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The molecular basis of sperm–oocyte membrane interactions during mammalian fertilization

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Gamete membrane interactions begin with adhesion (binding) of the sperm to the oocyte plasma membrane and culminate with fusion of the membranes of the gametes, thus creating the zygote through the union of these two very different cells. This review summarizes the molecular and cell biology of the cell–cell interactions between mammalian gametes. Recent research studies have provided new insights into the complexity of these interactions and into the importance of multimeric molecular networks and optimal membrane order in both sperm and oocytes for successful fertilization. Molecules that will be highlighted include cysteine-rich secretory protein 1 (CRISP1) and ADAMs [fertilin α (ADAM1), fertilin β (ADAM2) and cyritestin (ADAM3)] on sperm, and integrins, CD9, and other integrin-associated proteins on oocytes, as well as other molecules. The characteristics of these gamete molecules are summarized, followed by discussions of the experimental data that provide evidence for their participation in gamete membrane interactions, and also of the specific roles that these molecules might play. Insights from a variety of research areas, including gamete biology, cell adhesion, and membrane fusion, are put together for a tentative model of how sperm–oocyte adhesion and fusion occur. The clinical relevance of correct gamete membrane interactions is also noted.

Key words: ADAM/CD9/cell adhesion/integrin/membrane fusion

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Introduction

Mammalian sperm interact with oocytes on three different levels during fertilization: (i) the cumulus layer; (ii) the zona pellucida (ZP), which induces exocytosis of sperm acrosome contents; and (iii) the oocyte plasma membrane, which begins with adhesion (or binding) to the oocyte plasma membrane and concludes with fusion of the sperm membrane with the oocyte membrane. This review will focus on this third level, the interactions between gamete membranes. This interaction is preceded by acrosomal exocytosis, which is triggered by sperm binding to the ZP and is a critical prerequisite step to sperm–oocyte membrane interactions. It not only gives the sperm access to the perivitelline space where membrane interactions take place, but also exposes and modifies regions of the sperm surface to make the sperm capable of interacting with the oocyte membrane. Gamete membrane interactions involve a complex series of molecular interactions, beginning with initial attachment of the sperm to the oocyte, leading to firm cell–cell adhesion, and culminating in fusion of the two membranes, making one cell out of two. This review will address the cellular and molecular bases of these processes.

The main experimental method used to study the cellular and molecular mechanisms of sperm–oocyte interactions has been the IVF assay, examining sperm–oocyte interactions in experimentally manipulated conditions. For assessing gamete adhesion and fusion, ZP-free oocytes are usually used. The design of experiments with regard to insemination condition variables, assessment of sperm–oocyte adhesion and sperm–oocyte fusion, and interpretation of results from such assessments have been discussed (Evans, 1999). Data from IVF experiments have been augmented by other work, including studies of cellular interactions (such as with cells transfected to express gamete proteins of interest) and molecular interactions (such as analyses of specific protein–protein interactions).

Most of what is known about gamete interactions has been derived from animal models. Many of the molecules involved in fertilization processes that have been identified in mammalian model systems are conserved in humans. Studies of heterologous

systems (sperm and oocytes from different species) can also be performed. ZP-free hamster oocytes are commonly used for studies of membrane interactions because they fuse with sperm from nearly every mammalian species tested (Yanagimachi, 1988). It has generally been assumed that if a reagent (e.g. an antibody) inhibits the interaction of human sperm with hamster oocytes, then it would also inhibit the interaction of human sperm with human oocytes. While this is likely to apply in most cases, there are examples of 'false positives' [i.e. a reagent that inhibits in the heterologous system but not in the homologous system (Primakoff and Hyatt, 1986)] and 'false negatives' [i.e. a reagent that inhibits in the homologous system but not in the heterologous system (Okabe *et al*., 1988)]. Thus, care is necessary in the interpretation of results from the ZP-free hamster oocyte assays until more is known about the molecules involved in gamete membrane interactions. In addition, the fusibility of the hamster oocyte plasma membrane could be due to unique features of the biochemical nature of that particular membrane environment (lipids and/or proteins). As such factors are not yet completely understood, the relevance of the ZP-free hamster oocyte penetration test to bona fide human sperm–oocyte membrane interactions remains to be determined.

How have molecules involved in gamete membrane interactions been identified? The 'candidate molecule' approach has been applied, basing studies of sperm–oocyte interactions on studies of other cell–cell interactions. For example, oocytes and sperm have been examined for the expression of known cell adhesion molecules, such as integrins, extracellular matrix proteins, cadherins and immunoglobulin superfamily cell adhesion molecules. Data exist that support the role of integrins in sperm–oocyte interactions (see below). In addition, some sperm molecules were identified by what might be considered proteomic methods, used well before the word 'proteome' was coined. One example of this is the generation of a collection of anti-sperm monoclonal antibodies that are likely to cross-react with surface epitopes by immunizing mice with intact sperm or to sperm membrane proteins. These antibodies are then screened for the ability to bind to sperm and to perturb sperm function (such as sperm–zona binding, sperm–oocyte interaction, etc.). Details of some of the molecules identified by this and other methods are provided below.

Microscopic studies: key insights into sperm–oocyte membrane interactions

Early insights into gamete plasma membrane interactions came from elegant microscopic analyses, including light microscopy, scanning and transmission electron microscopy, and video microscopy (Yanagimachi, 1988, 1994; reviews). The oocyte plasma membrane is covered with microvilli; in rodent oocytes, the region overlying the meiotic spindle is free of microvilli, and sperm–oocyte fusion rarely occurs in this region (Ebenspaecher and Barros, 1984). Sperm interactions with the oocyte membrane also occur in a spatially restricted manner, with the inner acrosomal membrane—which is exposed after the acrosome reaction—contacting the oocyte membrane first (Huang and Yanagimachi, 1985). Subsequently, the equatorial segment and the posterior head of the sperm adhere to and then fuse with the oocyte membrane (Yanagimachi and Noda, 1970; Bedford *et al*., 1979). In the rodent, acrosome-intact sperm can adhere to the oocyte membrane, but only acrosome-reacted sperm fuse with the oocyte membrane (Yanagimachi and Noda, 1970; Phillips and Yanagimachi, 1982). In human sperm, there is evidence that the acrosome reaction is important for adhesion to the oocyte membrane (Talbot and Chacon, 1982; Bronson *et al*., 1999a). Initial attachments of the sperm to the oocyte membrane are reversible and appear to require sperm motility (Wolf and Armstrong, 1978), although sperm with poor motility can fuse with oocytes (Yanagimachi, 1988). Sperm tail movement decreases or stops within a few seconds of sperm–oocyte fusion (Wolf and Armstrong, 1978). Electron microscopy shows that the inner acrosomal membrane is later engulfed by the oocyte through a process that appears similar to phagocytosis (Huang and Yanagimachi, 1985). The sperm tail is also eventually incorporated into the oocyte (Hirao and Hiraoka, 1987).

Molecules that mediate gamete membrane interactions

Sperm-associated cysteine-rich secretory protein 1 (CRISP1) (DE, AEG-1 and ARP)

The protein known as DE was identified in rat epididymal protein lysates in an analysis of proteins expressed in the epididymis in an androgen-dependent manner (Cameo and Blaquier, 1976). The protein complex gets its name from two bands, D and E, on a denaturing polyacrylamide gel. Proteins D and E co-purify, are antigenically related, and are likely variants of the same gene product based on the high amino acid identity between tryptic peptides of protein D and protein E, although there are some differences between D and E (including size, high-performance liquid chromatographic separation of tryptic peptides, monoclonal antibody recognition and epididymal expression patterns) (Brooks, 1982; Xu and Hamilton, 1996). The molecular basis of the differences between proteins D and E is not known.

