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Mouse Oviduct Specific Glycoprotein (OGP) is an Egg-Associated ZP3-Independent Sperm Adhesion Ligand

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B.A., Harvard University, 1997

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Abstract

Mouse Oviduct Specific Glycoprotein (OGP) is an Egg-Associated ZP3-Independent Sperm Adhesion Ligand

By Robert John Lyng

Mouse sperm-egg binding requires a multiplicity of receptor-ligand interactions, including an oviduct-derived, basic, high molecular weight, wheat germ agglutinin (WGA)-binding, glycoprotein that associates with the egg coat after ovulation. Herein we report the purification and identification of a sperm-binding ligand. WGA-reactive, high molecular weight glycoproteins isolated from superovulated mouse oviduct lysates competitively inhibit sperm-egg binding in vitro. Within this heterogeneous glycoprotein preparation, a distinct 220 kDa protein selectively binds to sperm surfaces. Sequencing of the 220 kDa protein identified it as Oviduct-Specific Glycoprotein (OGP). OGP was confirmed as a sperm-binding ligand by the following three results: specific immuno-depletion of OGP from the denatured oviduct lysates completely abolished the sperm-binding activity, the immunoprecipitated OGP was capable of competitively inhibiting sperm-egg binding, and natively purified OGP also competitively inhibits sperm-egg binding. As expected, the secretory cells of the oviduct fimbriae and infundibulum express OGP; however, contrary to previous reports, OGP is found associated with both the zona pellucida and the perivitelline space of oocytes. Western blot analysis and lectin affinity chromatography demonstrates that whereas the bulk of OGP remains soluble in the ampullar fluid, distinct OGP glycoforms associate with the cumulus matrix, zona pellucida and perivitelline space. In this regard, the OGP sperm-binding activity is restricted to a PNA-reactive glycoform that shows preferential binding to the sperm surface, zona pellucida and perivitelline space, relative to other more abundant glycoforms that do not localize to these domains nor have sperm-binding activity. Interestingly, pretreatment of 2-cell embryos, which do not normally bind sperm, with PNA-reactive OGP stimulates sperm binding. Finally, the sperm-binding activity of the PNA-reactive OGP is shown to be carbohydrate dependent.

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Chapter 1: Introduction

1.1 Introduction to Dissertation

Sperm-zona pellucida (ZP) binding remains a central focus of investigation in reproductive biology, due in part, to its central role in maintaining species-specific fertilization. Species-specificity generally suggests that sperm are not able to recognize and bind eggs from a different species, which is beneficial because it preserves species identity and avoids lethal combinations. Nevertheless, the term “species-specific” is somewhat a misnomer, in that there are several examples of gametes from different species that do interact, including donkey with horse, rat with mouse, human with old world primates, etc. It thus may be better to suggest “taxon-specificity” of sperm-ZP binding, or that the more distantly related two mammalian species are, the less likely sperm-ZP binding will occur. One relevant example is that human sperm do not bind mouse eggs (Rankin et al., 2003).

However, there are numerous mechanisms, in addition to species-specific gamete recognition and activation, which ensure that only gametes of the same species interact. These obstacles to inter-species fertilization include: geographical restrictions, behavior requirements, compatible genitalia, and fertility timing. A comprehensive review of the requirements for numerous model systems has recently been published (Vieira and Miller, 2006). In any event, a fuller understanding of sperm-ZP interactions is critical if we are to appreciate the mechanisms underlying species-specific fertilization as well as develop new avenues for diagnosing and treating reproductive disorders.

1.2 Mammalian Fertilization

1.2.1 Overview

Fertilization comprises a highly coordinated series of cellular and molecular interactions. Mature gametes are terminally differentiated, non-transcriptionally active cells, and therefore, the machinery required to accomplish these complex interactions must be inherent in these cells as a result of gametogenesis and/or provided to them as they traverse the reproductive organs. Control over these interactions is crucial to gamete viability, as well as the control of fertilization.

During murine oogenesis, the egg develops a protective extracellular coat called the zona pellucida (ZP) that facilitates species-specific sperm-egg recognition and binding (Bleil and Wassarman, 1980). The mouse ZP is composed of three glycoproteins, ZP1, ZP2, and ZP3. The three zona pellucida proteins are synthesized by the egg and deposited on the cell surface forming a mesh-like matrix (Florman and Wassarman, 1985). Upon ovulation, the egg, encased in its ZP, is deposited into the oviduct. The murine oviduct is composed of four major sections: fimbriae, infundibulum, ampullea, and isthmus. Ovulated eggs are collected by the fimbriae, transported through the infundibulum to the ampullea where fertilization occurs. Long thought to be a passive organ, the oviduct has recently been shown to secrete several factors that are believed to function in gamete transport, fertilization, and embryogenesis (Killian, 2004). Some of these factors are believed to associate with the ZP, and are hypothesized to play a role in fertilization (Buhi, 2002).

Similarly, after spermatogenesis, sperm must undergo several biochemical, morphological, and physiological maturations in both the epididymis and the female

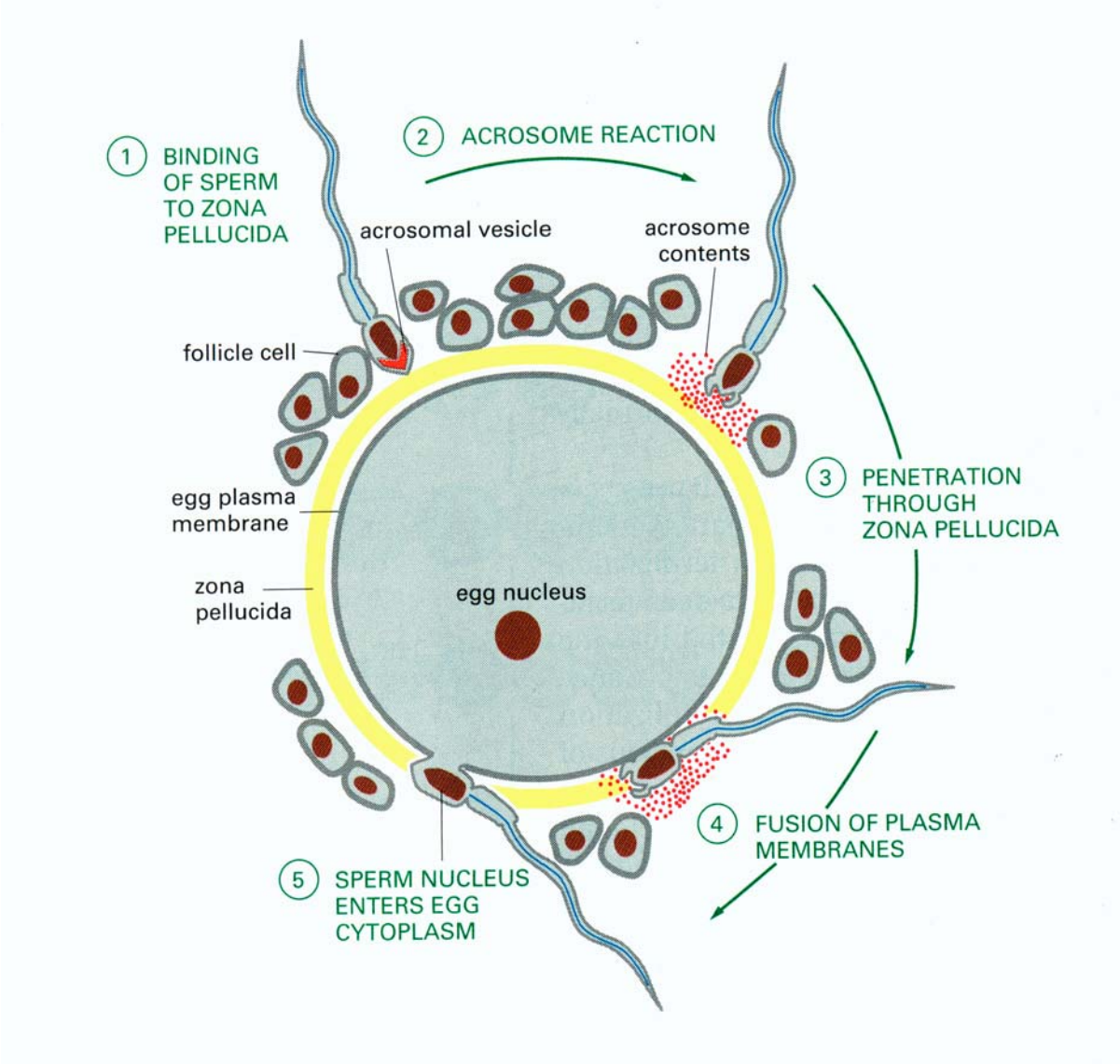
reproductive tract before they are able to recognize, bind, and fertilize an oocyte (Cross, 2004; Gadella, 2008). Within the epididymis, the sperm surface is remodeled, while environmental pH and epididymal glycoconjugates hold the sperm in a quiescent state (Cooper, 1995; Gibbons et al., 2005). Continued maturation occurs within the female reproductive tract in a process called "capacitation." Capacitation is defined as the events leading to the acquisition of fertilizing capability and is characterized by a series of biochemical, morphological, and behavioral changes (Yanagimachi and Usui, 1974). Capacitation can be replicated in vitro through conditioned media, and is characterized by: 1) an increase in sperm metabolism (Fraser and Herod, 1990), 2) an alteration in membrane fluidity and sterol ratio (Wolf et al., 1986), 3) hyperactivated motility (Ho and Suarez, 2001), and 4) an acquired ability to bind egg coats. Although the specific mechanisms behind these events remain largely unknown, recent findings have identified some key regulatory components (Travis et al., 2001). For example, successful capacitation begins with the shedding of epididymal glycoconjugates from the sperm surface. Epididymal glycoconjugates are thought to act as "decapacitating" factors that bind to the sperm surface and prevent premature capacitation and spontaneous acrosome reactions, which would result in an inability to fertilize eggs (Shur and Hall, 1982). Furthermore, capacitation requires the presence of albumin, Ca^{2+} and HCO_3^- (Davis, 1981; Lee and Storey, 1986; Yanagimachi and Usui, 1974). All three cofactors are found either in the seminal plasma or in the female reproductive track. Their presence modulates a series of processes, including cAMP synthesis and protein tyrosine phosphorylation. Albumin is thought to facilitate membrane fluidity by removing cholesterol from the sperm plasma membrane, whereas Ca^{2+} and HCO_3^- appear to

stimulate sperm adenylate cyclase activity. The increased cAMP levels lead to coordinated changes in protein phosphorylation and sperm motility (Gadella and Van Gestel, 2004; Lee and Storey, 1986; Litvin et al., 2003; Shadan et al., 2004; Wolf et al., 1986). As capacitated sperm reach the oviduct, they bind to the epithelium and are sequestered in a dormant state until ovulation (Cortes et al., 2004). Ovulation triggers the release of mature sperm. This coordinated release allows for constant replenishment of capacitated, mature sperm for transport to the ampullea.

Successful fertilization requires numerous specific interactions between the sperm and egg (Figure 1-1). First, sperm must encounter the cumulus cells, which surround and nurse the egg during oogenesis, and use mechanical and enzymatic means to penetrate this initial barrier (Shur et al., 2004b; Talbot et al., 2003). After transversing the cumulus layer, sperm are thought to bind to the ZP via a species-specific receptor-ligand interaction (Bleil and Wassarman, 1980). Sperm binding to the ZP stimulates the acrosome reaction, which releases hydrolytic enzymes from the acrosome vesicle that facilitate sperm penetration through the ZP (Gong et al., 1995; Youakim et al., 1994).

Sperm binding to the ZP also causes-hyper stimulation of the sperm that allows for more forceful and directed movement through the zona matrix (Ho and Suarez, 2001). After penetrating through the ZP, sperm arrive in the perivitelline space where they fuse with the egg plasma membrane. Although several potential candidates exist, the mechanisms behind sperm-oocyte membrane fusion remain a mystery. Two leading candidates that may facilitate membrane fusion are the egg tetraspanin protein CD9 (Kaji et al., 2000; Miyado et al., 2000; Miyado et al., 2008), and the recently discovered sperm immunoglobulin protein Izumo (Inoue et al., 2005; Miyado et al., 2008).

Figure 1-1. Schematic of sperm-egg interactions during fertilization. Reprint of image from Molecular Biology of the Cell, 4th Edition (p. 1152). Garland Publishing Inc. New York.


1 BINDING OF SPERM TO ZONA PELLUCIDA
2 ACROSOME REACTION
acrosomal vesicle
acrosome contents
follicle cell
egg plasma membrane
zona pellucida
egg nucleus
3 PENETRATION THROUGH ZONA PELLUCIDA
4 FUSION OF PLASMA MEMBRANES
5 SPERM NUCLEUS ENTERS EGG CYTOPLASM

Finally, polyspermy, or fertilization of one egg by more than one sperm, must be blocked, as it is lethal. Polyspermy is prevented by the release of egg cortical granules after fertilization. Enzymes within the granules alter the ZP and prevent a second sperm-egg adhesion event (Miller et al., 1993; Shur et al., 2004b).

Ongoing investigation of these events has lead to a new understanding of gamete surface biology, as well as the appreciation that sperm-egg recognition requires multiple, complex interactions (Lyng and Shur, 2007). Outlined below are several components that have been suggested to play critical roles during murine sperm-oocyte interactions. Although robust, this synopsis should not be considered all-inclusive. There exists several molecular players whose role in mouse has only been inferred and requires additional study, as well as, additional components have been observed in other model systems that have yet to be confirmed in mouse.

1.2.2 Murine Sperm-Oocyte Interactions

1.2.2.1 Sperm-ZP recognition and binding

1.2.2.1.1 Zona Pellucida 3 (ZP3)

Early biochemical analysis of the ZP demonstrated that soluble ZP3, but not ZP2 or ZP1, specifically bound to capacitated sperm and inhibited sperm-egg interactions in vitro (Bleil and Wassarman, 1980). Interestingly, ZP3 from fertilized eggs is unable to bind sperm, suggesting that the sperm-binding ligand is altered after fertilization (Bleil and Wassarman, 1980). Specific deglycosylation of soluble ZP3 disrupts sperm-ZP3 interaction, suggesting the recognition epitope resides in the oligosaccharides of ZP3 and

not the protein backbone. Subsequently, ZP3 *O*-linked oligosaccharides were shown to competitively inhibit sperm-ZP binding (Bleil and Wassarman, 1980), and α-linked galactose in particular has been suggested as the sperm-binding determinant, because treatment of soluble ZP3 with α-galactosidase abolishes sperm receptor activity (Bleil and Wassarman, 1988). However, the role of α-galactose has been questioned by the observation that eggs devoid of α-galactose residues are fertilized normally (Liu et al., 1997; Thall et al., 1995).

Sperm binding to ZP3 stimulates several intracellular signaling cascades within the sperm, which leads to acrosomal exocytosis (Bleil and Wassarman, 1980; Shi et al., 2001). The acrosome is a vesicle of degradative enzymes located in the head of sperm. Once a sperm binds to an egg, the acrosome fuses with the plasma membrane, releasing its enzymes so that they may degrade the ZP and allow for penetration. Acrosome reacted sperm are unable to bind ZP3, but are able to remain bound to the egg coat, signifying that secondary interactions must be available between the acrosome-reacted sperm and the ZP (Bleil and Wassarman, 1986).

1.2.2.1.2 β1,4-Galactosyltransferase 1 (GalT 1)

Although the identity of the sperm ZP receptor remains controversial, sperm surface β1,4-galactosyltransferase-1 (GalT I) satisfies a majority of the criteria for a ZP3 receptor. In mammals, GalT I is synthesized as both long and short isoforms that differ only by 23 amino acids at the cytoplasmic C-terminus (Hathaway et al., 2003). Both isoforms are found in the Golgi apparatus, where they function in carbohydrate biosynthesis, transferring galactose from UDP-galactose to terminal *N*-acetylglucosamine

(GlcNAc) residues on growing polysaccharide chains. However, the long isoform is also found on the plasma membrane of sperm and selected cell types. On the cell surface, glycosyltransferases have been proposed to function in cell-cell and cell-extracellular matrix adhesions (Lopez et al., 1985). The extracellular catalytic domain is able to bind to glycosides on adjacent structures forming a stable interaction, since free donor sugar substrates, which would be added to the carbohydrate chain causing the release of the glycosyltransferase, are not found in the extracellular fluids.

