

Focus on Fertilization

Molecular genetic approaches to studying fertilization in model systems

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Abstract

In a wide range of experimental systems, a variety of both forward and reverse genetic approaches are becoming available for the study of the molecules involved in fertilization. An integration of these methods with the antibody-based and biochemical studies traditionally used in fertilization research is enabling rapid advancements in our understanding of this process. We highlight some of the recent advances resulting from these genetic methods and their applications in these systems.

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Introduction

Despite the enormous wealth of information available about the biological process of fertilization, relatively little is known about the actual molecules and signaling pathways that mediate this critical event. Initial research on fertilization was hampered by the lack of many of the genetic, molecular, bioinformatic and imaging tools that we take for granted today. It has been only with the synthesis of biochemistry, genetics, cell biology, molecular biology and bioinformatics that we are really beginning to unravel the intricacies and complexities of the process of fertilization. A variety of systems are now being utilized to identify many molecules of interest using these integrated approaches (Table 1). Here, we will review the various genetic approaches/systems and their applications in our quest toward understanding this process. Finally, we review two recent papers that have successfully used a combination of these approaches to further our understanding of fertilization.

Early approaches

Early researchers trying to uncover the molecules involved in fertilization relied primarily on biochemical methods. This approach proved to be extremely useful, and yielded many interesting candidates. For example, the murine egg receptor for the sperm, zona pellucida glycoprotein ZP3, was identified by such approaches (Bleil & Wassarman 1980a,b). The generation of antibodies in heterologous systems, use of antibody suppression of fertilization, and classical protein chemistry helped to identify a host of

molecules that were implicated in fertilization in a multitude of organisms including mice, rats, pigs, sea urchins and humans (Oikawa & Yanagimachi 1975, Dunbar & Shivers 1976, Tsunoda & Chang 1976, Yanagimachi *et al.* 1976). To summarize these studies, sperm preparations from a donor organism (e.g. hamster) were injected into a recipient organism (e.g. mouse) that would then generate antibodies against the sperm antigens. These antibodies were then isolated, purified and tested for their ability to inhibit fertilization in the donor, as well as in other organisms. Such antibodies became valuable tools to identify the actual molecules they bound that mediated fertilization. Indeed, so successful were these strategies, that antibody-based strategies were even being touted as 'contraceptive vaccines' (Isojima *et al.* 1986). Both species-specific antibodies as well as antibodies that affected fertilization in a wide variety of animals were generated by such techniques (Isojima *et al.* 1984, Koyama *et al.* 1985, Elce *et al.* 1986).

These early model systems had the advantages of having well-established *in vitro* fertilization protocols in conjunction with standard antibody techniques. What was lacking, however, was *in vivo* genetic evidence to determine the precise roles of these putative candidates in fertilization. In fact, even before the establishment of such genetic evidence, questions were being raised as to the roles of these molecules. Thus, the inhibition of murine fertilization by antibodies against murine ZP2 was found to be mostly due to steric hindrance, rather than the actual abolition of a function required for fertilization (East *et al.* 1984).

Table 1 Summary of genes/loci discussed.

Gene/locus	Organism	Gamete	References
ZP3	Mouse	Oocyte	Bleil & Wassarman (1980a,b)
<i>t</i> -haplotype	Mouse	Sperm	Olds-Clarke & Carey (1978)
<i>Unfertilizable</i>	<i>Xenopus</i>	Oocyte	Kubota & Asada-Kubota (1993)
<i>casanova</i>	<i>Drosophila</i>	Sperm	Perotti <i>et al.</i> (2001)
<i>misfire</i>	<i>Drosophila</i>	Sperm	Ohsako <i>et al.</i> (2003)
<i>sneaky</i>	<i>Drosophila</i>	Sperm	Fitch & Wakimoto (1998)
<i>spe-9</i>	<i>C. elegans</i>	Sperm	Singson (2001)
<i>pph-4.1</i>	<i>C. elegans</i>	Sperm	Sumiyoshi <i>et al.</i> (2002)
<i>Mos</i>	Mouse	Oocyte	Stein <i>et al.</i> (2003)
<i>TRPC2</i>	Mouse	Sperm	Jungnickel <i>et al.</i> (2001), Leypold <i>et al.</i> (2002)
<i>TRPC4</i>	Mouse	Sperm	Freichel <i>et al.</i> (2001), Tiruppathi <i>et al.</i> (2002)
<i>trp-3</i>	<i>C. elegans</i>	Sperm	Xu & Sternberg (2003)
<i>SED1</i>	Mouse	Sperm	Ensslin & Shur (2003)

