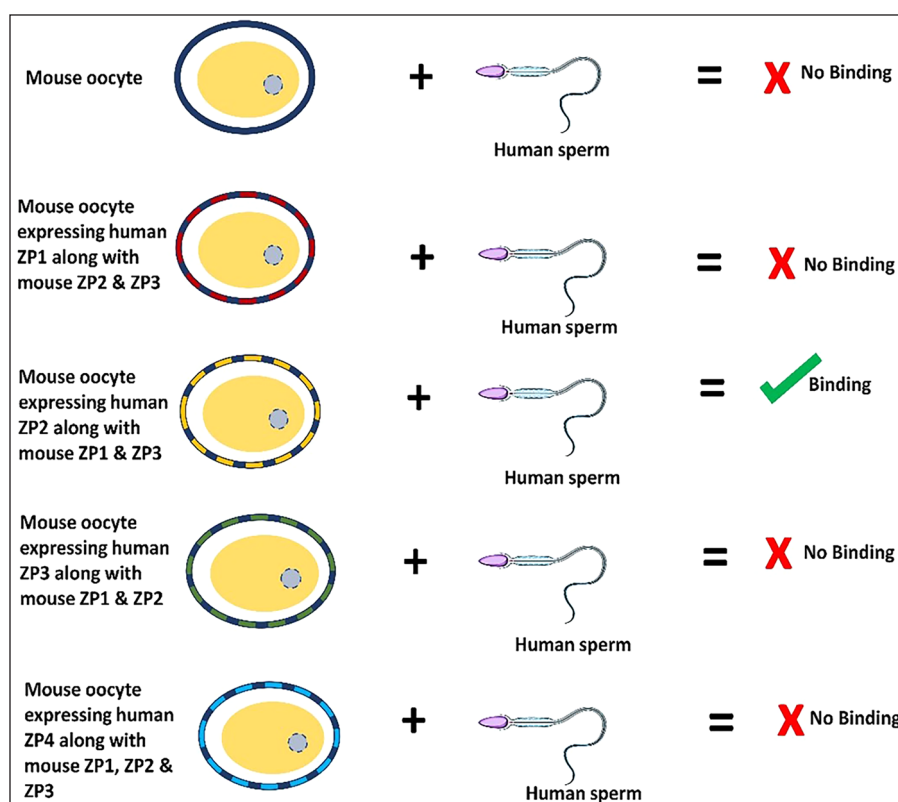


Figure 5: Schematic diagram showing human sperm-binding with different transgenic mouse oocytes having humanized zona pellucida (ZP).

triggers oligomerisation of cleaved ZP2, which changes the physiochemical properties of the zona, otherwise known as zona hardening, and prevents embryonic lethal polyspermy.^[27,51] In transgenic mice carrying a mutated ovastacin cleavage site (¹⁶⁵LA↓DEN¹⁷⁰→¹⁶⁵LGAAN¹⁷⁰), the ZP2 protein did not undergo post-fertilisation cleavage, and sperm continued to bind to the ZP even at the two-cell stage despite successful fertilisation and cortical granule exocytosis. Similarly, in *Astl*^{null} mice lacking ovastacin, ZP2 remained uncleaved after fertilisation, leading to persistent sperm binding at the two-cell stage and subsequent polyspermy.^[44,51] This impaired fertility was evident from the reduced litter sizes in both *Astl*^{null} females and females with the ZP2 cleavage site mutation.

Mutations in the Human ZP2 Gene and Infertility

Since ZP2 plays such a crucial role in the various steps of folliculogenesis, oocyte maturation, fertilisation and embryo implantation, any change in the structure or function of the protein can result in infertility. The common ZP dysmorphologies observed in infertile female patients include abnormalities such as dark ZP, an irregularly shaped ZP, and thin or absent ZP. These dysmorphologies led to a decrease (44% vs 70%) in clinical pregnancy success rates, indicating a strong impact of ZP morphology on reproductive outcomes.^[54]

Understanding the genetics behind these abnormalities in ZP is significant for the success of IVE.^[55–57] Since similar phenotypes were also observed in *Zp2* KO female mice, it is possible that human females with ZP dysmorphologies may harbour mutations in the *ZP2* gene. Several studies have already reported mutations in the *ZP2* gene of infertile female patients whose oocytes have abnormal morphologies^[68–72] [Table 1].