Consistent with a role for sperm GalT 1 in mediating binding to the egg coat, free UDP-galactose can dissociate bound, acrosome-intact sperm from the ZP (Lopez et al., 1985). Furthermore, in the presence of UDP-[^{3}H]galactose, sperm transfer [^{3}H]galactose to ZP3 oligosaccharides within the intact ZP (Miller et al., 1992). These results suggests that GalT I is a sperm receptor for the ZP3 oligosaccharide sperm-binding ligand. Genetic experiments supporting this interaction include the demonstration that overexpression of GalT I on mouse sperm causes increased binding of soluble ZP3 (Youakim et al., 1994), and ectopic expression of mouse GalT 1 on *Xenopus leaves* oocytes leads to an ability to bind mouse ZP3 (Shi et al., 2001). On the other hand, transgenic mice lacking the long isoform of GalT I do not bind soluble ZP3 and do not undergo a ZP3 dependent acrosome reaction (Lu and Shur, 1997) (Rodeheffer and Shur, 2002).

The GalT 1/ZP3 interaction also contributes to acrosomal exocytosis (Youakim et al., 1994). Binding of GalT I to the multivalent ZP3 causes aggregation of GalT I , which triggers the acrosome reaction through a heterotrimeric G-protein coupled signal transduction pathway (Gong et al., 1995). Overexpressing GalT 1 in sperm results in accelerated G-protein activation and an increase acrosome reactions (Youakim et al.,

1994). Likewise, ectopic expression of GalT 1 on *Xenopus* oocytes leads to GalT 1-dependent G-protein activation (Shi et al., 2001).

1.2.2.1.3 SED1

Integral and peripherally-associated sperm proteins with ZP binding properties have been identified in numerous species. An investigation into boar sperm proteins that bind the ZP identified p47, a sperm protein with homology to milk fat globule membrane protein E8, known as MFG-E8 (Ensslin et al., 1998)

The homologous mouse gene was cloned and named SED1 (***S***ecreted ***E***GF ***D***iscoidin-domain 1). In addition to being found in various organs, SED1 is secreted by principle cells of the initial segment of the epididymis. As sperm progress through the epididymis, SED1 binds to the sperm head, specifically to the plasma membrane domain overlying the acrosome - the area that initially interacts with the egg ZP. Numerous biochemical and genetic experiments have shown SED1 is required for sperm-egg binding. For instance, recombinant SED1 binds sperm and competitively inhibits sperm-egg binding in vitro. Recombinant SED1 binds to ZP3 and ZP2 of unfertilized eggs, but does not bind to fertilized eggs. Furthermore, SED1 knockout males are sub-fertile, and SED1-null sperm do not bind eggs in vitro (Ensslin and Shur, 2003).

SED1 contains two N-terminal EGF repeats, and two C-terminal discoidin/F5/8 type C domains. Through truncation studies it was determined that the discoidin/C domains are required for gamete binding. Discoidin/C domains are known to bind to membrane bilayers as well as to extracellular matrices. Structural studies show that these domains have two hypervariable hairpin loops that intercalate into lipid bilayers or

carbohydrate structures depending on the amino acid sequence of the hairpin loops. Two models for SED1 function in sperm-egg binding have been proposed(Shur et al., 2004a). SED1 may function as a monomer, with each discoidin domain mediating binding to each gamete. Alternatively, the EGF repeats may facilitate dimerization such that SED1 may function as an oligomer during sperm-ZP binding (Shur et al., 2004a).

1.2.2.1.4 Cyritestin

The ADAM (**A D**isintegrin and **A M**etalloprotease) family of proteins, which includes fertilin α, fertilin β, cyritestin, ADAM 4, and ADAM 5, have been a major focus in fertilization because they are expressed in the mouse testes and contain an integrin binding domain (a disintegrin) that has been proposed to function in cell-cell adhesion. (Bigler et al., 1997; Linder and Heinlein, 1997; Yuan et al., 1997) Originally, the heterodimeric fertilin α/β complex was thought to participate in sperm-egg plasma membrane fusion. Fertilin α knock-out studies, however, have shown that fertilin α/β does not participate in fusion, but does help direct cyritestin to the sperm surface (Kim et al., 2006; Nishimura et al., 2004)

Although cyritestin-null males exhibit normal spermatogenesis, mating behaviors, and migration of sperm through the female reproductive tract, they are infertile. In vitro studies has shown that cyritestin-null sperm are unable to bind to the ZP but can bind and fertilize ZP-free eggs. Furthermore, a synthetic peptide that comprises a portion of the cyritestin disintegrin loop competitively inhibits sperm-ZP interactions (Shamsadin et al., 1999). The ZP binding partner of the cyritestin disintegrin domain remains unknown.

1.2.2.1.5 Glycodelins and Fucosyltransferase 5 (FUT5)

Glycodelins-A, -F, and –S belong to the lipocalin protein family and have been known to modulate human sperm function and inhibit sperm-ZP binding (Oehninger et al., 1995). Recently, a fourth glycodelin, glycodelin-C, has been identified and stimulates sperm-ZP binding (Yeung et al., 2006).

According to a model proposed by Yeung and colleagues, sperm bind glycodelin-S, found in the seminal plasma, before they are deposited in the female reproductive tract. Glycodelin-S binding maintains sperm in a quiescent state. As sperm transverse the cervix, glycodelin-S is rapidly displaced by glycodelin-A and -F, which are secreted by oviductal glands (Chiu et al., 2005). Glycodelin-A is known to suppress sperm-ZP binding through its carbohydrate moieties (Chiu et al., 2003), while providing immunosuppressive activity through its protein backbone (Jayachandran et al., 2004). Glycodelin-F, the major glycodelin in follicular fluid, also interacts with sperm and inhibits ZP binding in a carbohydrate-dependent manner, but also is capable of inhibiting progesterone induced acrosome reactions (Chiu et al., 2003). As sperm penetrate the cumulus cells surrounding the egg, glycodelin-A and –F are though to be displaced by glycodelin-C, which has the same protein backbone as –A and –F but has altered glycosylation states. Glycodelin-C association with the sperm surface promotes sperm-ZP binding (Chiu et al., 2007a).

Investigation into the sperm-associated receptor for glycodelin-A and ZP proteins identified fucosyltransferase 5 (FUT5). Anti-FUT5 antibodies and purified FUT5 prevent glycodelin-A binding to sperm and eliminate glycodelin-A’s ability to inhibit sperm-ZP binding. Furthermore, investigations of FUT5 as a ZP-receptor demonstrate FUT5

strongly binds to intact and solubilized ZP (Chiu et al., 2007b). Yeung and colleagues propose a model in which glycodelin-A binds to FUT5, preventing erroneous binding of FUT5 to its recognition signal. As sperm pass through the cumulus layer, glycodelin-C displaces glycodelin-A on the sperm surface thereby supporting and potentially promoting sperm-ZP binding (Chiu et al., 2007b). Although the majority of evidence for FUT5 as a ZP-receptor has been completed in human, initial evidence suggest similar roles for glycodelin-A, and thus FUT5 in mouse.

1.2.2.1.6 Arylsulfatase-A and SGG glycolipid

Lipid rafts have been implicated as highly specialized plasma membrane domains capable of organizing cell adhesion complexes and cell signaling molecules (Marmor and Julius, 2001). Mouse sperm possess lipid rafts on their head and flagellum. The presence of lipid rafts on the head suggests a possible role in sperm-egg interactions. Present within these rafts is the sperm-specific sulfoglycolipid, sulfogalactosylglycerolipid (SGG) (Bou Khalil et al., 2006; Lingwood, 1985). SGG and related sulfoglycolipids bind several adhesion molecules (Roberts et al., 1986), and thus have been proposed to be involved in sperm-egg binding.

Various in vitro studies have demonstrated a role of sperm SGG in mouse sperm-ZP binding. Fluorescently labeled SGG liposomes, but not control GG liposomes, bind to ovulated unfertilized eggs. A significant decrease in SGG liposome binding is observed when fertilized eggs are assayed. Secondly, incubation of SGG liposomes in a sperm-egg binding assay significantly reduces the number of sperm bound to egg coats (White et al., 2000). Researchers speculate that the sulfate moiety of SGG defines its functional

domain, which interacts with ZP glycoproteins through electrostatic interactions. Arylsulfatase A, which is present in epididymal fluid and deposits on the head during transport, was also shown to interact with SGG and bind to ovulated, unfertilized eggs (Tantibhedhyangkul et al., 2002).

The interaction of Arylsulfatase A with SGG, their ZP binding capabilities, and their localization to lipid rafts implicates these domains as organizational centers for the ZP binding machinery. This hypothesis is further supported by the findings in boar and guinea pig sperm that lipid rafts aggregate over the sperm head as a function of capacitation (Bou Khalil et al., 2006). Tanphaichitr and colleagues suggest that as a result of capacitation when cholesterol is removed from the plasma membrane, SGG dominated lipid rafts coalesce, localizing binding machinery to a domain strong enough support interaction with the ZP, without destroying the plasma membrane due to sperm motility (Furimsky et al., 2005).

1.2.2.2 Sperm-ZP secondary interactions

1.2.2.2.1 Zona Pellucida 2 (ZP2)

The proposed role of ZP2 in secondary binding stems from early investigations into the role of the individual ZP glycoproteins. Unlike ZP3, ZP2 does not inhibit acrosome-intact sperm binding to egg coats in vitro, but does bind to acrosome-reacted sperm (Bleil and Wassarman, 1980). As a result, it was proposed that ZP2-binding sites are exposed as a result of the acrosome reaction that subsequently interacts with the ZP during penetration. Researchers suggest that this secondary ZP2-dependent binding

provides a weak interaction between sperm and the ZP, allowing sperm penetration but preventing sperm detachment from the egg coat (Bleil and Wassarman, 1986).

Efforts to identify the sperm receptor responsible for ZP2 interaction have proven difficult. However, one candidate that meets several of the criteria for a secondary-receptor is proacrosin/acrosin. Proacrosin/acrosin is found in the acrosomal vesicle, is exposed as a result of the acrosomal exocytosis, and binds to sulphate groups on ZP2. In support of proacrosin/acrosin as a ZP2 receptor, solubilized ZP2 was shown to bind less effectively to proacrosin-null sperm (Gaboriau et al., 2007; Howes et al., 2001). However, the role of proacrosin/acrosin remains unclear, since acrosin-null sperm still are able to fertilize eggs (Baba et al., 1994).

1.2.2.2.2 sp56

Originally identified by ZP3 affinity chromatography of sperm proteins, sp56 was shown to bind ZP3 through a photoaffinity cross-linking experiment where ZP3 peptides, containing the previously identified sperm-binding domain, interacted with sperm and co-migrated with sp56 (Cheng et al., 1994). As a result, it was suggested that sp56, which contains “sushi domains” and is a member of the C3/C4 binding protein superfamily, plays a role in initial sperm-ZP recognition and binding.

Subsequent research challenged this theory, by demonstrating that sp56 is localized to the acrosomal matrix and not present on the surface of sperm (Kim et al., 2001). These results suggest a secondary binding role for sp56, which is exposed after the acrosome reaction and stabilizes sperm adhesion to the ZP as the sperm penetrate the egg coat (Buffone et al., 2008). Proponents of a role for sp56 in initial sperm-ZP binding

have since argued that capacitation allows for fusion points between the outer acrosomal membrane and the plasma membrane, thus revealing portions of the acrosomal matrix to the exterior of the sperm. Accordingly, these fusion "pores" would reveal sp56 and allow it to function in initial binding to the ZP (Kim and Gerton, 2003).

Regardless of the sequence in which sp56 interacts with the ZP, most data are consistent with a role for sp56 in fertilization. Recent investigations have demonstrated that recombinant sp56 is capable of binding ZP-intact eggs, but not 2-cell embryos. Furthermore, recombinant sp56 has been shown to reduce the number of sperm in vitro that reach the perivitelline space, thus suggesting a role in sperm penetration (Buffone et al., 2008).

1.2.2.2.3 Zonadhesin

Zonadhesin was first identified in pig by affinity chromatography of sperm proteins on porcine ZP (Hardy and Garbers, 1994). A type 1 transmembrane protein, zonadhesin is composed of various putative domains capable of functioning in cell adhesion (Hardy and Garbers, 1995). Interestingly, post-translational processing of zonadhesin, including proteolysis and glycosylation, creates products that possess varying avidities for pig ZP (Hickox et al., 2001). Localization studies determined its presence in the leading edge of the acrosomal matrix of acrosome-intact sperm, where it is hypothesized to function in binding to the ZP during acrosomal exocytosis (Bi et al., 2003).

Similar to the porcine homologue, analysis of mouse zonadhesin cDNA suggests a large, transmembrane mosaic protein with numerous domains capable of facilitating or

participating in cell-cell adhesion. These putative domains include: four full and twenty-one partial D-domains homologous to von Willebrand factor; three tandem repeats of MAM domains, a mucin domain; and a domain homologous to epidermal growth factor (EGF). It has been hypothesized that amino acid differences between mouse, pig, and other mammalian models contributes to the species specificity of zonadhesin binding to the ZP (Gao and Garbers, 1998).

1.2.2.3 Sperm-egg plasma membrane binding and fusion

1.2.2.3.1 CD9

CD9 is a member of the tetraspanin superfamily and is hypothesized to play a role in cell migration, cell adhesion, and cell fusion through a proposed interaction with integrins and other plasma membrane proteins (Hemler, 2003). Its expression on the plasma membrane of eggs suggests a similar role in fertilization. As predicted, CD9 knockout females are infertile, and CD9 (-/-) oocytes support sperm binding, but sperm fail to fuse and fertilize the egg (Kaji et al., 2000; Miyado et al., 2000).

CD9 facilitates fusion through two mechanisms. First, deletion of CD9 causes significant malformation of the microvilli on the oocyte plasma membrane. Thus, it is thought that CD9 regulates microvilli length and organization, and that sperm initially interact with the microvilli prior to fusion (Runge et al., 2007). Second, CD9 containing vesicles accumulate in the perivitelline space (PVS) and transfer CD9 to sperm, suggesting that transfer of CD9 to the sperm prepares it for fusion (Barraud-Lange et al., 2007). Furthermore, CD9 deficiency reduces the number of CD9 containing vesicles in

the PVS and reduces fertilization at the fusion step (Miyado et al., 2000). Overall, these data suggest that sperm interaction with CD9-containing vesicles is a prerequisite to fusion. Indeed, CD9 vesicles from wild-type oocytes, when incubated with sperm, are able to restore fusion to CD9-null eggs. This process appears to be conserved, because a similar effect was found using hamster eggs to pretreat the sperm (Miyado et al., 2008).

1.2.2.3.2 Izumo

The search for sperm-related proteins involved in sperm-egg plasma membrane binding and fusion had been disappointing until the discovery of Izumo, an immunoglobulin superfamily protein on sperm (Inoue et al., 2005). Izumo knockout males exhibit normal mating behavior, normal sperm motility, and sperm are capable of traversing the female reproductive tract. However, Izumo (-/-) males are sterile. In vitro, Izumo (-/-) sperm bind and penetrate the ZP, as well as adhere to ZP-free oocytes, but do not fertilize the eggs. Antibodies against Izumo do not bind to acrosome-intact sperm, but bind to acrosome-reacted sperm, thus suggesting Izumo is revealed after acrosomal exocytosis. Furthermore, anti-Izumo antibodies do not prevent sperm-ZP binding and penetration or sperm-egg binding, but do inhibit fertilization in vitro. Together, these results illustrate that sperm-egg plasma membrane binding and fusion are separate events, and that Izumo functions specifically during membrane fusion (Inoue et al., 2005).