One of the first instances of the use of a genetic model system to study fertilization was with *t*-haplotype mice. Mice that had this particular haplotype were found to have distorted segregation ratios – sperm from males that were heterozygous for the *t*-haplotype were found to have unequal distribution of progeny that carried either the *t* or the wild-type haplotype (Olds-Clarke & Carey, 1978). This implied that there was something on the surface of these *t*-haplotype-bearing sperm that affected fertilization, and there were a flurry of papers addressing this issue (Mcgrath & Hillman 1980, Tucker, 1980). Such genetic systems were rather rare, however, and another approach used to augment biochemistry was the use of pharmacological agents. Once again, systems in which *in vitro* fertilization was possible proved indispensable. Studies in algae and in sea urchins using known pharmacological inhibitors and monoclonal antibodies showed the importance of calcium ions in the process of egg activation and fertilization (Snell *et al.* 1982, Trimmer *et al.* 1986).

Current approaches

Current genetic approaches can be viewed under two broad headings (Fig. 1). In the first approach, no prior knowledge of the molecules involved in the process of fertilization is assumed. The organisms being used are randomly mutagenized; animals with interesting phenotypes are then collected and analyzed to determine whether the phenotype is worth studying. Finally, the gene that has been mutated in each case is cloned and its product is characterized (Fig. 1a). Such a combination of screens in which we have no previous information regarding the molecules involved in a particular process is referred to as 'forward genetics.'

In the reverse genetics approach, interesting candidate gene sequences are identified via their implication in a process, and are then analyzed using a variety of techniques to eliminate their function in the organism (Fig. 1b). Such techniques include homologous replacement of a wild-type locus by a mutated form of the protein (called targeted mutation, or homologous gene replacement) and

the use of sequence-specific double-stranded RNA molecules to knock down the expression of a particular gene, phenocopying mutations in that gene (termed RNA interference, or simply, RNAi).

In the frog *Xenopus laevis*, forward genetics uncovered a mutant called *unfertilizable* (*Uf*). Oocytes produced by this mutant could not be fertilized by sperm, either *in vivo* or *in vitro*. Careful analysis of the mutant phenotype indicated that the mutation caused a change in the external egg-jelly coat that prevented successful interactions with sperm (Kubota & Asada-Kubota 1993). The molecular nature of the mutation still awaits description.

Most of the forward genetics studies to date on fertilization have been carried out in the nematode *Caenorhabditis elegans* and in the fruit fly *Drosophila melanogaster*. An early screen performed in *Drosophila* resulted in the generation of a number of interesting flies containing dominant mutations causing decreased numbers of offspring (Szabad *et al.* 1989). These mutations specifically affected only females, and were therefore called female-sterile (Fs) mutations. A subset of these mutations was found to affect the process of fertilization. More recently, a mutation called *casanova* was discovered in flies (Perotti *et al.* 2001), which caused male sterility. Sperm from these mutant males appeared normal, had wild-type motility, and were transferred to the site of fertilization. However, close scrutiny of these sperm showed that they had severely decreased beta-*N*-acetylglucosaminidase activity, an enzyme involved in the production of the polysaccharides of glycoproteins. Even more interestingly, ultrastructural studies on these sperm indicated that the sub-cellular localization of this enzyme was affected in the sperm of *casanova* mutants, such that the sperm lacked the enzyme in the acrosome region, although localization to the terminal end of the tail was unchanged. This study further underlined the seemingly global theme of the role of polysaccharides in the process of sperm–egg interactions – a theme that has been well established by the use of lectins and enzymatic approaches in other model systems (Nicolson *et al.* 1975, Oikawa *et al.* 1975, Hirohashi & Lennarz 2001).

