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New insights into the molecular basis of mammalian sperm-egg membrane interactions

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1. ABSTRACT

Fertilization is the process by which two terminally differentiated cells, the sperm and the egg, merge to form a totipotent cell, the zygote. This review addresses one of the culminating steps in getting sperm and egg together: the cell-cell interactions that allow the two gametes to fuse and create the zygote. Based on cell biological and genetic studies, major players include CD9 on the egg and Izumo on the sperm, although other molecules are part of an ever-evolving discussion of models for the molecular mechanisms leading to sperm-egg fusion, since few molecules have been shown to be completely essential for sperm-egg union. This sets the stage for consideration of how genetic approaches impact the field – of how knockout mouse reproductive phenotypes translate to humans and other animals and also of how interactions between redundant, nonessential genes could affect reproductive processes such as gamete interaction ("synthetic infertility," analogous to synthetic lethality). We will address these issues, examine the molecular basis of sperm-egg union and how this field has evolved with modern approaches combined with classical studies, and also discuss basic research in gamete biology in light of its possible application to reproductive health.

2. INTRODUCTION TO FERTILIZATION AND GAMETE MEMBRANE INTERACTIONS

2.1. The steps of fertilization leading to sperm-egg fusion

Gamete plasma membrane interactions occur following sperm penetration of the cumulus layer and the zona pellucida (ZP), once the sperm reaches the space between the egg plasma membrane and the ZP, known as the perivitelline space. Sperm-ZP interaction is an important prerequisite step to sperm-egg membrane interactions, because sperm-ZP interaction induces the sperm to undergo the exocytosis of the acrosome vesicle on the head of the sperm (also known as the acrosome reaction). Acrosome exocytosis has two important results. First, enzymes released from the acrosome allow the sperm to penetrate the ZP and gain access to the perivitelline space. Secondly, the acrosome reaction appears to prime regions of the sperm head that participate in gamete binding and membrane fusion, as only acrosome-reacted sperm can bind and fuse with the egg plasma membrane. This likely involves the exposure and/or modification of portions of the sperm membrane that will participate in binding to and fusing with the egg membrane – the inner (proximal) portion of the membrane of the acrosome (the

inner acrosomal membrane) and a region known as the equatorial segment (addressed more below) (1-4). In addition to the acrosome reaction occurring in response to ZP binding, acrosome exocytosis can also occur spontaneously in a subset of sperm during capacitation (e.g., (5)), making it possible for ZP-free eggs to be fertilized *in vitro*.

The remarkable thing about this cellular merger is that it results in a very different single cell from the two starting cell types. The fusion of the sperm with the egg creates the zygote and initiates embryonic development in this cell. This involves the delivery of a Ca2+ release activity (phospholipase Czeta, perhaps others) upon gamete fusion at the sperm entry point into the egg cytoplasm (6, 7). Phospholipase C activity generates IP3 which then binds to IP3 receptors on the egg endoplasmic reticulum to trigger release of Ca2+ from into the cytosol. The increased cytosolic Ca2+ serves as the key signal to the egg to begin the developmental program, triggering events such as exit from meiotic arrest, progression to embryonic interphase, establishment of blocks to prevent fertilization by additional sperm, and activation of transcription from the new genome created from the maternal and paternal DNA (4, 7, 8).

2.2. Assays of sperm-egg interactions

The term "sperm-egg fusion" has often been used historically in the fertilization field to refer collectively to the entire process of gamete membrane interactions that culminate in sperm incorporation into the egg. In this review, we will use the term "sperm-egg interactions" or "sperm-egg membrane interactions" to describe the process of the sperm interacting (binding and fusing) with the egg plasma membrane. The egg plasma membrane is also called the oolemma or the vitellus; here, for simplicity, we will call it the egg membrane. From a cell biological standpoint, distinctions between the steps of gamete plasma membrane interactions can be made: sperm binding to the egg membrane (or sperm-egg adhesion) that then leads to sperm-egg membrane fusion and formation of a single cell, the zygote, from the two gametes. Other fields have distinct terms for the events leading to membrane fusion, with the process beginning with the interaction between the membranes, progressing to the membranes coming in close proximity to each other, and finally leading to mixing of the lipid bilayers. For example, intracellular vesicles are said to dock or tether prior to fusion, myoblasts undergo a process of adhesion and alignment leading to fusion, and membrane-coated virus particles attach to host cells and then progress to fusion (9-11). All of these phenomena (docking, adhering, attaching, binding) are analogous processes of cell membranes making contact prior to fusion and could be mediated by a variety of adhesion mechanisms.

The union of sperm and egg is an intricate process, particularly considering the size and morphology of the cells involved. Elegant microscopic analyses reveal several nuances of the merger of sperm and egg. Initial contact can occur through the inner acrosome membrane, a region of the sperm head exposed after acrosome exocytosis (see Section 2.1). Subsequent interaction occurs through the equatorial segment and the posterior region of the sperm head; electron micrographs suggest that membrane fusion incorporates these posterior regions of the sperm head into the egg, whereas the anterior portion of the sperm head appears to be engulfed by the egg (2-4, 12, 13). Studies using sperm with labeled membranes or immunofluorescence of sperm surface antigens reveal that the sperm membrane is incorporated into the egg membrane (14, 15). The entire sperm is incorporated, with tail incorporation occurring several hours after the head has been incorporated (16-19). It is unclear if tail incorporation involves membrane fusion (as seems to occur with the posterior regions of the sperm head) and/or engulfment (as appears to occur with the anterior sperm head). Scanning electron micrographs show that sperm tail incorporation apparently occurs through multiple contact points to the egg membrane, and egg microvilli are closely associated with the sperm tail (17, 18). Treatment of eggs with microfilament-disrupting drugs cytochalasin B or cytochalasin D perturbs tail incorporation (18-20), suggesting that the egg's cortical actin cytoskeleton could be part of the process that brings the sperm tail into the egg.

The assays used to analyze sperm-egg interactions have been described in the previous version of this review (21); in the interest of space and emphasis on more recent topics, these assays will not be re-reviewed here. Sperm-egg fusion is the culmination of gamete interactions and is straightforward to identify, whereas the intermediate step, sperm-egg adhesion, is a more operational definition. An adherent cell is defined as one that stays attached to a substrate (e.g., another cell or an extracellular matrix protein) after a defined series of washes (22, 23). Sperm-egg binding analyses are done with ZP-free eggs, as it is impossible to wash away loosely attached sperm in the perivitelline space of ZP-intact eggs. Biological and experimental factors affect day-to-day results from these sorts of assays, but data from sperm-egg binding assays, with well-designed studies that allow interpretation with respect to intra-experiment controls, can provide insights into the mechanistic basis of forming a zygote from the two individual gametes. Analyses of gamete fusion provide additional insight, as the occurrence of fusion indicates that the sperm bound properly and successfully to the egg.