1.2.2.3.3 Cysteine-Rich Secretory Protein-1 (CRISP-1)

Originally identified in rat, CRISP-1 is expressed in the epididymis (Cameo and Blaquier, 1976). Subsequently, several other CRISPs have been identified, including CRISP-2, which is synthesized in the testes (Kasahara et al., 1989); CRISP-3, which is produced in many tissues (Haendler et al., 1993); and CRISP-4, which is also expressed in the epididymis (Jalkanen et al., 2005). CRISP-1 has been rigorously investigated in rat, and has been shown to associate with the dorsal region of the sperm head where it remains until capacitation, at which time a majority is shed while a small portion relocates to the sperm head equatorial region (Rochwerger and Cuasnicu, 1992). It is hypothesized that the loss of CRISP-1 from the sperm head is correlated with sperm capacitation.

Its role in sperm-egg fusion has been inferred from the fact that pre-incubation of ZP-free rat eggs with rat CRISP-1 reduces fusion events, but does not prevent sperm-egg binding (Rochwerger et al., 1992). The reduction in fusion is most likely the result of exogenous CRISP-1 binding to the egg plasma membrane and masking complimentary binding sites (Cohen et al., 2008). Mouse transgenics support a role for CRISP-1 in sperm-egg fusion. CRISP-1 knockout males exhibit normal fertility in vivo, however, CRISP 1 (-/-) sperm have impaired fertilization capabilities in vitro. This deficiency, according to investigators, appears to result from a reduced ability of CRISP-1-null sperm to fuse with eggs under non-competitive fertilization assays, which is magnified in competitive assays with wild-type sperm (Da Ros et al., 2008). Investigators also suggest that additional CRISPs, specifically CRISP-2, play a role in sperm-egg plasma membrane binding and fusion (Busso et al., 2007).

Finally and most recently, investigators have proposed a role for CRISP-1 in sperm-ZP recognition, due to the fact anti-CRISP-1 antibodies prevent sperm-ZP binding (Da Ros et al., 2008). Due to limited studies into this potential role, its function in sperm-ZP binding remains unconfirmed.

1.2.2.3.4 SLLP-1

Sperm Lysozyme-Like Protein-1 (SLLP-1) was first observed in the acrosome of human sperm (Mandal et al., 2003). SLLP-1 is a c lysozyme-like protein that contains a functional substrate-binding domain, but is missing critical residues within the catalytic domain, rendering it catalytically inactive. The substrate binding domain interacts with GlcNAc residues and polymers of GlcNAc and sialic acid (Strynadka and James, 1996). It has been hypothesized that this substrate binding domain may facilitate cell-cell adhesions.

SLLP-1 has been studied in the mouse model, where it localizes to the anterior acrosome in non-capacitated sperm, and is retained in the equatorial region after capacitation and the acrosome reaction. The equatorial region has long been known to function in sperm-egg plasma membrane binding and fusion (Talbot and Chacon, 1982), thus its localization to this region implicates it in a similar role. To assess its function, investigators assayed the ability of sperm to fertilize eggs following treatment with recombinant SLLP-1 or anti-SLLP1 antibodies. In both cases, fertilization rates drop dramatically. Furthermore, recombinant SLLP-1 binds to the egg plasma membrane and prohibits sperm-egg binding but does not interfere with sperm-egg fusion (Herrero et al., 2005).

1.3 Sperm-ZP binding models

1.3.1 The carbohydrate-mediated model

The carbohydrate-mediated model of sperm-ZP recognition and binding suggests sperm recognize the egg coat in a carbohydrate-dependent manner. Accordingly, the sperm surface contains egg coat binding proteins, which interact with sugar moieties that are decorating proteins that comprise the egg coat, as oppose to interacting through protein-protein based mechanisms.

This model is supported by numerous in vivo and in vitro observations. First, it has long been known that the protein compositions of mammalian ZP are similar between species. All mammalians species contain homologues to the mouse ZP1, ZP2, and ZP3 genes. Some species may contain an additional ZP protein, named ZP4 in human (Lefievre et al., 2004). This, however, immediately raises the question of how species-specificity ("taxon-specificity") is achieved if all mammals are utilizing the same machinery. The use of variable glycosylation to create unique carbohydrate structures on the surface of proteins is one potential solution to this dilemma.

As mentioned previously, Bleil and Wassarman provided evidence that sperm-ZP binding is carbohydrate-mediated by showing that soluble ZP3 could inhibit sperm-ZP binding in vitro, even after ZP3 had been heat denatured and digested with proteases. Furthermore, they showed ZP3-derived glycans, but not deglycosylated ZP3, could inhibit sperm-ZP binding in vitro (Bleil and Wassarman, 1980). The functional glycans on ZP3 were shown to be *O*-linked glycans (Florman and Wassarman, 1985), and soluble ZP3-mediated inhibition could be ablated by pretreatment with α-galactosidase (Bleil and Wassarman, 1988). These results and in vitro sperm-ZP binding assays utilizing soluble

oligosaccharides implicate terminal α1,3-linked Gal and β1,4-linked Gal as potential ligands (Litscher et al., 1995).

However, the ability of terminal α1,3-linked Gal to serve as the initial binding epitope has been questioned, due to the fact that 1) α-galactosidase treated eggs exhibit normal sperm binding (Mori et al., 1997), 2) α-galactose residues are only present on inner portions of the ZP (Aviles et al., 2000b), and 3) female mice lacking α1,3 galactosyltransferase are fertile and produce eggs with normal sperm binding attributes (Liu et al., 1997; Thall et al., 1995). These results suggest that terminal α1,3 Gal may either not be functional in the organized ZP matrix, or may facilitate a sperm-egg interaction other than initial recognition and binding. Terminal β1,4 Gal remains a viable candidate, because global treatment of oocytes with β-galactosidase reduces sperm binding by 70% (Mori et al., 1997).

Similarly, pretreatment of oocytes with *N*-acetylglucosaminidase significantly reduces sperm-ZP binding, suggesting a role for GlcNAc residues in sperm-ZP recognition (Miller et al., 1993). GlcNAc residues have been shown to exist within the zona matrix on both *O*-linked and *N*-linked glycans (Dell et al., 2003; Mori et al., 1997). Shur and colleagues have suggested that the GlcNAc residues are associated with ZP3-linked polylactosaminoglycans (Lopez et al., 1985; Miller et al., 1992), however, others debate the existence of these glycans on the mouse ZP (Dell et al., 2003; Easton et al., 2000). Furthermore, the Shur laboratory has demonstrated that egg cortical granules contain high levels of *N*-acetylglucosaminidase that cleave GlcNAc from the ZP after fertilization and may act as a block to polyspermy (Miller et al., 1993; Miller et al., 1992).

Two additional carbohydrate moieties have been proposed as mediators of sperm-ZP interaction: mannose and fucosylated LacNAc, also known as the Lewis X antigen. Although their validity as initial egg-coat ligands requires additional investigation, their ability to inhibit sperm-egg binding in vitro strengthens the argument for a carbohydrate-mediated model. Mannose has been implicated by the following observations: (1) α-methyl mannose reduces sperm-egg binding in vitro (Lambert, 1984), (2) enzymatic removal of *N*-linked glycoproteins, including high mannose glycans, from the mouse ZP reduces sperm-egg binding (Cornwall et al., 1991), (3) mannose neoglycoproteins stimulate spontaneous acrosome reactions (Loeser and Tulsiani, 1999), and (4) inhibition of sperm surface α-D-mannosidase leads to a decrease in sperm-egg binding (Cornwall et al., 1991). In the case of fucosylated LacNAc, investigators utilized small oligosaccharides to inhibit sperm-egg binding. Fucosylated LacNAc inhibits sperm-egg binding at low concentrations, but never achieves greater than 45% inhibition. LacNAc, on the other hand, achieves greater than 70% inhibition at higher concentrations (Johnston et al., 1998). Although Lexis X structures have not been found within the mouse ZP (Aviles et al., 2000b), the LacNAc structure has been associated with Core 2 *O*-glycans, complex type tri- and tetraantennary *N*-glycans, and branched polylactosamine structures on ZP3 (Dell et al., 2003; Easton et al., 2000).

1.3.2 The protein-protein mediated model

The protein-protein mediated model of sperm-ZP recognition and binding suggests that initial binding is the result of specific protein-protein interactions that are independent of carbohydrate moieties. An extension of this model is the “supramolecular

structure" model, which suggests that sperm recognize the three dimensional architecture of the ZP. Overall, these models address gaps in the carbohydrate-mediated model, but for the most part, they have emerged as the result of a lack of consensus within the carbohydrate-mediated model, and not due to specific demonstration of a protein-protein interaction.

The development of ultra-sensitive biophysical analytical techniques and mouse transgenics has created several unexpected and unexplained findings that are in conflict with a traditional carbohydrate-mediated model. For instance, Wassarman and colleagues have asserted that *O*-linked glycosylation of two serine residues (Ser-329 and Ser-334) residing in exon 7 of the ZP3 gene is responsible for sperm-egg binding (Kinloch et al., 1991; Kinloch et al., 1995). Their findings show that conversion of these serine residues to alanines within a recombinant ZP3, expressed in F9 embryonal carcinoma cells, eliminates the recombinant ZP3's sperm-ZP binding inhibitory capabilities (Chen et al., 1998). However, proteomic analysis of native mouse ZP3 does not reveal any glycosylation of Ser-329 or Ser-334 (Boja et al., 2003).

Next, knockouts of either sperm receptors or glycosyltransferases proposed to create carbohydrate ligands on the ZP have all failed to ablate fertility and initial sperm-egg binding. For example, mice lacking GalT 1 are fertile and GalT 1 (-/-) sperm surprisingly bind to wild-type eggs at a higher level than wild-type sperm (Lu and Shur, 1997). Similarly, elimination of Core 2 *O*-glycans or branched *N*-glycans, through deletion of the genes responsible for their formation, does not compromise fertility (Ellies et al., 1998; Shi et al., 2004). Critics of the carbohydrate-dependent model also point to the fact that exoglycosidase digestion of the mouse ZP has a maximum inhibitory effect

of ~75% (Mori et al., 1997). It has been suggested that the residual 25% binding may be protein or supramolecular structure based and account for initial sperm binding and subsequent fertility (Clark and Dell, 2006).

Finally, in an attempt to distinguish between carbohydrate- and protein-mediated binding, investigators have "humanized" the mouse ZP by replacing the mouse ZP3 gene with the human ZP3 homologue. By doing so, they hypothesized mouse sperm would cease to bind the humanized mouse ZP. However, they observed that mouse sperm, but not human sperm, bind to the humanized ZP (Rankin et al., 1998). Comparison of the glycosylation states of the mouse ZP and the humanized mouse ZP provided a simple explanation that is consistent with a carbohydrate-mediated model; human ZP3 expressed in the mouse oocyte exhibit mouse *O*-glycan structures synthesized by mouse glycosyltransferases (Dell et al., 2003). Identical results were observed when both mouse ZP2 and ZP3 were replaced with human ZP2 and ZP3. However, one interesting observation from these studies is that humanized ZP2/ZP3 mouse eggs continue to bind sperm after fertilization. In these humanized mouse oocytes, ZP2 is not cleaved after fertilization, unlike the situation when mouse ZP2 is present. Researchers hypothesize that the lack of human ZP2 cleavage and continued sperm binding to fertilized eggs argues for a role of the ZP's supramolecular structure in initial sperm-egg binding (Rankin et al., 2003).

1.3.3 The multi-interaction model

The vast number of studies into the basis of sperm-egg binding has resulted in a list of potential molecular players that support carbohydrate-mediated (ZP3, GalT-1,

SED1, FUT-5), protein-mediated (Cyritestin, supramolecular structure), and electrostatic-mediated (AS-A, SGG) binding. In concert, these findings suggest that sperm-egg binding, and thus fertilization, may be achieved through numerous molecular interactions. As a result, this process is now believed to be extremely redundant. Moreover, binding must be considered the result of coordinated sperm-egg recognition machinery, and not just of a single interacting pair of molecules.

In no instance is that more clear than in the case of the GalT 1 deficiency. As noted previously, GalT 1 knockout mice are fertile in vivo, and GalT 1-null sperm bind eggs in vitro at higher levels than wild type sperm. Shur and colleagues have also demonstrated a role for GalT 1 in the regulation of the acrosome reaction (Shi et al., 2001; Youakim et al., 1994), and have shown that GalT 1-null sperm fail to undergo ZP-induced acrosome reactions. However, they do maintain a basal level of spontaneous acrosome reaction that affords them reproductive capabilities in vivo. Regardless of the role of GalT 1 on acrosome stability, the fact that GalT 1-null sperm do not bind soluble ZP3 in vitro, but do bind to the intact ZP, suggests there must be additional components associated with the sperm and the egg coat that facilitate binding. They propose a new two-step model of initial recognition and binding: a GalT 1/ZP3-independent adhesion followed by GalT 1/ZP3-dependent binding that leads to acrosomal exocytosis (Rodeheffer and Shur, 2004). This model is reminiscent of leukocyte interactions with the endothelium, where initial leukocyte binding and subsequent tight adhesion are mediated by distinct receptor-ligand interactions (Ellies et al., 1998).

1.4 Emergence of a ZP3-independent ligand

1.4.1 Oviduct participation during fertilization

The composition of the mouse ZP has been studied extensively (Aviles et al., 2000a; Aviles et al., 2000b; Bleil and Wassarman, 1986; Bleil and Wassarman, 1988; Williams et al., 2006). As a result, it was unlikely the unidentified ZP3-independent ligand was a component of the ZP matrix (e.g. ZP1, ZP2, and ZP3). However, characterization of the mouse ZP has relied primarily on ovarian sources, leaving open the possibility that the ZP may be altered either structurally or compositionally after ovulation.

For several decades the oviduct has been recognized as an active organ in reproduction; participating in gamete transport (Suarez, 1987), sperm capacitation (McNutt and Killian, 1991), fertilization (Staros and Killian, 1998), and early embryo development (King et al., 1994; Nancarrow and Hill, 1995). Numerous reviews on oviductal function are available in the literature (Buhi et al., 2000; Killian, 2004; Leese, 1988). Compositional analysis of oviductal fluid has been completed in several model systems and demonstrates the presence of numerous albumins, globulins, enzymes, protease inhibitors, and growth factors (Killian, 2004). However, determining the function of individual oviductal components has proven difficult. Regardless, specific components of the oviductal fluid have been observed to interact with sperm and eggs in vivo and in vitro (McCauley et al., 2003; McNutt and Killian, 1991; Staros and Killian, 1998). Secreted oviductal proteins may provide an opportunity to account for discrepancies in our current models of sperm-ZP binding.

1.4.2 Isolation and characterization of a ZP3-independent ligand

Given that the oocyte surface changes dramatically during transit through the oviduct, our laboratory hypothesized that additional adhesion factors may be deposited on the egg coat after ovulation, and which may facilitate sperm binding in a ZP3-independent manner. Analysis of ovulated egg coats demonstrated two compositional components: a matrix fraction, composed of the three ZP glycoproteins, and a peripheral fraction, composed of loosely associated proteins. The matrix fraction competitively inhibited ZP binding by wild-type, but not GalT 1-null, sperm, due to the inability of ZP3 to bind GalT 1-null sperm. In contrast, the peripheral fraction inhibited both wild-type and GalT 1-null sperm-ZP binding. Inhibition was shown not to result from egg cortical granule contamination (Rodeheffer and Shur, 2004). Sperm-binding activity in the peripheral fraction could be depleted by WGA-beads, but not by incubation with BS-1 (GS-1)-beads. 2D SDS-PAGE characterization of the ovulated egg coat proteins reveals the ZP3-independent ligand to be a basic, high molecular weight WGA-reactive glycoprotein. Proteins isolated from the basic portion of an isoelectric focusing gel of ovulated egg coats, which does not contain ZP3, were capable of competitively inhibiting sperm-ZP binding in vitro. Unfortunately, the amount of ZP3-independent ligand that could be extracted from ovulated egg coats was not sufficient for sequence analysis, and thus its identity remained unknown (Rodeheffer and Shur, 2004).