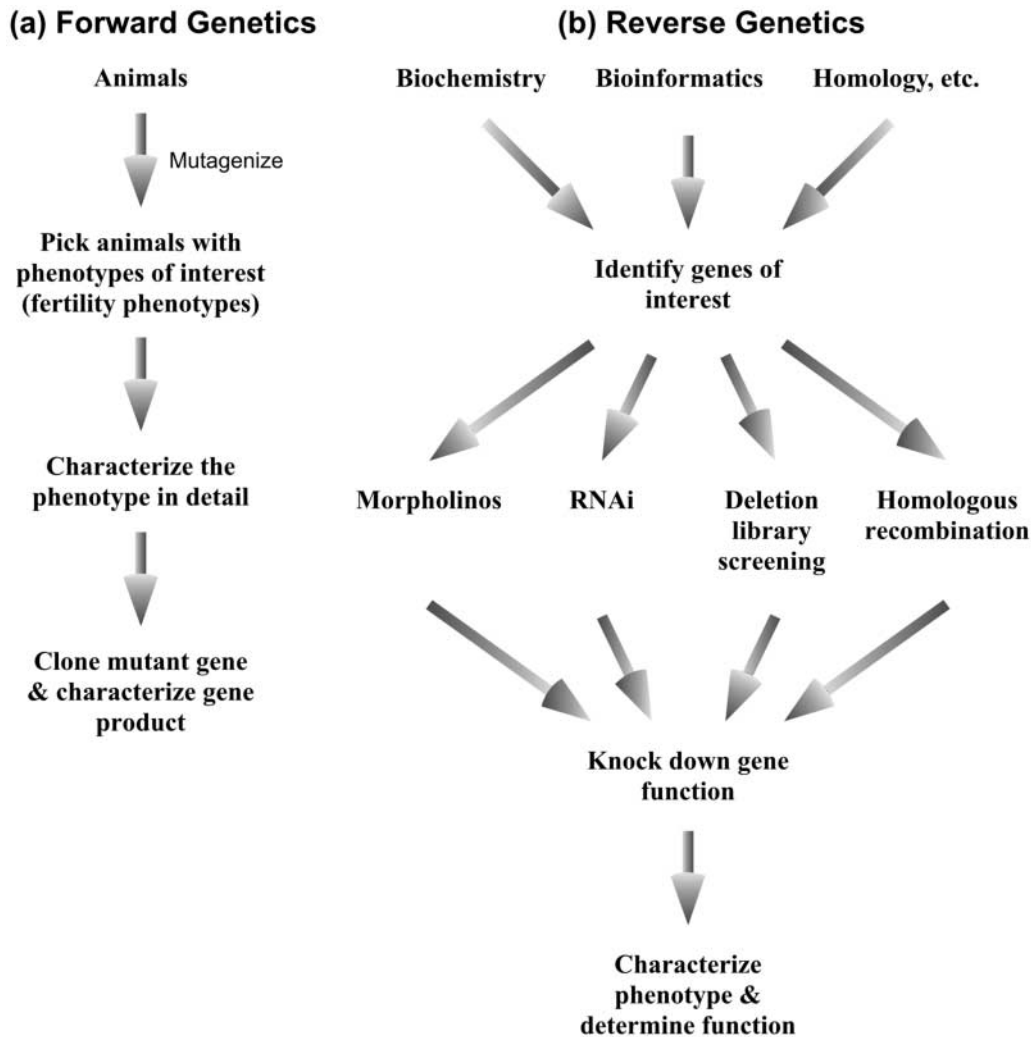


Figure 1 Types of genetic approaches. (a) The forward genetic approach proceeds from a phenotype to the gene responsible for that phenotype. (b) In the reverse genetics approach, a gene sequence of interest is first identified, and then analyzed via methods to eliminate its function.