2.3. The impact of genetic approaches

The field of reproductive biology, including fertilization and sperm-egg interactions, has charged forward with an increasing number of mice engineered with knockouts of candidate genes involved in reproductive processes (i.e., reverse genetics; for a 2002 synopsis, see (24)), as well as with forward genetic approaches, such as screens of randomly mutagenized mice for infertile phenotypes (such as the NIH-sponsored ReproGenomics program based at the Jackson Laboratories (25); see also reprogenomics.jax.org). The mutagenesis projects bring the power of an unbiased approach to identify molecules involved in reproductive processes, as did the approach of the late 1970s and early 1980s of generating batteries of monoclonal antibodies to gamete surface antigens. This

antibody approach led to the identification of some sperm proteins such as ADAM2 and Izumo; both of these were first identified as antigens of monoclonal antibodies that inhibited the fertilization of ZP-free eggs (26, 27) (discussed more below, Sections 3.1.1 and 3.2.3).

Forward and reverse genetic approaches have set the stage for focus on an infertile phenotype. Certainly, this is the most practical endpoint for which to screen in a large mutagenesis project (versus screening for a more subtle phenotype, such as abnormal sperm motility). An infertile phenotype in a knockout or mutagenized mouse from classic multi-week mating trials has the power of demonstrating an essential role of a molecule in a process of interest, or, on the flip side, that a molecule is not essential and/or has complementary molecules that work in redundant pathways. With regard to redundancy, genetic model systems (yeast, *C. elegans*) are leading in efforts to understand this phenomenon, using screens to identify complex genetic interactions between nonessential genes that result in synthetic lethality – i.e., combinations of mutations that individually have no effect on viability but result in a lethal phenotype when combined (e.g., see ref. (28)). In the future, it may be possible to address experimentally the concept of "synthetic infertility" in mammals, as genetic redundancy is an issue affecting certain reproductive processes, such as ovulation (29) and aspects of fertilization.

With consideration of applications of basic research to human reproductive health, it should be emphasized that a genetic deficiency that does not have a dramatic *in vivo* effect on murine reproduction possibly could have a noticeable effect on fertility in humans, particularly in females. Considering that mice ovulate nearly eight times as frequently as female humans, and ovulate more than one egg at a time, a genetic deficiency that causes a modest reduction in litter size in mice might manifest itself very differently in humans, such as increased time for a woman to achieve a pregnancy. This is a significant consideration for human reproductive health, particularly in light of the fact that the clinical definition of infertility is failure to conceive after 12 months of unprotected intercourse.

Finally, it should also be noted gene knockouts may affect more than just one molecule, such as in the cases in which the knocked-out molecule works in concert with others. One example of this is the knockout of each of the three ZP components, ZP1, ZP2, or ZP3. Developing oocytes from each of these knockouts have abnormal zonae pellucidae (varying in severity), indicating that that deletion of one ZP component affects the ability the remaining two to assemble the ZP matrix (30-32). Examples pertinent to sperm-egg interactions are the ADAM knockouts; deletion of one ADAM affects the surface expression of a subset of other ADAMs (see Section 3.2.3). There also have been fortuitous and unanticipated discoveries, such as studies of knockouts of ZP1, ZP2 or ZP3 combined with transgenic rescue with the human orthologs demonstrating that the structure of the zona pellucida mediates successful sperm-ZP interaction, rather than a specific peptide or carbohydrate moiety on an individual ZP component (33). The knockouts of ADAM proteins in sperm have produced similar interesting findings; with data suggestive of roles for these proteins in multiple processes that affect sperm function (see Section 3.2.3).

3. THE MOLECULAR BASIS OF MAMMALIAN SPERM-EGG INTERACTIONS

The considerations above frame how we will discuss the molecules proposed to be involved in sperm-egg interactions. The first subsection here will focus on molecules that appear to be essential or nearly essential for sperm-egg interaction, based on detailed analyses of knockout phenotypes as well as complementary data (e.g., antibody studies). The next subsection will address molecules that may also participate in gamete membrane interactions, but the knockouts either do not lead to a fertility-impairing loss of gamete membrane interaction function or there are added complexities in the phenotype, or no knockout data are yet available.

3.1. Major players: Molecules that are crucial for sperm-egg interaction

3.1.1. Izumo on sperm

The sperm protein that has come to be known as Izumo has a fairly long history in fertilization research. This sperm protein was first was characterized through studies screening a battery of anti-sperm monoclonal antibodies for the ability to disrupt a process associated with fertilization. The monoclonal antibody OBF13 interacted with a testis-specific antigen, labeled the head of capacitated sperm, and inhibited sperm-egg binding in IVF assays with ZP-free eggs (27, 34). But it was almost two decades later when the antigen for OBF13 was characterized (35). Liquid chromatography tandem mass spectrometry of the OBF13-immunoreactive species from mouse sperm lysates identified ten peptide sequences, which were used to identify the cDNA sequence. The gene for the OBF13 antigen, now named Izumo (pronounced IZ-mo, for a Japanese shrine to marriage), encodes a novel member of the large family of immunoglobulin superfamily (IgSF) proteins. Mouse Izumo contains one immunoglobulin-like domain with a putative N-glycoside link motif (35). A polyclonal antibody to recombinant mouse Izumo identifies a 56.4 kD protein in testis and sperm lysates but not in other tissues, in agreement with the data with the OBF13 antibody. Interestingly, Izumo appears to be exposed on the sperm surface after acrosome exocytosis (see Section 2.1). Izumo knockout mice are healthy; females appear completely normal, while *Izumo*-/- males are infertile, despite the fact these males have normal mating behavior and ejaculation, and sperm motility and migration into the oviduct are also normal. *In vitro* assays with *Izumo*-deficient sperm showed that the sperm can penetrate the ZP, but they accumulate in the perivitelline space, suggestive of defective interaction with the egg plasma membrane. Sperm from *Izumo*-/- males are able to bind ZP-free eggs, and eggs fertilized by intracytoplasmic sperm injection (ICSI) with *Izumo*-/- sperm develop into normal embryos that implant and develop to term. These results strongly suggest that Izumo is essential for sperm-egg fusion.

