Zona Pellucida

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All mammalian oocytes and eggs are surrounded by a relatively thick extracellular matrix (ECM), the zona pellucida (ZP), that plays vital roles during oogenesis, fertilization, and preimplantation development. Unlike ECM surrounding somatic cells, the ZP is composed of only a few glycosylated proteins, ZP1–4, that are unique to oocytes and eggs. ZP1–4 have a large region of polypeptide, the ZP domain (ZPD), consisting of two subdomains, ZP-N and ZP-C, separated by a short linker region, that plays an essential role in polymerization of nascent ZP proteins into crosslinked fibrils. Both subdomains adopt immunoglobulin (Ig)-like folds for their 3-dimensional structure. Mouse and human ZP genes are encoded by single-copy genes located on different chromosomes and are highly expressed in the ovary by growing oocytes during late stages of oogenesis. Genes encoding ZP proteins are conserved among mammals, and their expression is regulated by cis-acting sequences located close to the transcription start-site and by the same/similar trans-acting factors. Nascent ZP proteins are synthesized, packaged into vesicles, secreted into the extracellular space, and assembled into long, crosslinked fibrils that have a structural repeat, a ZP2-ZP3 dimer, and constitute the ZP matrix. Fibrils are oriented differently with respect to the oolemma in the inner and outer layers of the ZP. Sequence elements in the ZPD and the carboxy-terminal propeptide of ZP1–4 regulate secretion and assembly of nascent ZP proteins. The presence of both ZP2 and ZP3 is required to assemble ZP fibrils and ZP1 and ZP4 are used to crosslink the fibrils. Inactivation of mouse ZP genes by gene targeting has a detrimental effect on ZP formation around growing oocytes and female fertility. Gene sequence variations in human ZP genes due to point, missense, or frameshift mutations also have a detrimental effect on ZP formation and female fertility. The latter mutations provide additional support for the role of ZPD subdomains and other regions of ZP polypeptide in polymerization of human ZP proteins into fibrils and matrix.

zona pellucida | mammalian oogenesis | gene expression | proteins | zona pellucida domain | polymerization | fibrils | gene targeting | gene mutations | female fertility

1. Introduction

Extracellular matrix (ECM) that surrounds most animal cells can affect cellular adhesion and migration, cell-to-cell communication, as well as gene expression, differentiation, and morphogenesis [1]. ECM consists of proteoglycans (e.g., hyaluronic acid, heparin-, chondroitin- , and keratin-sulfate) and fibrous proteins (e.g., collagens, elastins, fibronectins, and laminins) [2][3][4]. On the other hand, ECM of mammalian oocytes and eggs, the zona pellucida (ZP), is composed of a unique set of glycosylated proteins, ZP1–4, that differ from proteins present in somatic cell ECM [5][6][7].

Each ZP protein has a zona pellucida domain (ZPD) that consists of ≈270 amino acids (aa), 8 or 10 conserved cysteine (Cys) residues present as intramolecular disulfides, and two subdomains, ZP-N and ZP-C. These subdomains adopt immunoglobulin (Ig)-like folds and are connected to each other by a short, protease-sensitive linker region [7][8][9][10][11]. Subdomain ZP-N is involved in polymerization of nascent ZP proteins into fibrils, as well as in polymerization of many other ZPD-containing proteins, such as tectorin, uromodulin, mesoglein, and cuticlins, into fibrils and matrices. Mutations in ZPD genes can result in severe human pathologies such as vascular disease, renal disease, deafness, cancer, or infertility. Although the ZP and somatic cell ECM consist of different proteins, they have certain properties in common, such as viscoelasticity that can affect cellular behavior. It has been proposed that ZP proteins self-aggregate into fibrillar structures via cross-β-sheets, similar to the structure of amyloids.

A ZP first appears as oocytes begin to grow, continues to thicken as oocytes increase in size, and is from ≈2 to ≈20 µm thick for fully grown oocytes from different mammals, e.g., the human egg ZP (*hZP*; ≈18 µm width) is about 3 times thicker than the mouse egg ZP (*mZP*; ≈6 µm width). The ZP is a viscoelastic ECM permeable to large macromolecules, (e.g., antibodies, enzymes, and small viruses) and consists of long, crosslinked fibrils that are polymers of ZP proteins. A variety of agents that do not break covalent bonds dissolve the ZP indicating that its components are held together by non-covalent interactions.