Another interesting mutant discovered recently in flies is the *misfire* mutant (Ohsako *et al.* 2003). Although sperm from these mutants successfully enter eggs, they fail to undergo the next series of steps that are essential for proper fertilization, including nuclear decondensation and the first nuclear division. This phenotype is very similar to the *sneaky* mutation (Fitch & Wakimoto 1998), and taken together, the characterization of the molecular nature of the defects in these mutants should greatly enhance our understanding of the later steps of fertilization that occur after the sperm enter the egg.

The model organism *C. elegans* has also been firmly established as an excellent system in which to study fertilization. Despite the fact that *C. elegans* sperm are morphologically distinct from mammalian sperm (*C. elegans* sperm are amoeboid and move by crawling), they must perform the same basic functions as sperm from other organisms – they must locate and localize to the site of fertilization; correctly recognize, fuse with and activate the egg; and initiate development (Singson 2001). The generation of a series of mutants in *C. elegans* has served to delineate genetically the pathway for sperm development

and function (L'Hernault, 1997). The *spe-9* mutant is one of a collection of mutants (now termed the *spe-9* class, after the first member to be cloned) that make sperm specifically impaired in their ability to fertilize oocytes (Singson 2001). Genetic screens are also underway to generate mutants that have defects in the egg that prevent it specifically from being fertilized by sperm. The cloning and characterization of these mutants will help elucidate the molecular pathways leading to a successful fertilization event.

A number of other vertebrate organisms are also being established as tractable genetic model systems. Large-scale forward mutagenesis in mice (Nolan *et al.* 2000), zebrafish (Solnica-Krezel *et al.* 1994, Haffter *et al.* 1996) and medaka fish (Loosli *et al.* 2000) have all yielded a multitude of interesting developmental phenotypes and it is only a matter of time before such screens are used to study the molecular mechanisms of fertilization in these organisms

In addition to the above-mentioned forward genetic approaches, reverse genetics methods are being increasingly utilized for determining gene function during

fertilization. Given the large amounts of data being generated from genomics studies, the prospect of taking an interesting candidate gene and quickly identifying its role via reverse genetics is an attractive one, and the use of reverse genetic approaches is likely to increase. We will discuss some of these methods and how they may be used/are being utilized to further our understanding of the molecules involved in fertilization.

Knock-down methods: RNAi and morpholinos

RNAi

RNAi is the phenomenon whereby double-stranded (ds) RNA exposure causes a sequence-specific gene silencing in an organism. RNAi was first discovered in *C. elegans*, when Fire *et al.* (1998) showed that injection of dsRNA into the gonad of adult worms was a much more potent inhibitor of endogenous gene function than either sense or anti-sense single-stranded RNA alone. This phenomenon has subsequently been shown to occur following soaking (Tabara *et al.* 1998) or simply feeding *C. elegans* dsRNA or bacteria engineered to express it (Timmons & Fire 1998, Kamath *et al.* 2001, Timmons *et al.* 2001). Exposure to dsRNA triggers gene silencing both systemically in the treated animal, and in its F1 progeny. The phenomenon also occurs in other organisms such as *Drosophila* (Kennerdell & Carthew 2000) and appears to be related to gene silencing in other organisms such as the post-transcriptional gene silencing of plants and quelling of *Neurospora* (Romano & Macino 1992).

RNAi has become a powerful technique for the analysis of gene function, and is becoming increasingly employed as a quick loss-of-function approach. In a recent study by Sumiyoshi *et al.* (2002), RNAi was used to test the *in vivo* function of protein phosphatase-4.1 (PPH-4.1) in *C. elegans*, where results suggest that this serine/threonine protein phosphatase is essential for mitotic spindle formation and formation of spindles in sperm meiosis. It should be noted, however, that at least *C. elegans* sperm seem refractory to RNAi (for reasons that are not entirely clear); this may limit the usefulness of the technique for studying such functions as spermatogenesis in the worm. Whether this is also true in other organisms remains to be seen.

