Roles of Sperm Proteins

Chunghee Cho

K-JIST, Department of Life Science

I. Abstract

One of recent advances of mammalian fertilization is the understanding of the molecular basis of fertilization. Several proteins localized in sperm nucleus or on sperm surface are necessary for the fertilization process. Protamines, sperm nuclear proteins, are required for normal sperm function that leads to fertilization. Fertilin and cyritestin are sperm surface proteins and essential for sperm-egg binding. Fertilin is also required for sperm transport in the female reproductive tracts. Metalloproteses on sperm plasma membrane are found to play a role in sperm-egg fusion. The functional analysis of these proteins provides a new insight into the molecular mechanisms underlying mammalian fertilization and male fertility.

II. Introduction

Sperm have a long way to travel and undergo a series of events for fertilization. After spermatogenesis, a sperm head leaving the testis becomes tightly packed. Ejaculated sperm having entered the uterus are rapidly transported into the oviduct. In the oviduct, eggs are surrounded by the cumulus mass. Sperm undergo capacitation and pass through the cumulus mass to reach the surface of zona pellucida, an egg coat made of glycoproteins. When sperm adhere to the zona pellucida, they bind to one of three zona glycoproteins and this binding induces sperm acrosome reaction. Subsequently, sperm penetrate the zona pellucida and sperm head makes contact with egg plasma membrane. The sperm head bound to the egg plasma membrane is incorporated into the egg cytoplasm via membrane fusion.

Several proteins present in sperm have been proposed to be important for the fertilization process. First, protamines are the major DNA-binding proteins in the nucleus of mammalian sperm. They are small basic proteins rich in arginine and contain abundunt cysteine residues. Humans and mice have two protamines (*Prm1* and *Prm2*). Protamine gene expression occurs only from the haploid genome present in spermatids. Protamines have been proposed to have a role in packaging sperm DNA. Second, fertilin and cyritestin, members of the ADAM (A Disintegrin And Metalloprotease) family, are found on the plasma membrane of mammalian sperm head. The typical domain strucutre of an ADAM includes a signal sequence followed by pro-, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor-like, transmembrane and cytoplasmic tail domains. Among the 29 known ADAMs, 12 show testis specific expression and two of them (fertilin β, ADAM 2 and cyritestin, ADAM 3) have been studied to define their functions in fertilization.

Fertilin β and cyritestin are made as precursors in spermatogenic cells and are processed before sperm maturation is complete. Both precursors are composed of the multiple domains found in all ADAM family membrane proteins as described above. Proteolytic processing removes the pro- and metalloprotease domains, leaving an N-terminal disintegrin domain on mature sperm. It has been proposed that the disintegrin domains of fertilin and cyritestin mediate sperm-egg plasma membrane binding. Finally, sperm-egg fusion occurs after binding. Sperm-egg plasma membrane fusion mechanism is not well understood. Zinc metalloproteases are necessary for some intercellular fusion events. Results from yeast fusion studies suggest that cellular fusion events, potentially related to mammalian gamete fusion, involve zinc metalloproteases. Metalloproteases have previously been reported to have important roles during fertilization. However, the proposed metalloproteases are posited to act on sperm during acrosome reaction, not during sperm-egg fusion. Thus it remains to be determined if metalloproteases are directly involved in sperm-egg fusion.

III. Experimental Methods

1. Gene Targeting

A specific exon of the fertilin β (*Ftnb*), cyritestin (*Cryn*) or protamin (*Prm*) genes was disrupted by homologous recombination in 129 embryonic stem (ES) cells. The $Ftnb^{+/-}$, $Cryn^{+/-}$ or $Prm^{+/-}$ ES cells were injected into C57BL/6 blastocysts and chimeric mice were produced from these blastocysts. Chimeric mice were directly analyzed (for protamine) or were mated to produce heterozygous or homozygous mutant mice (for fertilin or cyritestin).

2. *In Vitro* Fertilization

To analyze sperm proteins, two types of *in vitro* fertilization assays were employed. The first assay is a sperm-egg zona pellucida binding assay. The second assay is a sperm-egg plasma membrane binding and fusion assay. In this assay, egg zona pellucida was removed by chymotrypsin digestion. Sperm were obtained from the epididymis or uterus from females mated with male mice.

3. Sperm Analysis

Various parameters were evaluated to analyze sperm. The numbers of epididymal or ejaculated sperm were counted. The morphology of sperm were evaluated by microscopic observation using phase contrast, Normaski, and fluorescence optics. Sperm motility was evaluated by computer assisted sperm analysis (CASA). Acrosome reaction was evaluated by Commassie blue staining.