The peptide sequences of proteins D and E in the rat (Xu and Hamilton, 1996; Cohen *et al*., 2000b) and the mouse epididymal protein (MEP)-7 epididymal antigen in the mouse (Rankin *et al*., 1992) match the deduced amino acid sequence of previously characterized cDNA clones, an androgen-regulated sperm-coating epididymal protein in rat and rat acidic epididymal glycoprotein (AEG)-1 (Brooks *et al*., 1986; Charest *et al*., 1988). With the identification of additional related proteins in several mammalian species, this family has come to be known as the CRISP family. Family members include DE-like proteins expressed in the epididymis (Haendler *et al*., 1993; Eberspaecher *et al*., 1995; Krätzschmar *et al*., 1996a; Sivashanmugam *et al*., 1999), another family member, CRISP2, expressed primarily in the testis (also known as Tpx-1) (Kasahara *et al*., 1989; Foster and Gerton, 1996; Krätzschmar *et al*., 1996a; O'Bryan *et al*., 1998), and another, CRISP3, with variable tissue distribution (Mizuki and Kasahara, 1992; Haendler *et al*., 1993; Krätzschmar *et al*., 1996a; Schambony *et al*., 1998a). Cysteine-rich carboxy-terminal portions are present in all CRISPs, and the overall amino acid identities between CRISP family members range from ~30% to >80%. Epididymal proteins D and E are believed to be two forms of rat CRISP1. Mammalian CRISPs are closely related to certain toxins in reptiles. The vertebrate CRISPs are more distantly related to pathogenesis-related proteins (PR1) in plants and

similar proteins in fungi; venom polypeptides in insects; and a *Xenopus* sperm chemoattractant protein that is synthesized in the oviduct and deposited with oocyte jelly coat (Foster and Gerton, 1996; Hayashi *et al*., 1996; Olson *et al*., 2001).

CRISP1/DE has been studied most extensively in the rat and, unless otherwise noted, the data discussed below refer to that species. CRISP1 is synthesized by the epididymis and associates with rat sperm during epididymal transit. Expression of the D and E forms of CRISP1 protein varies by epididymal region (Brooks, 1982; Moore *et al*., 1994), although the differences do not seem to be due to alternative splicing of CRISP1 mRNA (Klemme *et al*., 1999; Roberts *et al*., 2001). Most association of CRISP1 with sperm is evident in sperm retrieved from the distal corpus and cauda epididymis (Moore *et al*., 1994). There appear to be two 'levels' of CRISP1 association with sperm. The majority of CRISP1 is loosely associated with rat sperm, and is released during capacitation *in vitro* (Cohen *et al*., 2000b) or by biochemical extraction treatments (Kohane *et al*., 1980; Rankin *et al*., 1992; Krätzschmar *et al*., 1996a). A fraction of CRISP1 may be tightly associated with rat sperm (Wong and Tsang, 1982; Moore *et al*., 1994; Cohen *et al*., 2000b), although the amount of CRISP1 that remains associated with sperm and/or the strength of the association may vary by species (Rankin *et al*., 1992; Krätzschmar *et al*., 1996a; Cohen *et al*., 2001). Other CRISP family proteins expressed in the male reproductive tract may associate with sperm in some species (Schambony *et al*., 1998b). It is not known how rat CRISP1 associates with the sperm membrane; there are data to indicate that rat sperm-associated CRISP1 is not attached through a glycosylphosphatidylinositol (GPI) linkage, directly or indirectly (i.e. to a GPI-linked binding partner) (Moore *et al*., 1994). Immunofluorescence data show that sperm-associated CRISP1 is localized on the dorsal region of the acrosome of cauda epididymal rat and mouse sperm (Rochwerger and Cuasnicu, 1992; Cohen *et al*., 2000a); with one antibody, it is detected on the tail of cauda epididymal and ejaculated rat sperm (Moore *et al*., 1994; Xu *et al*., 1997). On capacitated and acrosome-reacted rat sperm, CRISP1 is detected on the equatorial segment (Rochwerger and Cuasnicu, 1992). In ejaculated sperm from the horse, human and rhesus monkey, CRISP proteins are detected on the principal piece and mid-piece of the tail as well as the posterior (post-acrosomal) region of the sperm head (Hayashi *et al*., 1996; Schambony *et al*., 1998b; Sivashanmugam *et al*., 1999).

CRISP1 has been implicated in rodent gamete membrane interactions. Anti-DE antibodies inhibit fertilization of rat oocytes, either when mixed with rat sperm before the sperm are used for artificial insemination (Cuasnicu *et al*., 1984) or in IVF (Cuasnicu *et al*., 1990). Additionally, CRISP1 protein purified from rat epididymal extracts binds to the plasma membrane of rat and mouse oocytes, and rat oocytes and mouse oocytes treated with purified rat CRISP1 show reduced levels of fertilization when mixed with sperm in IVF (Rochwerger *et al*., 1992; Cohen *et al*., 2000a). Male rats immunized with CRISP1 show reduced fertility in mating trials, as well as the presence of anti-CRISP1 antibodies in epididymal and vas deferens fluids; sperm from these immunized males could adhere to but not fuse with ZP-free oocytes (Ellerman *et al*., 1998). In agreement with this, purified CRISP1 was found to inhibit rat sperm–oocyte fusion but was apparently without effect on sperm–oocyte adhesion (Rochwerger *et al*., 1992; Ellerman *et al*., 1998). A CRISP protein might also participate in human gamete membrane interactions. A human epididymal protein with some homology to rat CRISP1 (38% amino acid identity) has been described as AEG-related protein (ARP) or the human orthologue of rat CRISP1 (Hayashi *et al*., 1996; Krätzschmar *et al*., 1996a); a similar protein has also been characterized in the rhesus monkey (Sivashanmugam *et al*., 1999). There are conflicting data regarding how much ARP is tightly associated with human sperm. Some biochemical extraction data suggest that no ARP is tightly associated (Krätzschmar *et al*., 1996a), while other biochemical extraction data suggest that a subset of ARP is tightly associated (Cohen *et al*., 2001) and immunofluorescence detects ARP antigen on the heads and tails of ejaculated human and monkey sperm (Hayashi *et al*., 1996; Sivashanmugam *et al*., 1999). Human ARP is implicated in human gamete membrane interactions by the observation that human sperm treated with anti-human ARP antibodies show a reduced ability to fertilize hamster oocytes (Cohen *et al*., 2001). In addition, a recombinant form of this protein binds to the plasma membrane of human oocytes (Cohen *et al*., 2001). However, it is not known if there is a binding partner on the oocyte membrane for sperm-associated CRISP proteins, and the mechanism by which CRISP proteins participate in gamete membrane interactions remains uncharacterized.

Sperm ADAMs: fertilin β (ADAM2), fertilin α (ADAM1) and cyritestin (ADAM3)

Fertilin β was first implicated in gamete membrane interactions because it was identified as the antigen of an antibody, PH-30, that blocked fertilization of guinea pig oocytes (Primakoff *et al*., 1987). This antibody was made as described above, by immunizing mice with a guinea pig sperm membrane preparation to generate a battery of monoclonal antibodies that recognize sperm surface antigens (Primakoff and Myles, 1983). Fertilin α was identified and characterized with fertilin β, since these two proteins form a heterodimer (Primakoff *et al*., 1987; Waters and White, 1997; Cho *et al*., 2000). In turn, cyritestin was identified in the mouse and monkey by various cloning strategies (Barker *et al*., 1994; Wolfsberg *et al*., 1995; Heinlein *et al*., 1996). All have since been identified in rodents and primates, and fertilin β and fertilin α have also been identified in bovine and rabbit species.

The significant homologies and conserved domain structure of these and related proteins led to the identification of the molecular family known as ADAMs (an acronym for a disintegrin and a metalloprotease domain); the terms MDC (for metalloprotease-disintegrin-cysteine rich), metalloprotease-disintegrin and cellular disintegrin are also used in the literature. Fertilin α, fertilin β and cyritestin are known as ADAM1, ADAM2 and ADAM3 respectively. Approximately 30 members of this molecular family have been identified to date in vertebrates, expressed in a wide range of tissues and cell types. ADAM proteins are also present in invertebrates such as *Drosophila* and *Caenorhabditis elegans* (Primakoff and Myles, 2000). ADAM proteins, like the name suggests, have a specific domain structure: a signal sequence, prodomain, metalloprotease domain, disintegrin-like domain, cysteine-rich domain, an epidermal growth factor (EGF)-like repeat, and a transmembrane segment with a short cytoplasmic tail. The disintegrin-like domains of these proteins have generated great interest. These domains have homology to snake venom

ligands for the integrin family of cell adhesion molecules (Gould *et al*., 1990; McLane *et al*., 1998; Evans, 2001), suggestive of a role for these sperm proteins in cell adhesion (discussed below). Many of the snake venom polypeptides contain an RGD tripeptide, similar to that recognized by eight of the 24 known integrins (Evans, 2001; summary), although virtually no ADAMs have an RGD in this region. Nevertheless, this region in disintegrin domains is sometimes referred to as the 'disintegrin loop', since the RGD motif is on a loop structure in some snake disintegrins.