There appears to be a human ortholog/homolog of mouse Izumo. Antibodies to the putative human Izumo cross-react with a 37.2 kD protein in human sperm lysates, and these anti-human Izumo antibodies inhibit fusion (but not binding) of human sperm to ZP-free hamster eggs (35). Interestingly, the OBF13 monoclonal antibody has no effect on the fusion of mouse sperm with human eggs; one possible explanation of this result is that the OBF13 epitope is mouse-specific. In addition, *Izumo*-/- mouse sperm bind but do not fuse with ZP-free hamster eggs (whereas wild type mouse sperm do fuse with ZP-free hamster eggs, see Section 3.3 in the previous version of this review (21)).

3.1.2. CD9 on eggs

CD9 is a membrane protein that made a surprise appearance in the fertilization field in 2000 with the discovery that the severe subfertility of the CD9 knockout female mice was due to defective sperm-egg interactions (36-38), complementing work that showed that anti-CD9 antibodies had inhibitory effects in mouse IVF assays (39). CD9 is a membrane protein that is expressed in many cell types in the body, but, interestingly, the knockout only affects eggs. Mating trials show that 60% of *CD9*-/- females became pregnant, but achieving these pregnancies took nearly four times longer than it did for control mice, and the *CD9*-/- females had litters that were ~25% the size of litters for control females (40). IVF assays show that sperm binding to *CD9*-/- eggs appears to be unaffected, but sperm rarely fuse; between the three original knockout reports, only three of 246 *CD9*-/- ZP-free eggs were fertilized (36-38). When ZP-intact *CD9*-/- eggs were inseminated, sperm accumulate in perivitelline space. *CD9*-/- eggs can be fertilized and undergo normal embryonic development when sperm-egg membrane interactions are bypassed by ICSI (36, 38), demonstrating that the *CD9* deletion does not affect the egg's ability to progress into embryogenesis.

CD9 is a tetraspanin, a family of membrane proteins that plays roles in cell adhesion, cell motility and tumor cell metastasis (41, 42). Tetraspanins are small proteins (200-350 amino acids) and are so named because they have four transmembrane domains. On the extracellular face, these proteins have two loops, one large (69-132 amino acids) and one small (13-31 amino acids) (42, 43). Tetraspanins interact with other molecules in the plasma membrane, including other tetraspanins, integrins, growth factor receptors, IgSF proteins, complement regulatory proteins, and proteoglycans. Tetraspanins are thought to act as plasma membrane "molecular facilitators" that regulate plasma membrane functions through these *cis* interactions creating networks of proteins in the plane of the membrane, called "tetraspanin webs."

The exact mechanism(s) of CD9 participation in gamete membrane interactions remains unclear. Based on what is known about tetraspanins, it is likely that CD9 regulates the "fusibility" of the egg membrane, rather than functioning as a fusion-mediating protein (a fusogen). The large extracellular loop of CD9 is implicated in its function in fertilization, based on studies in which a mutated version of mouse CD9 was expressed in *CD9*-/- eggs; this work identified a tripeptide sequence, SFQ in positions 173-175, as required for CD9 function in sperm-egg fusion (44). In addition to this possible role for CD9 in regulating the ability of the mouse egg membrane to undergo fusion with the sperm, CD9 or a CD9-containing complex may also be involved in certain aspects of the egg supporting sperm binding, as sperm-egg binding is reduced with eggs incubated with anti-CD9 antibodies or with recombinant large extracellular loop (39, 45). Anti-CD9 antibodies also inhibit the binding of recombinant forms of the sperm proteins ADAM2 and ADAM3 (see Section 3.2.3) to eggs when these sperm proteins are immobilized on beads (46, 47), while anti-CD9 antibodies have no effect on the binding of soluble, monovalent ADAM2 to mouse eggs (47). Based on this result, it has been speculated that that CD9 may function in adhesion strengthening of the sperm's interaction with the egg membrane, following initial binding and leading to membrane fusion. Interestingly, another tetraspanin, CD151, has been shown to function in adhesion strengthening of cell interactions with laminin (48).

Based on the data summarized above, the primary model for how CD9 functions in the egg is through *cis* interactions with other egg proteins, regulating egg membrane order in some way and thus the ability of the membrane to support sperm binding and/or fusion. However, an additional model is that CD9 binds a ligand on sperm (and it is certainly possible that CD9 could function both as a membrane organizer and as a receptor). This model of CD9 interacting with a ligand on sperm is based on the finding that the large extracellular loop of CD9 interacts *in vitro* with pregnancy-specific glycoprotein PSG17, and this interaction is dependent on the same SFQ tripeptide that is required for sperm-egg fusion (49). PSG17 is a member of carcinoembryonic antigen (CEA) subfamily of the IgSFs, but PSG17 has not been detected on sperm and thus is less relevant to fertilization. But Izumo is an IgSF member, like PSG17, and so this has led to the speculation that CD9 functions in fertilization by interacting with Izumo and/or other sperm IgSF proteins. There are no data as yet that support this hypothesis, but it is interesting to note that there are potential similarities to CD81, another tetraspanin. CD81 interacts with the E2 envelope protein of Hepatitis C virus, and this interaction is dependent on a phenylalanine residue in the large extracellular loop of human CD81 (50). Finally, as will be addressed below, CD9 in mouse eggs appears to function with at least one other tetraspanin, and eggs of other species may utilize different tetraspanins to regulate egg membrane function (Section 3.2.1).

3.2. Additional players to consider: Other molecules that could participate in sperm-egg interaction

The section above highlighted the molecules that are strongly implicated in murine sperm-egg interaction based on knockout mice being infertile (Izumo) or having severely reduced fertility (CD9). This subsection will address molecules that may participate in gamete membrane interactions (some of which were highlighted the 1999 version of this review), but the knockout does not lead to a loss of gamete membrane function that reduces

fertility, the knockout has added complexities in the phenotype, or no knockout data are yet available.