1.4.3 Implications

The likelihood that sperm-ZP binding involves multiple adhesion mechanisms complicates the traditional approaches used to assess the basis of sperm-egg interactions.

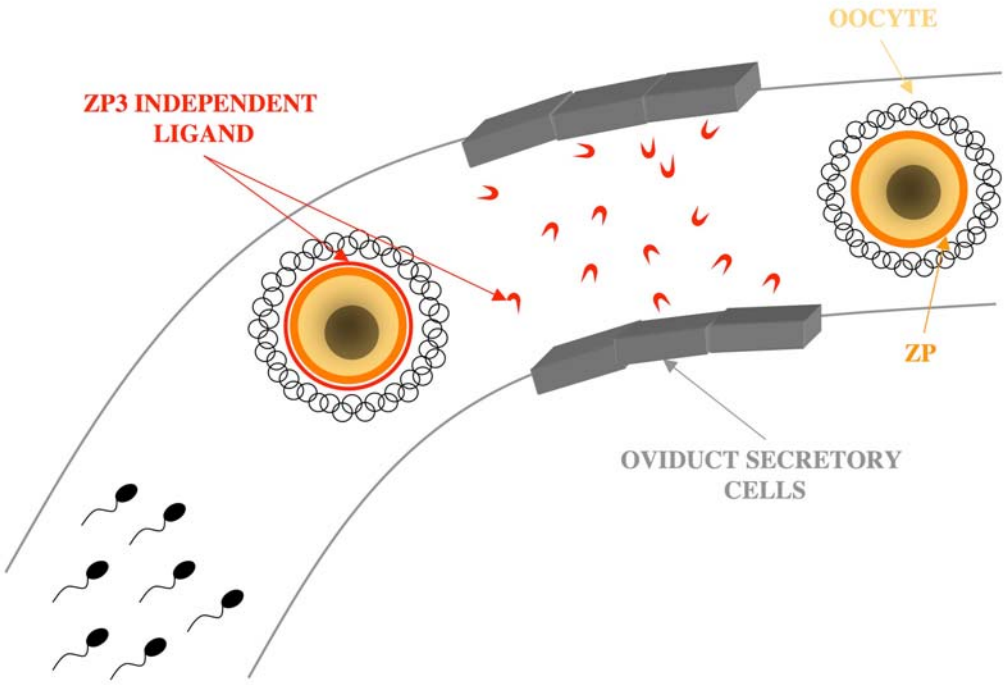
Previous findings must be challenged and scrutinized, particularly when data eliminate a factor as playing a role in sperm-egg binding. For instance, the biochemical analysis of available sugar moieties of the egg coat was completed on the ZP matrix alone. This analysis failed to include carbohydrate structures on any oviduct-derived components that associate with the egg coat after ovulation. As a result, there exists an additional pool of carbohydrate structures that may function in binding, which could explain the inhibitory nature of oligosaccharides not found within the zona matrix (Aviles et al., 1999; Aviles et al., 2000b). Similarly, the role of several carbohydrate moieties in sperm-ZP binding has been criticized because knockouts of specific glycosyltransferases do not ablate sperm-ZP binding and fertility. For example, the role of hybrid *N*-linked glycans in sperm-egg binding has been refuted, since elimination of ovarian *Mgat1*, a glycosyltransferase responsible for such glycans, does not impair fertility (Shi et al., 2004). Additionally, Core 2-null mice, which lack *O*-glycans attributed to binding, also remain fertile (Ellies et al., 1998). Such investigations, however, do not account for any compensation by glycan residues of secreted oviductal proteins. Furthermore, the ability of humanized hZP2 mouse eggs to support sperm binding, even after fertilization, has been cited as support for the protein-mediated model (Rankin et al., 2003). If the ZP3-independent ligand is removed from the egg coat after fertilization, the possibility exists that its removal from the ZP surface is dependent on ZP2 cleavage. Thus, in the absence of ZP2 cleavage, as in the hZP2 egg, the ZP3-independent ligand may remain available for sperm binding. Finally, oviduct-specific glycoprotein (OGP), a chitinase protein family member secreted by the oviduct, was determined not to be essential for fertilization in mouse (Araki et al., 2003). Previous reports in various species, however, demonstrate

that OGP interacts with eggs and spermatozoa and has a positive effect on fertilization (Martus et al., 1998; McCauley et al., 2003; O'Day-Bowman et al., 2002). Again, the inconsistency may reside in the potential redundancy of interactions.

1.5 Goals

It is now believed that sperm-egg binding in the mouse requires a multiplicity of receptor-ligand interactions, one of which is an oviduct-derived WGA-reactive, high molecular weight glycoprotein that associates with the egg coat at ovulation. Our ultimate goal is to purify and identify this sperm-binding ligand. Based on data showing that the ZP3-independent ligand associates with the egg coat after ovulation, we hypothesize the ligand is expressed and secreted from the oviduct (Figure 1-2). As a result, we predict the superovulated oviduct would be a rich source of the ZP3-independent ligand. Our approach seeks to use functional assays to locate and enrich the ZP3-independent ligand from mouse oviducts (Chapter 3). After identification, the expression, localization, and functional domains of the ligand will be investigated (Chapter 4).

Figure 1-2. Model of ZP3-independent ligand association with the egg coat. Ovulated oocytes, surrounded by cumulus cells, enter the oviduct. The oocyte passes by oviductal secretory cells that are secreting the ZP3-independent ligand, which associates with the zona pellucida prior to the introduction of sperm.


OOCYTE
ZP3 INDEPENDENT
LIGAND
ZP
OVIDUCT SECRETORY
CELLS

Chapter 2: Materials and Methods

2.1 Sperm-egg binding assay

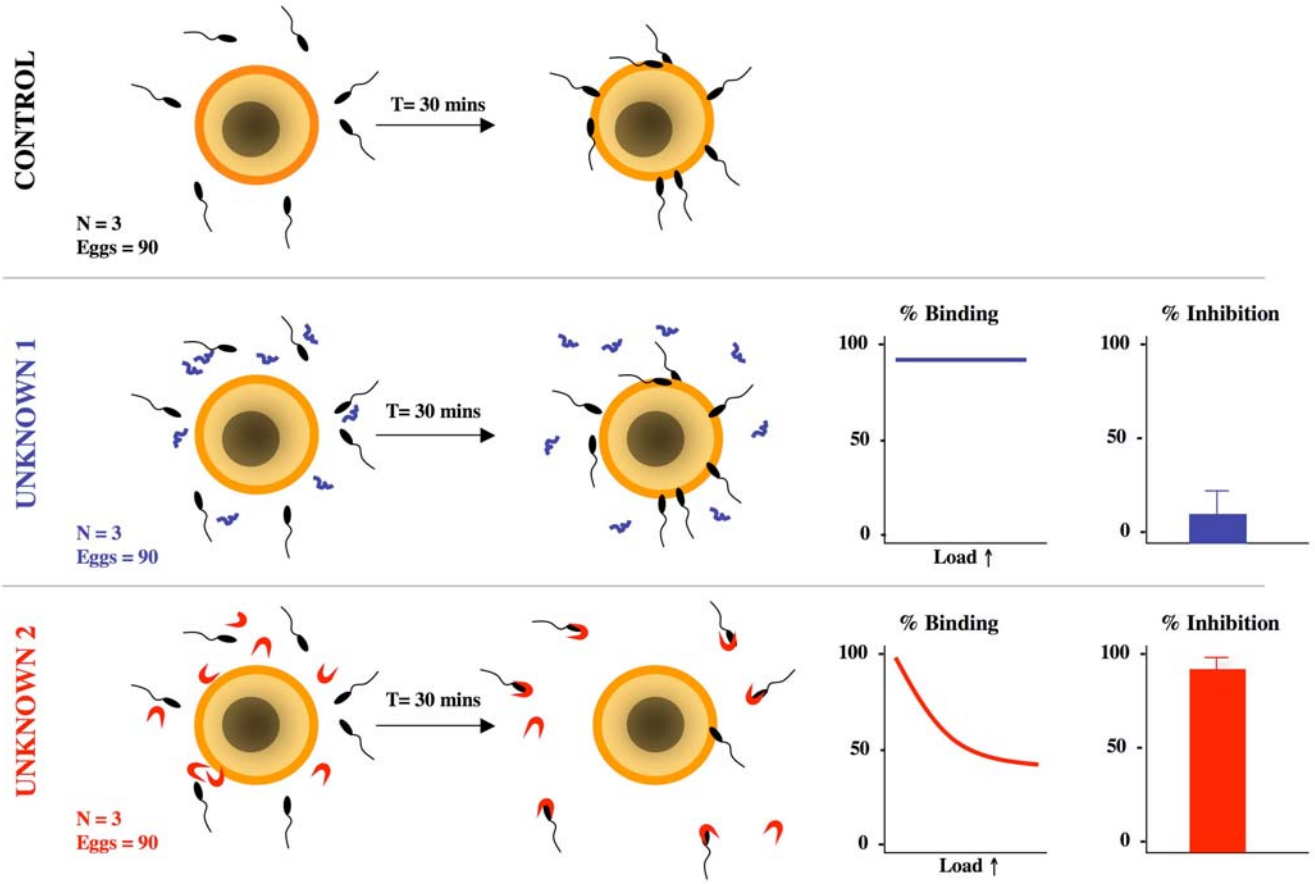
All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. Eight-week old CD-1 female mice (Charles River; Wilmington, MA) were superovulated by hormone injection using 7.5 I.U. of pregnant mare's serum (PMS) and human chorionic gonadotropin (hCG), 48 hours apart. Cumulus-oophorous masses were collected from the oviducts of superovulated females. The masses were transferred into 0.2% hyaluronidase in 1X phosphate-buffered saline (PBS). Cumulus free eggs were then washed through 3 drops of modified Krebs-Ringer buffer (mKBRT; 120 mM NaCl, 2 mM KCl, 2 mM CaCl2, 10 mM NaHCO3, 0.36 mM NaHPO4H2O, 1.2 mM MgSO4H2O, 5.6 mM glucose, 1.1 mM sodium pyruvate, 25 mM TAPSO, 18.5 mM sucrose, 1X penicillin/streptomycin (Gibco; Carlsbad, CA), and 6 mg/ml BSA (fatty acid free), pH 7.4), via a glass pipette approximately twice the diameter of the egg. Two-cell embryos were collected into dmKBRT (but not washed) from the oviducts of superovulated CD-1 females that were mated 15 hours earlier. The caudae epididymides of CD-1 males or GalT 1-null males were dissected into dmKBRT and shredded. The epididymides were incubated at atmospheric CO2 at 37°C for 15 minutes to release the sperm, which were collected after filtration (Nitex; Sefar America; Kansas City, MO). The sperm were further capacitated for 45 minutes and number of sperm was determined. 40,000 sperm were then co-incubated with 25-35 ovulated cumulus-free eggs and 3-5 two-cell embryos (as a control for non-specific binding) in 50 μl drops of dmKBRT for 30 minutes at 37°C. Eggs and embryos were washed through sequential drops of

dmKBRT until ~1 sperm remained bound to the two-cell embryos. The gametes were fixed in 4% paraformaldehyde in PBS. The number of sperm bound to each egg and two-cell embryo was counted using phase-contrast optics (Figure 2-1). The average number of sperm bound/two-cell embryos (non-specific binding) was subtracted from the average number of sperm bound/eggs. The data presented are the average of at least three experiments (s.e.m.), each of which contained triplicate droplets for each experimental parameter, unless otherwise noted.

2.2 Western and lectin blot analysis of oviduct lysates

Eight-week old CD-1 female mice were superovulated as above. Six females were sacrificed at 0, 12, 14, 16, 18 hours post hCG injection. Oviducts were dissected from the ovary and uterus into ice-cold 500 μl PBS, pH 7.4. Oviducts were homogenized, and the insoluble debris was removed by centrifugation for 1 hour at 13,000 rpm. Oviducts and ovaries from six non-hormonally injected, random cycling, females were also obtained. Samples were denatured with 2X loading buffer (0.125 M Tris, 2% glycerol, 2% SDS, 0.5% β-mercaptoethanol, 20 mM DTT) at 95°C for 3 minutes. Lysates were fractionated on a 7.5% Criterion SDS-PAGE gel (Bio-Rad Laboratories; Hercules, CA). Proteins were transferred to PVDF (Millipore; Billerica, MA) and blocked in 1% BSA, TBS-T (0.1% Tween 20, 0.8% NaCl, 0.002% KCl, 25 mM Tris, pH 7.4). Membranes were incubated with a 1:2000 dilution of goat anti-OGP polyclonal antibody (Santa Cruz; Santa Cruz, CA) and subsequently in a 1:1000 dilution of donkey anti-goat IgG-horseradish peroxidase (HRP) (Santa Cruz). Membranes were incubated with 1 μg /ml of biotinylated lectins. Blots were washed and subsequently probed with a 1:50,000 dilution of

Figure 2-1. Sperm-egg binding assay. Sperm are co-incubated with 25-35 ovulated cumulus-free eggs and 3-5 two-cell embryos (as a control for non-specific binding) in 50 μl drops of dmKBRT for 30 minutes at 37°C. Eggs and embryos are washed through sequential drops of dmKBRT until ~1 sperm remained bound to the two-cell embryos. The number of sperm bound to each egg and two-cell embryo are counted using phase-contrast optics. The average number of sperm bound/two-cell embryos (non-specific binding) is subtracted from the average number of sperm bound/eggs and compared to control binding. Unless otherwise noted, the data presented in this thesis are the average of at least three experiments (s.e.m.), each of which contained triplicate droplets for each experimental parameter.


CONTROL
T= 30 mins
N = 3
Eggs = 90
UNKNOWN 1
T= 30 mins
N = 3
Eggs = 90
% Binding
100
50
0
Load ↑
% Inhibition
100
50
0
UNKNOWN 2
T= 30 mins
N = 3
Eggs = 90
% Binding
100
50
0
Load ↑
% Inhibition
100
50
0

streptavidin-HRP (Zymed, South San Francisco, CA). After washing, the chemiluminescence signal was assayed (GE Healthcare; Fairfield, CT) and band density was quantified using spot densitometer software (Alpha Innotech Corp; San Leandro, CA).

2.3 ZP3-independent ligand and Oviduct-Specific Glycoprotein (OGP) purification

Twenty-four 8-week old CD-1 female mice were superovulated as above and sacrificed at 16 hours hCG injection. Oviducts were placed in 1 ml ice-cold Lectin Affinity Buffer (10 mM Phosphate Buffer, 150 ml NaCl, 0.25 mM CaCl2, pH 7.4). Oviducts were homogenized, and the insoluble debris was removed by centrifugation.

Denaturing purification - Oviduct lysates were denatured with 2X loading buffer and fractionated on a 5.0% Criterion SDS-PAGE gel. Electrophoresis conditions were optimized so that the separation between the 250 kDa and 150 kDa molecular weight markers was significant enough to properly select various ranges. Excised gel pieces were minced and eluted in a Bio-Rad Electroluter according to the manufacturer‘s instructions. Electroluted proteins were collected and dialyzed 3 times in a 10,000 MWCO Slide-A-Lyzer (Pierce Biotechnology; Rockford, IL) in 500 ml 8 M urea, 10 mM phosphate buffer, pH 7.4 at 4°C, followed by 3 times into 10 mM phosphate buffer.