The ADAM protein family has dual functionality. Some family members have important proteolytic activities and others function as cell adhesion molecules (Black and White, 1998; Primakoff and Myles, 2000), although the specific functions of many of these ADAM proteins remain to be fully characterized. This review will focus exclusively on ADAM proteins involved in adhesion between sperm and oocyte during fertilization, since much has been learned about how fertilin β, fertilin α and cyritestin participate in this cell–cell interaction. However, some data suggest the possibility that metalloprotease activity (perhaps of an ADAM or another protein) may play a role in gamete membrane interactions (Díaz-Pérez *et al*., 1988; Correa *et al*., 2000) and there are ADAM family members that are candidates for this (Zhu *et al*., 1999, 2001).

Fertilin α, fertilin β and cyritestin undergo proteolytic processing between the metalloprotease and disintegrin domains, so that only the disintegrin domain, the cysteine-rich domain and the EGF-like repeat remain on the surface of the mature sperm (Figure 1). Fertilin α is processed intracellularly during spermatogenesis in the testis (Lum and Blobel, 1997). Cyritestin and fertilin β are cleaved during epididymal transit of the sperm (Blobel *et al*., 1990; Linder *et al*., 1995; Hunnicutt *et al*., 1997; Lum and Blobel, 1997; Yuan *et al*., 1997; Frayne *et al*., 1998). These sperm ADAM proteins are localized to regions of the sperm head (Phelps *et al*., 1990; Hardy *et al*., 1997; McLaughlin *et al*., 1997; Yuan *et al*., 1997).

Numerous functional studies have provided evidence that fertilin α, fertilin β and cyritestin participate in sperm–oocyte adhesion. As noted above, an anti-fertilin β monoclonal antibody, PH-30, blocked sperm–oocyte fusion of guinea pig oocytes (Primakoff *et al*., 1987). Since that original study in 1987, multiple types of reagents, including antibodies, peptides and isolated proteins, have been used to examine the roles of these proteins in gamete membrane interactions. Antibodies that cross-react with these proteins bind to sperm and inhibit fertilization in IVF assays (Primakoff *et al*., 1987; Hardy *et al*., 1997; Yuan *et al*., 1997). Recombinant forms of fertilin α, fertilin β and cyritestin bind to the mouse oocyte plasma membrane, and in IVF assays these proteins inhibit sperm–oocyte binding and reduce the incidence of fertilization (Evans *et al*., 1997a,b, 1998; Bigler *et al*., 2000; Zhu *et al*., 2000; Takahashi *et al*., 2001; Wong *et al*., 2001; Eto *et al*., 2002; Zhu and Evans, 2002). In a similar fashion, peptides corresponding to the fertilin β disintegrin loop inhibit sperm binding and reduce fertilization in the mouse (Almeida *et al*., 1995; Evans *et al*., 1995b; Yuan *et al*., 1997; Gupta *et al*., 2000; Zhu *et al*., 2000; McLaughlin *et al*., 2001), guinea pig (Myles *et al*., 1994) and human and non-human primate sperm with ZP-free hamster oocytes (Gichuhi *et al*., 1997) and with homologous oocytes (Bronson *et al*., 1999b; Mwethera *et al*., 1999). Fertilin β disintegrin loop peptides and antibodies against the fertilin β disintegrin loop also inhibit the binding of recombinant fertilin β to oocytes (Evans *et al*., 1997a; Bigler *et al*., 2000), indicating that fertilin β uses its disintegrin loop to bind to cognate binding partners on the oocyte surface. There are also similar data supporting the role of the mouse cyritestin disintegrin loop (Linder and Heinlein, 1997; Yuan *et al*., 1997; McLaughlin *et al*., 2001; Takahashi *et al*., 2001).

Structure–function studies of the disintegrin loops of mouse fertilin β and cyritestin have revealed similar functional motifs: ECDV in fertilin β (Bigler *et al*., 2000; Zhu *et al*., 2000; Takahashi *et al*., 2001) and QCD with some involvement of flanking sequences in cyritestin (Takahashi *et al*., 2001). Recent experimental data suggest that fertilin β and cyritestin could recognize the same receptor (discussed in more detail below) (Eto *et al*., 2002). Structure–function analysis of mouse fertilin α has also been performed. Unlike fertilin β, a recombinant form of fertilin α that lacks the disintegrin domain or has a truncated disintegrin domain inhibits sperm–oocyte binding (Evans *et al*., 1997b, 1998; Wong *et al*., 2001). This suggests that the cysteine-rich domain and/or the EGF-like repeat of fertilin α participate in fertilin α-mediated cell adhesion; this is similar to what has been observed for ADAM12 (Iba *et al*., 1999). A recombinant form of the fertilin α disintegrin domain also binds to mouse oocytes and inhibits sperm–oocyte binding (Wong *et al*., 2001), demonstrating that this domain is also involved in fertilin α-mediated adhesion. Interestingly, peptides from the fertilin α disintegrin loop do not inhibit sperm–oocyte binding (Yuan *et al*., 1997) or the binding of recombinant fertilin α disintegrin domain to oocyte (Wong *et al*., 2001), raising the possibility that other amino acids are involved in the interaction of the fertilin α disintegrin domain with cognate binding partners on the oocyte surface.

Important insights have come from studies of fertilin β and cyritestin knockout mice. Sperm from both fertilin β and cyritestin knockout mice show reduced binding to the oocyte plasma membrane, although some of the few sperm that bind are able to fuse with the oocyte membrane and fertilize and activate oocytes (Cho *et al*., 1998; Nishimura *et al*., 2001). Surprisingly, sperm from these knockout male mice have some other, unanticipated defects. In addition to adhering poorly to the oocyte plasma membrane, sperm from fertilin β and cyritestin knockout mice also adhere poorly to the ZP (Cho *et al*., 1998; Shamsadin *et al*., 1999; Nishimura *et al*., 2001), and fertilin β knockout sperm show deficient migration from the uterus to the oviduct (Cho *et al*., 1998). Considering that fertilin β knockout males mate normally and their sperm have normal motility, this suggests a defect in sperm migration to the oviduct or interaction with the oviduct walls. [Cyritestin knockout sperm, in contrast, have normal transit into the oviduct (Shamsadin *et al*., 1999; Nishimura *et al*., 2001).] Does this mean that fertilin β or cyritestin is involved with ZP binding, or that fertilin β is involved in migration to or interaction with the oviduct? This is one possible interpretation, but there are others. Analysis of protein expression profiles of fertilin β and cyritestin knockout sperm reveals that: (i) fertilin β knockout sperm lack fertilin α and have greatly reduced amounts of cyritestin; and (ii) cyritestin knockout sperm lack fertilin α and have ~50% of the wild-type amount of fertilin β (Nishimura *et al*., 2001). These multiple molecular deficiencies are likely to contribute to the defects in