Dai *et al.* identified two homozygous mutations, c.1695-2A>G and c.1691_1694 (C566Wfs*5), in the *ZP2* gene of two infertile females.^[58] The oocytes retrieved from these patients showed an abnormally thinner ZP matrix and enlarged PVS. Expression of recombinant ZP2 with these mutations showed protein truncation, resulting in the loss of the ZP-C sub-domain, CFCS, TMD, and C-terminal domain. Similarly, Jia *et al.* reported three mutations in the *ZP2* gene: c.1695-2A>G, c.1813G>T (V611F) and c.1924C>T (R642X).^[59] In these females, the cumulus oocyte complexes retrieved were either found empty after denudation or had oocytes with abnormal or no ZP. Transcriptomic studies involving a gene-edited (*Zp2*^{mut/mut}) rat model having the c.1924C>T (R642X) mutation revealed differential expression of many genes.^[60] Pathway enrichment analysis revealed enrichment in multiple signalling pathways, especially in the transforming

Table 1: Summary of mutations reported in the human *ZP2* gene.

cDNA change	Amino acid change	Domain	Genotype	Phenotype	References
c.428T>C	V143A	N-terminal domain	Heterozygous	ZP abnormality/EFS/degenerated oocytes	[68]
c.474T>A	D158E	N-terminal domain	Heterozygous	ZP abnormality/EFS/degenerated oocytes	[68]
c.836_837delAG	E279Vfs*6	N-terminal domain	Homozygous	Oocytes with thin ZP and enlarged PVS	[63]
c.1115G>C	C372S	ZP-N domain	Homozygous	Oocytes with thin or no ZP	[69]
c.1235_1236del	Q412Rfs*17	ZP-N domain	Homozygous	Oocytes with thin ZP	[62]
c.1421T>C	L474P	Between ZP-N and C domain	Heterozygous	Oocytes with thin ZP	[70]
c.1447C>T	P483S	Between ZP-N and C domain	Heterozygous	ZP abnormality/EFS/degenerated oocytes	[68]
c.1543C>T	P515S	ZP-C domain	Heterozygous	COCs with immature oocytes or oocytes without ZP	[65]
c.1599G>T	R533S	ZP-C domain	Heterozygous	COCs with either degenerated or ruptured oocytes and crackled ZP	[64]
c.1616C>T	T539M	ZP-C domain	Heterozygous	Immature oocytes with no ZP or EFS	[61]
c.1691_1694dup	C566Wfs*5	ZP-C domain	Homozygous	Degenerated oocytes with thin ZP and EFS	[58]
c.1695-2A>G	C566Hfs*5	ZP-C domain	Homozygous	Degenerated oocytes with thin ZP and EFS	[58]
c.1696T>C	C566R	ZP-C domain	Heterozygous	COCs with either degenerated or ruptured oocytes and crackled ZP	[64]
c.1856T>A	I619N	ZP-C domain	Heterozygous	COCs with degenerated, immature or zona free oocytes	[71]
c.1859G>A	C620Y	ZP-C domain	Heterozygous	Oocytes with thin or absent ZP	[70]
c.1888T>G	C630G	ZP-C domain	Heterozygous	ZP abnormality/EFS/degenerated oocytes	[68]
c.1925G>A	R642Q	Near CFCS	Heterozygous	COCs with degenerated, immature or zona free oocytes	[71]
c.1924C>T	R642X	Near CFCS	Homozygous	Oocytes with thin or no ZP or EFS	[60]
c.1831G>T	V611F	ZP-C domain	Compound heterozygous	Oocytes with abnormally thin ZP	[59]
c.1695-2A>G	-	Splice site mutation			
c.1924C>T	R642X	Near CFCS	Compound heterozygous	EFS	[59,72]
c.1695-2A>G	C566Hfs*2	Splice site mutation			

COCs: Cumulus-oocyte complexes, EFS: Empty follicle syndrome, PVS: Perivitelline space, CFCS: Consensus furin cleavage site, ZP: Zona pellucida.

growth factor- β (TGF- β) signalling pathway, and protein expression analysis showed downregulation of ACRV2B, SMAD2, p38MAPK and BCL2 proteins. The absence of the ZP matrix probably disrupts various signalling pathways like TGF- β signalling between oocytes and surrounding granulosa cells, leading to apoptosis and/or impaired developmental potential of the oocytes.

Another novel heterozygous mutation, c.1616C>T (T539M), in *ZP2* was identified in an infertile woman with abnormalities like oocyte maturation defect or oocytes with thin or no ZP.^[61] Using CRISPR-Cas9, *Zp2*^{WT/T541M} and *Zp2*^{T541M/T541M} female mice were generated to characterise the effect of this mutation on fertility. It was found that heterozygous females

ovulated oocytes with thin ZP but exhibited normal fertility, whereas homozygous females were completely infertile as their oocytes lacked ZP and underwent degeneration.^[61]