3.2.1. Other tetraspanins on eggs

Mouse eggs express not only CD9 but also at least one other tetraspanin, CD81. *CD81*-/- female mice have reduced fertility due to an apparent sperm-egg fusion defect, but this phenotype is not as severe as in *CD9*-/- mice (40). In mating trials, only 60% of *CD81*-/- females became pregnant, whereas 100% of wild-type controls did. In those *CD81*-/- females that became pregnant, times to pregnancy and litter sizes were similar to those for wild-type females (40). In the same study, ~60% of *CD9*-/- females became pregnant, similar to the *CD81*-/- females; however, achieving these pregnancies *CD9*-/- females took longer and litter sizes were smaller than in control females or *CD81*-/- females (40). An IVF experiment showed that only 1/34 eggs from *CD81*-/- females was fertilized, whereas 41/45 wild-type eggs were fertilized (40). IVF assays have also been performed with eggs treated with anti-CD81 antibodies or with recombinant CD81 large extracellular loop. The recombinant protein and some anti-CD81 antibodies are reported to inhibit sperm-egg binding and fusion to a moderate extent (45, 46), although one antibody study showed no inhibitory effect (40).

Mouse egg CD9 is likely to function in conjunction with CD81. CD9/CD81 double knockout female mice are completely infertile, suggesting that CD9 and CD81 play complementary roles in fertilization (40). Overexpression of CD81 in *CD9*-/- eggs partially rescues the *CD9*-/- fusion-defective phenotype (51); this may be due to the fact CD9 and CD81 are not completely functionally redundant in eggs, or that an appropriate level of CD81 expression is crucial.

Interestingly, human eggs may rely on different tetraspanins than do mouse eggs. Human sperm-egg fusion is partially inhibited by treating eggs with an antibody to another tetraspanin CD151 (52), whereas *CD151*-/- mice do not show any defects in reproductive capacity (53). In addition, while anti-CD9 antibodies inhibit sperm binding and fusion to mouse or pig ZP-free eggs (39, 54), two different anti-CD9 antibodies had no effect on the fusion of human sperm with human ZP-free eggs (52).

3.2.2. GPI-anchored proteins on eggs

In addition to egg tetraspanins, GPI-anchored proteins on the egg plasma membrane have been implicated in sperm-egg interactions by knockout studies. GPI-anchored proteins are attached to cell surface lipid bilayer by a covalent bond of the GPI moiety of the protein. Female mice with an oocyte-specific knockout of PIG-A, a subunit of an N-acetyl glucosaminyl transferase that participates in first steps of GPI synthesis (55), are infertile (56). Mating studies show that sperm accumulated in the perivitelline space of eggs, and sperm binding and fusion to ZP-free PIG-A-deficient eggs was significantly reduced (56). In agreement with these results, treatment of mouse eggs with PI-PLC (an enzyme that cleaves GPI-anchored proteins) significantly reduces sperm-egg binding and fusion (57). Based on these data, GPI-anchored proteins on the egg are essential for fertilization. What remains unknown is which egg GPI-anchored protein(s) are crucial and what role(s) egg GPI-anchored proteins could be playing in fertilization. A specific GPI-anchored protein may interact with a ligand on the sperm. It is also possible that the effect of the PIG-A deficiency is more general, that the absence of GPI-anchored proteins in the membrane alters the composition and/or organization of the plasma membrane so that sperm interactions are not favored. GPI-anchored proteins are enriched in lipid microdomains (rafts), raising the possibility that the microdomain structure of the egg plasma membrane could be perturbed in the absence of GPI-anchored proteins. These different explanations are underscored by the fact that GPI-anchored proteins are involved in diverse cellular processes, including signal transduction, membrane trafficking and cell adhesion (58, 59). Thus, the role of GPI-anchored proteins in fertilization needs to be further defined.

3.2.3. ADAM (A Disintegrin and A Metalloprotease) proteins on sperm

The sperm protein ADAM2 (fertilin beta), one of the founding members of the ADAM protein family, was identified by a fertilization-blocking antibody, PH-30, in a research approach very similar to the methods that led to the identification of Izumo. Since those PH-30 antibody studies and work in other systems that identified additional ADAMs, the family of ADAM proteins has expanded to nearly 40 members, several of which are expressed exclusively in the testes, and in some cases, specifically in the male germ cells. The earlier version of this review (21) provides a discussion of the earlier studies of sperm ADAMs, and the model that the disintegrin (integrin ligand-like) domain of sperm could interact with an egg integrin. Here we will focus on more recent work. Four of the ADAMs expressed in male germ cells (ADAM1a, ADAM1b, ADAM2, and ADAM3) have been knocked out, and examination of the phenotypic characteristics of the *ADAM* knockouts (summarized in Table 1, compiled from (60-65)) has produced two intriguing overall findings. First, some (but not all) of these knockouts led to a male infertile phenotype, but the male infertility was due to more than just a defect in sperm-egg interactions. Multiple sperm functions are impaired in these knockouts: reduced migration of sperm to the oviduct through the uterotubal junction (UTJ), reduced binding to the ZP, and/or reduced binding or fusion to the egg plasma membrane. Second and, quite unexpectedly, ADAMs were found to have an inter-dependent post-translational mechanism of regulating surface expression, so that the surface expression of one or more ADAM proteins on sperm appears to be affected by the gene disruption and the lack of expression of one ADAM.

Our discussion here will focus on the data related to sperm-egg membrane interactions. (Other aspects of the phenotypes have been nicely addressed in other reviews as well as in the discussions of relevant papers (64, 66-69).) One interpretation of these data on sperm-egg binding and fusion is that no single ADAM is essential, although there appears a correlation between the ability of sperm to bind and fuse with the egg membrane and the levels of ADAM

Table 1. Summary of phenotypic characteristics of ADAM and related knockout mice

	Knockout			
Phenotype	*ADAM1a*$^{-/-}$	*ADAM1b*$^{-/-}$	*ADAM2*$^{-/-}$	*ADAM3*$^{-/-}$
Male infertile in mating trials?	Yes	No	Yes	Yes
Sperm function				
UTJ migration	Reduced	Normal	Reduced	Normal
ZP binding	Significantly reduced	Normal	Significantly reduced	Significantly reduced
PM binding	no data	no data	Significantly reduced	Significantly reduced
PM fusion	Normal	Normal	Reduced	Normal
ADAM surface expression				
ADAM1b	Normal	(KO - none)	Significantly reduced	Slightly reduced
ADAM2	Slightly reduced	Very low	(KO - none)	Slightly reduced
ADAM3	Significantly reduced	Normal	Significantly reduced	(KO - none)
ADAM5	no data	no data	Significantly reduced	Reduced
ADAM27	no data	no data	Normal	Normal
ADAM32	no data	no data	Normal	Normal
ADAM7	no data	no data	Reduced	Reduced
Fertilin complex formation				
ADAM1a/2	None	Normal	None	Normal
ADAM1b/2	Normal	None	None	Normal