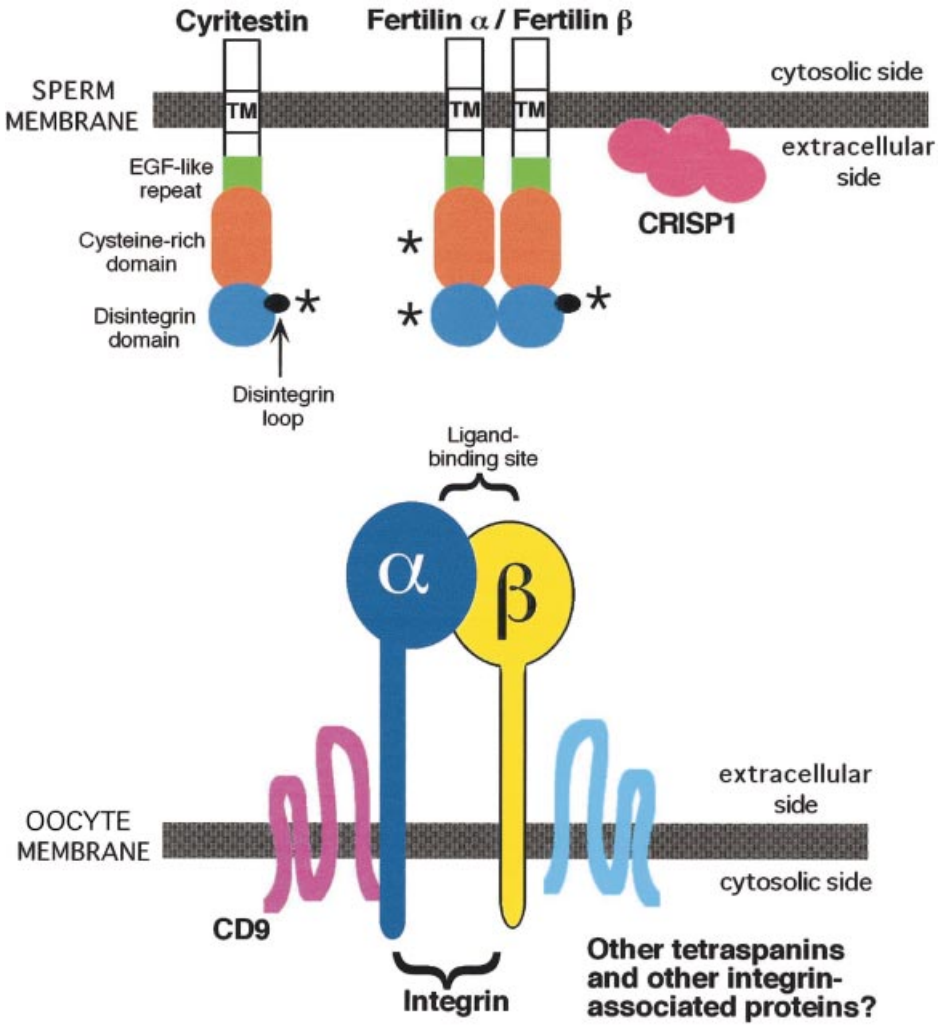

Figure 1. Schematic diagram of sperm and oocyte molecules known to participate in gamete membrane interactions. The diagram shows the approximate relationship of sperm and oocyte adhesion molecules and associated membrane proteins. As noted in the text, CRISP1 is not an integral membrane protein of the sperm, but a protein that associates with the sperm (by an as yet uncharacterized mechanism) during epididymal maturation. It is not known if there is a binding partner for CRISP1 on the sperm or on the oocyte (it is drawn as a peripheral membrane protein on the sperm, rather than receptor-bound). Fertilin α and fertilin β are shown associated as a dimer (Primakoff *et al*., 1987; Waters and White, 1997; Cho *et al*., 2000). Asterisks next to domains of fertilin α, fertilin β and cyritestin indicate the domains that participate in adhesion mediated by these molecules (Evans *et al*., 1998; Bigler *et al*., 2000; Zhu *et al*., 2000; Takahashi *et al*., 2001; Wong *et al*., 2001). A single integrin is shown in the oocyte plasma membrane as an α/β heterodimer. This integrin could be one of several integrins ($\alpha_9\beta_1$ and possibly $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_6\beta_1$; see text for details). The ligand binding site of integrins is composed of regions in the globular heads of both the α and β subunits (additional information in Evans, 2001); the heads are presented on extended stalks (Xiong *et al*., 2001). EGF = epidermal growth factor.

gamete membrane interactions and possibly also to other sperm function deficiencies. It can also be speculated that the ZP binding defect and/or oviductal transit are disrupted due to protein expression abnormalities. In other words, sperm from fertilin β knockout males that lack fertilin β, fertilin α and cyritestin could also lack a protein critical for interaction with the ZP or the oviduct.

Why might sperm from these knockout mice have abnormal protein expression profiles? Some insight comes from a different knockout mouse, lacking a chaperone protein (called calmegin) expressed in the endoplasmic reticulum of developing sperm. Sperm from calmegin knockout males have almost exactly the defects in sperm function as do sperm from fertilin β knockout males: reduced transit into the oviduct, reduced adhesion to the ZP, and reduced adhesion to the oocyte plasma membrane (Ikawa *et al*., 1997, 2001). Sperm from calmegin knockout males also have greatly reduced levels of fertilin β (Ikawa *et al*., 2001), indicating that this knockout also has an abnormal protein expression profile. Calmegin appears to function as a chaperone to help protein folding; it interacts with newly synthesized proteins (including fertilin α and fertilin β) in the endoplasmic reticulum during spermatogenesis (Ikawa *et al*., 1997, 2001). This suggests that in a calmegin knockout mouse, fertilin α and fertilin β are left to 'fend for themselves' in the endoplasmic reticulum, and this in turn leads to abnormalities, including the disappearance of fertilin β from sperm (Ikawa *et al*., 2001). It can be speculated that other proteins are also adversely affected, by misfolding and/or by misexpression. These problems could then lead to the fertilization function defects observed in calmegin knockout male mice. The angiotensin-converting enzyme (ACE) knockout also has a

similar phenotype. ACE is present in the blood, is expressed in the testis, and is also present in epididymal fluid. Sperm from ACE knockout mice have defects in transit up the oviduct and in ZP binding (Hagaman *et al*., 1998). Whilst it is unclear exactly why the ACE knockouts have these defects, one possibility is that sperm from these mice might have an abnormal protein profile on their surfaces due to defects in epididymal maturation.

To date, functional fertilin α and cyritestin genes have not been identified in humans. Fertilin α and cyritestin genes were determined to be non-functional pseudogenes (Jury *et al*., 1997, 1998; Frayne and Hall, 1998; Grzmil *et al*., 2001), having numerous point mutations that introduce frameshifts and premature stop codons and thus being unable to produce functional protein. In fact, several human testis-specific ADAM genes are intronless or non-functional pseudogenes (Cerretti *et al*., 1999; Frayne *et al*., 1999; Poindexter *et al*., 1999). The fact that humans are essentially fertilin α and cyritestin knockouts (lacking functional fertilin α and cyritestin genes) and yet have a different phenotype from cyritestin knockout mice suggests at least two possibilities. It could be that fertilin α and cyritestin are not essential for human gamete membrane interactions, and are also not required for a proper sperm protein expression profile. While fertilin α forms heterodimers with fertilin β in guinea pig, bovine and mouse sperm (Primakoff *et al*., 1987; Blobel *et al*., 1990; Waters and White, 1997; Cho *et al*., 2000), it could be that fertilin β functions by itself in human sperm, as a monomer (or homomultimer) without fertilin α. Alternatively, it could be that some other protein—perhaps another ADAM protein—plays the same role and substitutes for fertilin α and cyritestin in human sperm. Thirteen members of the ADAM family are expressed predominantly in the testis [fertilin β, cyritestin, ADAM5 (tMDCII), ADAM6, ADAM16 (xMDC16), ADAM18 (tMDCIII), ADAM20, ADAM21, ADAM24 (testase 1), ADAM25 (testase 2), ADAM26 (testase 3), ADAM29, ADAM30]. Five of these testis ADAMs (fertilin β, cyritestin, ADAM5, ADAM16, ADAM18) are known to be expressed as proteins on male germ cells and/or mature sperm in at least one species. Several ADAMs, including some that are expressed in the testis, are similar to and closely related to fertilin α or cyritestin (Poindexter *et al*., 1999; Yoshinaka *et al*., 2002; for phylogenetic trees of ADAM family members), and two fertilin α genes, dubbed ADAM1a and ADAM1b, were recently identified in the mouse (Nishimura *et al*., 2002). An additional candidate is ADAM15, as recombinant mouse ADAM15 disintegrin domain was recently shown to bind to oocytes and inhibit sperm–oocyte binding (Eto *et al*., 2002). It should be noted that this effect could be non-specific, since ADAM15, fertilin β, cyritestin, fertilin α and other ADAMs have been proposed to recognize the same receptor, the integrin $\alpha_9\beta_1$ (discussed in more detail below). It is not known if ADAM15 protein is expressed on sperm; ADAM15 mRNA is detected in Northern blots of human and mouse testis and many other tissues (Krätzschmar *et al*., 1996b; Lum *et al*., 1998). ADAM5 is not a candidate to participate in human gamete membrane adhesion, as it is also a pseudogene in humans (Frayne *et al*., 1999).