The ZP plays vital roles during oogenesis, fertilization, and preimplantation development. For example, it supports the health and growth of oocytes and follicles during oogenesis, provides species-restricted receptors for binding of free-swimming sperm to eggs during fertilization, undergoes both physical and biological changes that help to prevent polyspermy following fertilization, and protects preimplantation embryos as they traverse the female reproductive tract on their way to the uterus. In this context, it has been demonstrated that either inactivation of *mZP* genes or mutation of *hZP* genes can have a deleterious effect on ZP formation during oogenesis and can result in female infertility.

2. *mZP* Genes and Female Fertility and *hZP* Genes and Female Fertility

2.1. *mZP* Genes and Female Fertility

Results of experiments in which antisense oligonucleotides directed against either *mZP2* or *mZP3* mRNAs were injected into growing mouse oocytes strongly suggest that *mZP2* and *mZP3* are dependent upon each other for incorporation into the ZP [12]. To extend these observations, gene targeting was used to establish mouse lines in which *mZP* genes were inactivated by either homologous recombination or insertional mutagenesis and the fertility of the mice was assessed (**Table 1**).

Table 1. Phenotypes of *ZP1,2,3* Null Female Mice.

Genotype	Fertility	Zona Pellucida	References
Wild-type	Fertile	Normal	-
ZP1$^{-/-}$	Reduced Fertility	Abnormal	[13]
ZP2$^{-/-}$	Infertile	None	[14]
ZP3$^{-/-}$	Infertile	None	[15][16]
ZP3$^{+/-}$	Fertile	Thin	[17]

2.1.1. mZP2 and mZP3 Homozygous Nulls Are Infertile

Male mice that are homozygous nulls for *mZP1*, *mZP2*, or *mZP3* are as fertile as wild type males. On the other hand, female mice that are homozygous nulls for either *mZP2* (*mZP2*$^{-/-}$) or *mZP3* (*mZP3*$^{-/-}$) produce eggs that lack a ZP, and these females are completely infertile [14][15][16] (**Table 1**). Infertility is due to a scarcity of both growing oocytes and ovulated oviductal eggs in homozygous null mice. This suggests that the presence of both *mZP2* and *mZP3* is absolutely required for assembly of a ZP around growing oocytes and is consistent with results of antisense experiments mentioned above [12]. The paucity of growing oocytes and follicles in ovaries of *mZP3*$^{-/-}$ mice is reflected in weight differences of ovaries from 20-day-old wild type females, 1.0 ± 0.17 mg/ovary, and ovaries from *mZP3*$^{-/-}$ females the same age, 0.26 ± 0.1mg/ovary; a 4-fold difference due to retarded oocyte growth and follicle development [18][19]. The relatively small number of growing oocytes in ovaries from homozygous null mutant mice is not intimately associated with surrounding follicle cells and ovaries contain few, if any, Graafian follicles.

It has been shown that gap junctions are present between oocytes and surrounding follicle cells at sites where follicle cell processes traverse the ZP and contact the oolemma [20][21][22]. In the absence of a ZP around oocytes from *mZP2*$^{-/-}$ and *mZP3*$^{-/-}$ mice it is likely that formation of gap junctions is severely reduced, thereby compromising the electrical and metabolic coupling between oocytes and follicle cells that is necessary for oocyte growth, follicle development, and fertility [23][24][25]. The latter is consistent with the phenotype of female mice that are homozygous nulls for gap junction proteins, such as connexin-37 and -43; these mice are infertile, and their ovaries are deficient in growing oocytes and multi-layered follicles and [26][27]. Furthermore, note the finding that as oocytes grow follicle cells elaborate enormous numbers of new transzonal projections (filopodia) that contact the oocyte surface and increase oocyte–follicle cell communication [28]. Overall, these observations with *mZP2* and *mZP3* homozygous null females suggest that the ZP may serve as a kind of glue with which to stabilize gap junctions and other contacts between oocytes and innermost follicle cells.

2.2.2. mZP3 Heterozygous Nulls Are Fertile

Female mice that are heterozygous nulls for *mZP3* (*mZP3*$^{+/-}$) are as fertile as wild-type females, but their eggs have a thin ZP (ave. width ≈ 2.7 ± 1.2 μm) compared to the ZP of eggs from wild type females (ave. width ≈ 6.2 ± 1.9 μm) [17] (**Table 1**). The thin ZP contains about one-half the amount of *mZP2* and *mZP3* found in ZP of eggs

from wild type mice. These observations suggest that the width of the ZP is not a critical parameter for either binding of free-swimming sperm to the ZP or fertilization of eggs.

