A profile of fertilization in mammals

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Fertilization is defined as the process of union of two gametes, eggs and sperm. When mammalian eggs and sperm come into contact in the female oviduct, a series of steps is set in motion that can lead to fertilization and ultimately to development of new individuals. The pathway begins with species-specific binding of sperm to eggs and ends a relatively short time later with fusion of a single sperm with each egg. Although this process has been investigated extensively, only recently have the molecular components of egg and sperm that participate in the mammalian fertilization pathway been identified. Some of these components may participate in gamete adhesion and exocytosis, whereas others may be involved in gamete fusion. Here we describe selected aspects of mammalian fertilization and address some of the latest experimental evidence that bears on this important area of research.

In essence, once ovulated eggs and ejaculated sperm are present in the oviduct, fertilization in mammals encompasses at least five steps that take place in a compulsory order. There is some evidence to indicate that mammalian sperm may be drawn to the egg by a chemoattractant (for example, heat-stable peptides) emitted by follicle cells surrounding the egg (so-called 'sperm chemotaxis')[1,2], rather than simply by a chance encounter. In any event, sperm with an intact acrosome must first bind in a species-specific manner to the thick extracellular coat, or zona pellucida (ZP), of the egg (step 1; Fig. 1). Once bound to the ZP, sperm must undergo the acrosome reaction, or cellular exocytosis (step 2), and then penetrate the extracellular coat (step 3). Having reached the perivitelline space between the egg ZP and plasma membrane, sperm must bind to the plasma membrane (step 4) and then fuse with it (step 5). Fusion with a single sperm prevents the egg plasma membrane from fusing with further sperm that have penetrated the ZP. At this point, the egg has been fertilized and becomes a zygote, and free-swimming sperm are no longer able to bind to the ZP. This entire process takes roughly 90 min during *in vitro* fertilization of mouse eggs.

In recent years, each of the five steps that lead to fertilization has been studied extensively in mice and to a lesser degree in other mammals, including humans[3–5]. The ability to introduce genes into mice ('transgenic mice') and to disrupt specific genes by targeted mutagenesis in mice ('knockout mice') has made this system particularly useful for identifying gamete proteins that may participate in the fertilization pathway. Consequently, much of our recent knowledge in this area of research comes from work with mice. In this context, there is every indication that mice are, in fact, an appropriate model organism for investigating many, if not all, aspects of mammalian fertilization. Here we attempt to bring readers up to date on several aspects of mammalian fertilization, particularly the interaction of sperm with the egg ZP and plasma membrane, and the consequences thereof. It should be noted that some aspects of

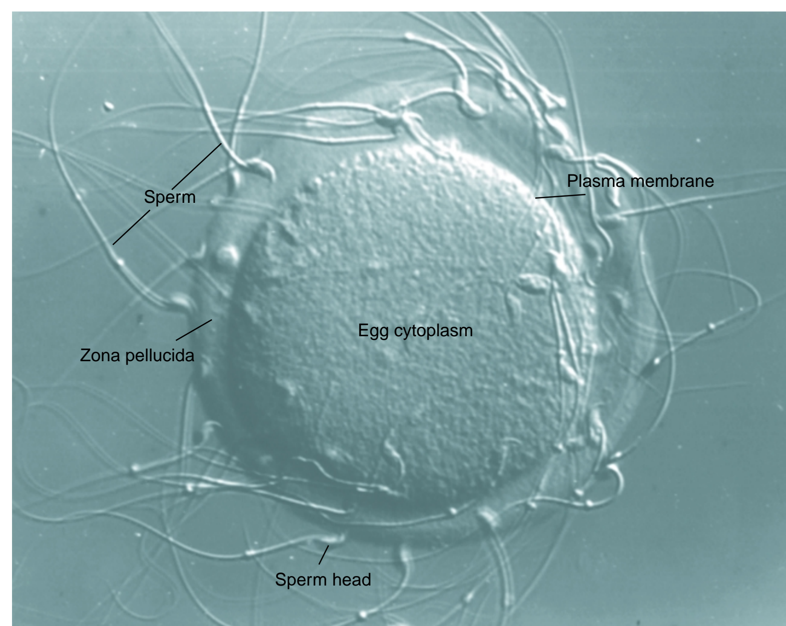

Figure 1 **Binding of sperm to the egg zona pellucida.** Light photomicrograph (Nomarski differential interference contrast) of mouse sperm bound to the ZP of an unfertilized mouse egg *in vitro*.