Oocyte integrins

Integrins as well as other cell adhesion molecules (e.g. cadherins, immunoglobulin superfamily members, etc.) have been speculated to mediate sperm–oocyte adhesion, as they mediate somatic cell adhesion. In particular, ever since the identification of an integrin ligand-like, disintegrin domain in fertilin β and cyritestin [and later in fertilin α (Lum and Blobel, 1997; Waters and White, 1997)], it was hypothesized that that these sperm ligands could bind to integrins on the oocyte membrane. Therefore, the issue of the role of oocyte integrins in gamete membrane interactions has two aspects: (i) whether oocyte integrins participate in gamete interactions in general; and (ii) whether oocyte integrins recognize sperm ADAMs.

Integrins are a family of heterodimeric cell adhesion molecules that mediate cell–cell and cell–extracellular matrix interactions. To date, 18 α subunits and eight β subunits have been identified, and these combine to make 24 different integrins. These 24 integrins can be divided into six subfamilies based on sequence homologies between the α subunits and the general characteristics of ligands recognized by integrin heterodimers containing each α subunit.

Several integrin subunits have been detected in mammalian oocytes at the mRNA or protein level, including α_2, α_3, α_4, α_5, α_6, α_v, α_9, α_M, β_1, β_2, β_3 and β_5 (Fusi *et al*., 1992, 1993; Anderson *et al*., 1993; Tarone *et al*., 1993; Almeida *et al*., 1995; Evans *et al*., 1995a; de Nadai *et al*., 1996; Linfor and Berger, 2000; Neilson *et al*., 2000; Stanton and Green, 2001). The involvement of the RGD-binding subfamily of integrins ($\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, $\alpha_{IIb}\beta_3$) has been suggested by the inhibition of interactions of human and hamster sperm with ZP-free hamster and human oocytes by RGD peptides (Bronson and Fusi, 1990; Ji *et al*., 1998). RGD peptides do not have a substantial inhibitory effect on mouse gamete interactions (Almeida *et al*., 1995; Evans *et al*., 1995b), although they do have a modest inhibitory effect on the binding of recombinant fertilin β to mouse oocytes (Zhu and Evans, 2002). Biochemical analyses have implicated α_v and β_1 integrin subunits on the pig oocyte in the recognition of isolated pig sperm membrane proteins (Linfor and Berger, 2000). A few anti-integrin antibodies have been reported to affect gamete interactions in IVF assays. Anti-β_1 antibodies have a moderate inhibitory effect on mouse, human and pig sperm–oocyte interactions (Evans *et al*., 1997a; Ji *et al*., 1998; Linfor and Berger, 2000) and also on the binding of recombinant fertilin β to mouse oocytes (Evans *et al*., 1997a). An anti-α_6 function-blocking monoclonal antibody, GoH3, inhibits mouse sperm–oocyte interactions in some (Almeida *et al*., 1995) but not all IVF assays (Evans *et al*., 1997a; Evans, 1999; Miller *et al*., 2000). Moreover, the findings that oocytes from α_6 knockout mice and oocytes with undetectable amounts of α_6 GoH3 epitope are capable of being fertilized strongly suggest that oocyte α_6 is not required for fertilization (Evans *et al*., 1997a; Miller *et al*., 2000). It should be noted that it is extremely difficult to study oocytes from integrin knockout mice, since many integrins are widely expressed in the body and thus many integrin knockouts die pre- or peri-natally, well before reaching sexual maturity (Hynes, 1996; De Arcangelis and Georges-Labouesse, 2000). As α_6 knockout mice die shortly after birth, the study of oocytes from the α_6 knockout mice required the isolation of ovaries from newborns, and transplanting these ovaries into the kidney capsule bursal cavity of ovariectomized mice to allow the ovaries to develop to the point where oocytes could be retrieved from them (Cox *et al*., 1996; Miller *et al*., 2000).

Despite these technical challenges, there are other ways to examine the possible role of oocyte integrins in gamete membrane interactions. One way is to consider data from other experimental systems. To date, eight different ADAM family members have been reported to interact with six different integrins (Table I), based primarily on assays of adhesion of integrin-expressing cells to ADAM proteins, in some cases combined with antibody and peptide inhibition studies. Members of three different integrin subfamilies appear to interact with ADAMs, with several of these integrins expressed by oocytes. With these findings in mind, what are the data that suggest these oocyte integrins could actually interact with an ADAM on sperm?

$\alpha_4\beta_1$ and $\alpha_9\beta_1$ are members of the same integrin subfamily; this subfamily has three members ($\alpha_4\beta_1$ $\alpha_4\beta_7$, $\alpha_9\beta_1$). These integrins recognize a diverse array of ligands, including vascular cell adhesion molecule (VCAM)-1, osteopontin, tenascin-C, fibronectin and Mad-CAM-1, and in several cases recognizing a conserved amino acid sequence motif containing an Asp residue in these proteins (Evans, 2001; summary). As shown in Table I, seven of the eight ADAMs thus far demonstrated to bind to an integrin, including the three sperm ADAMs (fertilin α, fertilin β, cyritestin), can interact with a member of the α_4/α_9 subfamily (Eto *et al*., 2000, 2002; Bridges *et al*., 2002). With regard to gamete membrane interactions, a member of the α_4/α_9 subfamily has been implicated by two results. Somatic cells expressing $\alpha_9\beta_1$ adhere to recombinant fertilin α, fertilin β and cyritestin (Eto *et al*., 2002). In addition, the binding of recombinant fertilin β to mouse oocytes is inhibited by a peptide sequence, MLDG, that perturbs adhesion mediated by the α_4/α_9 integrins (Zhu and Evans, 2002). The α_4 subunit has been reported to be present on hamster oocytes (de Nadai *et al*., 1996), but expression is weak or absent on human oocytes (Campbell *et al*., 1995; de Nadai *et al*., 1996) and mouse oocytes (J.P.Evans, unpublished data). α_9 cDNA is detected in a pool of expressed sequence tags from mouse oocyte cDNA libraries (Stanton and Green, 2001), although the protein has not been detected. Thus, the possibility remains that an integrin related to but distinct from $\alpha_4\beta_1$, $\alpha_9\beta_1$ and $\alpha_4\beta_7$ is expressed by oocytes and mediates adhesion to sperm ADAMs. The identity of this oocyte integrin remains to be definitely determined.

Table I. ADAM–integrin interactions

Integrin	Integrin subfamily (members)	ADAMs (reference)
$\alpha_9\beta_1$	α_4/α_9 subfamily ($\alpha_4\beta_1$ $\alpha_4\beta_7$, $\alpha_9\beta_1$)	Fertilin α (Eto *et al*., 2002) Fertilin β (Eto *et al*., 2002) Cyritestin (Eto *et al*., 2002) ADAM9 (Eto *et al*., 2002) ADAM12 (Eto *et al*., 2000) ADAM15 (Eto *et al*., 2000, 2002)
$\alpha_6\beta_1$	Laminin binding ($\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_7\beta_1$)	Fertilin β (Chen and Sampson, 1999; Bigler *et al*., 2000) Cyritestin (Takahashi *et al*., 2001) ADAM9 (Nath *et al*., 2000)
$\alpha_v\beta_3$	RGD binding ($\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, $\alpha_{IIb}\beta_3$)	ADAM15 (Zhang *et al*., 1998; Nath *et al*., 1999) ADAM23 (Cal *et al*., 2000)
$\alpha_v\beta_5$	RGD binding ($\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, $\alpha_{IIb}\beta_3$)	ADAM9 (Zhou *et al*., 2001)
$\alpha_5\beta_1$	RGD binding ($\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, $\alpha_{IIb}\beta_3$)	ADAM15 (Nath *et al*., 1999)
$\alpha_4\beta_1$	α_4/α_9 subfamily ($\alpha_4\beta_1$ $\alpha_4\beta_7$, $\alpha_9\beta_1$)	ADAM28 (Bridges *et al*., 2002)