Native purification - Lysates from 48 superovulated oviducts were fractionated on a Pharmacia FPLC system. Lysates were applied to a Superose 6 size separation column (GE Healthcare) at 0.3 ml/min in 50 mM HEPES, 50 mM NaCl, pH 7.4. Fractions were collected in 0.5 ml volumes and assayed for OGP by western blot analysis. OGP-positive

fractions were pooled and run over a MONO-Q ion exchange column (GE Healthcare). After sufficient washing, bound proteins were eluted with a 0.05-1 M NaCl gradient in 50 mM HEPES, pH 7.4. OGP-positive fractions were pooled and concentrated. The sample was resuspended in 5 ml Lectin Affinity Buffer and separated on various lectin columns (10 ml column, run at 0.5 ml/min) as indicated. Eluted proteins were concentrated and the free sugar was dialyzed against PBS. After dialysis for both denaturing and native purification, samples were concentrated to 30-50 µl. An aliquot of the fluid that passed through the concentrator was collected and served as a dialysis control in sperm-egg binding assays. Concentrated samples and dialysis controls were assayed for protein concentration using RC DC Protein Assay (Bio-Rad Laboratories).

For lectin depletion or enrichment - Oviduct lysates or native OGP fractions, in lectin affinity buffer, were added to 20 ml *Griffonia simplicifolia* (GS-1), Concanavalin A (Con A), *Ricinus communis* agglutinin-1 (RCA-1), *arachis hypogaea* (PNA), or wheat germ agglutinin (WGA) agarose columns (E.Y. Laboratories; San Mateo, CA). Depending on the desired purification, columns were used in a variety of sequential configurations. After sufficient washing of the columns with Lectin Affinity Buffer, bound proteins were eluted with 0.5 M melibiose, (GS-1), 0.2 M D-methyl mannose (Con A), 0.1 M lactose (RCA-1, PNA), 0.5 M N-acetyl-D-glucosamine (WGA), or 1 M NaCl. Eluted proteins were collected, dialyzed against PBS to remove free sugar or salt, concentrated by iCON concentrators (Pierce Biotechnology), and resolved by SDS-PAGE or assayed for biological activity.

2.4 Gamete interaction assays

Sperm-pull down assay - Distinct bioactive fractions were biotinylated at a 20:1 molar ratio using Pierce EZ-Link Sulfo-NHS-LC-LC-biotin (Pierce Biotechnology). After dialysis to remove free biotin, individual fractions were incubated with two million capacitated wild-type sperm at 12 μg/ml in mKBRT. After 30 minutes incubation at 37°C, the sperm were collected by centrifugation, the supernatant was discarded and the sperm pellet washed several times in mKBRT or PBS. After the final wash, the sperm were resuspended and all associated proteins extracted by either 1% Triton X-100, 1 M NaCl, or heat denaturation (70°C for 1 hour). The sperm were pelleted by centrifugation, and the extracted proteins within the supernatant were collected and prepared for 1D SDS-PAGE. The gel-separated proteins were transferred to PVDF and probed with streptavidin-HRP to identify any biotinylated species that were "pulled down" from the original supernatant by the sperm.

Exogenous OGP binding - The purified OGP glycoforms were biotinylated as described above and free biotin was removed by dialysis, after which each glycoform was added to a suspension of 40,000 capacitated sperm in mKBRT or to a 50 μl mKBRT droplet containing10-15 cumulus-free ZP-intact oocytes and four or five 2-cell embryos. Cumulus cells were removed from the oocytes by hyaluronidase treatment as described above. Controls were included for each glycoform. After co-incubation, the gametes and embryos were washed two-times in mKBRT, resuspended in 4% paraformaldehyde in PBS for 10 minutes, after which gametes and embryos were washed to remove excess paraformaldehyde, placed on microscope slides, and bound biotinylated OGP detected

with Texas-Red streptavidin (Molecular Probes, Carlsbad, CA). Gametes were imaged under conventional (60X magnification) and confocal (5 μm sections) microscopy.

2.5 OGP immunodepletion and immunoprecipitation

LE 200-250 kDa fractions, prepared as described above, were split into three identical 2 μg samples. The first sample, which served as a positive control for bioactivity, was stored at 4°C. The second sample was subjected to three 2-hour batch depletions with anti-OGP antibodies cross-linked to magnetic beads (Invitrogen; Carlsbad, CA). Immunobeads were removed and the depleted supernatant was collected. The third sample was treated identically to the OGP immunodepletion, however, the beads were prepared with a non-specific IgG and served as a control for the immunodepletion procedure. Proteins bound to the antibody-conjugated beads were recovered by acidic extraction and dialyzed against PBS. One half of each extracted sample was prepared for 1D SDS-PAGE and western blot analysis, and the other half was utilized in a sperm-ZP binding assay.

2.6 Immunohistochemistry and immunolocalization

Oviducts were isolated from superovulated CD-1 female mice, fixed overnight in Bouin's solution, and paraffin embedded. Sections (5 μm) were subjected to microwave "antigen retrieval" as described (Janssen et al., 1994). Sections were cooled, blocked in 5% milk TBS-T, and processed for immunocytochemistry using 1:100 primary anti-OGP antibody, 1:1000 Alexa Fluor 488-conjugated chicken-anti-goat (Molecular Probes). For

oocyte immunolocalization studies, “clutches” were obtained from superovulated CD-1 females and the cumulus cells were removed using 0.2% hyaluronidase in PBS. When indicated, mechanical removal of cumulus cells was achieved by repetitive pipetting of clutches through a small pore pipette. Cumulus-free eggs and 2-cell embryos were washed 3 times in mKBRT. Oocytes and 2-cell embryos were fixed in 50 µl 4% paraformaldehyde for 1 hour. After fixation, eggs were washed and blocked with 2% BSA in PBS for 1 hour and incubated with goat anti-OGP antibody or a non-specific goat IgG at room temperature for 30 minutes with mild shaking. Oocytes and embryos were washed 3 times in 2% BSA in PBS, incubated with 1:1,000 anti-goat Alexa Fluor 488 (Molecular Probes) for 30 minutes with mild shaking at room temperature, washed 3 times in 2% BSA in PBS, placed on slides, and imaged at 60X magnification.

2.7 Fractionation of the ampullar environment and cumulus-oocyte complexes

Oviducts were acquired from 48 superovulated CD-1 female mice, and washed 2 times in a vast excess of ice-cold PBS to remove any contaminating surface proteins. After washes, oviducts, one at a time, where transferred into 5 ml of fresh ice-cold PBS and their ampullae were pierced allowing the ampullar contents and cumulus-oocyte complexes to be expelled. The cumulus-oocyte complexes were immediately removed and placed in 1 ml PBS on ice, and the remaining oviducts were discarded. The cumulus-oocyte complexes were centrifuged at 4,000 rpm for 10 minutes at 4°C. The supernatant was collected, concentrated (final volumes: 20 µl for SED-PAGE, and 250 µl for chromatography analysis), and labeled “ampullary fluid.” The pelleted cumulus-oocyte complexes were washed 5 times in 1 ml PBS by inverting the microcentrifuge tube 10

times followed by centrifugation at 66 x g for 2 minutes. The wash supernatants were either collected individually and labeled by number, or pooled and labeled “washes”. Washed cumulus-oocyte complexes were resuspended in 250 µl of 0.2% hyaluronidase in PBS. Cumulus-free oocytes were washed 3 times in PBS and resuspended in 100 µl PBS. The washes containing 0.2% hyaluronidase, cumulus cells, and oocyte were collected and spun at 13,000 rpm for 5 minutes. The supernatant was collected, concentrated, and labeled “cumulus removal supernatant.” The cumulus cells were resuspended in 2X gel loading buffer. Cumulus-free oocytes were incubated at 70ºC for 1 hour to solubilize the ZP and all associated proteins. After incubation, zona-free eggs were spun at 66 x g for 5 minutes. The supernatant was collected, concentrated, and labeled “solubilized zona.” The zona-free oocytes were resuspended in 2X gel loading buffer. Samples were analyzed by 1D SDS-PAGE and anti-OGP immunoblot and lectin blot as described. For 2D SDS-PAGE analysis, samples were dialyzed into 50 mM HEPES, pH 7.4 overnight, acetone precipitated, and resolubilized in 1st dimension buffer as per the manufacturer’s instruction (Bio-Rad Laboratories). Samples were run on Bio-Rad IGP 3-10 non-linear strips (11 cm) using the Bio-Rad Protean IEF Cell system. The 2nd dimension was run on Criterion 10% IPG + 1 well gels (Bio-Rad Laboratories). Lectin affinity chromatography was performed as described above.

2.8 Deglycosylation of PNA-binding OGP

A preparation of the PNA-binding OGP glycoform was separated into three identical aliquots. The first aliquot served as an internal control for bioactivity. The second aliquot was heat-denatured for 10 minutes at 65°C. The final aliquot, as well as

the control glycoprotein bovine fetuin, were deglycosylated using the E-DEGLY kit, per manufacturers instructions (Sigma), which enzymatically removes N- and O-glycans. After deglycosylation, the glycosidases were inactivated by heat denaturation. To control for any affect of the denatured glycosidases, a control sample containing glycosidases but lacking OGP was heat-denatured. All samples were assayed for sperm-binding activity.

Chapter 3. Oviduct derived oviduct-specific glycoprotein (OGP) is identified as a sperm adhesion ligand

3.1 Hypothesis

The ability of GalT 1-null sperm to bind ovulated ZP, but not soluble ZP3, suggests that the ovulated egg coat contains adhesion factors other than ZP3 that mediate initial sperm binding. Analysis of ovulated egg coats demonstrates the presence of two distinct sperm-binding components: an insoluble matrix fraction, composed of the three ZP glycoproteins, that competitively inhibits ZP binding by wild-type, but not GalT 1-null sperm; and a peripheral fraction, composed of loosely associated proteins, that is able to inhibit both wild type and GalT 1-null sperm-ZP binding (Rodeheffer and Shur, 2004). Furthermore, bioactivity in the peripheral fraction is depleted by WGA lectin-beads, but not by BS-1 (GS-1) lectin-bead incubation. Using 2-D gel electrophoresis, a basic, high molecular weight (200-250 kDa) WGA-reactive glycoprotein associates with ovulated, but not ovarian egg coats. When extracted from the basic end of an iso-electric focusing gel, this protein species inhibits wild-type and GalT 1-null sperm from binding to eggs. Unfortunately, purification of this ligand from ovulated eggs is untenable due to the small levels of obtainable protein, leaving the identity of the factor unknown (Rodeheffer and Shur, 2004).

Based on these results, we hypothesize the ZP3-independent ligand is expressed in the oviduct, where it is secreted prior to or during ovulation, and associates with the egg coat and facilitates initial sperm binding. This hypothesis predicts that the oviduct would provide a significantly larger pool of the ZP3-independent ligand, as compared to

ovulated egg coats. Therefore, the ZP3-independent ligand was isolated from oviduct lysates and identified guided by the following specific aims:

1) to demonstrate that a protein with similar characteristics to the ZP3-independent ligand can be isolated from the oviduct,
2) to achieve suitable enrichment of the ZP3-independent ligand for sequence identification, and
3) to validate the identified candidate is a ZP3-independent ligand by analysis of the purified protein and by specific immuno-depletion.

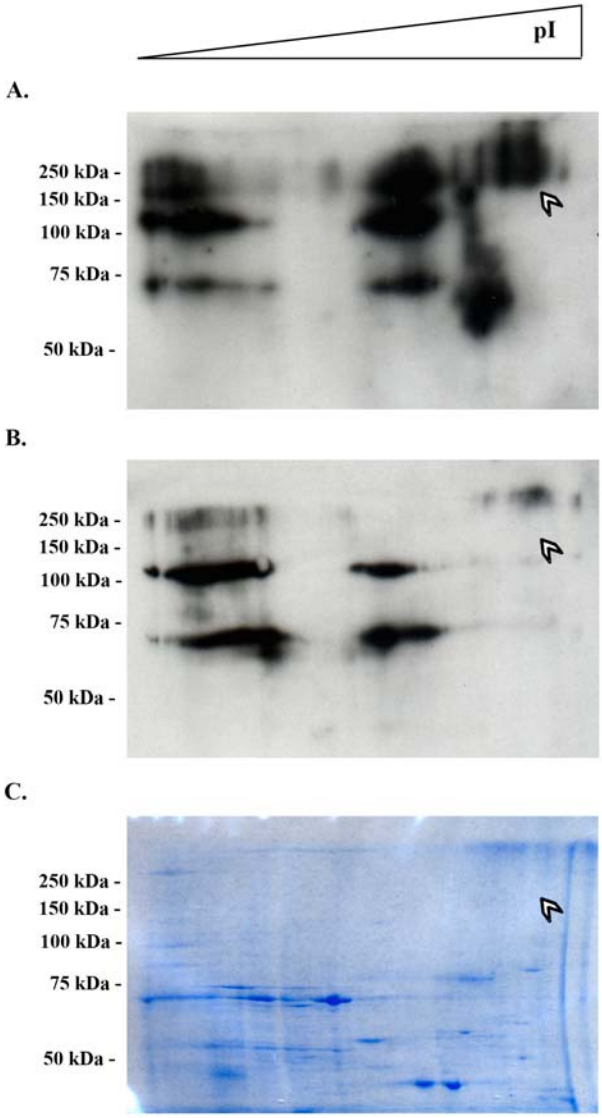
The results presented here show that the oviduct is a rich source of a ZP3-independent sperm-binding ligand. Specifically, a high molecular weight glycoprotein that inhibits sperm-ZP binding and demonstrates a sperm-specific interaction in vitro was isolated and identified as oviduct-specific glycoprotein (OGP). Targeted purification of native OGP and specific immuno-depletion validated OGP as a sperm adhesion ligand. Although OGP is reminiscent of the ZP3-independent ligand previously characterized using GalT 1-null sperm, previous studies demonstrate that GalT 1-null sperm can bind to OGP-null oocytes in vitro (Rodeheffer and Shur, 2004). Thus, it appears that OGP is one of several ZP3-independent sperm-binding ligands. Furthermore, results show that OGP is expressed as a heterogeneous population of distinct isoforms that show isoform-specific distribution and function.

3.2 Results

3.2.1 Superovulated oviduct lysates contain glycoproteins reminiscent of the ZP3-independent ligand

Based on our hypothesis, we predicted that lysates of superovulated oviducts would contain basic, high molecular weight glycoproteins that are WGA-, but not GS-1-reactive. Consistent with this, protein and lectin analysis of 2D SDS-PAGE fractionated soluble oviductal proteins collected from superovulated females demonstrate numerous proteins ranging in pI and molecular weight. Most pertinent is the observation that the basic, high molecular weight region (i.e. proteins above 150 kDa) contains several glycoproteins that are WGA-reactive and GS-1-non-reactive (Figure 3-1 A,B). Unfortunately, none of these lectin-defined regions produced a band visible by protein stain that could be immediately assessed for inhibition of sperm-ZP binding or for sequence identification (Figure 3-1 C).