Oocyte studies have benefited from the technique as well. In another recent experiment, Stein *et al.* (2003) for example, used the ZP3 promoter to drive expression of a long hairpin dsRNA construct targeted to *Mos* mRNA to knock down this gene in mouse oocytes. Similar approaches should allow for the study of any oocyte-synthesized gene.

In addition to targeted single-gene studies, large-scale systematic RNAi screens are now being performed (Gartner 2003, Kamath & Ahringer 2003, Kamath *et al.* 2003). The screening of an ovarian cDNA library by Piano *et al.* (2000), for example, enabled the identification of 81 genes with essential roles in embryogenesis, while avoiding the

problem of maternal contribution masking gene knockout effects. Kamath *et al.* (2003) constructed an RNAi library of 16 757 bacterial clones containing approximately 86% of the *C. elegans* genome and identified mutant phenotypes for 1722 genes, two-thirds of which were not previously identified with a phenotype. Another recent genome-wide screen of the *C. elegans* genome identified 27 genes involved in transposon silencing by looking for genes that when knocked down promoted transposition of Tc1 (Vastenhouw *et al.* 2003). Although RNAi has proven its worth as a technique for the rapid analysis of gene function, as with any non-mutant loss-of-function technique, careful interpretation of results is required. Phenotype severity may vary within an experimental group, for example, and not every organism exposed to dsRNA may show a phenotype at all, confounding results and making careful controls a must. Additionally, it is important to distinguish between actual loss-of-function phenotypes and artifactual effects resulting from the exposure method itself (injection, for example).

With the development of genome-wide RNAi libraries available for gene screening combined with the ease and rapidity of the technique, we expect to see increased adoption of the method in the future.

Morpholinos

Morpholinos are DNA analogs that contain a morpholine moiety in place of the riboside moiety, and phosphorodiamidate intersubunit linkages in place of phosphodiester linkages (Summerton & Weller 1997). Usually 25-mers, morpholinos act by selectively blocking translation of target mRNA (presumably by interfering with ribosome binding) when complementary to 5' leader sequences or to the first 25 bases 3' to the start site.

Although first developed for clinical therapeutic applications (Summerton & Weller 1997), morpholinos are fast becoming a loss-of-function method of choice for identifying gene function in model organisms. From their first use in *Xenopus* embryos to examine β -catenin signaling activity in blastulas (Heasman *et al.* 2000), they are now being utilized extensively in zebrafish (Nasevicius & Ekker 2000), as well as in other organisms such as chick (Kos *et al.* 2003), mouse (McCaffrey *et al.* 2003) and sea urchin (Amore *et al.* 2003). Although morpholinos have not, as of this writing, been used to identify molecules with roles in fertilization specifically, it should only be a matter of time before the method will prove itself useful for furthering our understanding of this process.

Since morpholino-induced phenotypes are not genetic mutations, the same caveats apply to the interpretation of morpholino experiments as they do to other knock-down methods such as RNAi. For example, phenotypes may be due to non-specific side-effects, may represent severities ranging from partial loss of function to null, and may be dependent upon factors such as morpholino concentration, diffusion, rate and amount of new

mRNA transcription, localization of targeted mRNA and amount and stability of protein whose mRNA is targeted at the time of injection (Heasman 2002). By employing proper controls and confirming results with other methods of gene study, morpholinos are poised to become a valuable tool in the search to identify gene functions at fertilization.