2.2.3. mZP1 Homozygous Nulls Exhibit Reduced Fertility

Female mice that are homozygous nulls for *mZP1* (*mZP1*$^{-/-}$) are fertile, but exhibit reduced fertility compared to wild type mice due to early loss of preimplantation embryos in oviducts [13] (**Table 1**). This loss is attributable to a ZP that is not crosslinked and, consequently, extremely fragile as cleavage-stage embryos traverse the reproductive tract on their way to the uterus. The presence of *mZP2* and *mZP3* in growing oocytes of *mZP1*$^{-/-}$ mice supports formation of heterodimers that can assemble into long fibrils. However, in the absence of *mZP1* the fibrils are not crosslinked, creating an unusually porous ZP matrix that even permits follicle cells to enter the perivitelline space between the ZP and plasma membrane. New insights into the structural basis of *hZP1*/*hZP4* crosslinking of the human ZP have recently been reported [29].

2.2. *hZP* Genes and Female Fertility

Female infertility has increased dramatically over the past 25 years and today it is estimated that ≈10% of married women worldwide are infertile. Nearly 65% of human infertility cases can be attributed to either male or female factors and ≈50% of infertility cases have a genetic component. In this context, some early evidence suggested that there might be a causal relationship between gene sequence variations (GSV) in *hZP* genes and female fertility [30][31][32]. For example, it was found that there was ≈1.5 times more GSV in *hZP1* and *hZP3* of women who were unsuccessful in in vitro fertilization (IVF) trials compared to women with proven fertility [30]. This finding has now been extended by a large number of case studies carried out to assess whether GSV in *hZP1-4* have an effect on female fertility. Results of these studies with human IVF patients are summarized below and in **Table 2**.

Table 2. *hZP1*–4 Mutations in Infertile Human Patients.

hZP1 Mutations	Location of Mutation	Status of Zona Pellucida	References
G57Dfs*9	exon-1, SC in NI befroe TD	none	[33]
R61C	exon-1, NI befroe TD	none (?)	[34]
W83R	exon-2, NI befroe TD	abnormal/none	[35]
E67>X	exon-2, SC in NI befroe TD	none	[36]
RI09H	exon-3, NI befroe TD	none	[37]
H701fs*52	exon-3, SC between NI and TD	none	[38]

hZP1 Mutations	Location of Mutation	Status of Zona Pellucida	References
Q292>X	exon-5, SC in ZP	none	[39]
I386>X	exon-7, SC between ZP-N and ZP-C(linker)	none	[39]
I390fs404X	exon-7, SC between ZP-N and ZP-C(linker)	none	[40][41]
I390Tfs*16	exon-7, SC between ZP-N and ZP-C(linker)	none	[33][34]
R410W	exon-7, between ZP-N and ZP-C(linker)	none	[38]
W471>X	exon-8, SC in ZP-C	abnormal/none	[35]
C478>X	exon-9, SC in ZP-C	none	[38]
V570M	exon-11, between CFCS and EHP	none	[38]
D592Gfs*29	exon-12, SC between CFCS and TMD	none	[38]
***hZP2*Mutations**			
C372S	exon-11, ZP-N	thin/none	[38]
Q412Rfs*17	exon-11, ZP-N	thin	[42]
R533S	exon-15, ZP-C	normal/none	[35]
C566R	exon-16, ZP-C	abnormal/none	[35]
R698>X	exon-19, SC between CFCS and TMD	very thin/none	[43]
***hZP3*Mutations**			
A134T	exon-2, ZP-N	none	[37][44]
S173C	exon-3, ZP-C	none	[45]
R255G	exon-5, ZP-C	none	[38]
R349L>X	exon-8, SC at CFCS	very thin/none	[43]
***hZP4*Mutations**			

hZP1 Mutations	Location of Mutation	Status of Zona Pellucida	References
D100N	exon-3, NI	thin, irregular	[46]
V444L	exon-10, ZP-C	thin, irregular	[46]

Abbreviations: CFCS, concensus furin cleavage-site; EHP, external hydrophobic patch; h, human.