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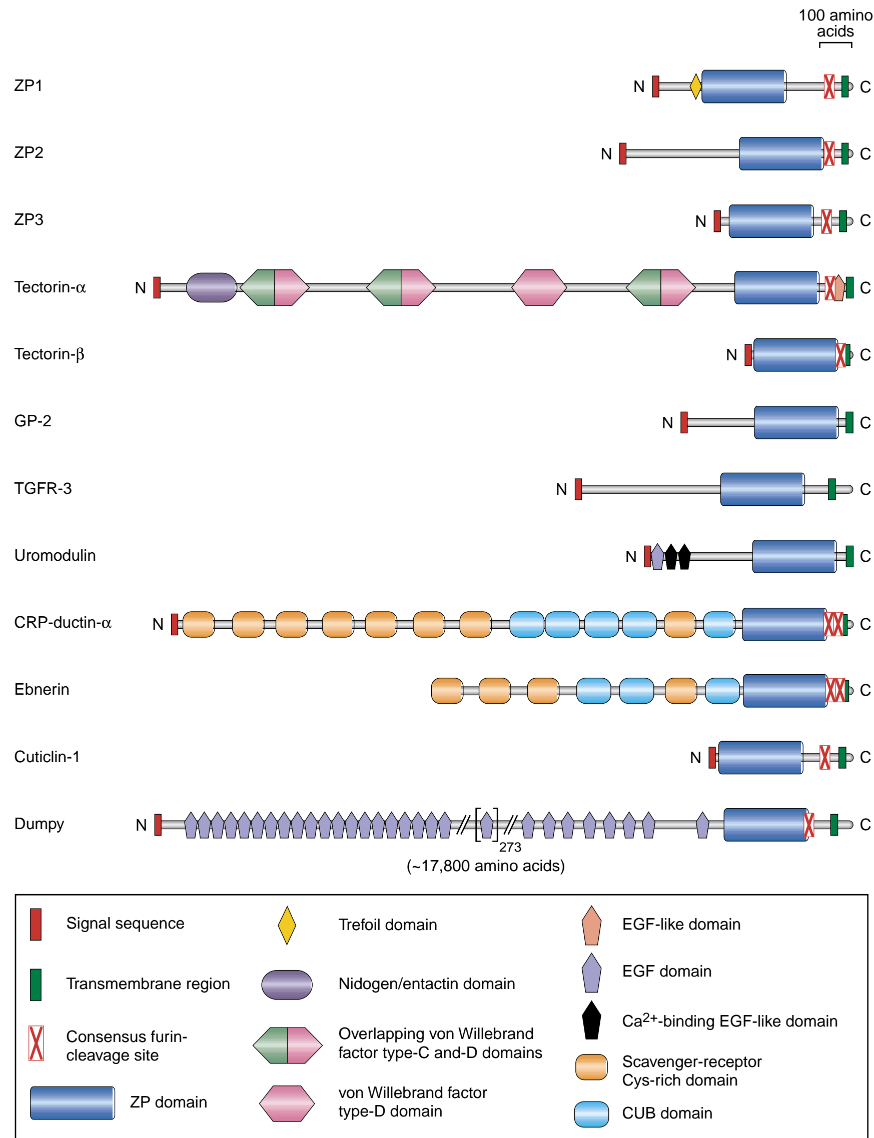

Figure 2 **Modular architecture of representative proteins containing ZP domains.** The primary sequence of each protein is shown as a grey bar, drawn to scale (with the exception of the central region of the gigantic Dumpy protein) and with both the amino and carboxy termini marked. Domains were identified using SMART[79] (http://smart.embl-heidelberg.de/); putative signal peptides were predicted using SignalP[80] (http://www.cbs.dtu.dk/services/signalp); transmembrane regions were predicted using PHDhtm[81] (http://cubic.bioc.columbia.edu/predictprotein/) and Tmpred[82] (http://www.ch.embnet.org/software/TMPRED_form.html). In three distinct regions within tectorin-α, von Willebrand factor type-C and type-D domains were identified by SMART which overlap in sequence, giving rise to eight alternative representations of the protein; these are summarized in the figure using a mixed symbol for each unresolved assignment. Proteins ZP1-3, tectorin-α and -β[83], GP-2 (ref. 84), TGFR-3 (ref. 85), uromodulin[86], CRP-ductin-α[87] and ebnerin[88] are all found in vertebrates; cuticlin[89] and Dumpy[90] were identified in *Caenorhabditis elegans* and *Drosophila melanogaster*, respectively.

fertilization, such as sperm chemotaxis and capacitation, and the cortical and zona reactions, are not discussed in detail here.