IV. Results and Discussion

1. Protamines

To determine if protamines are necessary for an essential process of fertilization, gene targeting approach was employed. The coding sequence of one allele of either *Prm1* or *Prm2* was disrupted in ES cells derived form 129-strain mice, and the ES cells were injected into blastocysts from C57BL/6-strain mice. Male chimeras produced 129-genotype sperm with disrupted *Prm1* or *Prm2* alleles, but failed to sire offspring carrying the 129 genome. Morphological and biochemical studies were carried out to determine how the mutations were interfering with transmission of the 129 genotype during reproduction. First, 129 sperm were morphologically abnormal. Abnormalities seen most frequently were sperm with the flagellum tightly wrapped around the head or with elongated heads having a reduced ventral flexure. Second, 129 sperm showed altered pattern in acridine orange staining, indicative of altered sperm chromatin integrity. Third, the nuclei of 129 sperm were less resistant to chemical disruption than nuclei from wild type sperm, indicating that chromatin assembly is incomplete in 129 sperm. Finally, 129 sperm were defective in motility. Therefore, a decrease in the amount of either protamine disrupted nuclear formation and normal sperm function.

These results indicate that both protamines are essential and that haploinsufficiency caused by a mutation in one allele of *Prm1* or *Prm2* prevents genetic transmission of both mutant and wild-type alleles. Haploinsufficiency is known to affect a variety of processes in diploid cells. Because protamines are expressed in haploid spermatids, it might seem that a mutation in one allele would not affect transcription or translation in the half of the spermatid population with an intact allele. Cytokinesis is incomplete in spermatogenic cells, however, allowing spermatids to share mRNA and proteins through cytoplasmic bridges and to be phenotypically diploid. Many genes are expressed in spermatids and it remains to be determined if a recessive mutation in other genes inherited from the mother, arising spontaneously, or produced by gene targeting, affect transmission of both alleles in the male. Although haploinsuffciency has not been shown previously to disrupt reproduction, protamine-2 deficiency correlates with infertility in humans. This suggests that single-copy mutation in genes encoding protamines or other essential sperm proteins may be a cause of infertility in men with apparently normal sperm production.

2. Fertilin and Cyritestin

To determine the roles of fertilin and cyritestin, fertilin $\beta^{-/-}$ and cyritestin$^{-/-}$ mice were produced by gene targeting. These mice are viable and develop normally. However, male mutant mice, not female mutants, were infertile *in vivo* (98% for fertilin $\beta^{-/-}$ and 100% cyritestin$^{-/-}$). Sperm from these mice were normal in number, morphology, motility and rate of spontaneous acrosome reaction, indicating that infertility is due to defects in other sperm functions. First, fertilin $\beta^{-/-}$ sperm, not cyritestin$^{-/-}$ sperm, were unable to migrate

from the uterus to the oviduct. This suggests that fertilin adhesion activity to epithelium in the uterotubal junction is required for sperm to achieve competence to progress into the oviduct Second, sperm lacking fertilin β or cyritestin were shown to be completely defective in sperm-egg zona binding. The role of fertilin and cyritestin in sperm-zona binding could be due to regulation of other unknown sperm molecules directly involved in zona binding. Finally, sperm from mice lacking fertilin β or cyritestin were found to be deficient in sperm-egg plasma membrane binding. This result is consistent with a direct role of fertilin or cyritestin in interaction between sperm ADAM and egg integrin.

The striking difference between the two ADAM knockouts in fertilization performance is that cyritestin mutant sperm migrate into the oviduct while fertilin mutant sperm do not. The nature of sperm migration is unknown though now it is clear that sperm, in addition to motility, must have some property or receive some signal that makes them competent to move into the oviduct. Sperm lacking fertilin or cyritestin are defective in binding to the zona pellucida. Impairment of binding to the zona pellucida is not due to altered acrosome reaction or motility in mutant sperm. Although fertilin or cyritestin may have a direct role in zona binding, certain problems remain to be clarified in this hypothesis: (1) there is no evidence that an integrin exist in zona pellucida; (2) it is a carbohydrate structure on zona pellucida protein to which sperm bind and it is unclear how a disintegrin active site loop would bind to carbohydrate; (3) several different sperm proteins have been proposed to function as zona adhesion molecules. Neither fertilin nor cyritestin shares sequence homology with any of previously identified candidate proteins for zona binding. An alternative model is that sperm adhesion to zona pellucida depends on unique protein assemblies in the sperm cell membrane. In the absence of fertilin or cyritestin, these assemblies become nonfunctional, losing proteins that are directly required for zona binding. Relevant to previous hypothesis about fertilin and cyritestin function, the findings that the mutant sperm are defective in sperm-egg plasma membrane binding indicate that both proteins have a key role in plasma membrane adhesion. Fertilin β and cyritestin may act either sequentially or concurrently, and the activity of both is required for successful adhesion of a motile sperm to the egg plasma membrane. Although binding through fertilin or cyritestin is important in sperm-egg plasma membrane adhesion, they are not required for sperm-egg fusion. A possible explanation for the differential effects on these two processes is that the mutant sperm can accomplish another type of adhesion that leads directly to fusion.

