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## INVITED RESEARCH HIGHLIGHT

Reproductive Health

# Unraveling the intricacies of mammalian fertilization

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**I**t is imperative to understand the molecular basis of various steps involved during fertilization. In the manuscript by Bianchi *et al.*<sup>1</sup> a novel protein, Juno on egg membrane (oolemma) has been characterized that binds to sperm specific protein, Izumo-1. Monoclonal antibodies against Juno inhibited *in vitro* fertilization. Juno knock-out female mice failed to deliver litters on mating. It is rapidly shed from oolemma after fertilization, suggesting its role in preventing polyspermy. Taken together these studies will help in our understanding of sperm-egg recognition mechanisms and also facilitate development of new fertility treatment regimens and novel contraceptives.

In mammals, fertilization is a highly synchronized process that involves the adhesion followed by fusion of two highly differentiated haploid germ cells, egg and spermatozoon, leading to the production of a single cell embryo. Understanding the intricacies leading to successful fertilization is one of the most exciting questions for biologists and clinicians which will facilitate development of new treatment options to either overcome infertility or novel contraceptives. During fertilization, initially spermatozoa bind to the zona pellucida (ZP), an extracellular glycoproteinaceous coat that surrounds all mammalian oocytes. ZP serves as a “gate-keeper” to regulate sperm binding to the egg by acting as taxon-selective substrate. Extensive biochemical and cell biology analyses led to characterization of multiple ligands (not exhaustive list) such as  $\beta$ -1,4-galactosyltransferase, ZP glycoprotein-3 receptor (ZP3R formerly designated as sp56) zonadhesion, SED1 (secreted protein that contains notch-like epidermal growth factor

repeats and discoidin/F5/8 type C domains), a disintegrin and metalloprotease 3 (ADAM3), *etc.* associated with capacitated spermatozoa that are involved in the binding of the spermatozoa to the ZP.<sup>2–4</sup> PH20, proacrosin *etc.* exposed on acrosome-reacted spermatozoa play an important role in the continued binding and subsequent penetration of the acrosome-reacted spermatozoa through the ZP matrix.<sup>2–4</sup> Genetic ablation studies using knock-out or transgenic animals revealed that several of the above proteins are not essential for fertilization and thus may only provide supportive role. Initial studies suggested that ZP3 acts as the ligand for binding of capacitated sperm to ZP.<sup>5</sup> However, recent studies suggest that in addition to ZP3, other zona proteins such as ZP1, ZP2 and ZP4 also plays a role in sperm-egg binding.<sup>6–8</sup>

Once the acrosome-reacted spermatozoa complete its journey through ZP matrix and reach perivitelline space, second level of recognition and binding of egg membrane (oolemma) with spermatozoa membrane is critical, which is also associated with their fusion leading to accomplishment of fertilization. In a recent issue of nature, the manuscript by Bianchi *et al.*<sup>1</sup> unravel exciting information that will further our understanding on the ligands involved in the binding of the spermatozoa to the oolemma. Previous studies showed that Izumo-1 (named after a Japanese marriage shrine) knock-out male mice are sterile and sterility in these animals is primarily due to the failure of fusion of the sperm with the oolemma.<sup>9</sup> Production of sperm and rest of the functional attributes were normal from Izumo-1-deficient male mice. In this manuscript, the authors identified and characterize Izumo-1 binding partner from egg oolemma. It binds to folate receptor 4, a GP1-anchored protein expressed on egg oolemma, which do not bind folate, and has been renamed by authors as “Juno” after the Roman goddess of fertility and marriage.