Step 1 — species-specific binding of sperm to eggs

It is reasonably well documented in the literature that binding of sperm to the egg ZP is most often species-specific[3,6]. In general, when eggs and sperm come from different mammalian species, binding of sperm to the ZP does not occur *in vitro* (for example, guinea pig or human sperm and mouse eggs). This restriction can usually be overcome by removing the ZP (for example, with the use of either proteases or low-pH buffers), thereby allowing sperm to bind directly to the egg plasma membrane. For example, the so-called 'hamster test', which is frequently used in *in vitro* fertilization (IVF) clinics to assess the fertilizing ability of sperm, uses ZP-free hamster eggs and human sperm[7]. Human sperm will not bind to hamster eggs with an intact ZP. Therefore, just as the extracellular coat of eggs from non-mammalian species prevents binding of foreign sperm[8,9], the ZP serves as a barrier to sperm from heterologous mammalian species. This indicates that the ZP may possess receptors ('sperm receptors') that are recognized by sperm from the same species and that sperm may possess proteins ('egg-binding proteins'; EBPs) that are compatible with eggs from the same species. The occasional binding of sperm from one species to eggs

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Conservation of the ZP domain

Each ZP glycoprotein has a distinct function in fertilization in mice. For example, mZP3 is a structural component of the ZP, a sperm receptor, and an inducer of the acrosome reaction. On the other hand, the three mZP proteins share a highly conserved sequence of ~260 amino acids, called the 'ZP domain', with more than 100 other protein sequences (Fig. 2). In almost all cases, this domain contains eight conserved cysteine residues and is located at the carboxy terminus. ZP domains are found in ZP glycoproteins of eggs from all mammals examined so far, from mice to humans, as well as in glycoproteins of egg extracellular coats (vitelline envelope, VE) from a variety of non-mammals, including fish, birds and amphibians. Furthermore, ZP domains are found in many other extracellular proteins, including tumour-growth factor (TGF)-β receptor type III (TGFR-3)/endogin, uromodulin, tectorins, ebnerin/CRP-ductin/vomeroglandin/hensin, cuticlin (*Caenorhabditis elegans*) and Dumpy *(Drosophila)*.

from another (for example, hamster sperm to mouse eggs and vice versa) could be attributable to sperm receptors and EBPs that share some common binding determinants.

Egg mZP3 oligosaccharides and sperm binding

mZP3, one of three mouse ZP glycoproteins (mZP1–3), was identified as a sperm receptor 20 years ago[10]. Although mZP1–3 are found exclusively as components of the egg ZP, proteins with homologous sequences are widely distributed in nature (Fig. 2). Today, mZP3 is designated as a sperm receptor on the basis of several lines of evidence[5,11]. Paramount among these is the ability of nanomolar concentrations of purified egg mZP3, but not embryo mZP3, to prevent binding of sperm to eggs *in vitro*. This ability, together with several other properties of the glycoprotein[5], has established mZP3 as a mouse sperm receptor. Evidence indicates that acrosome-intact sperm recognize and bind to specific O-linked oligosaccharides[12] located on serine residues (serine 332 and 334) near the carboxy terminus of mZP3 polypeptide[13,14]. Consistent with this is the finding that certain oligosaccharides, at micromolar concentrations, also inhibit binding of mouse sperm to eggs *in vitro*[15–17]. Therefore, as in many other instances of cellular adhesion[18,19], binding of sperm to the egg ZP is a carbohydrate-mediated event.

