

Marriage shrines and worms impacting our understanding of mammalian fertilization

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ABSTRACT

Genetic approaches in *C. elegans* are complementing the biochemical and antibody based strategies traditionally used to study the molecular underpinnings of fertilization in other organisms. A pair of worm studies, one based on forward genetics and one based on reverse genetics, converge on the sperm immunoglobulin superfamily molecule SPE-45. Loss of *spe-45* function leads to the production of sperm that cannot fertilize wild-type eggs. This is a strikingly similar phenotype as those seen in mice lacking the immunoglobulin superfamily protein Izumo1. This work sets the stage for leveraging the power of the *C. elegans* model system to learn more about Izumo-like molecular function but also for the discovery of additional deeply conserved components of fertility pathways.

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Most of us have seen generic images of sperm and eggs interacting. What surprises most people is that our understanding of the genetic and molecular underpinnings of fertilization is quite limited. We do not have microscopes that can show us individual molecules such as cell receptors or hormones in action. The many ethical and experimental limitations to studying human fertility directly add additional challenges.

A little more than a century ago, research on fertilization was conducted mostly on marine invertebrates whose gametes are relatively easy to obtain.¹ This tradition has lived on with the biochemical purification of many molecules hypothesized to function on the sperm or egg surface during fertilization.² In mammals, sperm and eggs are difficult to obtain and manipulate.³ However, biochemical and antibody based approaches have still identified candidate molecules with a role in gamete interactions during mammalian fertilization.⁴ Unfortunately, genetic evidence for a direct role in gamete interactions at fertilization exists for only a few genes.^{3,5} At present, the only mammalian sperm protein both genetically and biochemically verified to be necessary and sufficient for binding to the egg is Izumo1 (named after a

Japanese shrine dedicated to marriage).^{6,7} The paucity of fertility gene discovery in marine invertebrates and mammals could be due to the challenges of applying mutant screening strategies and the difficulty of maintaining strains of animals with fertility defects.

Recently, the nematode worm *Caenorhabditis elegans* has been emerging as a model system for understanding the molecular underpinnings of fertilization.^{4,8} Both forward and reverse genetic strategies have been used to identify molecules required for worm fertilization.^{9–11} Most of the molecules discovered in *C. elegans* sit on the surface of sperm or eggs and have features that support the idea that they are involved in sperm-egg signaling, binding, and/or fusion.⁸ However, the sequences of these genes have not matched up very well with the few other sperm and egg molecules discovered in traditional fertilization model systems. The lack of known deep molecular conservation of fertility genes has now changed with the publication of 2 complementary reports in *Current Biology* describing the discovery of the *spe-45* gene in *C. elegans*.

Through differing approaches, one forward genetics and one reverse genetics, our laboratory and the

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laboratory of Dr. Steven L'Hernault converged on the finding that *C. elegans* have an Izumo-like protein that is required for fertilization.^{12,13} Harnessing the genetic power of *C. elegans*, our lab carried out a forward genetic screen to isolate *C. elegans* mutants defective in fertilization and generate a more complete inventory of the molecules required for gamete interactions. The first mutant that we characterized from our screen was a temperature-sensitive allele that we mapped to the predicted gene *F28D1.8*. At the same time, Nishimura et al. took a reverse genetic approach and asked whether *C. elegans* have an Izumo-like protein required for fertilization, specifically addressing the hypothesis that some fertility molecules are conserved between mammalian and non-mammalian model systems. Their search of the *C. elegans* genome and follow up phenotypic analysis led them to *F28D1.8*. We later showed that the allele isolated from our screen fails to complement the deletion allele of *F28D1.8* characterized by Nishimura et al., confirming that the same gene was responsible for the sterile phenotype observed in both labs. Originally named *oig-7* based on the predicted protein structure, *F28D1.8* has been renamed *spe-45* due to its sperm-specific function.

SPE-45 is functionally similar to Izumo1

Loss of *spe-45* function in *C. elegans* produces a sterile phenotype restricted to male germline function.^{12,13} Hermaphrodites lacking *spe-45* are self-sterile, which could be due to a defect in sperm, eggs, or both, but 2 pieces of evidence suggest only sperm are affected. First, males lacking *spe-45* are unable to produce cross progeny when mated to hermaphrodites, as one would expect if sperm function is impaired. Second, *spe-45* hermaphrodites can produce progeny when mated to males that make wild-type sperm, indicating that they have fertilization- and developmentally-competent eggs. Loss of *spe-45* function does not result in any noticeable somatic defects. Thus, SPE-45 is required for fertility and acts exclusively in the sperm.