$\alpha_6\beta_1$ is a member of the subfamily of integrins that primarily recognize laminins, and has been suggested to participate in fertilization (Almeida *et al*., 1995; Bigler *et al*., 2000; Takahashi *et al*., 2001). It is also implicated as a receptor for fertilin β and cyritestin based on a chemical cross-linking study with a fertilin β peptide (Chen and Sampson, 1999) and from studies with an anti-α_6 function-blocking monoclonal antibody (Almeida *et al*., 1995; Bigler *et al*., 2000; Takahashi *et al*., 2001). However, other attempts to inhibit interactions of fertilin β and other ADAMs with oocytes and other cells using the same anti-α_6 antibody have been unsuccessful (Evans *et al*., 1997a; Eto *et al*., 2002; Zhu and Evans, 2002). It is a formal possibility that fertilin β recognizes a site on $\alpha_6\beta_1$ that is not blocked by this anti-α_6 antibody. However, as noted above, α_6 is not required for fertilization (Miller *et al*., 2000). More recently, there has been speculation that oocyte $\alpha_6\beta_1$ has a role in ADAM binding perhaps not as a direct binding partner but as a component of a multi-molecular complex in the oocyte plasma membrane (Bigler *et al*., 2000; Takahashi *et al*., 2001); this is addressed in more detail in the section below on oocyte tetraspanins.

Finally, $\alpha_V\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ are members of the RGD-binding subfamily that mediate adhesion to ligands such as fibronectin, vitronectin and fibrinogen by binding to an RGD motif in the ligand in most cases. Members of the RGD-binding integrin subfamily are expressed by oocytes (see above) and are implicated in fertilization by studies using RGD peptides in IVF assays (Bronson and Fusi, 1990; Ji *et al*., 1998) and other work (Linfor and Berger, 2000). With regard to the potential role of these integrins in the recognition of a sperm ADAM, RGD peptides have a moderate inhibitory effect on the binding of fertilin β to mouse oocytes (Zhu and Evans, 2002). While one possible explanation for this is that an RGD-binding integrin contributes to fertilin β binding, it is also possible that the RGD peptide is weakly perturbing an α_4/α_9 integrin on the oocyte surface (as these integrins recognize sequences in some ligands that have some weak similarities to RGD motifs). Therefore, the exact roles of RGD-binding integrins in gamete sperm ADAM-mediated adhesion as well as the identification of specific family members on oocytes that could be involved remain to be fully characterized.

Oocyte tetraspanins

Tetraspanins (also known as tetraspans or TM4SF proteins) are a family of proteins with the conserved structural features of four membrane-spanning regions (hence the name) and two extracellular loops (one large, one small), with a series of other conserved residues (Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, 2001). It should be noted that not every protein with four membrane-spanning regions is a bona fide tetraspanin. To date, 28 mammalian tetraspanin family members

have been described, and several of these are implicated in a variety of cellular and physiological processes, such as cell adhesion, motility, proliferation and differentiation (Boucheix and Rubinstein, 2001; Hemler, 2001). Tetraspanins in general do not appear to function as receptors for extracellular ligands, but they do associate in the plane of the lipid bilayer with other membrane proteins, including other tetraspanins, integrins, immunoglobulin superfamily members, proteoglycans, complement regulatory proteins, growth factors receptors and others (Hemler, 1998; Berditchevski and Odintsova, 1999; Woods and Couchman, 2000; Boucheix and Rubinstein, 2001; Hemler, 2001). These intramembrane (or lateral or *cis*) associations form complexes referred to as tetraspanin webs.

One member of this family, CD9, plays a key role in gamete membrane interactions. Although CD9 is widely expressed in the body, the phenotype of the knockout mouse shows specific effects on female fertility. CD9 knockout females appear to ovulate normally, and yields of oocytes from super-ovulated animals are similar to yields from knockouts. Oocyte maturation to metaphase II also appears to be normal. However, oocytes from CD9 knockout mice are rarely fertilized (Kaji *et al*., 2000; Le Naour *et al*., 2000; Miyado *et al*., 2000). Sperm are observed in the perivitelline space of ZP-intact oocytes fertilized *in vivo* or *in vitro*, apparently unable to undergo sperm–oocyte fusion and lead to fertilization and oocyte activation (Le Naour *et al*., 2000; Miyado *et al*., 2000). Sperm are able to adhere to the plasma membrane of ZP-free CD9 knockout oocytes, but very rarely fuse with the oocyte membrane [between three reports, only three of 246 ZP-free oocytes were fertilized (Kaji *et al*., 2000; Le Naour *et al*., 2000; Miyado *et al*., 2000)]. However, CD9 knockout females did, on rare occasions, conceive and give birth to pups. In addition, oocytes from CD9 knockout mice could be fertilized by ICSI and these embryos developed to term (Miyado *et al*., 2000). If the expression of CD9 is induced in CD9 knockout oocytes (via microinjection of CD9 mRNA), the ability of the oocytes to be fertilized can be restored (Zhu *et al*., 2002). Using this method, a structure–function analysis of CD9 has been performed. Two mutated forms of CD9, a point mutation of amino acid 174 and the triple mutation of amino acids 173–175 [F174A and SFQ(173–175)AAA respectively] very poorly rescue the ability of CD9 knockout oocytes to be fertilized (Zhu *et al*., 2002), suggesting that the SFQ motif in this region has a critical function in mouse oocyte CD9.

The observation that sperm adhere to the CD9 knockout oocyte plasma membrane but do not fuse is suggestive of a role for CD9 in sperm–oocyte fusion. Questions have been raised as to whether CD9 may play a role, direct or indirect, in sperm–oocyte adhesion. IVF results with anti-CD9 antibodies are conflicting; ZP-free oocytes treated with anti-CD9 monoclonal antibodies have been reported to have reduced numbers of bound sperm (Chen *et al*., 1999; Takahashi *et al*., 2001), whereas another report states that sperm binding was unaffected by an anti-CD9 antibody (Miller *et al*., 2000). Additionally, treatment of oocytes with a recombinant form of the large extracellular loop of CD9 leads to a reduction in sperm–oocyte fusion when the oocytes are inseminated, with no effects on sperm–oocyte binding (Zhu *et al*., 2002). Other data do suggest a role for CD9 in sperm–oocyte adhesion. ZP-free oocytes treated with anti-CD9 antibodies show reduced levels of binding of the sperm ligands cyritestin (Takahashi *et al*., 2001), fertilin α (Wong *et al*., 2001) and fertilin β (Chen *et al*., 1999; Zhu and Evans, 2002). The inhibitory activity of anti-CD9 antibodies on the binding of fertilin α and fertilin β depends on the presentation of the sperm ligand to oocytes in the binding assays. The binding of multimeric forms (immobilized on small beads) of fertilin α and fertilin β is inhibited by an anti-CD9 antibody, whereas soluble monomeric forms of these sperm ligands still appear to bind (Wong *et al*., 2001; Zhu and Evans, 2002). Similar inhibition of binding of beads coated with cyritestin and fertilin β has been observed with a different anti-CD9 antibody (Chen *et al*., 1999; Takahashi *et al*., 2001). While the significance of this is unclear, it could be suggestive of a role for CD9 to strengthen adhesions mediated by these sperm ADAMs rather than the initial molecular interaction of sperm ligand to oocyte receptor. The connection between this and the finding that sperm can bind to CD9 knockout oocytes is not known, although it is likely that the action of function-blocking antibodies is distinctly different from the effect of having no CD9 present in the oocyte membrane. Furthermore, results with different anti-CD9 monoclonal antibodies (Chen *et al*., 1999; Miller *et al*., 2000; Takahashi *et al*., 2001; Wong *et al*., 2001; Zhu and Evans, 2002) as well as structure–function analysis of CD9 (Zhu *et al*., 2002) raise the possibility that different portions of CD9 may have functions in adhesion and fusion.