The specificity of the interaction between sperm and mZP3 depends in part on information encoded in the mZP3 polypeptide, which presumably designates particular amino acid residues for glycosylation, as well as on the nature and distribution of glycosyltransferases and glycosidases in growing oocytes, where mZP3 is synthesized[18,20]. In this context, changes in the structure of mZP3 oligosaccharides could affect species-specific binding of sperm to eggs. This may account for results of recent experiments in which mice carrying the human sperm-receptor gene *(hZP3)* as a transgene were produced using females that were homozygous-null for *mZP3* *(mZP3*$^{-/-}$*)*[21]. Eggs from *mZP3*$^{-/-}$ females lack a ZP and the females are infertile[22,23], whereas eggs from *mZP3*$^{-/-}$ animals carrying *hZP3* as a transgene possess a thick ZP that consists of mZP1, mZP2, and hZP3. It is intriguing that human sperm fail to bind to the ZP of eggs from these transgenic mice, whereas mouse sperm bind and the females are fertile. This behaviour could reflect the presence of 'mouse-like', rather than 'human-like', O-linked oligosaccharides on hZP3 synthesized by mouse oocytes. Direct structural analysis of the oligosaccharides by mass spectrometry, in the manner recently reported for bulk mZP3 O-linked oligosaccharides[24], could resolve this issue once and for all.

An abundance of candidate EBPs on sperm

Although the search for mammalian EBPs has been extensive in recent years, it remains a highly contentious area of research. As many as two-dozen different sperm proteins have been implicated in species-specific binding of sperm to eggs[4,5,25]. These include a variety of enzymes (such as β-galactosyltransferase and α-fucosyltransferase, protein tyrosine kinase (ZRK) and phospholipase A_2) and lectin-like proteins (such as mannose- and galactose-binding proteins and spermadhesins), as well as several other sperm proteins (such as zonadhesin and sperm protein-56 (SP56)). In addition, results of recent experiments with homozygous-null mice have implicated two members of the ADAM (so-called because they contain a disintegrin and a metalloprotease domain) family of proteins[26], sperm β-fertilin[27] and cyritestin[28], as potential EBPs (see below).

Why is there such a large number and variety of presumptive EBPs? It is now generally held that, for a given species, more than one kind of EBP may be involved in binding of sperm to eggs. In addition, it is often proposed that sperm from different mammalian species use different EBPs during fertilization. This situation has been described recently[29] as reflecting that "a high degree of pathway specificity may be achieved through a sequence of steps, each of which has only moderate selectivity". However, the number and variety of candidate EBPs surely reflects difficulties in accurately assigning such a function to sperm proteins. It is very unlikely that all of the candidates are *bona fide* EBPs. One must also question whether the kinds of protein (enzymes, lectins, etc.) that act as EBPs vary so much among mammalian species. This seems not to be the case for the many species of sea urchins, in which alleles at the *bindin* locus (which encodes a sea-urchin sperm EBP) strongly affect species-specific fertilization[30]. In this instance, eggs from a given species select sperm with a bindin phenotype that is compatible with their sperm receptors, otherwise speciation would not be maintained. Surely, the analogous situation in mammals cannot have deviated too far from this paradigm.

Step 2 — mZP3, Ca^{2+}, G proteins and the acrosome reaction

Shortly after binding to the egg ZP, sperm undergo cellular exocytosis, the acrosome reaction. The acrosome is a relatively large, Golgi-derived, lysosome-like organelle that overlies the nucleus in the apical region of the sperm head[31]. Although the acrosome is surrounded by a continuous membrane, it is usually described as consisting of an 'inner' and 'outer' membrane; the former overlies the nucleus and the latter underlies the plasma membrane. The acrosome reaction involves multiple fusions between outer acrosomal membrane and plasma membrane at the anterior region of the sperm head, extensive formation of hybrid membrane vesicles, and exposure of inner acrosomal membrane and acrosomal contents[3]. Only sperm that have completed the acrosome reaction can penetrate the ZP and fuse with the egg plasma membrane. It is now generally accepted that mZP3 is the natural agonist that initiates the acrosome reaction upon binding of sperm to eggs[29,32,33]. The plasma membrane overlying the sperm head is capable of binding to thousands of copies of mZP3 in the ZP[34], and such binding is apparently sufficient to induce the acrosome reaction. A variety of evidence indicates that multivalent interactions between sperm and mZP3 may be required for induction of the acrosome reaction[5,25].