Figure 3-1. Superovulated oviduct lysates contain WGA-, but not GS-1-reactive basic high molecular weight glycoproteins. 2-D SDS-PAGE fractionation of 150 μg of superovulated oviduct lysates and subsequent analysis by A) WGA blot, B) GS-1 blot, and C) Coomassie blue protein stain. Images are representative of two experiments. Arrows indicate a basic, high molecular weight region that is WGA, but not GS-1, reactive, which does not correlate with a specific protein species.


pI
A.
250 kDa -
150 kDa -
100 kDa -
75 kDa -
50 kDa -
B.
250 kDa -
150 kDa -
100 kDa -
75 kDa -
50 kDa -
C.
250 kDa -
150 kDa -
100 kDa -
75 kDa -
50 kDa -

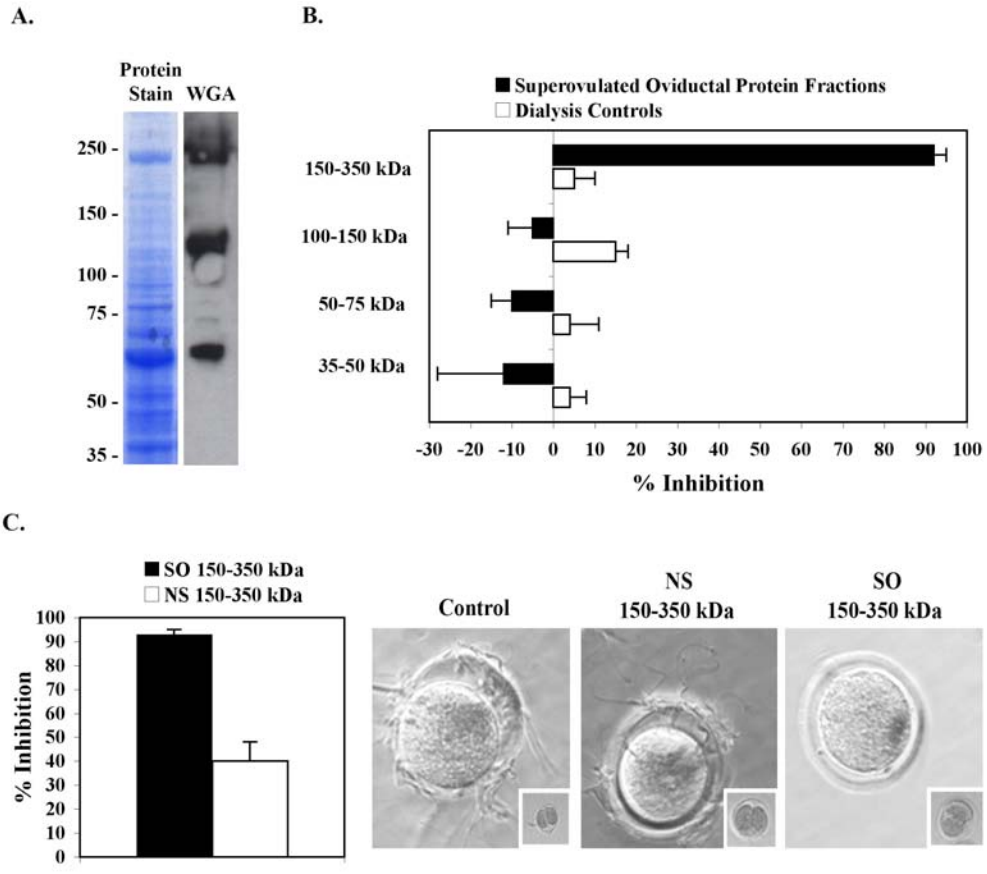
3.2.2 WGA-reactive high molecular weight fractions of superovulated oviduct lysates are capable of inhibiting sperm-egg binding

The lack of a visible protein species after 2D SDS-PAGE with similar lectin binding characteristics as the ZP3-independent ligand, required that superovulated oviductal proteins be concentration prior to their evaluation as sperm-adhesion molecules in the sperm-ZP inhibition assay. Unfortunately, the entire oviduct lysate could not be assayed due to non-specific agglutination of sperm. Therefore, forty-eight superovulated oviduct were homogenized and lysates were resolved by 1D SDS-PAGE. WGA-reactive species were identified by lectin blot at 150-350 kDa, 100-150 kDa and 50-75 kDa (Figure 3-2 A). Corresponding areas of the gel from multiple lanes were excised, extracted, and assayed for sperm-binding activity as indicated in the Materials and Methods. Additionally, a WGA-non-reactive region (37-50 kDa) was prepared in parallel to control for non-specific effects of sample preparation. Finally, to control for residual SDS and other buffer contaminants that would adversely affect the assay, an aliquot of the dialysate was collected and assayed as well. Pre-incubation of wild-type sperm with 4 μg protein from the high molecular weight region (150-350 kDa) specifically inhibited sperm binding to cumulus-free oocytes (Figure 3-2 B), whereas none of the other WGA-reactive or non-reactive regions had sperm-binding activity. Dialysis controls also did not perturb sperm-ZP binding, suggesting the bioactivity of the 150-350 kDa range was not an artifact of sample preparation. Furthermore, inhibition was not due to the general presence of WGA-reactive, GlcNAc-terminating glycoproteins as the 100-150 and 50-75 kDa fractions lacked bioactivity.

Since a number of oviduct glycoproteins show hormone-dependent expression, we

examined the possibility that the ZP3-independent ligand activity is hormonally regulated (Buhi, 2002; Buhi et al., 2000). High molecular weight (150-350 kDa) glycoproteins from superovulated and non-stimulated oviducts were obtained by identical methods and assayed for sperm-binding activity. Samples prepared from superovulated oviducts had more than twice the specific activity as samples prepared from non-stimulated, randomly cycling females (Figure 3-2 C). These results indicate that the sperm-binding activity present within the 150-350 kDa range is not the result of nonspecific effects from residual SDS or the purification protocol, and is upregulated during hormonal stimulation and ovulation.

Figure 3-2. High molecular weight proteins (150-350 kDa) from superovulated oviductal lysates demonstrate specific inhibition of sperm-ZP binding. A) 1D SDS-PAGE separation of 150 μg protein from superovulated oviduct lysates and subsequent protein stain and WGA lectin blot analysis. Specific WGA-positive and WGA-negative regions were selected for enrichment. Protein stain and lectin blot are representative of at least three experiments. B) Forty-eight oviducts were obtained from superovulated CD-1 females, homogenized, separated by 1D SDS-PAGE, and the indicated regions of the gel were excised. Following dialysis to remove contaminants, 4 μg of each sample was added droplets of capacitated sperm, and after 10-minute incubation, cumulus-free oocytes were added. Proteins isolated from the high molecular weight range showed strong competitive inhibition of sperm-ZP binding, whereas none of the other WGA-reactive polypeptide species did. C) The high molecular weight proteins (150-350 kDa) from superovulated (SO) oviduct lysates (■) demonstrate significantly higher sperm-binding activity than those from non-stimulated (NS) control oviducts (□). For B and C, each bar represents the mean ± s.e.m. of 3 experiments, each conducted in triplicate. The level of sperm binding is shown in representative oocytes and 2-cell embryos after three rounds of washing.

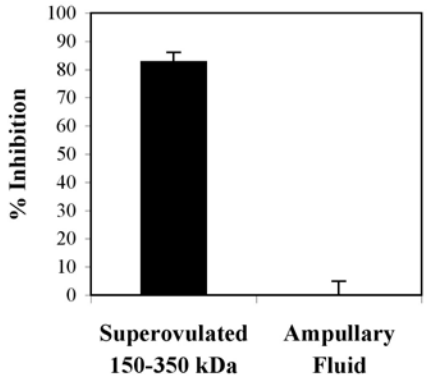

A.
Protein Stain
WGA
250 -
150 -
100 -
75 -
50 -
35 -
B.
Superovulated Oviductal Protein Fractions
Dialysis Controls
150-350 kDa
100-150 kDa
50-75 kDa
35-50 kDa
-30 -20 -10 0 10 20 30 40 50 60 70 80 90 100
% Inhibition
C.
SO 150-350 kDa
NS 150-350 kDa
% Inhibition
100 90 80 70 60 50 40 30 20 10 0
Control
NS 150-350 kDa
SO 150-350 kDa

3.2.3 Ampullary fluid from superovulated oviducts does not provide sufficient levels of ZP3-independent ligand for analysis

Although the high molecular weight proteins of hormonally stimulated oviducts appeared to provide the best opportunity for elucidating the identity of the ZP3-independent ligand, protein stain and WGA blot analysis of the fraction demonstrated it to be highly heterogeneous (data not shown). Since fertilization occurs in the ampullea portion of the oviduct, it was determined whether the soluble contents collected from the ampullea possess ZP3-independent ligand activity.

Twenty-four oviducts from superovulated female mice were used to collect the high molecular weight range of proteins as described above. An additional twenty-four superovulated oviducts were washed in several rounds of PBS and placed in a minimal amount of buffer, where the ampullea was pierced with fine tip tweezers to release the cumulus-oocyte complexes. The cumulus-oocyte complexes were removed and discarded. The ampullea were pressed to express the ampullary fluid. All insoluble components were removed and the remaining soluble preparation was concentrated. Overall, 10 μg of ampullary protein was obtained and assayed, along with 4 μg of the superovulated oviduct 150-350 kDa fraction (Figure 3-3). Unlike the enriched high molecular weight oviductal proteins, the ampullea fluid did not inhibit sperm-ZP binding.

Figure 3-3. Bioactivity observed in the enriched superovulated oviduct high molecular weight proteins (150-350 kDa) is not recapitulated utilizing secreted proteins from the ampullea. Twenty-four oviducts were obtained from both superovulated CD-1 females, which were homogenized, separated by 1D SDS-PAGE, and the high molecular range proteins were extracted. Following dialysis to remove contaminants, 4 μg of each sample was added to a single KBRT droplet for a final concentration of 80 μg/ml. Simultaneously, 24 superovulated oviducts were collected, washed, and their ampullea were pierced to remove cumulus-oocyte complexes and ampullary fluid. The oviducts and cumulus-oocyte complexes were discarded and the ampullary fluid was collected and concentrated. 10 μg of ampullary fluid was added to a single KBRT droplet for a final concentration of 200 μg/ml. Capacitated sperm were pre-incubated in these droplets for ten minutes prior to the introduction of cumulus-free oocytes. Quantitative results from one experiment, which was performed in triplicate, are shown. Each bar represents the mean ± s.e.m.

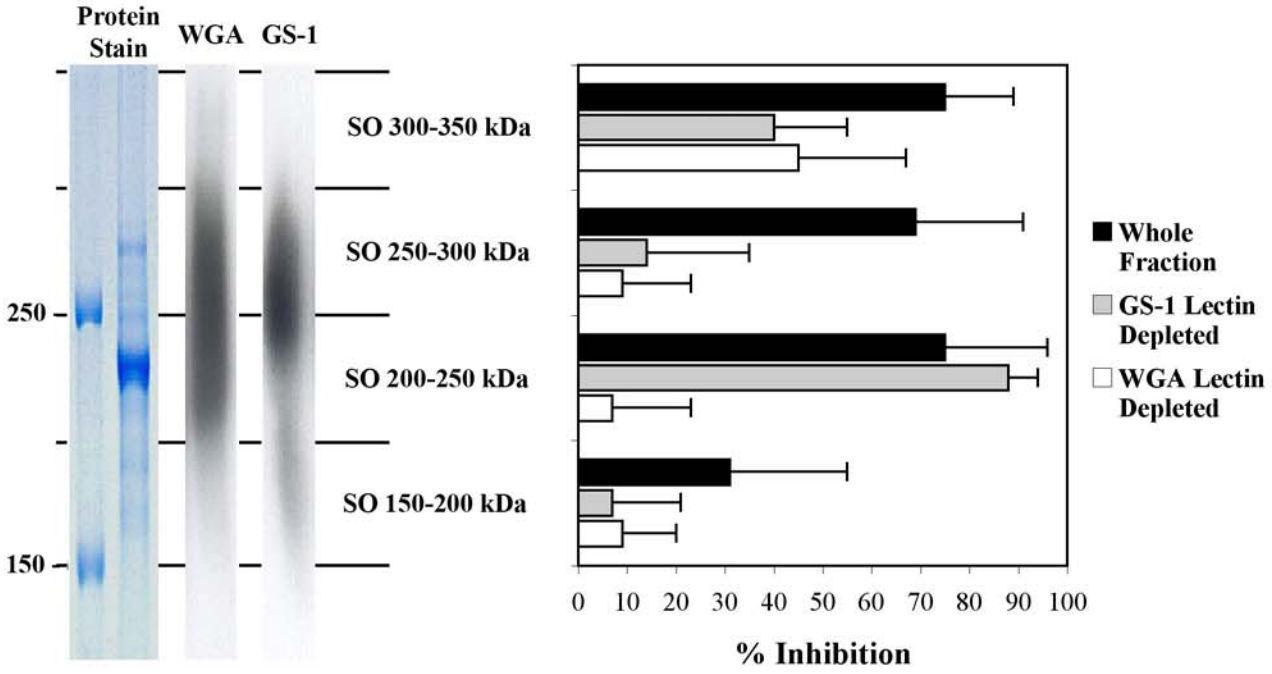

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
Superovulated 150-350 kDa
Ampullary Fluid

3.2.4 Superovulated oviductal proteins residing in the 200-250 kDa range demonstrate similar characteristics to the ZP3-independent ligand

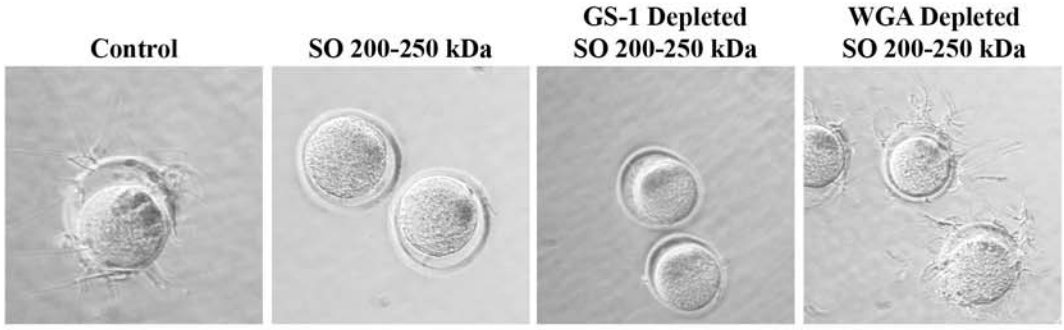
Since bioactivity could not be identified in total, unfractionated soluble ampullea proteins, the ZP3-independent ligand was characterized using the high molecular weight protein fraction isolated from superovulated oviduct lysates. Unfortunately, the heterogenous nature of this fraction prevented accurate identification of the ZP3-independent ligand. To further resolve the biologically-active species in the high molecular weight range, the 150-350 kDa region was divided into four equal ranges: 150-200 kDa, 200-250 kDa, 250-300 kDa, and 300-350 kDa, each of which was extracted and assayed for sperm-binding activity. Analysis of proteins in these four narrow molecular weight ranges demonstrated strong bioactivity in the three largest molecular weight fractions. To determine if these bioactivities possessed similar lectin characteristics as the previously identified ZP3-independent ligand, the fractions were depleted by WGA or GS-1 agarose beads. Only the bioactivity in the 200-250 kDa fraction was eliminated by WGA-agarose depletion, but not GS-1-agarose depletion, similar to the ZP3-independent ligand (Figure 3-4).

Figure 3-4. Bioactivity residing in soluble superovulated oviductal proteins within the molecular weight range of 200-250 kDa is reminiscent of the ZP3-independent ligand. A) 1D SDS-PAGE separation of 12 μg of enriched superovulated oviduct (SO) 150-350 kDa proteins and subsequent protein stain and WGA- and GS-1- lectin blot analysis. Biological activity of enriched superovulated proteins, defined by 150-200 kDa, 200-250 kDa, 250-300 kDa, and 300-350 kDa molecular weight ranges, before and after WGA- or GS-1- lectin agarose bead depletion. The starting concentration for each protein range in each droplet was 20 μg/ml. Each bar represents one experiment, completed in triplicate. B) Representative oocytes from the SO 200-250 kDa proteins both before and after WGA- or GS-1- lectin agarose bead depletion.

A.