So what is wrong with *spe-45* sperm? We can observe sperm within the spermathecae of *spe-45* hermaphrodites and dissected *spe-45* males release large numbers of spermatids that are morphologically indistinguishable from wild-type; so production of sperm is not the problem. Likewise, *spe-45* sperm do not display any defects in sperm activation. When *C. elegans* sperm are activated, they form a pseudopod, acquire

motility, and become fertilization-competent.¹⁴ Sperm dissected out of *spe-45* hermaphrodites appear identical to the activated spermatozoa dissected from wild-type hermaphrodites. Male *spe-45* sperm can also be activated *in vitro* with activation rates and efficiencies that are comparable to controls. Finally, mating experiments additionally confirm that *spe-45* sperm are phenotypically normal up until the point of fertilization. By mating *spe-45* males to feminized worms that don't produce any self-sperm, transfer and proper migration of sperm to the spermathecae (the site of fertilization) can be observed. The sperm from *spe-45* males can even out-compete the self-sperm of fertile hermaphrodites, as seen by a reduction in the number of self-progeny even though no cross-progeny are produced.

Despite having no observable defects up to the point of contact, *spe-45* sperm fail to fertilize eggs. We were unable to observe any paternal DNA in the eggs dissected from the uteri of *spe-45* hermaphrodites. Another indicator that these eggs are not fertilized is their single large DNA mass. Unfertilized eggs undergo endomitotic DNA replication, but since sperm are responsible for delivering the centrosomes, the DNA remains as a single mass in the absence of sperm entry. Nishimura et al. also showed that the self-sperm of *spe-45* hermaphrodites are retained in the spermathecae past the point when wild-type animals have normally depleted their store of sperm. Since sperm production is limited to a specific stage of larval development, hermaphrodites have a finite supply of self-sperm for fertilizing their eggs. As progeny are produced this supply of sperm is used up. Consequently, wild-type unmated hermaphrodites have almost no sperm remaining in the spermathecae by the third day of adulthood. In contrast, unmated *spe-45* hermaphrodites still had numerous sperm in their spermathecae as 3-day adults. This is consistent with *spe-45* sperm being unable to fertilize eggs and thus not being depleted in the manner observed in fertile animals.

Overall, we and Nishimura et al. showed that SPE-45 is required specifically for *C. elegans* sperm to successfully fertilize eggs. The phenotype that we observed for *spe-45* animals is incredibly similar to the phenotype of mice deficient for the immunoglobulin super-family protein Izumo1. In the absence of Izumo1, mice are completely healthy, females are fertile, but males are sterile.⁷ In addition, sperm from

Izumo1-deficient males have normal morphology, can migrate to the egg, undergo the acrosome reaction, and penetrate the zona pellucida (egg coat).⁷ So just like sperm from *spe-45* worms, sperm from *Izumo1*-deficient mice appear completely normal up to the point of contacting the plasma membrane of the egg, but fertilization fails to occur. Based on these data, we propose that SPE-45 and Izumo1 are carrying out similar functions mediating sperm-egg binding and/or fusion. But it takes more than phenotypic similarities to drive the hypothesis that these proteins are functional homologs demonstrating deep molecular conservation in the process of fertilization.

SPE-45 has a similar molecular architecture to Izumo1

After the identification of Izumo1 in mice, Nishimura et al. predicted that *C. elegans* might also have an Izumo1-like protein that functions during fertilization. However, no Izumo1 orthologs are detectable in the *C. elegans* genome by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). This led them to carry out a more in depth search based on the known domain structure and expression pattern of Izumo1. Mouse Izumo1 contains a single transmembrane domain, a single immunoglobulin (Ig)-like domain, and shows testis-specific expression (Fig. 1).⁷ Using the SMART (Simple Modular Architecture Research Tool) program¹⁵, they identified 8 genes in the *C. elegans* genome that

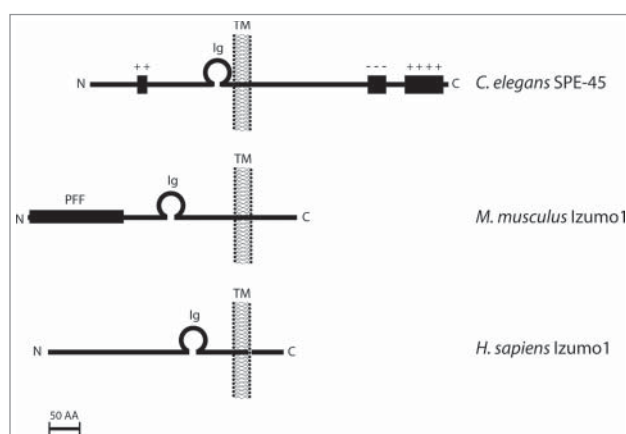


Figure 1. Schematic diagram showing the topology of Izumo-like proteins from *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens*. *C. elegans* SPE-45 has positively and negatively charged regions indicated by + and - respectively. PFF refers to the “putative functional fragment” shown to be sufficient for binding to the egg.⁶ Ig, immunoglobulin-like domain; TM, transmembrane domain; AA, amino acid.

meet the criteria of having one transmembrane domain and one Ig-like domain. Expression data shows that only one of these 8 genes, *F28D1.8*, is enriched in the male germline.^{12,16} The fact that a single gene, *F28D1.8* (now known as *spe-45*), has the same molecular attributes and expression pattern as *Izumo1*, led us and our colleagues to conclude that *C. elegans* does possess an Izumo1-like protein (Fig. 1). The similarity in knockout phenotype, as discussed above, points to a deep evolutionary conservation of this particular molecule as a mediator of fertilization. Together the evidence strongly supports the hypothesis that SPE-45 is orthologous to mouse Izumo1.