Several of the same kinds of molecule that participate in secretion by somatic cells[35] participate in initiation of the acrosome reaction. These include several signal-transducing components, including G proteins, inositol-3,4,5-triphosphate (IP3) and IP3 receptors, phospholipase C, Ca^{2+} and voltage-sensitive Ca^{2+} channels[29,36]. For example, mZP3 stimulation of sperm activates G proteins (G_{i1}, G_{i2} and $G_{q/11}$), depolarizes sperm plasma membrane (from ~−60 mV to ~−30 mV), activates Ca^{2+} channels (T type), and increases pH (by ~0.3 units) and intracellular Ca^{2+} concentration (from ~150 nM to ~400 nM). Activation of a pertussis-toxin-sensitive G protein complex has

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Table 1 Sites of glycoprotein synthesis

Source	Ovary*	Liver
Mammals (ZP)	+	–
Fish (VE)	+	+
Birds (VE)	+	+
Amphibians (VE)	+	–

* Oocytes and/or follicle cells
VE, vitelline envelope

Sites of synthesis of ZP and VE glycoproteins

An interesting feature of mammalian ZP and non-mammalian VE glycoproteins is their site of synthesis (Table 1). Whereas mZP1–3 are synthesized and secreted by growing mouse oocytes[72], homologous VE glycoproteins from fish, birds and amphibians may be synthesized by growing oocytes, surrounding follicle cells, and/or the liver (for example, in several species of fish[73–76]). In the latter case, expression of VE glycoproteins, like yolk proteins, is under oestrogen control, and nascent VE glycoproteins are presumably transported through the blood and delivered exclusively to the ovary. In chickens, for example, a VE homologue of ZP3 is synthesized by follicle cells and a homologue of ZP1 is synthesized by the liver[77,78]. Whether or not specific receptors that recognize nascent VE glycoproteins are present in ovaries remains to be determined.

been attributed to aggregation of β-galactosyltransferase on the sperm head[37], and entry of Ca^{2+} through store-operated channels is thought to result from depletion of IP3-sensitive Ca^{2+} stores[33,38]. Both of these events are triggered by exposure of sperm to mZP3. A modified version of a recent model for the ionic events of mZP3 signal transduction in sperm that lead to the acrosome reaction[33] is presented in Fig. 3. Finally, in this context, results of recent studies indicate that at least two components that are essential for intracellular membrane fusion in somatic cells, Rab3A GTPase and SNAREs, may be present in mammalian sperm and may participate in membrane fusion during the acrosome reaction[39–41].

Step 3 — penetration of the egg ZP by sperm

Acrosome-reacted sperm remain bound to the ZP, apparently by binding to mZP2 (refs 34, 42), and must now penetrate the ZP to reach and fuse with the egg plasma membrane. Penetration of the ZP is probably achieved by a combination of sperm motility and enzymatic hydrolysis[3,43], the latter being catalysed by an acrosomal serine protease called acrosin[44]. However, although sperm from mice that are homozygous-null for acrosin (*Acr*$^{-/-}$) exhibit a delay in dispersal of acrosomal proteins during the acrosome reaction and in penetration of the ZP[45,46], they successfully penetrate the ZP and fertilize eggs[47]. These findings indicate that, at least in mice, acrosin is not essential for fertilization. On the other hand, results of a recent study indicate that the action of other acrosomal serine proteases may substitute for that of acrosin in *Acr*$^{-/-}$ mice[48], thus raising the possibility that acrosin is indeed used for penetration of the ZP by sperm from wild-type animals.

Steps 4 and 5 — ADAMs, integrins and interaction of sperm with eggs

Acrosome-reacted sperm bind to and fuse with eggs by using plasma membrane at the postacrosomal region of the sperm; this region is capable of fusion only after the acrosome reaction has taken place[3]. Binding of sperm to the egg plasma membrane is thought to be mediated by a member of the ADAM family of transmembrane proteins on sperm and integrin α6β1 receptors on eggs[4,5,25]. Two mouse-sperm ADAM proteins in particular, the heterodimer fertilin (-α, ADAM-1; -β, ADAM-2) and cyritestin (ADAM-3), have been studied in some detail and are thought to interact with integrin in the egg plasma membrane through their disintegrin domains[26,49]. For example, a variety of peptide mimetics of fertilin-β and cyritestin disintegrin domains, at ~100–500 μM, prevent sperm from binding to and fusing with egg plasma membrane *in vitro*. In a recent investigation, this type of approach was extended by producing recombinant disintegrin-domain-containing constructs in insect and bacterial cells[50]. These recombinant proteins bound to egg plasma membrane and, like antibodies against fertilin-β[51–53], prevented sperm from binding to and fusing with the eggs. However, point mutations within the disintegrin domain of fertilin-β reduced its ability to inhibit sperm–egg interactions. These and other observations strengthen the proposal that fertilin-β participates, through its disintegrin domain, in binding of sperm to integrin in the egg plasma membrane.