Monoclonal antibodies against recombinant Juno prevented the binding of recombinant Izumo-1 to the egg membrane suggesting Juno as the main receptor on oocyte for binding to Izumo-1. The interaction of Izumo-1 with Juno has been further confirmed by using surface plasmon resonance. Juno contains a single globular domain, whereas Izumo-1 has N-terminus “Izumo domain” and an “immunoglobulin superfamily domain.” Employing recombinant proteins and “avidity-based extracellular interaction screen” it has been shown that “Izumo domain” of Izumo-1 binds to Juno. Further, Juno binds to only Izumo-1 and no other paralogs (family members) such as Izumo-3 and -4. Employing recombinant Izumo-1 and Juno from several species such as humans, pigs and opossum, the authors further showed that interaction of Izumo-1 and Juno is conserved within mammals. In addition to Juno-Izumo-1 interacting partners, other studies have shown the relevance of integrin  $\alpha_6\beta_1$  and a tetraspan membrane protein-CD9 on oolemma and fertilin (heterodimer of fertilin- $\alpha$  [ADAM1B] and fertilin- $\beta$  [ADAM2]) and cysteine-rich secretory protein-1 on sperm membrane that also play an important role in binding of sperm to oolemma and fusion.<sup>2,4</sup> Fertilin binds to  $\alpha_6\beta_1$  leading to sperm-egg binding and membrane fusion.<sup>10</sup> Fertilin- $\beta$  deficient mice are infertile.<sup>11</sup> Using CD9-knock-out female mice, it has been shown that CD9 is critical for the fusion of sperm membrane with oolemma.<sup>12,13</sup>

Using two different sets of experimental approaches, authors have established that Juno is essential for the accomplishment of fertilization. First, monoclonal antibodies against Juno potently inhibited *in vitro* fertilization. Second, mating studies of Juno-deficient (Juno<sup>-/-</sup>) female mice with male mice of proven fertility failed to produce any litters. Juno<sup>-/-</sup> knock-out female mice exhibited normal ovulation and mating

behavior. Eggs recovered by super ovulation from these mice at embryonic day 0.5 revealed more number of sperm within perivitelline space as compared to the wild-type, suggesting that failure to complete fertilization may be due to inhibition in either binding or fusion of spermatozoa with the oolemma. However, failure to observe any syncytia formation in co-culture of cells expressing either Juno or Izumo-1 suggested that Izumo-1-Juno interaction play a role only in adhesion and not fusion.

To prevent polyspermy leading to the formation of nonviable polyploid embryos, based on previous studies, two different models have been proposed. "ZP2-cleavage model" suggest that the cleavage of ZP2 at <sup>166</sup>LA<sup>1</sup>DE<sup>169</sup> by ovastacin, a metalloendoprotease, released following corticle granule exocytosis renders the ZP nonpermissive for gamete recognition.<sup>3,8</sup> In addition, "ZP3 glycan-release model" suggests that the release of glycosidase subsequent to corticle granule exocytosis lead to the release of O-glycans from ZP3 Ser<sup>332</sup> and Ser<sup>334</sup> residues leading to formation of ZP3f and thus account for the inability of sperm to bind to the ZP.<sup>3,8</sup> Using transgenic mice that are either deficient in ZP2 cleavage (Zp2<sup>mut</sup>) or release of O-glycan from ZP3 (Zp3<sup>mut</sup>), it was demonstrated that two cell embryos from Zp2<sup>mut</sup> mice bind sperm whereas Zp3<sup>mut</sup> failed to do so thereby suggesting the relevance of ZP2 cleavage in avoiding polyspermy.<sup>8</sup> Does Juno have any role in the prevention of polyspermy? Authors in this manuscript showed that Juno is rapidly shed from the egg membrane after fertilization.

Juno was weakly detectable in zona-intact fertilized eggs at telophase II and undetectable at the pronuclear stage. On the contrary, expression of Juno in oolemma is not lost in intra-cytoplasmic sperm injection-fertilized or parthenogenetically-activated eggs, which do not exhibit an effective polyspermy block. Thus Juno not only acts as interacting partner for Izumo-1 to accomplish fertilization but also play an important role in avoidance of polyspermy.

The studies presented in this manuscript have convincingly shown that Izumo-1-Juno interaction is responsible for binding of acrosome-reacted spermatozoa to egg membrane. Subsequent to fertilization, shedding of Juno as vesicles from egg membrane also play an important role in avoidance of polyspermy. In future, it may be relevant to investigate; if, Juno in the egg membrane interact with other proteins such as  $\alpha_6\beta_1$  and CD9 that have also been shown to play an important role in sperm-egg binding and fusion. None the less these investigations will facilitate development of treatment modalities for infertility and at the same time new contraceptives.

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