Data summarized from (60-65). Normal: levels similar to controls levels (~90-100% of controls), Slightly reduced: levels in the range of ~70-90% of control levels, Reduced: levels in the range of ~25-60% of control levels, Significantly reduced: levels detectable but very low, in the range of ~1-20% of control levels.

proteins. The *ADAM2*-/- knockout has the most serious defects in gamete membrane interactions and has the lowest overall levels of several ADAM proteins on the sperm surface. On the other hand, *ADAM1a*-/- and *ADAM1b*-/- sperm can fuse with the egg membrane; *ADAM1a*-/- sperm have very low ADAM3 (also known as cyritestin), but have near-normal levels of ADAM2, while *ADAM1b*-/- sperm have very low levels of ADAM2 with near normal levels of ADAM3. Clearly, none of these four ADAMs is essential for the sperm to be able to fuse with the egg membrane, but the data *en toto* suggest that ADAMs could function in redundant roles; this would be in agreement with structure-function data that suggest that certain ADAMs have similar adhesion-mediating functional domains (46, 70-72). Admittedly, there are some remaining issues, such as conflicting data on the localization of ADAM3 on sperm (73, 74). Furthermore, the *ADAM2*-/- loss-of-function in sperm-egg binding and fusion is not as dramatic as the loss of function in *Izumo*-/- knockout, suggesting that Izumo ranks higher in the hierarchy of crucial sperm proteins than the ADAMs. In sum, ADAMs are candidates to consider in sperm-egg interactions, although there are clearly additional sperm proteins of significance. ADAMs also are intriguing for reasons beyond gamete interactions, based on the apparent defects in ADAM knockouts in protein trafficking during spermatogenesis.

One additional update should be made on ADAM1, also known as fertilin alpha. ADAM1 was once considered a potential sperm ADAM to participate in gamete interactions, but new data argue against this function, at least in the mouse. The mouse has been found to have two genes that encode two ADAM1 isoforms, ADAM1a and ADAM1b (62). Both proteins are expressed in male germ cells, but only ADAM1b is localized on the surface of epididymal sperm (75). Functional studies used ADAM1a, as these were performed before ADAM1b was discovered. Recombinant ADAM1a supports cell adhesion and interacts with several integrins (72), but the function of ADAM1a in sperm cannot be sperm-egg adhesion since ADAM1a is not expressed on the sperm surface (75). Instead, ADAM1a may have a novel function in developing sperm. Based on the *ADAM1a*-/- phenotype, the appearance of ADAM3 on the sperm surface may depend on the formation of ADAM1a/ADAM2 complex in the endoplasmic reticulum of male germ cells. ADAM1b is not essential for fertilization, but the presence of the ADAM1b and ADAM2 on the sperm surface is correlated with their heterodimerization in male germ cells (64). Taken together, the data on ADAM1 suggest that it may participate in the transport of proteins to the sperm surface. ADAM1 genes have not been studied extensively in other species, although the macaque appears to have two ADAM1 isoforms (76). The *ADAM1* genes in human and gorilla (although not other primates) are non-functional, with several insertions, deletions, and termination codons (77, 78). Some form of ADAM1 forms a heterodimer with ADAM2 on the surface of guinea pig and bovine sperm (26, 79, 80).

3.2.4. Integrins on eggs

Integrins are a well-characterized family of cell adhesion molecules, and the identification of an integrin ligand-like (disintegrin) domain in ADAM2 (81) led to the hypothesis that a sperm ADAM(s) binds to an integrin(s) on the egg. Recent knockout studies, complementing the prior antibody studies, have raised a number of questions about this model for sperm-egg interactions. Integrins are heterodimeric membrane proteins, made up of an alpha and a beta subunit. There are 18 alpha subunits and eight beta subunits that combine to make 24 different combinations. Several integrin subunits have been detected in mammalian eggs (mouse, human, pig) at the mRNA or protein level (alpha2, alpha3, alpha4, alpha5, alpha6, alphav, alpha9, alphaM, beta1, beta2, beta3 and beta5 (see (82) for references). For the integrin subunits that are expressed in eggs and for which the mouse knockouts do not have an embryonic/perinatal lethal phenotype (alpha2, alphaM, beta2, beta3, beta5), there have been no reports of reproductive deficiencies (83). Other integrin subunits of interest (alpha3, alpha4, alpha5, alpha6, alphav, alpha9, beta1) have knockouts that are embryonic/perinatal lethal,

and thus other approaches must be taken to assess the effect of the gene deletion in eggs, either a cell-specific conditional knockout (with transgenic mouse expressing Cre recombinase specifically in oocytes) or dissection of the ovaries from knockout newborns and then implantation of the ovaries in the kidney capsule, to allow the oocytes to develop. These approaches have been taken to assess the effects of deletion of alpha6, alpha3, and beta1 in eggs, and none of these integrin subunits were found to be essential for fertilization (84, 85).

On the other hand, there are several studies in multiple species showing reduced sperm-egg interactions when eggs were treated with anti-integrin subunit antibodies or with integrin-blocking peptides (47, 52, 85-93). The effects observed in these studies vary, depending on the antibody and on the IVF conditions. Several anti-integrin antibodies have partial to significant inhibition of sperm-egg interactions, but complete ablation of sperm-egg binding and/or fusion was not observed in any of these studies. The results with integrin-blocking reagents (antibodies or peptides) could be due to incomplete or insufficient blocking of the target integrin by the antibody or peptide, and/or the ability of sperm-egg interactions to by-pass the targeted integrin, either by an integrin ligand on sperm (e.g., ADAMs) utilizing a different egg binding partner (integrin or non-integrin) or through a different ligand on sperm (such as Izumo; Section 3.1.1). In contrast, anti-CD9 antibody studies and analysis of the *CD9*-/- phenotype reveal a near complete requirement of CD9 for sperm-egg fusion (see Section 3.1.2 above), although the CD9/CD81 double knockout has even more dramatic effects on fertility (see Section 3.2.1 above), raising the question of whether more dramatic effects could be observed with the disruption of multiple integrins. Such studies are admittedly more complicated with integrins, as this is a large molecular family and thus far there is no "dominant" integrin subunit, thus differing from how CD9 appears to be the "dominant" tetraspanin in mouse eggs.