Whereas fertilin-β supports binding of sperm to egg plasma membrane, fertilin-α has been implicated in the subsequent step of fertilization, fusion of sperm and egg[5,54,55]. Fertilin-α contains a hydrophobic sequence that has been likened to a virus-like fusion peptide and that, in certain respects, behaves in a corresponding manner[56,57]. For example, synthetic peptides that correspond to the putative fusion peptide of fertilin-α interact with liposomes, induce lipid mixing of large unilamellar vesicles, and release encapsulated dyes from lipid vesicles[58,59]. Despite this supportive evidence, a role for fertilin-α in sperm–egg fusion remains a controversial, although attractive, proposal.

Mice have been produced that are homozygous-null for either fertilin-β (*fertilin-β*$^{-/-}$)[27] or cyritestin (*cyrn*$^{-/-}$)[28], and their phenotypes have been determined. Although the fertility of *fertilin-β*$^{-/-}$ and *cyrn*$^{-/-}$ male mice was reduced ~50-fold or more compared to that of wild-type mice, this resulted from failure of mutant sperm to bind to the egg ZP, not from an inability to fuse with egg plasma membrane. Sperm from *fertilin-β*$^{-/-}$ mice also exhibited severely reduced migration from the uterus to the oviduct, less than 5% of wild-type values, whereas sperm from *cyrn*$^{-/-}$ mice did not exhibit this defect. Another interesting feature of the *fertilin-β*$^{-/-}$ phenotype was the ability of sperm to fuse with egg plasma membrane *in vitro*, despite significantly reduced levels of fertilin-α on mutant sperm[27]. Together, these findings indicate that fertilin-β, fertilin-α and cyritestin may not be essential participants in the gamete-fusion pathway[60]. On the other hand, the surface organization of mutant sperm that lack any one of these proteins may not resemble that of wild-type sperm, and this could give rise to the observed effects on adhesion.

Step 5 continued — CD9 and fusion of sperm with eggs

CD9 is a member of the tetraspan superfamily of integral plasma-membrane proteins that associate with each other, as well as with a subset of β1 integrins, including integrin α6β1 (refs 61, 62). Results of several recent investigations indicate that CD9 in the egg plasma membrane has a vital function in sperm–egg fusion in mice[63–67]. In three of these studies[64–66] *CD9*$^{-/-}$ mice were produced by targeted mutagenesis and homologous recombination in embryonic stem cells. Homozygous-null females, but not males, exhibited severely reduced fertility, that is, infrequent pregnancies and reduced litter sizes compared with wild-type females. This reduced fertility was shown to be due to greatly impaired sperm–egg fusion (*CD9*$^{+/+}$, ~100%; *CD9*$^{-/-}$, ~21%), rather than to a reduction in binding of sperm to the egg plasma membrane, 6 h after combining sperm and ZP-free eggs *in vitro*[65]. Furthermore, embryos developed normally

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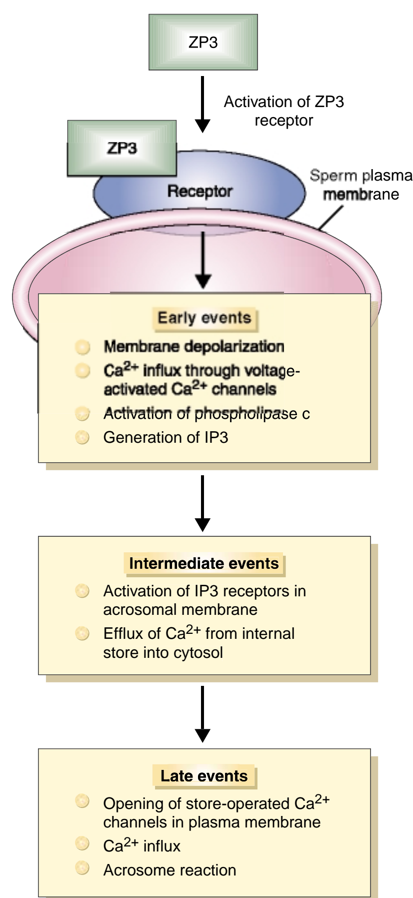