Deletion library screening

Although, with careful interpretation, knock-down methods such as those just discussed can provide a relatively quick and easy way to assess gene function, they do not provide the researcher with the actual germ-line disruptions necessary for further genetic manipulations and downstream studies such as genetic crosses, enhancer/ suppressor screens, etc. (Liu *et al.* 1999). Deletion library screening is one powerful, large-scale method for deriving actual mutations in target genes known only via their sequence and is used extensively in the nematode *C. elegans*, where other targeted mutagenesis methods such as homologous recombination and transposon insertion are not well established. In this method, chemical mutagens are used to induce mutations in a population of organisms (Yandell *et al.* 1994, Jansen *et al.* 1997, Liu *et al.* 1999), which are then screened by PCR for deletions within a particular target gene (Fig. 2).

The power of such a strategy was utilized in a recent study to demonstrate the importance of transient receptor potential (TRP) channels in the process of fertilization (Xu & Sternberg 2003). A large body of previous work had implicated these TRP channels in a number of processes including visual transduction, nociception, sexual behavior and fertilization (Harteneck *et al.* 2000, Montell *et al.* 2002a). TRP channels are a large and diverse superfamily of proteins that function as cation channels and have been found in every multicellular organism whose genome sequence has been searched (Montell *et al.* 2002b). These channels are regulated in a variety of ways and play a role in a number of different pathways. The TRP channels can be broadly classified into three major sub-families: TRP-canonical (TRPC), TRP-vanilloid (TRPV) and TRP-melastatin (TRPM) channel types. Except for their role in visual transduction in *Drosophila*, little is known about either the mechanisms by which these channels are activated and regulated, or the precise nature of the functions they mediate. Although antibody studies had implicated the murine TRPC2 channel in the acrosome reaction of sperm *in vitro* (Jungnickel *et al.* 2001), sperm from mice in which this molecule is mutated are still completely fertile (Leybold *et al.* 2002). Similarly, sperm from mice mutant for TRPC4 were also fertilization competent (Freichel *et al.* 2001, Tirupathi *et al.* 2002).

Thus, researchers looked elsewhere for clues as to how the TRP channels might be working. The *C. elegans* genome contains a number of TRPC channels, one of which,