Tetraspanins, as noted above, are components of multi-molecular complexes or networks in the plasma membrane. These networks include integrins, other types of tetraspanins, and other membrane proteins. Several different integrins are expressed by oocytes (see details above), and at least seven different tetraspanins (CD9, CD81, CD82, CD151, SAS, Tspan-3 and Tspan-5) and various tetraspanin-associated proteins are potentially expressed in mouse or human oocytes (Neilson *et al*., 2000; Stanton and Green, 2001). The participation of some of these network components in fertilization is beginning to be examined. CD81 is expressed on the mouse oocyte membrane, but anti-CD81 antibodies do not inhibit sperm or recombinant cyritestin binding to oocytes, whereas anti-CD9 antibodies do inhibit these interactions (Takahashi *et al*., 2001). However, there is some suggestion that CD81 knockout mice may have fertility problems [reduced reproductive capacity after repeated back-crosses (Deng *et al*., 2000)], although the cause of this is not known. Antibodies that cross-react with CD98, another type of protein that associates with integrins and tetraspanins, inhibit sperm or recombinant cyritestin binding to oocytes (Takahashi *et al*., 2001). Finally, as discussed above, the integrin $\alpha_6\beta_1$ has been implicated in oocyte interactions with fertilin β and cyritestin on sperm (Bigler *et al*., 2000; Takahashi *et al*., 2001), although studies of oocytes from α_6 knockout mice demonstrate that α_6 expression by oocytes is not required for fertilization (Miller *et al*., 2000). In other cell types, $\alpha_6\beta_1$ can interact with several members of the tetraspanin family (CD9, CD63, CD81, CD82, CD151) and many tetraspanins can interact laterally with at least one member of the laminin-binding family of integrins [$\alpha_6\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_7$ and $\alpha_7\beta_1$ (Berditchevski, 2001; Boucheix and Rubinstein, 2001)]. CD9 and $\alpha_6\beta_1$ can associate in the mouse oocyte membrane (Miyado *et al*., 2000), although oocytes lacking α_6 still express CD9 on their plasma membranes and fertilization of these oocytes is inhibited with anti-CD9 antibodies (Miller *et al*., 2000). Relatively little is known about tetraspanin complexes in the oocyte membrane, but

further characterization of these complexes in oocyte membranes should shed light on the mechanisms of action of these molecules in mediating adhesion and fusion with sperm.

Other candidate molecules

Fertilin α and fertilin β are two of the best characterized antigens identified by screening a battery of sperm surface monoclonal antibodies. However, it is worth noting that although many more gamete antigens have been identified by this method, these antigens and/or their role in fertilization has not been characterized as extensively as those of the PH-30 antigen. Examples include the sperm antigens for monoclonal antibody MH61 (Okabe *et al*., 1990) [which has been identified as CD46/membrane cofactor protein (MCP) (Okabe *et al*., 1992)], monoclonal antibody MN9 [the antigen of which has been named equatorin (Toshimori *et al*., 1992, 1998)] and others (Saling *et al*., 1985; Okabe *et al*., 1988; Allen and Green, 1995; Noor and Moore, 1999). CD46 is one protein that can laterally associate with integrins and tetraspanins (Lozahic *et al*., 2000), although CD46 was not detected in the membranes of human oocytes (Fenichel *et al*., 1995). There are other candidate molecules hypothesized to be involved in gamete membrane interactions. These include extracellular matrix molecules, sulphoglycolipids and components of the complement pathway, including C3b and its receptors CD46/MCP and the integrin $\alpha_M\beta_2$, and also C1q and its receptors (Evans, 1999; review). A GPI-anchored protein(s) on oocytes has been implicated by a study in which oocytes treated with phosphatidylinositol-specific phospholipase C (which removes GPI-anchored proteins) show a greatly reduced ability to support sperm adhesion and fusion (Coonrod *et al*., 1999). A 94 kDa protein on oocytes has been implicated by studies in which oocytes treated with proteases show a greatly reduced ability to support sperm adhesion and fusion, and reappearance of a 94 kDa protein correlated with recovery of the ability of the oocytes to be fertilized (Kellom *et al*., 1992). A metalloprotease (on either sperm or oocyte) has been implicated by studies in which treatment of gametes with metalloprotease inhibitors at the start of insemination leads to decreased fertilization (Correa *et al*., 2000).

A model of how mammalian sperm–oocyte adhesion and fusion may occur

Gamete membrane interactions involve cell adhesion and then membrane fusion. Fertilin α, fertilin β and cyritestin on sperm and integrins on oocytes appear to mediate the adhesion process. CRISP1 may be involved in adhesion or fusion. CRISP1 protein purified from rat epididymal extracts binds to the oocyte plasma membrane and inhibits sperm–oocyte fusion in IVF of ZP-free rat oocytes, apparently without affecting sperm–oocyte adhesion (Rochwerger *et al*., 1992). However, CRISP1 does not have homology to proteins known to be involved in membrane fusion events, and current speculation places CRISP1 upstream of fusion (Cuasnicu *et al*., 2001). Fertilin α was originally a candidate to mediate membrane fusion between the gametes (Blobel *et al*., 1992), but the finding that the fertilin α-lacking sperm from fertilin β and cyritestin knockout mice are still capable of membrane fusion has dispelled this theory (Shamsadin *et al*., 1999; Nishimura *et al*., 2001). CD9 has been implicated in certain types of membrane fusions (Schmid *et al*., 2000; Boucheix and Rubinstein, 2001), but it is not known if CD9 in the oocyte has a direct role in facilitating gamete fusion, or has an upstream action such as by enhancing sperm interactions or modulating the oocyte membrane environment to make it 'fusion competent'. Results with two different monoclonal antibodies (Chen *et al*., 1999; Miller *et al*., 2000; Takahashi *et al*., 2001; Wong *et al*., 2001; Zhu and Evans, 2002) as well as structure–function analysis of CD9 (Zhu *et al*., 2002) raise the possibility that different portions of CD9 may have functions in sperm adhesion or gamete fusion, although this remains to be fully elucidated. Nevertheless, one intriguing theme that has emerged is the importance of multimeric complexes and a proper membrane environment on both sperm and oocyte for gamete membrane interactions to be successful. In the case of the oocyte, CD9 appears to be a key component of these multimeric complexes. In the case of sperm, insight into the importance of multimeric membrane protein complexes has come from analyses of protein expression profiles of sperm from the fertilin β and cyritestin knockout mice (Nishimura *et al*., 2001). Disruption of the membrane environment by an antibody treatment, a gene knockout removing a key component or other means, appears to lead to suboptimal membrane order that is less capable of supporting gamete adhesion and/or fusion.

The adhesion of sperm to oocyte is frequently cited as likely to be analogous to the mechanism by which leukocytes interact with endothelial cells. In this cell–cell interaction system, the adhesions occur in a step-wise fashion, starting with initial attachments (also called rolling or tethering) and leading to firm adhesion bringing the two membranes into close apposition; finally, the leukocytes undergo extravasation, traversing the endothelium to exit from the bloodstream. In the leukocyte–endothelium system, distinct ligand–receptor pairs with specific kinetics and affinities mediate each of the specific adhesion steps. Rolling is mediated by selectin–carbohydrate interactions that have rapid kinetics, followed by firmer adhesions supported first by the integrin $\alpha_4\beta_1$ on the leukocyte binding endothelial VCAM-1, and then interactions of $\alpha_L\beta_2$ and $\alpha_M\beta_2$ with intracellular adhesion molecules (Brown, 1997; Worthylake and Burridge, 2001). Sperm–oocyte membrane interactions also appear to occur in a step-wise fashion, in terms of the spatial domains of the sperm head that interact with the oocyte (see above) and possibly also in terms of the molecules involved.