Figure 3 **Events associated with the ZP3-mediated acrosome reaction in mammalian sperm.** Adapted in part from the model for ionic events in ZP3 signal transduction[33].

when sperm were microinjected directly into the cytoplasm of eggs from *CD9*$^{-/-}$ females, indicating that CD9 affects only sperm–egg interaction, and not early development. Further evidence indicates that CD9 may be present on the egg plasma membrane in association with integrins, as an antibody against α6 integrin co-immunoprecipitates CD9 from egg lysates, and antibodies against CD9 prevent fusion of sperm with eggs.

How does CD9 affect sperm–egg fusion? The simplest explanation, on the basis of the bulk of the literature[5], is that CD9 in the egg plasma membrane is intimately associated with integrin α6β1, to which fertilin-β binds. CD9 would thereby regulate the interactions between integrin and fertilin that are ultimately responsible for sperm–egg fusion[63]. This is consistent with the proposal that CD9 facilitates membrane fusion in other kinds of cellular systems (for example, myoblast fusion during muscle differentiation[68]). However, some recent evidence (see below) indicates that a somewhat different assortment of egg and sperm proteins may be involved in gamete fusion.

First, sperm that lack fertilin-α, the subunit of heterodimeric fertilin that is proposed to be directly involved in sperm–egg fusion, are capable of fusing with eggs at about 50% of the rate of wild-type sperm[69]. Second, eggs that lack integrin α6β1 are fully functional in terms of allowing sperm to bind to and fuse with the plasma membrane[67]. These findings indicate that, whatever the function of CD9 in sperm–egg fusion, it may not require the participation of either egg integrin α6β1 or sperm fertilin-α. This idea is consistent with reports that some primates, including humans, possess a mutated, single-copy fertilin-α gene that encodes a non-functional protein[70,71]. On the other hand, we cannot dismiss the possibility that other egg and sperm proteins are able to take over the wild-type functions of integrin α6β1 and fertilin-α in homozygous-null mice. This is all the more likely as fertilin and integrin α6β1 are members of very large protein families, several members of which are present on eggs and sperm. A great deal of work remains to be done before these issues can be satisfactorily resolved.

Concluding remarks

A large number of mammalian egg and sperm gene products have been identified as participants in sperm–egg interactions during fertilization. Interestingly, virtually all of these gamete products have counterparts in somatic cells. Even ZP glycoproteins, which are components of a unique egg organelle, have counterparts (that is, proteins that contain ZP domains) elsewhere, from the extracellular coat of zebrafish eggs, to ears (tectorins) and noses (vomeroglandin), to worm cuticles (cuticlin; Table 1). In addition, all of the molecules that are involved in signalling in sperm during the acrosome reaction were initially found in somatic cells and were implicated in membrane-fusion events. These findings are unsurprising, as all of the events described for fertilization, such as cell–cell adhesion and cellular exocytosis, occur elsewhere.

Finally, it should be apparent that our current view of many aspects of mammalian fertilization is influenced to a large extent by results of experiments using knockout mice (for example, *mZP3*$^{-/-}$, *acrosin*$^{-/-}$, *fertilin-β*$^{-/-}$, *cyritestin*$^{-/-}$, *integrin-α6*$^{-/-}$ and *CD9*$^{-/-}$ mice). These mice have proved useful for examining each of the five steps of mammalian fertilization. Although in some instances the phenotype of homozygous-null mice is consistent with results of other experimental approaches (for example, *mZP3*$^{-/-}$), in others it is not (for example, *acrosin*$^{-/-}$ and *fertilin-β*$^{-/-}$). On the basis of a relatively small number of examples, it seems that alterations in the protein composition of sperm membranes by targeted mutagenesis can have profound, and often unexpected, effects on sperm–egg interactions. It seems likely that some of these phenotypes are misleading. In the case of sperm, it is clear that much more basic research needs to be carried out on the organization and behaviour of proteins in sperm membranes. Hopefully, with such background information, results of studies of EBPs and sperm from knockout mice can be interpreted in a more reliable manner. □

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This review is dedicated to the memory of Paul Sigler, a friend.

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