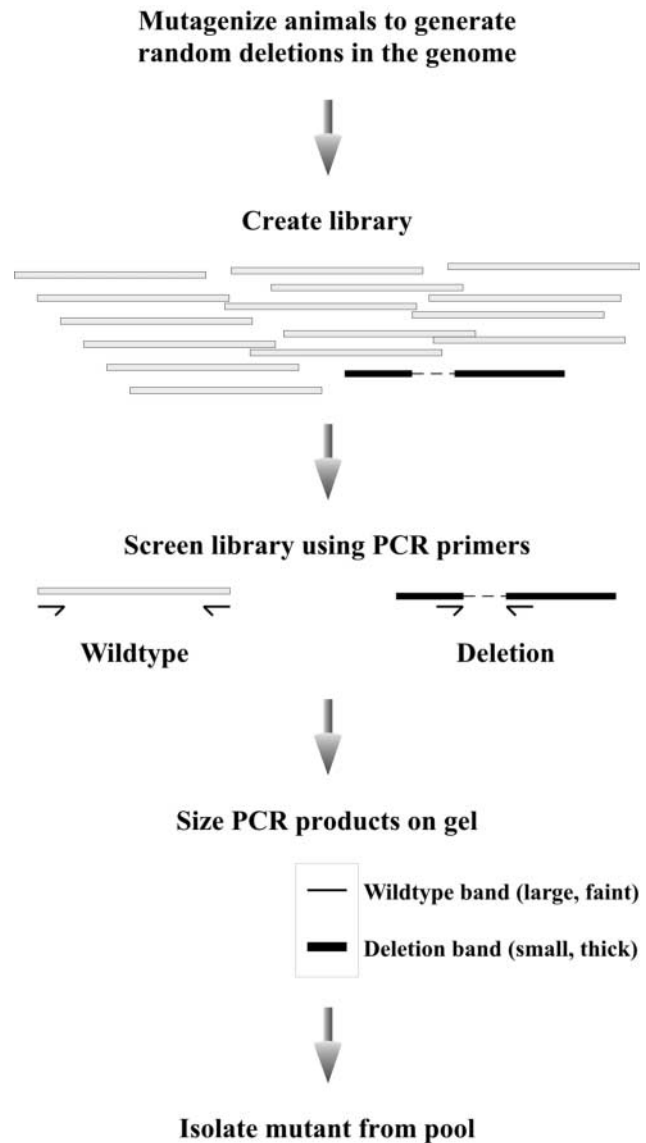


Figure 2 Deletion library screening. Organisms are first mutagenized to induce DNA deletions. A DNA library (shown here as a schematic representation of genome fragments with the deletion-containing gene of interest in bold) is next created by lysing the animals, extracting and pooling their DNA. PCR is used to screen the library for deletions in the gene of interest. Deletion-containing populations are sub-divided to isolate a single organism homozygous for the deletion.

TRP-3, was found to be sperm enriched. In an attempt to determine the function of this molecule, a well-designed series of experiments was performed (Xu & Sternberg 2003). First, a deletion library was screened for mutations in the *trp-3* locus. This screening resulted in the identification of two *trp-3* alleles, both of which caused recessive sterility in the worms. The sterility was not due to defects in the oocyte, since the oocytes appeared to be normal in every functional assay. Furthermore, wild-type sperm could fertilize the oocytes of *trp-3* mutants, confirming that the defect in *trp-3* mutants lay in sperm function.

Sperm from *trp-3* mutants showed no gross morphological abnormalities, underwent spermatogenesis like wild-type sperm, made their way to the spermatheca (the site of fertilization in *C. elegans*) and were stored in numbers large enough to ensure fertilization. Sperm from mutant males could suppress the self-fertility of hermaphrodites with wild-type sperm, confirming that *trp-3* sperm were capable of successful sperm competition – which in worms is independent of a sperm's ability to fertilize the egg, but dependent on both motility and sperm size. Although Xu and Sternberg claim to have shown that mutant sperm bind the oocyte, they show only a single sperm in close proximity to an oocyte as evidence for this. Considering that the sperm and the oocytes are released from the gonad by cutting open the worm and spewing out its contents, it is conceivable that the juxtaposition of the single sperm and the oocyte is mere coincidence. Still, in every conceivable functional assay, *trp-3* mutant sperm were indistinguishable from wild-type sperm. Despite this, and the fact that these sperm contact the oocyte, they cannot successfully complete fertilization, meaning that these sperm must be defective specifically in a molecule required for fertilization.

Knowing the nature of the TRP channel that they had mutated, Xu & Sternberg (2003) chose to study the dynamics of calcium exchange in wild-type and mutant sperm. They found that store-operated calcium exchange (SOCE; the influx of calcium into cells whose calcium stores have already been depleted) was decreased in mutant spermatozoa as compared to wild-type. In fact, TRP-3 increased calcium influx into cells even when expressed in a mammalian cell line. However, SOCE was unaffected in mutant spermatids. This seeming paradox was reconciled by antibody staining. In spermatids, TRP-3 is found in membranous organelles (MOs), whose fusion with the plasma membrane is an obligatory step in spermiogenesis in *C. elegans*. This is evinced by the co-localization of TRP-3 with an MO marker, 1CB4 (Okamoto & Thomson 1985). After the MOs fuse with the plasma membrane and at the completion of spermiogenesis, 1CB4 is found only on the cell body, whereas TRP-3 is found on both the cell body and the pseudopod, no longer co-localized with 1CB4. This suggests sub-cellular localization as a mechanism of regulating the activity of TRP-3 and explains why the SOCE defect is seen only in mature spermatozoa. TRP-3 might be activated downstream of phospholipase C (PLC), since *in vitro* studies involving expression of TRP-3 in mammalian cells have found that activation of PLC β leads to an increased influx of calcium into cells in a TRP-3-dependent manner.