2.2.1. Infertile Women and Mutant *hZP1* Genes

One study revealed a homozygous frameshift deletion of 8 bp in *hZP1* of women who were infertile and whose eggs lacked a ZP [40]. The deletion was predicted to result in a premature stop codon (SC) in *hZP1* and synthesis of a truncated form of *hZP1*; Ile390*fs*404X, a 404 aa polypeptide for mutant *hZP1* versus a 638 aa polypeptide for wild type hZP1. Truncated *hZP1* had the N-terminal SS, TD, and first half of the ZPD, but was missing the CTP essential for protein secretion [47][48][49][50]. As oocytes from *mZP1* homozygous null mice have a ZP, albeit a very loose and porous ZP, it was surprising that oocytes from these women lacked a ZP. However, subsequently it was reported that accumulation of truncated hZP1 in the oocyte's cytoplasm apparently interfered with secretion of nascent hZP3 and hZP4 and thereby prevented assembly of a ZP around growing oocytes [41]. An alternative explanation for the observation has recently been put forward that does not involve interference with secretion of nascent ZP proteins by truncated ZP1, but rather by affecting the crosslinking function of ZP1 [29].

Other studies also have attributed female infertility to GSV in *hZP1*. A heterozygous missense mutation in exon-3 of *hZP1* was identified in an infertile patient whose oocytes lacked a ZP [37]. The mutation resulted in His replacing Arg109 at the N-terminus of ZP1. Similarly, a compound heterozygous mutation consisting of a point mutation and deletion in *hZP1* was identified in an infertile woman whose oocytes lacked a ZP [39]. The mutation in exon-5 resulted in synthesis of *hZP1* stopping at Glu292 and a 2 bp deletion in exon-7 also resulted in a premature SC and synthesis of *hZP1* stopping at Ile386.

Several additional studies also led to identification of GSV in *hZP1* in infertile females who had abnormal oocytes. For example, a missense mutation in exon-2, Trp83Arg, was found in a patient with degenerated oocytes and an abnormal or no ZP, and in another patient a nonsense mutation with a premature SC in exon-8, Trp471>X, had a similar phenotype [35]. A compound heterozygous mutation, Arg61Cys and Ile390Thr*fs**16, was found to be associated with abnormal oocytes and no ZP since replacement of Arg61 with Cys was predicted to be deleterious to hZP1 and a frameshift mutation introducing an SC in exon-7, Ile390*fs*404X, resulted in a 234 aa deletion at the C-terminus of hZP1 [33][34]. In another case, two frameshift mutations in *hZP1* resulted in premature SCs in exon-1, Gly57Asp*fs**9, and exon-7, Ile390Thr*fs**16, and apparently disrupted interactions between *hZP* proteins and caused degeneration of oocytes [33]. Missense mutations, Val570Met and Arg410Trp, were identified in two infertile females that had no oocytes or oocytes lacking a ZP [38]. Similarly, a compound heterozygous mutation with premature SCs in exon-9, Cys478>X, and exon-12, Asp592Gly*fs**29, and a frameshift mutation in exon-3, His170Ile*fs**52, were identified that possibly resulted in a truncated hZP1 that interfered with ZP formation [38]. A mutation in exon-2 of *hZP1*, G199>T (E67>X), resulted in a truncated protein of 67 aa that impaired secretion and

ZP assembly [36]. In certain cases, GSV in *hZP1* affected its ZPD, a region of all ZP proteins considered critical for proper secretion of nascent ZP proteins and proper assembly of a ZP around growing oocytes [8][10][11][47].

2.2.2. Infertile Women and Mutant hZP2, hZP3, or hZP4 Genes

GSV in *hZP2* and *hZP3* of infertile women can also result in synthesis of *hZP* proteins that are unable to undergo normal secretion and assembly during oocyte growth. An infertile woman was found to have a heterozygous missense mutation in *hZP2*, exon-19 Arg698>X, with insertion of an SC at aa 698 and a heterozygous frameshift mutation in *hZP3*, exon-8 Arg349Leu>X, followed by an SC [43]. Both mutations resulted in the synthesis of truncated ZP proteins, hZP2 lacking a TMD and hZP3 lacking a CTP. Three other cases of GSV in *hZP2* have been described in which Cys372 was changed to Ser, Arg533 to Ser, and Cys566 to Arg [35][38]. All these changes occurred at conserved aa residues in the ZPD of hZP2, aa 371–637. A homozygous frameshift mutation in *hZP2* gave rise to a deletion variant in exon-11, c.1235_1236del, resulting in an altered aa and a truncated *hZP2*, Gln412Arg*fs**17 [42]. The mutation was located within the N-terminal half of the ZPD and was predicted to impede the interaction between *hZP2* and *hZP3*, resulting in a thin ZP. A heterozygous missense variant in exon-2 of *hZP3* also was identified as a change of Ala134 to Thr; a change proposed to cause empty follicle syndrome and female infertility [37][44]. A similar missense mutation in exon-5 of *hZP3*, Arg255Gly, was found in a female with primary infertility [38]. In both cases the mutations occurred in the ZPD of *hZP3*, aa 45–304. A heterozygous mutation in exon-3 of *hZP3*, C518>G (S173C), located in the ZP-C subdomain, changed a highly conserved Ser to Cys and resulted in oocytes that lacked a ZP [45]. Two separate heterozygous variants were found in *hZP4*, a G298>A mutation in exon-3 (D100N) and a G1330>C mutation in exon-10 (V444L), and in each case the oocytes were surrounded by a thin and irregular ZP [46]. The mutations affected 2 highly conserved aa and caused impaired assembly and function of the ZP.