A new finding with regard to egg integrins is that different species' eggs may rely on different receptors to varying extents. For example, an anti-alpha6 function-blocking monoclonal antibody (GoH3) has been reported to have some (87) or no (84, 90) effect on mouse sperm-egg interactions (although this antibody does bind to alpha6 on the mouse egg membrane). However, this same antibody has a modest (93) to significant (52) inhibitory effect on sperm interactions with human ZP-free eggs. The basis of this, as well as the role of integrins in fertilization in general, awaits further analysis, but these potential species differences should be kept in mind with integrins, tetraspanins (Section 3.2.1) and possibly others to be identified.

3.2.5. CRISPs on sperm

The protein D/E gained attention as a candidate to be involved in gamete interactions based on the findings that anti-D/E antibodies inhibit sperm-egg fusion (94, 95) and isolated D/E binds to the egg membrane and blocks fertilization (96, 97). Rat D/E, which is secreted from the epididymal epithelium and associates with the sperm surface, actually is two proteins, D and E. These are products of the same gene, *Cysteine-rich Secretory Protein* (*Crisp) 1*, with the D and E variants having some antigenic differences (98, 99). Recent work has shed new light on the CRISP family, members of which have also been known as Acidic Epididymal Glycoprotein (AEG) in the rat or AEG-related protein (or *AEG-like-1*, for the gene) in the human. The human AEG-related protein is known as CRISP1, but its orthologs in mouse and rat are CRISP4. Anti-human CRISP1 antibodies (made to recombinant protein) inhibit human sperm fusion with ZP-free hamster eggs, and recombinant human CRISP1 protein binds to human eggs (100). The functional data on the involvement of CRISP(s) in rat and mouse fertilization include a study using recombinant CRISP1 (101), studies using antibodies made in rabbits immunized with minced gel pieces containing Proteins D and E (94, 102, 103), and studies using isolated epididymal proteins (96, 97). The isolated proteins were prepared from epididymal cytosol by a three-step purification procedure that results in an enrichment of proteins that migrate with proteins D and E (102). With the insights that have come from recent studies of the CRISPs, it seems possible that these protein preparations could be either pure CRISP1 or may be combinations of CRISPs. For example, CRISP4 in the rat and mouse is expressed by the epididymis, with CRISP1 and CRISP4 in the rat epididymis having very similar regional distribution (104). Thus, it is possible that CRISP4 co-purified with CRISP1 and perhaps other epididymal proteins in the preparations used in IVF studies or as immunogens for antibodies. Like CRISP1, CRISP4 associates with rat sperm (104) and thus CRISP4 could be a candidate to participate in rat and mouse sperm-egg interactions, as is CRISP1. Peptides corresponding to a region of CRISP1 that is conserved in other CRISPs (FYVCHYCPGGNY) have an inhibitory effect on the interaction of rat sperm with ZP-free eggs (105). Whether one or more of the CRISPs are essential for fertilization remains to be fully determined.

3.2.6. Candidates from proteomic approaches: SLLP1, SAMP14, and SAMP32 on sperm

Recent "discovery-driven" proteomic approaches have identified some candidates on sperm; examples of these are Sperm Acrosomal Membrane-Associated (SAMP) 14, SAMP32, and Sperm Lysosomal-Like Protein (SLLP) 1. Each of these was identified in human sperm, and appears to be testis-specific. As the names indicate, SAMP14 and SAMP32 are associated with the sperm acrosome, both remaining on the inner acrosomal membrane after the acrosome reaction. SLLP1 also is associated with the acrosome, being present in the acrosomal matrix (106). Antibodies to recombinant forms of each of these proteins reduce the binding and fusion of human sperm to ZP-free hamster eggs (106-108). (See the earlier version of this review (21) for information on use of heterologous IVF experimental systems.) SLLP1 has also been characterized in the mouse as well, with either anti-SLLP1 antibodies or recombinant SLLP1 in the IVF medium having inhibitory effects on fertilization, with the recombinant protein having particularly robust effects (109).

Each is a novel protein. SLLP1 has homology to conventional lysozymes; these are able to bind oligosaccharides such as N-acetylglucosamine, and thus SLLP1 could potentially function as a binding site for N-acetylglucosamine on the egg membrane, in the ZP, and/or the cumulus layer (106). SAMP14 is a GPI-anchored protein, based on sequence predictions and sensitivity to cleavage by phosphatidylinositol phospholipase C. SAMP14 also has homology to the urokinase plasminogen activator receptor superfamily (108); these proteins that can participate in cell interactions or in the localization proteolytic activity by binding the protease urokinase plasminogen activator. SAMP32 has a domain with homology to a surface antigen expressed at the infectious stage of the lifecycle of a malaria-causing parasite, and another domain with homology to a family of membrane-bound peptidases in fission yeast (107). The roles that these proteins could play in fertilization have yet to be clearly defined.

3.2.7. Enzyme activities implicated in sperm-egg interactions

The use of inhibitors to various enzymes in IVF assays has revealed potential roles for certain enzymatic activities in sperm-egg fusion. Zinc metalloprotease activity has been implicated by the finding that mouse sperm-egg fusion is reduced in the presence of various metalloprotease inhibitors (110). Reagents that disrupt the action of enzymes that mediate thiol-disulfide exchange in proteins (protein disulfide isomerases, PDIs) also reduce the incidence of sperm-egg fusion (111, 112). The most significant effects of these metalloprotease and PDI inhibitors are observed when the inhibitor is present during the insemination, rather than when the gametes were pretreated; this suggests that the enzymes act during the process of gamete interaction. The lack of an effect of these inhibitors on sperm-egg binding suggests that the enzymes are linked with gamete fusion. The results with N-ethyl-maleimide (NEM) and 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB) (111, 112) suggest that sulfhydryl groups may be a common element important for fusion systems. The NEM sensitivity of a cytosolic factor required for vesicle transport and fusion proved to be a critical tool for the identification of NSF (NEM-sensitive factor) which is involved in vesicle fusion (113), and PDI activity and remodeling of extracellular disulfide bonds play a role in certain viral fusion events (114, 115).