A hypothetical model for how gamete membrane adhesion leads to fusion is shown in Figure 2. This model is based on membrane fusion events between cells and virus particles (Lentz *et al*., 2000; Eckert and Kim, 2001). In many virus–cell interactions, membrane fusion is mediated by a viral fusion protein in the membrane of the viral envelope. The viral fusion protein contains a hydrophobic subdomain, called a fusion peptide, which is folded within the fusion protein so that its hydrophobic amino acids are not exposed to the aqueous environment until a conformation change occurs to expose it. In the hypothetical model in Figure 2, panel A shows adhesion mediated by receptor–ligand pairs labelled 1 and 2; the adhesion between receptor–ligand pair 2 has induced bending of the lipid bilayers, bringing the two membranes into closer apposition. A putative fusion protein is shown with the hydrophobic fusion peptide concealed. (Please note that the two plasma membranes shown are purposely not identified as sperm and oocyte, since the mechanism could work with these proteins in either gamete.) In

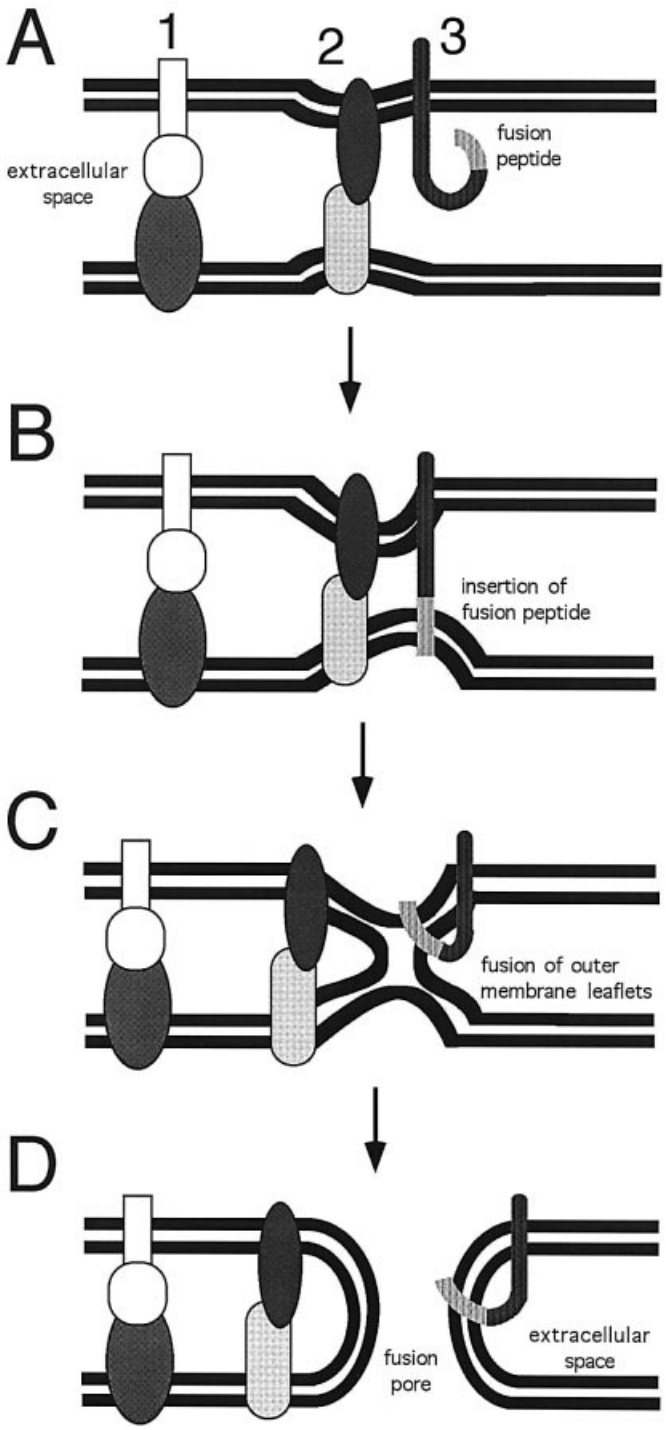

Figure 2. A hypothetical model for how gamete membrane adhesion and fusion occur. (**A**) Adhesion mediated by receptor–ligand pairs labelled 1 and 2; the adhesion between receptor–ligand pair #2 has induced bending of the lipid bilayers. A putative fusion protein is shown with the hydrophobic fusion peptide concealed (labelled 3). (**B**) The fusion protein undergoes a conformational change to expose the fusion peptide, which then inserts in the opposing bilayer. (**C**) Hemifusion, the fusion of the outer leaflets of the two membranes. (**D**) The formation of a fusion pore (fusion of outer and inner leaflets) between the two membranes. See text for more details.

panel B, the fusion protein has undergone a conformational change to expose the fusion peptide, which then inserts in the opposing bilayer. Next, the facing outer leaflets of two lipid bilayers intermingle, a state called hemifusion (shown in panel C). Finally, panel D shows the formation of an opening, called a fusion pore, between the two membranes, connecting the cytoplasms of the two cells. The fusion pore then expands, ultimately incorporating one membrane into the other. It should be emphasized that this is only a possibility for how mammalian gamete membrane interactions occur, although recent analysis of invertebrate sperm proteins has identified hydrophobic regions that might function by a mechanism somewhat similar to that of viral fusion peptides (Ulrich *et al*., 1998; Glaser *et al*., 1999; Kresge *et al*., 2001).

A second model for membrane fusion comes from studies of membrane fusion between intracellular vesicles with other membranes during trafficking, mediated by transmembrane proteins known as soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins and cytoplasmic accessory proteins that regulate SNARE function (Lentz *et al*., 2000; Chen and Scheller, 2001). A v-SNARE on a vesicle interacts with a t-SNARE on a target membrane, and these are believed to intertwine and form bundles of α-helices, bringing the vesicle and target membranes into close apposition, analogous to the way in which a viral fusion protein brings membranes close together after insertion of the fusion peptide, leading to formation of the fusion pore. It is possible that there are SNARE-like proteins on the extracellular surface of the sperm and oocyte that mediate gamete fusion. However, it should be emphasized that the SNARE model is based on membrane fusion events that occur on the interior of cells, with fusion being initiated by the membrane proteins and lipids that face the cytoplasm. Sperm–oocyte (and other cell–cell) fusion events are different in this regard, occurring on the extracellular surfaces of membranes and thus initiated by membrane proteins and lipids that face the extracellular space. While some SNARE machinery has been detected in gametes and is postulated to mediate acrosome and cortical granule exocytosis, it is unclear if it participates in plasma membrane fusion (Ramalho-Santos *et al*., 2000). Finally, metalloprotease activity may play a role in gamete membrane fusion (Díaz-Pérez *et al*., 1988; Correa *et al*., 2000)

Concluding thoughts

The advancement of our knowledge of the molecular basis of events surrounding fertilization, including gamete membrane interactions, has paled in comparison with the speed with which assisted reproductive technologies have been developed. The most obvious procedure that affects gamete membrane interactions is ICSI, which actually bypasses membrane interactions altogether. Other clinical procedures may impact fertilization as well, such as by affecting oocyte or sperm maturation. For example, expression of CRISP1 mRNA and protein in the rat epididymis is reduced after androgen ablation (Cameo and Blaquier, 1976; Roberts *et al*., 2001), after depletion of testicular factors from epididymal fluid (Turner and Bomgardner, 2002) and after vasectomy (Turner *et al*., 1999), and is not recovered after vasovasostomy (Turner *et al*., 2000). This may have implications for male infertility and for patients hoping to recover fertility after vasovasostomy. It is not known (but is conceivable) that other epididymal functions, such as processing of fertilin β or cyritestin,

might also be adversely affected by vasectomy and not recovered after vasovasostomy.

Proper gamete membrane interactions may do much more than simply merge two cells into one. These cellular processes introduce the paternal DNA, the sperm centriole and perhaps an oocyte activating factor to the oocyte in a precisely regulated fashion. While ICSI does result in oocyte activation, plasma membrane interactions between gametes may be important for correct temporal and spatial patterns of calcium signalling upon oocyte activation (Tesarik *et al*., 1994; Nakano *et al*., 1997; Sato *et al*., 1999; Deguchi *et al*., 2000) and/or for patterning of early embryonic development (Piotrowska and Zernicka-Goetz, 2001). Thus, plasma membrane interactions between the gametes as well as other events around the time of conception could contribute to the success (or failure) of embryo and fetal development.

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