Apart from these, several other mutations in the *ZP2* gene (c.1599G>T (R533S), c.1696T>C (C566R), c.1235_1236del (Q412Rfs*17) and c.836_837delAG (Q279Vfs*6)) have been reported in different infertile females whose oocytes show a common phenotype of having crackled or thin ZP and undergo degradation.^[62-64] Some mutations like c.1925G>A (R642Q), c.1856T>A (I619N), and c.1115G>C (C372S) were found to be associated with Empty Follicle Syndrome (EFS) in females. Another *ZP2* mutation, c.1543C>T (P515S), was also found in an infertile female with oocyte maturation

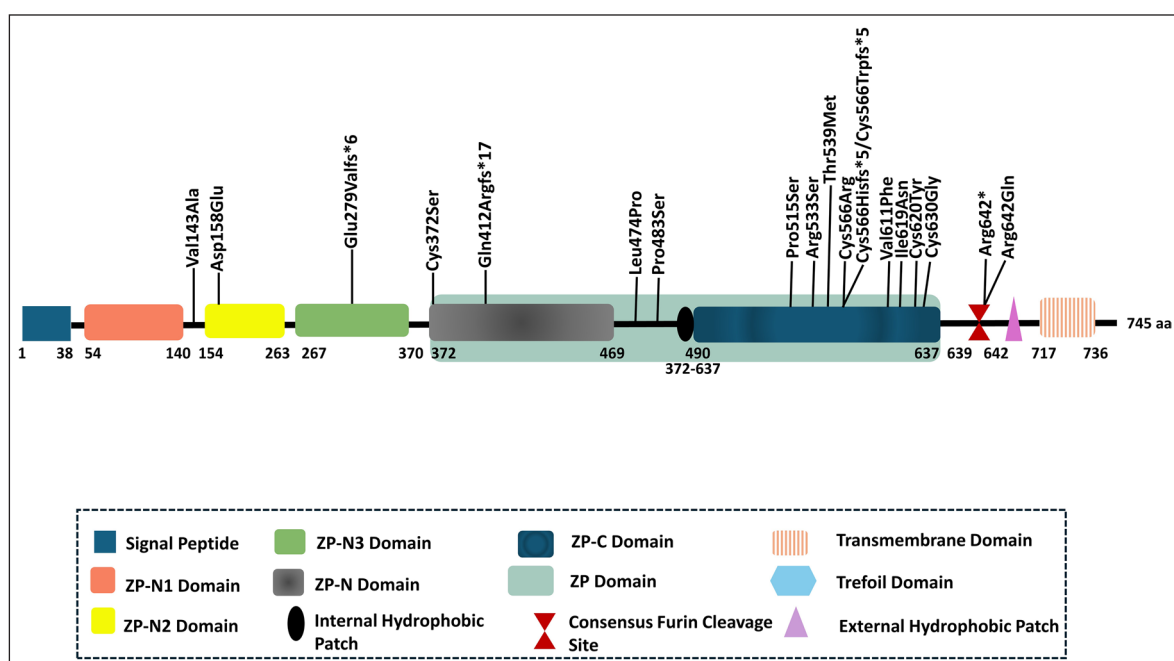


Figure 6: Reported mutations in the *ZP2* gene of infertile females.

arrest.^[65] Since ZP2 is an important structural component of ZP, improper synthesis of the mature protein can disrupt ZP assembly and consequently impair fertility.

The location of all the reported *ZP2* mutations identified in infertile females is illustrated in Figure 6. *In silico* studies have also demonstrated that single nucleotide polymorphisms (SNPs) in the human *ZP2* gene can be detrimental to female fertility.^[66] Collectively, these studies suggest that mutations in the *ZP2* gene can be one of the underlying causes of female infertility, and the phenotypes that have been associated so far include EFS, ZP-thin oocytes, ZP-free oocytes and oocyte maturation defects.^[67]

CONCLUSION

The ZP2 glycoprotein is a crucial component of the ZP, playing a fundamental role in sperm binding, oocyte protection, and fertilisation. Genetic mutations in the *ZP2* gene disrupt ZP integrity, leading to defective oocyte maturation and compromised fertility. Studies in humans and animal models highlight the essential nature of ZP2 in maintaining reproductive function, with mutations often resulting in infertility due to abnormal folliculogenesis, impaired sperm-oocyte interaction, and increased oocyte degeneration. Infertile females carrying SNPs in the NTR region of ZP2 may benefit from intracytoplasmic sperm injection (ICSI) over conventional IVF, as defective sperm-zona binding in such cases can lead to IVF failure. ICSI represents a more effective treatment strategy than

conventional IVF because, by directly injecting sperm into the oocyte cytoplasm, ICSI circumvents the requirement for sperm-ZP interaction. For patients with EFS, *in vitro* maturation (IVM) of oocytes may provide some advantage. With the increasing affordability and accessibility to genome sequencing, incorporating molecular diagnostics into personalised fertility treatment is going to be a key aspect of future reproductive care.

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