3. Summary Points

(i) The ZP is an atypical ECM that surrounds all mammalian oocytes, eggs, and preimplantation embryos and plays vital roles during oogenesis, fertilization, and preimplantation development. A ZP appears around growing oocytes during oogenesis while arrested in the dictyate stage of meiosis and is shed by expanded blastocysts just prior to implantation in the uterus. The presence of a ZP is required for normal oocyte growth, follicle development, and species-restricted fertilization. The ZP also assists in prevention of polyspermic fertilization and protects cleavage-stage embryos as they traverse the female reproductive tract on their way to the uterus.

(ii) The ZP is composed of either three or four proteins, ZP1-4, each with a unique polypeptide chain that is heterogeneously glycosylated with both N- and O-linked oligosaccharides. ZP2-4 are monomers and ZP1 is a dimer interconnected by a disulfide bond. ZP1-4 are encoded by single-copy genes located on different chromosomes that are only expressed by oocytes during their growth phase. *ZP* genes are hypomethylated in oocytes where they are expressed, as compared to somatic cells where they are not expressed. Genes encoding ZP proteins are conserved such that a *ZP* gene promoter from one mammal (e.g., human) can utilize the transcriptional machinery of oocytes from a different mammal (e.g., mouse). Expression of *ZP* genes in growing

oocytes is regulated by *cis*-acting sequences located close to the transcription start-site and by *trans*-acting factors, certain of which are restricted to growing oocytes.

(iii) ZP proteins are synthesized, packaged in unusually large secretory vesicles, and secreted into the extracellular space. There ZP2 and ZP3 form heterodimers that, in turn, polymerize into long fibrils which exhibit a structural repeat. ZP fibrils are crosslinked by ZP1 and/or ZP4. ZP1-4 each have a ZPD that consists of two subdomains, ZP-N and ZP-C, that have an Ig-like 3-dimensional structure. The presence of both ZP2 and ZP3, but not ZP1 or ZP4, is required for assembly of ZP fibrils and matrix during oocyte growth. Polymerization of ZP proteins is regulated by sequence elements such as the CTP, EHP, and IHP that prevent premature polymerization of nascent ZP proteins in growing oocytes. Proteolytic cleavage of inhibitory sequence elements results in exposure of polymerization elements, such as subdomain ZP-N, and assembly of crosslinked ZP fibrils. ZP proteins possess some amyloid-like structural and physical features and have been proposed to be functional amyloids.

(iv) Failure to assemble a ZP around growing oocytes during oogenesis results in female infertility. Infertility is due to a paucity of growing oocytes and antral follicles in ovaries which results in very few, if any, ovulated eggs in oviducts. Such a situation occurs when *mZP* genes are inactivated by either homologous recombination or insertional mutagenesis or when *hZP* genes undergo point, missense, or frameshift mutations. In many instances, these *hZP* gene mutations result in the insertion of premature SCs, synthesis of truncated ZP proteins lacking sequence elements required for protein polymerization, and failure to assemble a normal ZP around growing oocytes. In the absence of a ZP, the stability of gap junctions between oocytes and surrounding follicle cells is reduced, thereby compromising transfer of nutrients, metabolites, and other molecules essential for oocyte and follicle growth and development.

The role of the ZP during mammalian oogenesis, fertilization, and preimplantation development has been of interest to clinicians and research scientists since the middle of the 19th century. An arsenal of contemporary methodology, from biochemistry to genetics to X-ray crystallography, has been employed during the past 40–50 years in order to reveal many significant features of ZP structure and function. Consequently, today we have a much deeper understanding and appreciation of the composition, genesis, evolution, and role of this unique and remarkable ECM. It is likely that further X-ray crystallographic, electron cryo-microscopic, advanced imaging, and other contemporary experimental approaches will provide answers to a variety of remaining questions about the ZP and its essential roles during mammalian development.

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