3.2.8. Antigens implicated in sperm-egg interactions by fertilization-blocking antibodies

There are a number of antibodies like OBF13 (the antibody that identified Izumo, Section 3.1.1) that perturb sperm-egg binding and/or fusion, but the antigens of these antibodies remain unidentified or only partially characterized (116-122). One of potential interest is sperad, an IgSF member like Izumo (Section 3.1.1). A monoclonal antibody that recognizes this protein inhibits guinea pig sperm fusion with ZP-free hamster eggs (118). However, conflicting data on the localization (suggesting either exposure after the acrosome reaction (118, 123), or loss after the acrosome reaction (124)) make sperad's putative role in sperm-egg interaction unresolved. There are possible explanations for the conflicting data (such as the epitope of the fertilization-blocking monoclonal antibody is a form of the protein that migrates to the equatorial region during or after acrosome reaction) but further analysis is required to determine if sperad plays a role in sperm-egg interactions.

4. SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Eight years have brought incredible advances in this field. In the 1999 version of this review (21), one of the most important egg molecules in sperm-egg interaction, CD9, had not yet been characterized as having a role in fertilization. Izumo was merely the mystery antigen of an anti-sperm antibody that inhibited gamete membrane interactions (27), and was only briefly noted in a section addressing the fertilization-blocking antibodies collectively. With these advances and others, there are many exciting directions and applications for future work in this field.

A diagram illustrating what we know about gamete membrane interactions is shown in Figure 1; obviously there are still a significant number of unknowns here. Defining functional roles more precisely and placing molecules in context with each other will be the central process of building a model for how sperm-egg membrane interactions occur. For many candidate molecules, defining the exact roles that the molecules play will be the next important step -- which molecules are the adhesion molecules (involved in binding), which are the fusogens (i.e., molecules that participate in the actual process of membrane fusion rather than membrane attachment/adhesion processes leading up to fusion), and what supporting roles are crucial. As noted above, CD9 and CD81 may regulate mouse egg membrane order and/or membrane protein function, but other tetraspanins may be important for similar functions in other species' eggs. Izumo on sperm likely mediates fusion or close membrane apposition. ADAMs on sperm may interact with integrins on eggs, although knockouts show that individual ADAMs or integrin subunits are not essential, suggesting that there are redundant family members and/or unrelated molecules (e.g., CRISPs on sperm) that function in redundant ways.

Elucidation of these issues likely will require data from a variety of approaches, including examining molecules in context (i.e., in the gametes) and out of this context. Adhesion molecules can be studied using assays that define specific adhesion functions (e.g., early attachment or adhesion strengthening). The characterization of would-be fusogens can use structural analyses and assays of liposome fusion, as has been done with sp18, an abalone sperm protein that is thought to mediate sperm-egg fusion in this mollusk species (125, 126). Another approach is to express the putative fusogen in cells that normally would not fuse and demonstrate that expression of this protein renders the cells capable of fusion. An elegant example is a study of epithelial cell fusion in *C. elegans*. In *eff-1* (epithelial fusion failure) mutant worms, epidermal cells are unable to undergo