3. Metalloproteases

To determine if, after sperm-egg binding has occurred, there is a step involving zinc metalloprotease action that is necessary for sperm-egg fusion, *in vitro* sperm-egg plasma membrane binding and fusion assay was performed. First, zinc chelator, 1,10-phenanthroline inhibited sperm-egg fusion but did not decrease sperm-egg binding. Second, Ro31-9790, a synthetic zinc metalloprotease inhibitor, blocked sperm-egg fusion. Finally, tissue inhibitor of metalloprotease-3 (TIMP-3), a biological inhibitor of zinc metalloproteases, also inhibited sperm-egg fusion but not sperm-egg binding. These results indicate a role in gamete fusion for zinc metalloproteases that act after plasma membrane binding and before sperm-egg

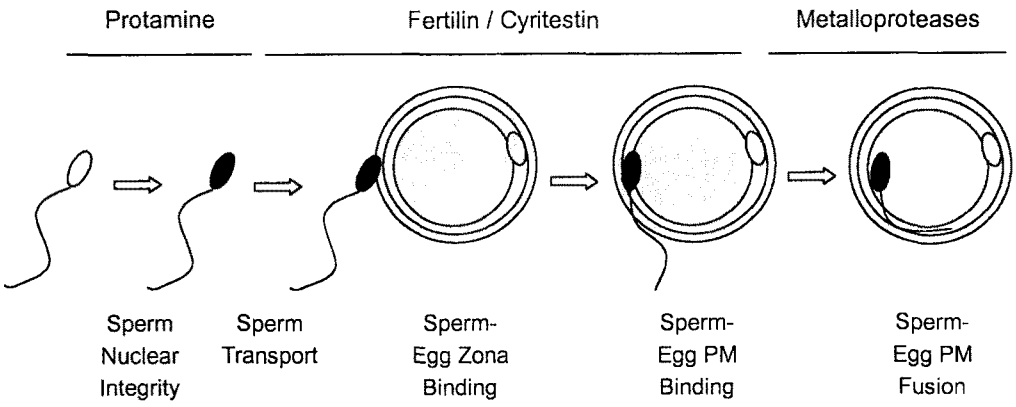

Figure 1. Roles of sperm proteins

membrane fusion.

Sensitivity to Ro31-9790 and to TIMP-3 suggests that the inhibited gamete metalloproteases could be related to the matrixin or adamalysin families. Most metalloproteases of the matrixin family are secreted enzymes, although the recently identified subset of membrane-type matrix metalloproteases (MT-MMPs) contains a transmembrane domain. The sperm metalloproteases identified in the present study, presumably a membrane protein since the activity was inhibited at the surface of sperm, may be comparable to the MT-MMP group of matrix metalloproteases. A subset of the adamalysins, the ADAMs, is a family containing plasma membrane anchored metalloproteases. Unlike ADAM 2 (fertilin) and ADAM 3 (cyritestin), these ADAM proteins could be present on sperm surface with metalloprotease domain. To answer directly if a particular ADAM or MMP, or any other sperm surface metalloprotease, is the identified metalloprotease with a role in gamete fusion will require further investigation. Other questions include what are the substrates of the gamete surface metalloprotease, what factors regulate its activity, and what is its specific contribution to the membrane fusion process?

V. Conclusion

Normal amounts of protamines are essential for sperm nuclear integrity that is required for normal sperm function. Fertilin and cyritestin are key molecules in the development and/or performance of multiple sperm functions such as sperm transport and sperm-egg binding. After sperm-egg binding, gamete membrane fusion requires zinc-dependent metalloproteases (Figure 1).

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