Taken together the data indicate that TRP-3 is an important player in the fertilization pathway in *C. elegans*. TRP-3 is at least partially regulated by sub-cellular localization and is sequestered in intra-cellular compartments (MOs) prior to spermiogenesis. Following spermiogenesis, TRP-3 is found distributed throughout the plasma membrane, both on the cell body as well as on the pseudopod.

Here, it is activated by an as-yet-unknown mechanism, possibly involving PLC β , which by allowing an influx of calcium, enables sperm to successfully fertilize the oocyte (Jungnickel *et al.* 2003).

This study lays the foundation for further investigation into the biological significance of TRPC channels. Enhancer and suppressor screens can be performed in the *trp-3* mutant background to reveal molecules that activate, regulate and affect TRP-3 functions. The role of TRPC channels can now be reinvestigated in mammalian fertilization, not only for their ability to influence the acrosome reaction, but also for gamete adhesion and fusion. It would also be interesting to see what effects knocking out the other *C. elegans* TRPC channels may have on the worm.

Homologous recombination

Although at least some genes required for gametogenesis have been identified via random mutagenesis in the mouse (Ward *et al.* 2003), the larger, more complex genomes of the organisms commonly utilized to study more complex biological processes make this method relatively unpractical (Capecchi, 2001). Thus, although homologous recombination has recently been utilized in systems such as plants (Hanin & Paszkowski 2003) and *Drosophila* (Rong, 2002, Rong & Golic 2001), the majority of gene-targeting attempts using homologous recombination have been carried out in the mouse – disrupting target genes in order to determine function. Much of our current understanding of the molecules involved in fertilization comes from such knockout studies and other genetic manipulations in mice (Rankin *et al.* 1998, 2003, Wassarman *et al.* 2001, Primakoff & Myles 2002).

The recent identification of SED1 as a protein required for mouse sperm to bind the egg ZP may serve as an example (Ensslin & Shur 2003). The authors first used reverse genetics (homology-based cloning) to identify SED1 based on its homology to p47, a porcine sperm surface protein first identified biochemically by affinity chromatography on a ZP3 column (Ensslin *et al.* 1998). Antibodies to SED1 revealed its localization on the surface of sperm, at the area initially responsible for ZP binding – the acrosome (Wassarman *et al.* 2001, Primakoff & Myles 2002). In competitive inhibition assays both anti-SED1 immunoglobulin G (IgG) and recombinant SED1 were shown to inhibit sperm binding to the ZP, and truncated fusion protein experiments suggested a requirement for SED1's discoidin/C domain for this inhibition.

To test the ability of SED1 to bind to the ZP directly, Ensslin & Shur added intact and truncated SED1-conjugated beads to ZP sources. Both intact and truncated forms of the protein bound directly to unfertilized oocytes, with higher binding levels observed in SED1 constructs containing both of the protein's discoidin/C domains. Neither intact nor truncated SED1 bound to embryos. Subsequent experiments using recombinant SED1 bound to

purified ZP showed that SED1 binds specifically to ZP2 and ZP3, but not to ZP1.

To determine more directly the function of SED1 in the mouse, homozygous SED1 null mice were created via homologous recombination. By comparing litter sizes of mice mated with either wild-type or SED1 null mutants, Enslin & Shur (2003) showed reduced male fertility in SED1 nulls, as evidenced by their siring smaller broods than wild-type.

The ability of SED1 null sperm to bind the ZP *in vitro* was also compared with that of wild-type sperm. SED1 null sperm were shown to bind at near-background levels, despite normal sperm morphology, number, motility and level of basal acrosome reactions. Their results are consistent with the proposed function of SED1 in sperm–egg binding, as suggested by the antibody and biochemical approaches. The use of a reverse genetics approach (the creation of SED1 null mutants) established the biological relevance of this molecule in sperm–egg adhesion.

Conclusion

As genomic studies continue to present interesting genes, the use of genetic approaches for assessing gene function and establishing biological relevance is likely to increase. An integrated approach, combining these methods with the more traditional biochemical and antibody-based methods used to study the molecules of fertilization, will continue to help further our understanding of this fascinating biological process.

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