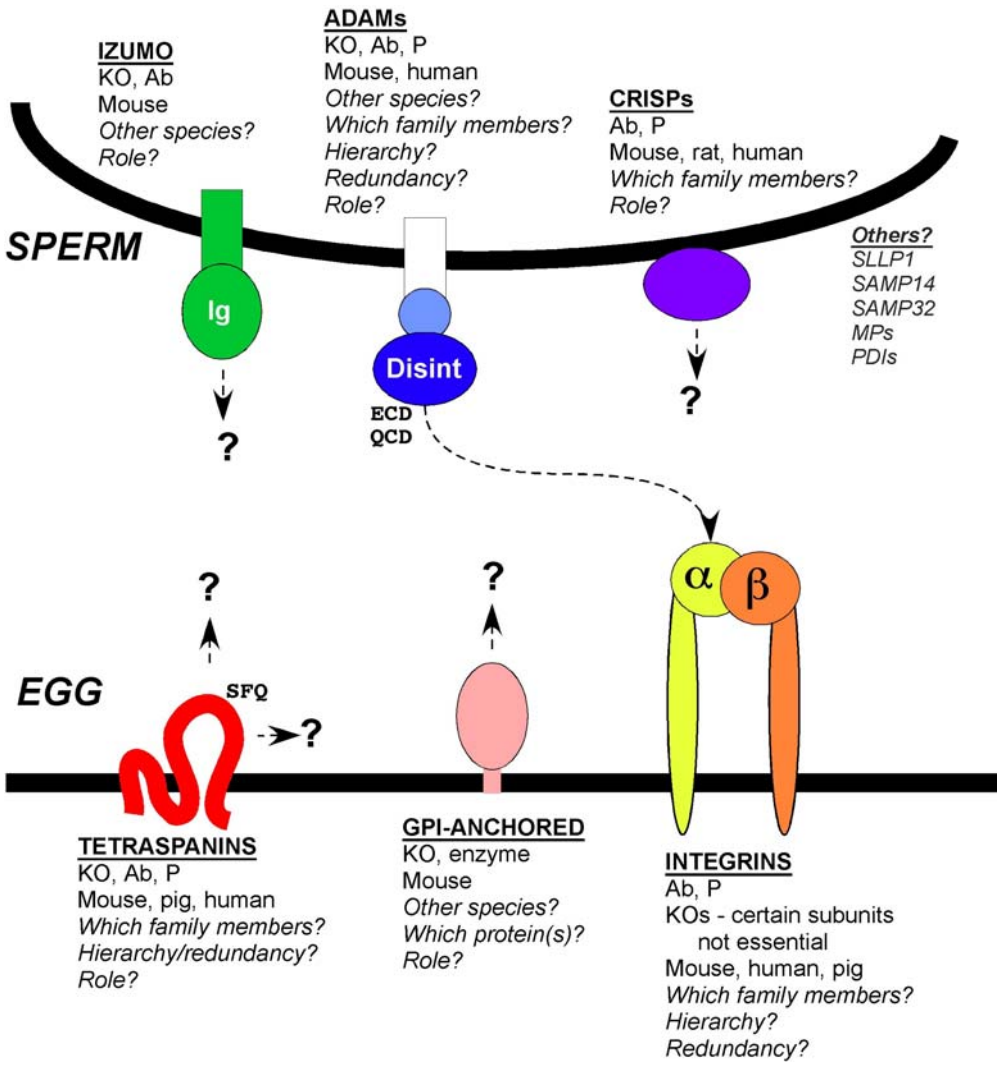

Figure 1. This diagram illustrates the molecules proposed to participate in sperm-egg membrane interactions and summarizes our knowledge base, what is known and what remains to be determined on how these molecules might function. The first line of the text under each molecule's heading indicates the data that provide evidence for the role of the indicated molecule or molecular family (KO, mouse knockout; Ab, antibody perturbation; P, protein or peptide perturbation); for GPI-anchored proteins on the egg, "enzyme" refers to evidence from studies using treatment of eggs with PLC ((57); Section 3.2.2). The next line of text indicates the species in which the molecule or molecular family has been implicated to function in fertilization (i.e., not just expression in an indicated species). For the molecules on which structure-function analysis has been performed (tetraspanins, ADAMs), the key functional amino acids that have been identified are shown in Courier font in the diagram (SFQ in mouse CD9 (44), ECD in mouse ADAM2 (70, 71), QCD in mouse ADAM3 (46)). A dotted line shows the proposed molecular interactions between ADAMs and integrins. Uncharacterized interactions are indicated with a question mark, including whether tetraspanins interact in *cis* with other egg proteins and/or with sperm proteins in *trans*. The issues of redundancy and hierarchies (i.e., multiple members of a molecular family participating, with certain family members being dominant) are addressed in the text (Sections 3.2.1, 3.2.3, 3.2.4). Other abbreviations: Ig (on Izumo), Immunoglobulin-like domain; Disint (on ADAM), disintegrin domain.

normal cell-cell fusion, while ectopic expression of EFF-1 in worms was sufficient to promote fusion between cells that normally do not fuse (127-129). On the other hand, data such as these must be interpreted with care as such approaches can identify fusogens and also molecules that are cofactors for fusion but not necessarily *bona fide* fusogens. An example of this is work in which expression of a human cDNA in cells that were not able to allow HIV-1 entry (mouse cells expressing CD4) rendered these cells capable of being infected by HIV-1 and capable of fusing with cells expressing the HIV-1 envelope glycoprotein (130). The product of this cDNA was first dubbed "fusin" but was then characterized to be a chemokine receptor that functions as a coreceptor to support fusion (131). In a similar fashion, IVF assays of Izumo and CRISP1 function indicate the perturbation/absence of either of these proteins

affects sperm-egg fusion but not binding, but these proteins could prove to be cofactors that facilitate fusion or to be involved in membrane attachment leading to fusion. This latter role has been proposed in recent reviews discussing these sperm proteins (132, 133). Finally, for antibodies that have been shown to inhibit sperm fusion with hamster eggs (e.g., antibodies against SAMP14, SAMP32, sperad), it will be important to demonstrate that these antibodies have an inhibitory effect when homologous gametes are used. There is a precedent for an antibody inhibiting fusion with hamster eggs but not eggs of other species (134) and thus it is possible that such antibodies could identify molecules that are relevant for tests with ZP-free hamster eggs, but not fertilization in general.

Discussions of the appropriateness of different experimental approaches lead to the bigger question of what is the best assay or experimental endpoint. Knockout mouse studies clearly are extremely useful and informative. However, a challenge with mouse knockouts is that discovery of infertile phenotype requires that the mouse be viable and that the molecule of interest is expressed exclusively in the gamete, as is the case with Izumo, or that a molecule affects gamete fusion and is not essential for other critical processes in the body, as was the case with CD9. The CD9 knockout phenotype was remarkable in this sense, as CD9 is widely expressed in the body and yet has thus far only been identified to have an essential function in the egg. In other cases in which the molecule of interest is expressed in somatic cells, gene deletion studies could require performing a gamete-specific disruption.

The question of whether a knockout mouse must have an infertile phenotype to be interesting is actively debated. Clearly, infertility is one of the most exciting phenotypes, but infertility is not the only phenotype that is indicative of a molecule's function in a reproductive process. Other assays, such as limited duration mating trials or IVF assays, may reveal phenotypes that could not be detected in the context of *in vivo* fertilization occurring in traditional multi-week mating trials. There may be relatively few knockouts that affect reproduction and only reproduction, necessitating the use of conditional knockouts. There is probably considerable redundancy in reproductive processes, requiring consideration of "synthetic infertility" interactions between redundant, nonessential genes (Section 2.3).

A simple but important point to make here is that knockouts thus far are performed in mice but not other species. Since some recent data suggest that different species' eggs may rely on different molecules to varying extents (Sections 3.2.1 and 3.2.4), this issue could be important to consider for future candidate molecules as well. It is certainly possible that a certain knockout mouse could be fertile but the molecule could still be important in fertilization in another species. For studies in species besides the mouse, alternatives to genetic approaches will continue to be a fundamental part of research, and insights into the function of a molecule or molecular family can come from a variety of experimental approaches: antagonist treatment (antibody, peptide, recombinant protein, etc.), a gene deletion, or a loss-of-function mutation discovered in a patient or in an animal model.

All of the issues above become increasingly worthy of consideration when one makes the leap from basic science to translational research that could impact human reproductive health, reproduction of economically important animal species, or population control of pest animal species. A genetic deficiency that causes a mild reproductive phenotype in mice (e.g., modest reduction in litter size) might very well manifest itself in humans as couples facing 12+ months of trying to conceive with no success (Section 2.3). The identification of a potent antagonist of gamete function or interaction could be the foundation for contraceptive development. With regard to the assisted reproductive procedure ICSI, there possibly are issues with the egg-to-embryo transition when injecting a sperm rather than the sperm binding and fusing in the normal fashion, as the key signal of egg activation, Ca2+ oscillations, differs in embryos fertilized by ICSI as compared to embryos fertilized by conventional IVF, and alterations in Ca2+ oscillations can affect gene expression and developmental potential (8, 135, 136). Improved understanding of ways to facilitate sperm-egg interactions could possibly lead to improved outcomes, if the sperm were able to fertilize an egg "the old-fashioned way" rather than by being injected into the egg cytoplasm. Although the field of sperm-egg membrane interaction still awaits a precise model for how this important step of fertilization occurs, this area will continue to be an exciting one in biology with important implications for reproductive health.

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Abbreviations: UTJ: Uterotubal junction, ZP: Zona pellucida, PM:egg plasma membrane; ICSI, intracytoplasmic sperm injection

Key Words: Fertilization, Gamete Biology, Gamete, Sperm, Egg, Cell Adhesion, Membrane Fusion, Membrane Interactions, Tetraspanin, Immunoglobulin Superfamily, IgSF, A Disintegrin and A Metalloprotease,ADAM, integrin, Cysteine-rich Secretory Protein,CRISP, Review

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