Protein Stain
WGA
GS-1
250
150
SO 300-350 kDa
SO 250-300 kDa
SO 200-250 kDa
SO 150-200 kDa
0 10 20 30 40 50 60 70 80 90 100
% Inhibition
Whole Fraction
GS-1 Lectin Depleted
WGA Lectin Depleted

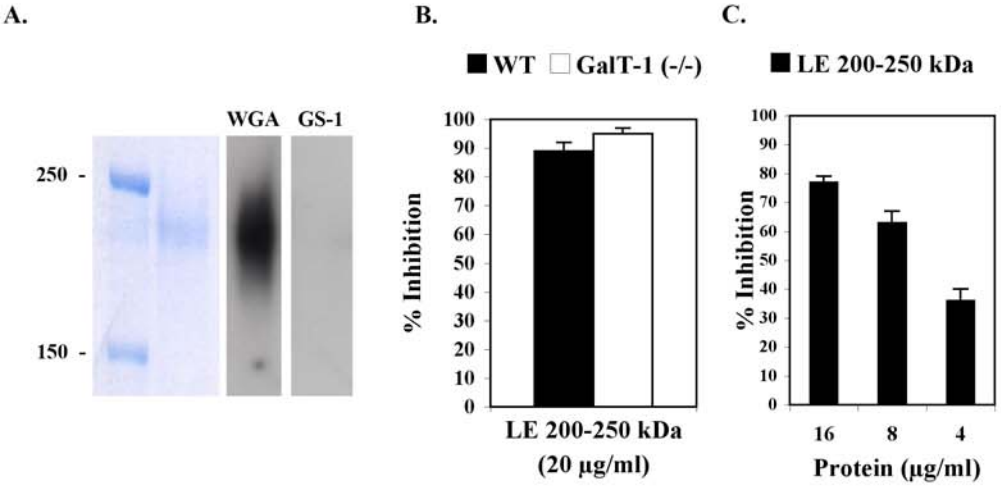
B.


Control
SO 200-250 kDa
GS-1 Depleted SO 200-250 kDa
WGA Depleted SO 200-250 kDa

3.2.5 Lectin enriched (LE) 200-250 kDa proteins from superovulated oviduct lysates inhibit ZP binding of wild-type and GalT 1-null sperm

Lectin binding characteristics and a narrowed molecular weight range were used to further enrich the bioactive ZP3-independent ligand for subsequent identification. Superovulated oviduct lysates were passed over a GS-1-agarose column and adsorbed to a WGA-agarose column. The WGA reactive proteins were eluted with 0.5 M GlcNAc or 1M NaCl and prepared for 1D SDS-PAGE. After gel separation, the 200-250 kDa range was excised and the proteins were extracted and prepared for assessment. The enriched sample, designated LE (lectin enriched) 200-250 kDa, was analyzed by 1D SDS-PAGE and lectin blot. As expected, the enriched sample is represented by a major Coomassie-stained polypeptide of ~220 kDa that is WGA, but not GS-1, reactive (Figure 3-5 A). The LE 200-250 kDa species showed strong bioactivity against both wild-type and GalT 1-null sperm at a concentration of 20 μg/ml (Figure 3-5 B), suggesting a ZP3/GalT 1 independent interaction. Inhibition was dose dependent, with a linear range of inhibition between 4-16 μg/ml (Figure 3-5 C).

Figure 3-5. Enriched 200-250 kDa range proteins from GS-1 lectin agarose depleted, WGA-lectin agarose bead pull downs of superovulated oviduct lysates competitively inhibits wild-type and GalT 1-null sperm-egg binding in a dose dependent manner. A) 1D SDS-PAGE separation of 1 μg of lectin enriched (LE) 200-250 kDa proteins and subsequent protein stain, WGA-, and GS-1- lectin blot analysis. B) Biological activity of LE 200-250 kDa proteins against wild-type (WT) [■] and GalT-1-null [□] sperm. The starting LE 200-250 kDa concentration in each droplet was 20 μg/ml. C) Dose dependent evaluation of LE 200-250 kDa protein inhibition of WT sperm. Each bar represents the mean ± s.e.m of n=3 experiments, completed in triplicate.

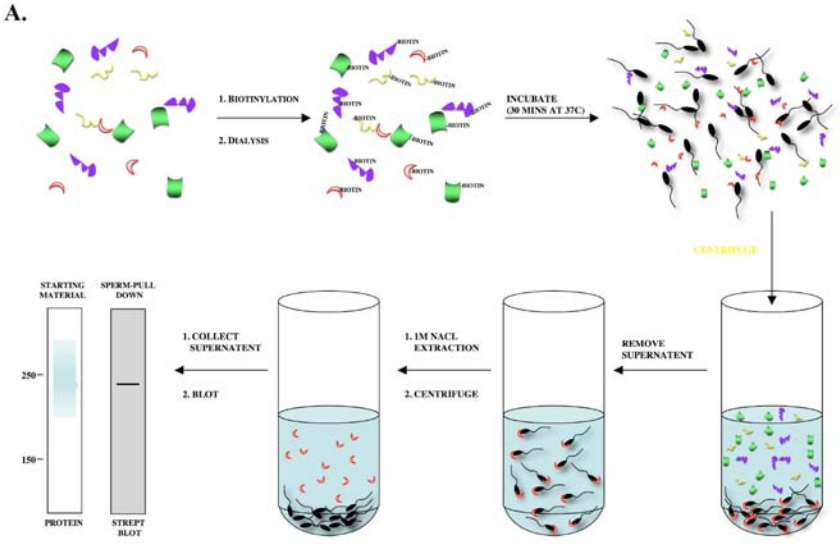

A.
WGA
GS-1
250 -
150 -
B.
WT
GalT-1 (-/-)
% Inhibition
100
90
80
70
60
50
40
30
20
10
0
LE 200-250 kDa
(20 µg/ml)
C.
LE 200-250 kDa
% Inhibition
16
8
4
Protein (µg/ml)

3.2.6 A 220 kDa glycoprotein demonstrates a sperm-specific interaction

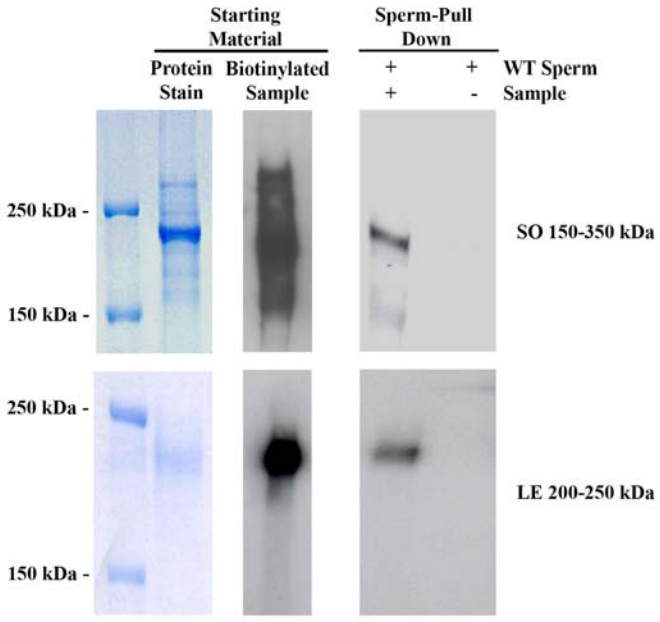
The ability of the LE 200-250 kDa species to competitively inhibit sperm-ZP binding implies that the bioactive species is binding to the sperm, occupying ZP recognition sites, and preventing those sites from interacting with the intact ZP. To explore whether there was a direct interaction between sperm and any species within the bioactive fractions, we used a pull down assay with sperm. Several distinct bioactive fractions (SO 150-350 kDa, SO 200-250 kDa, LE 200-250 kDa) were used as starting material for the sperm-pull down assay. As shown in Fig. 4, a ~220 kDa protein is extracted from the sperm surface following pre-incubation with SO 150-350 kDa, despite the presence of numerous protein in the starting material, as well as with the enriched LE 200-250 kDa sample. Identical results were obtained with the SO 200-250 kDa fraction (data not shown).

Figure 3-6. Sperm-pull down analysis reveals a single sperm-interacting protein of approximately 220 kDa molecular weight present in bioactive fractions. A) Schematic of the sperm-pull down assay as outlined in the Materials and Methods. B) Protein stain and streptavidin-blot of biotinylated high molecular weight (150-350 kDa) and LE 200-250 kDa proteins isolated form superovulated oviducts. Following incubation of sperm with the biotinylated factions, sperm were pelleted, washed and extracted with either NaCl or detergent, which releases a distinct 220 kDa molecular weight band from sperm, despite the large number of proteins present in the starting material.

A.


1. BIOTINYLATION
2. DIALYSIS
INCUBATE
(30 MINS AT 37C)
CENTRIFUGE
REMOVE
SUPERNATENT
1. 1M NACL
EXTRACTION
2. CENTRIFUGE
1. COLLECT
SUPERNATENT
2. BLOT
STARTING
MATERIAL
SPERM-PULL
DOWN
250
150
PROTEIN
STREPT
BLOT

B.


Starting
Material
Sperm-Pull
Down
Protein
Stain
Biotinylated
Sample
+
+
WT Sperm
+
-
Sample
250 kDa -
150 kDa -
SO 150-350 kDa
250 kDa -
150 kDa -
LE 200-250 kDa

3.2.7 The ZP3-independent ligand is identified as Oviduct-Specific Glycoprotein

The 220 kDa band, visualized by Pierce Imperial Protein Stain, was excised from a 1D SDS-PAGE of the LE 200-250 kDa fraction, and subjected to nano-electrospray ionization mass spectrometry (nanoESI-MS). Peptide analysis showed multiple sequence matches to human cytokeratin-1 (16 unique sequences, ion score: 1077), mouse myosin-11 (20 unique sequences, ion score: 1039) and mouse oviduct-specific glycoprotein (OGP) (16 unique sequences, ion score 702) (Figure 3-7 A). None of the other potential candidate proteins were represented by more than 1-2 peptide sequences, other than the laminin B and C chains, which were represented by 5-6 peptide sequences. Cytokeratin was eliminated as a candidate because it was of human origin, as was myosin-11 because myosin-free fractions retained bioactivity. OGP remained a potential candidate because its presence within the LE 200-250 kDa fraction was validated by western blot analysis (data not shown).

To confirm that OGP is the bioactive species, OGP was immuno-depleted from the LE 200-250 kDa fraction and bioactivity assessed (Figure 3-7 B,C). Consistent with previous results, the LE 200-250 kDa fraction exhibited high bioactivity, producing more than 80% inhibition of wild-type sperm binding to egg coats. Mock depletions with control IgG beads showed a slight decrease in binding that is most likely due to non-specific protein loss as judged by western blot analysis of OGP. In contrast, depletion with anti-OGP beads resulted in undetectable levels OGP by western blot and a significant reduction in bioactivity. Furthermore, material recovered from the anti-OGP immuno-beads was able to competitively inhibit sperm-oocyte binding, whereas parallel

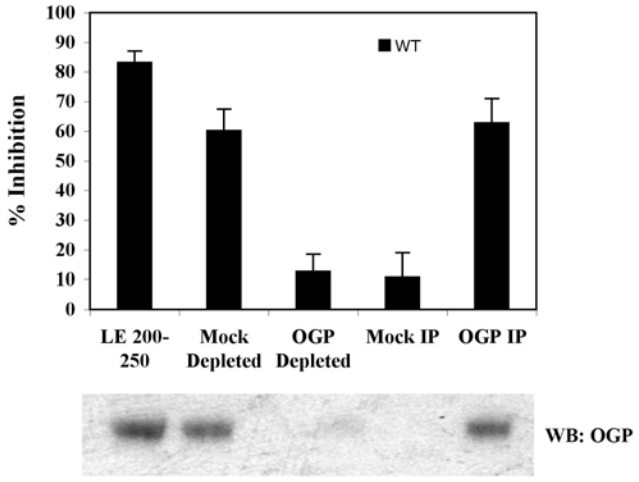
incubations with material removed from control beads had negligible activity.

Figure 3-7. NanoESI sequence analysis of the 220 kDa band and immuno-depletion studies confirms OGP is the bioactive protein. A) NanoESI mass spectrometric analysis of the 220 kDa band identifies 16 peptide sequences that exist within the polypeptide sequence of oviduct-specific glycoprotein (OGP). The boxes indicate matches. B) Quantitative analysis of biological activity, i.e. competitive inhibition of sperm-ZP binding, by the LE 200-250 kDa fraction before and after depletion with OGP or control antibodies, as well as of the recovered immunoprecipitated material. Each bar represents the mean of four assays ± s.e.m. The relative amount of OGP in each fraction is illustrated by the accompanying western blot, which is representative of two assays. Representative oocytes are shown following each assay condition.

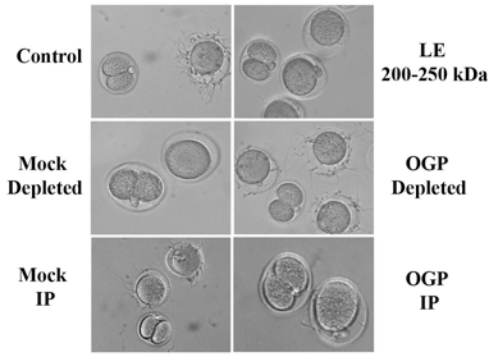
A.

MGRLLLLAGLVLLMKHSDGTAYKLVCYFTNWAHSRPGPASIMPHDLDPFLCTHLIFAFASMSNNQIVAKNLQDENVLYPEFNKLKERNR
ELKTLLSIGGWNFGTSRFTAMLSTLANREKFIDSVISFLRIHGFDGLDLFFLYPGLRGSPPHDRWNFLFLIEELQFAFEREALLTQHPR
LLLSAAVSGIPSIIHTSYDALLLGRRLDFINVLSYDLHGSWEKFTGHNSPLFSLPEDSKSSAYAMNYWRKLGTPADKLIMGFPTYGRNF
YLLKESKNGLQTASMGPASPGKYTKQAGFLAYYEVCSFVQRAKKHWIDYQYVPYAFKGKEWLGYDDTISFSYKAMYVKREHFGGAMVWT
LDMDDVRGTFCGNGPFPLVHILNELLVQTESNSTPLPQFWFTSSVNASGPGSENTALTEVLTTDTIKILPPGGEAMTTEVHRRYENMTT
VPSDGSVTPGGTASPRKHAVTPENNTMAAEAKTMSTLDFFSKTTTGVSKTTTGISKTTTGVSKTTTGVSKATAGISKTIPEISKATAGV
SKTTTGVSKTTTGISKTITGVSKTTTGISKTTTGISKTTTGVSKITTGVSKTTTGISKTTTGISQTTTGISKTTTDISKTTTGISKTTP
GISKTTPGMTVIVQTQANEAETTATMDHQSVTPTEMDTTLFYLKTMTPSEKETSRKKTMVLEKATVSPREMSATPNGQSKTLKWASLIT
EVETYSQDG

B.



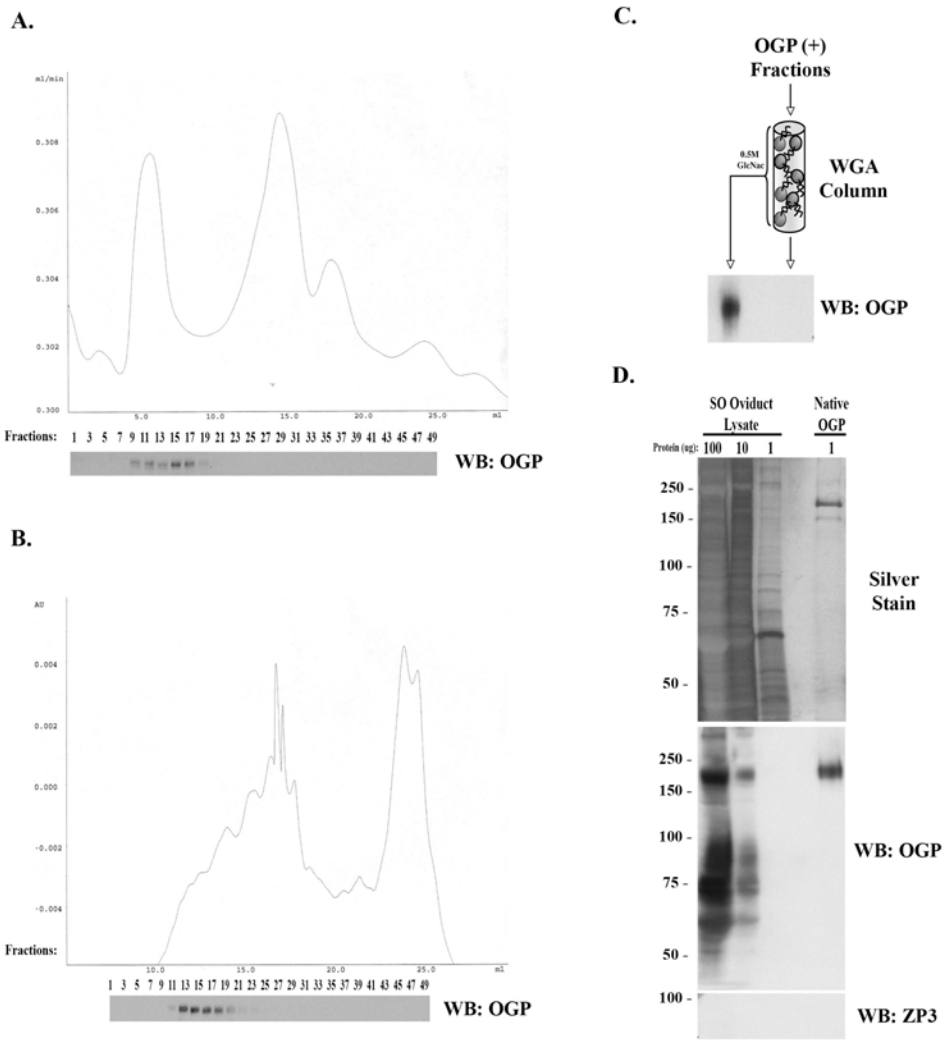
C.