Since reproductive proteins are known to evolve quite rapidly,¹⁷ the low sequence identity between the 2 proteins and the inability to find *spe-45* through a straightforward BLAST search, is not surprising. But despite the limited sequence identity, Nishimura et al. showed that a chimeric SPE-45 protein containing the mouse Izumo1 Ig-like domain in place of the SPE-45 Ig-like domain can function in place of the wild-type protein. This is in contrast to a SPE-45 protein where the Ig-like domain had been replaced with the Ig-like domain of a somatic *C. elegans* protein, which could not rescue the *spe-45* phenotype. Thus, the Ig-like domain of Izumo1 and SPE-45 share a functional specificity that cannot be explained by the general presence of an Ig-like domain, lending further support to the orthologous nature of these 2 proteins.

Moving forward worms will continue to impact our understanding of fertilization

Questions still remain regarding the mechanism of SPE-45 function. Based on the *spe-45* phenotype and the role of Izumo1, we predict that SPE-45 is likewise important for sperm adhesion with the egg. As such, we expect that SPE-45 should localize to the plasma membrane of the sperm where it would be available to interact with surface molecules on the egg. Unfortunately, localization of the protein has not been determined up to this point, despite numerous attempts. It is our hope that alternative strategies will eventually answer this persisting question.

Dissection of the functional domains of SPE-45, besides the Ig-like domain, can also complement our knowledge of Izumo1. It is much easier to test *in vivo* function of chimeric or truncated forms of the protein in *C. elegans* compared to mammalian systems. For

example, we do not currently know if the intracellular region is important for function. Nishimura et al. also hypothesized that additional extracellular regions may act in the formation of SPE-45 multimers. Izumo1 is proposed to dimerize after initial binding with its receptor on the egg, so this may be another common attribute of both proteins.^{6,18,19} As the functionally important regions of SPE-45 are identified, they may help direct future studies of Izumo1 in mammals.

Finally, one of the critical aims for the future is to identify the SPE-45 binding partner(s) on eggs. It took nearly a decade after the identification of Izumo1 to find its binding partner—Juno—on mammalian eggs.²⁰ By applying the same techniques that led to the discovery of Juno, it should take significantly less time to find the *C. elegans* egg receptor for SPE-45. And while the presence and function of Izumo1/SPE-45 on sperm is conserved, we are likely to discover that the egg binding partner differs between taxa.

Ig-like domains probably evolved to mediate interactions at the cell surface.²¹ Additionally, Ig-like cell adhesion molecules are one of the most ancient and diverse families of cell adhesion proteins.²² So it is easy to see how a member of this protein class was co-opted early in the evolution of sexual reproduction to mediate sperm binding to an egg. In addition to our finding of SPE-45 in *C. elegans*, a recent study identified potential Izumo1 orthologs in birds, fish, and reptiles lending more support to the deep evolutionary conservation of this molecule.²³ That same study however, suggests that Juno is only present in mammals. Immunoglobulin superfamily (IgSF) proteins are known to interact with cell surface proteins of multiple different classes.²² As such, it presently appears that different species have evolved to utilize different proteins on the egg to interact with the sperm's Izumo1-like protein. Growing evidence also suggests that fertilization is not mediated by a single ligand-receptor pair, but by a complex network of multiple proteins. Even if Juno became an egg receptor specifically during mammalian evolution, we may find that the binding partner of SPE-45 still has a homolog with a supporting role in mammalian fertilization.

C. elegans has the largest repertoire of genes that have been genetically verified to be essential for fertilization.⁸ The discovery of *spe-45* and the deep molecular conservation that it implies between *C. elegans* fertilization and mammalian fertilization indicates the broader significance of gene discovery in our worm

model system. *C. elegans* lends itself particularly well to forward genetic screens making it a powerful tool for the continued discovery of fertilization molecules with genetic validation. Since the same cellular events - gamete recognition, adhesion, and fusion - underlie successful fertilization across taxa is it unlikely that SPE-45/IZUMO1 is the only example of conserved molecules mediating these events. As more genes are discovered they can complement ongoing studies in other model systems and provide new candidates for study in other organisms.

Abbreviation

Ig immunoglobulin

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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