3.2.8 Purification of native OGP from superovulated oviducts

OGP was purified under non-denaturing conditions to confirm its ability to function as a ZP3-independent sperm-binding ligand. Enrichment of native OGP was accomplished through a combination of size separation, ion exchange, and lectin affinity chromatography. Superovulated oviduct lysates were applied to a Superose 6 size separation column, eluted, and fractions were collected. OGP positive fractions were determined by western blot analysis and compared to the protein elution profile (Figure 3-8A). OGP positive fractions were combined and applied to a MONO-Q ion exchange column (Figure 3-8B). Eluted fractions were assayed for the presence of OGP, and positive fractions were combined and dialyzed against lectin affinity buffer. After dialysis, the sample was applied to a 20 ml WGA-agarose column and bound glycoproteins were eluted (Figure 3-8C). The non-bound fraction for each column was collected and assayed for OGP to confirm that the entire pool of OGP was acquired (data not shown). The Superose, MONO-Q and WGA enriched material behaves as one predominant silver-stained polypeptide of ~220 kDa that shows strong reactivity with anti-OGP antibodies (Figure 3-8D). Although the oviduct preparations are believed to be free of contaminating oocytes, the presence of any contaminating ZP3 was ruled out by western blot analysis.

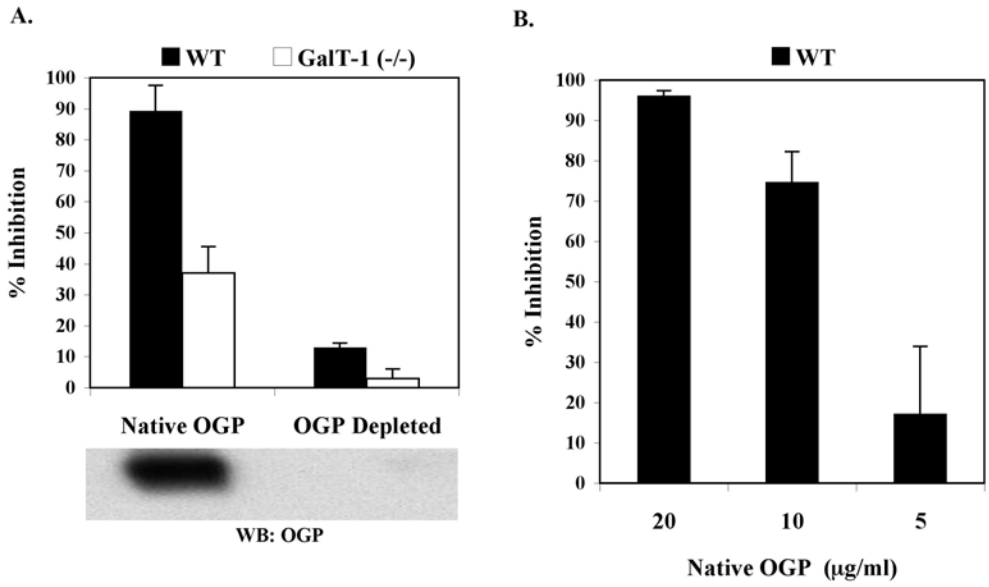
Figure 3-8. Purification of native OGP by size selection, ion exchange and lectin affinity chromatography. A) Superovulated oviduct lysates were created as described and applied to a Superose 6 size separation column under the control of a GE AKTA FPLC system. B) OGP positive fractions are collected and applied over a MonoQ ion exchange column. Proteins were eluted with a 0.05-1.0 M NaCl gradient. C) The OGP positive fractions were applied to a WGA-affinity column and eluted with 0.5 M GlcNAc. D) 1D SDS-PAGE separation of the original oviduct lysate and 1 μg of the purified protein followed by silver stain, OGP, and ZP3 western blot analysis. Blots are representative of several purification experiments, and demonstrate significant enrichment of the OGP-reactive 220 kDa protein.


A.
ml/min
0.308
0.306
0.304
0.302
0.300
5.0
10.0
15.0
20.0
25.0
ml
Fractions: 1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
WB: OGP
B.
AU
0.004
0.002
0.000
-0.002
-0.004
10.0
15.0
20.0
25.0
ml
Fractions:
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
WB: OGP
C.
OGP (+)
Fractions
0.5M
GlcNac
WGA
Column
WB: OGP
D.
SO Oviduct
Lysate
Native
OGP
Protein (ug): 100 10 1 1
250 -
150 -
100 -
75 -
50 -
Silver
Stain
250 -
150 -
100 -
75 -
50 -
WB: OGP
100 -
WB: ZP3

3.2.9 Native OGP competitively inhibits sperm-ZP binding in a sperm-specific manner

Native OGP was assayed for sperm-binding activity in competitive sperm-ZP binding assays. As expected, native OGP (20 μg/ml) competitively inhibited wild-type sperm-ZP binding by nearly 90%; however, identical concentrations inhibited GalT 1-null sperm binding by only ~35%. Although OGP showed dose-dependent inhibition of sperm-ZP binding for both sperm genotypes (Figure 3-9 B and data not shown), the decreased bioactivity against GalT 1-null sperm suggests that the loss of GalT 1 may somehow influence the affinity of OGP binding to the sperm surface. In either event, bioactivity towards both wild-type and GalT 1-null sperm could be removed by anti-OGP depletion (Figure 3-9 A).

Figure 3-9. Highly enriched native OGP exhibits differential inhibition of wild-type (WT) and GalT 1-null sperm. A) Bioactivity assessment of native OGP against WT (■) and GalT 1-null (□) sperm, before and after specific OGP immuno-depletion. The starting OGP concentration in each droplet was 20 μg/ml. Each bar represents the mean ± s.e.m of n=3 experiments, each completed in triplicate. The relative amount of OGP in each fraction is illustrated in the western blot, which is representative of several experiments. B) Native OGP shows a dose-dependent inhibition of wild-type sperm-ZP binding. Each bar represents the mean ± s.e.m. of n=3 experiments, each completed in triplicate.


A.
WT
GalT-1 (-/-)
% Inhibition
100
90
80
70
60
50
40
30
20
10
0
Native OGP
OGP Depleted
WB: OGP
B.
WT
% Inhibition
100
90
80
70
60
50
40
30
20
10
0
20
10
5
Native OGP (μg/ml)

Chapter 4: Oviduct-specific glycoprotein (OGP) exists as distinct isoforms, as defined by pI and lectin reactivity, which exhibit distinct localization and function

4.1 Hypothesis

Analysis of proteins peripherally associated with the ovulated egg coat revealed the presence of a sperm-binding glycoprotein unrelated to ZP3. A high molecular weight protein fraction isolated from superovulated oviduct lysates recapitulated this bioactivity. A 220 kDa protein species, with biochemical characteristics reminiscent of the ZP3-independent ligand, was shown to directly interact with sperm. This species was subsequently identified as oviduct-specific glycoprotein (OGP). Targeted purification of native OGP from superovulated oviduct lysates, in addition to specific immuno-depletion of OGP, demonstrated an ability of OGP to inhibit sperm-ZP interactions in vitro.

If OGP is involved in sperm-egg binding, we predict that it would be expressed in appropriate locations within the oviduct, present on the ZP surface, and removed after fertilization. Furthermore, purified native OGP should interact with the sperm plasma membrane, specifically at the acrosomal cap, as well as the ZP of oocytes. These predictions were tested through three specific aims:

1) to assess OGP expression and localization within the superovulated oviduct and ovulated oocytes
2) to assess whether purified native OGP demonstrates a specific interaction with gametes, and
3) to analyze the functional domain of native OGP.

Immunohistochemistry and western blot analysis of superovulated oviducts demonstrate that OGP expression is induced by follicle stimulating hormone, but not by luteinizing hormone. As with other mammals, OGP expression and localization within the superovulated oviduct is regionally defined, and for the first time in mouse its association with the ovulated oocyte ZP is demonstrated, along with a previously identified localization in the perivitelline space. Additionally, OGP is removed from the ZP of 2-cell embryos, representing a potential block to polyspermy. Furthermore, distinct OGP glycoforms have been identified with respect to localization, pI, lectin reactivity, and function. A distinct OGP glycoform that comprises a minor fraction of the total OGP shows specific interaction with sperm heads and the oocyte ZP, and is capable of inhibiting sperm-egg binding in vitro.

4.2 Results

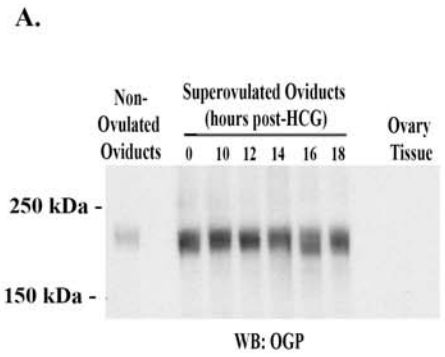
4.2.1 OGP expression and localization in superovulated oviducts

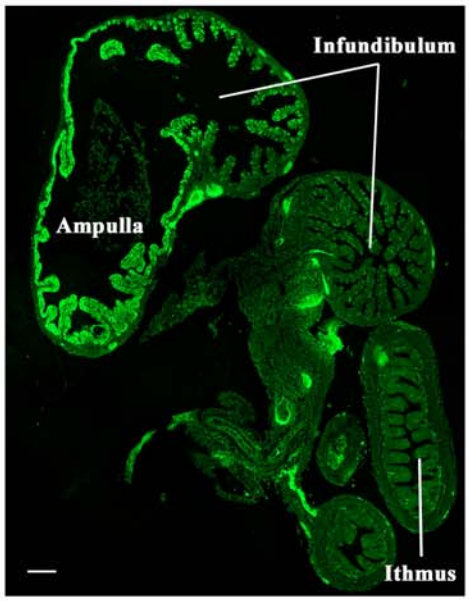
Although OGP expression within the oviduct has been documented in several model systems, to our knowledge, no investigations have analyzed OGP expression in the mouse oviduct utilizing specific anti-mouse OGP antibodies. Since earlier results indicated that the sperm-binding activity of the 150-350 kDa oviduct polypeptides showed hormone-dependent specific activity, we examined the expression of OGP before and after hormone injection. For comparison, non-stimulated oviducts and ovarian tissue were collected. Because hormonal priming results in increased vascularization within the oviduct, OGP expression was normalized to “oviduct equivalents” rather than to protein

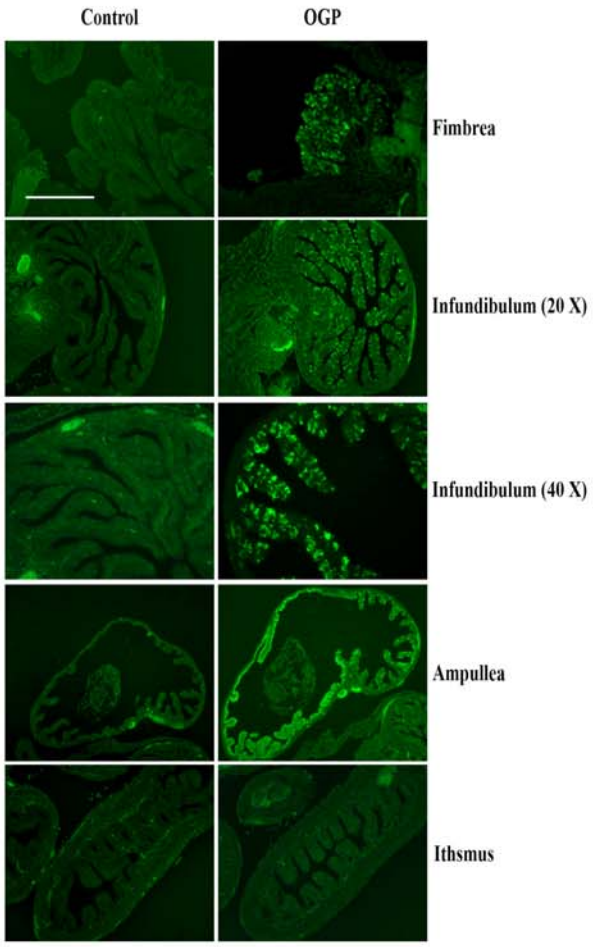
concentration. Western blot analysis shows a 2.2-fold increase in the 220 kDa OGP isoform following PMS stimulation, and remained consistently elevated during hCG exposure (Figure 4-1 A).

OGP was localized in superovulated oviduct sections by indirect immunofluorescence. OGP immunoreactivity occurs in the fimbriae, infundibulum, and ampullea, whereas the isthmus showed only background reactivity (Figure 4-1 B,C). Distinct OGP-reactive cells occur within the fimbriae and infundibulum that are reminiscent of secretory, or “peg”, cells (Oliphant et al., 1984). Strong immunoreactivity is also observed in the ampullea, where the surface of the lumen appears coated by OGP. It is unclear whether such a coating exits, or if this reflects fixation of soluble OGP to the luminal surface. Regardless, OGP is clearly present within the secretory cells of the fimbriae and infundibulum where it is presumably secreted and associates with the newly ovulated oocyte, as well as in the ampullea where fertilization occurs. Unfortunately, this method was not sensitive enough to identify any interactions or localization that OGP maintains with the ovulated oocyte.

Figure 4-1. Oviduct specific glycoprotein (OGP) is hormonally regulated and expressed in specific regions of the mouse oviduct. A) OGP Western blot of non-hormonally primed and superovulated CD-1 mouse oviduct lysates as a function of time post hCG injection. Each lane represents 0.25 oviduct equivalents, and is representative of three experiments. B) Immunohistochemical analysis of OGP in 0.5 μ paraffin sections of superovulated CD-1 mouse oviducts (16 hours post hCG injection). OGP is detected in the secretory cells of the fimbriae and infundibulum, as well as associated with the ampullar epithelium, after which reactivity is negligible (isthmus). A higher magnification view of the secretory cells of the infundibulum is illustrated, as is a control section incubated with an irrelevant primary antibody. C) Merge of images into representative oviduct cross-section. Scale bars = 100 μm


A.
Non-Ovulated Oviducts
Superovulated Oviducts (hours post-HCG)
0 10 12 14 16 18
Ovary Tissue
250 kDa -
150 kDa -
WB: OGP


C.
Infundibulum
Ampulla
Ithmus


B.
Control
OGP
Fimbrea
Infundibulum (20 X)
Infundibulum (40 X)
Ampullea
Ithsmus

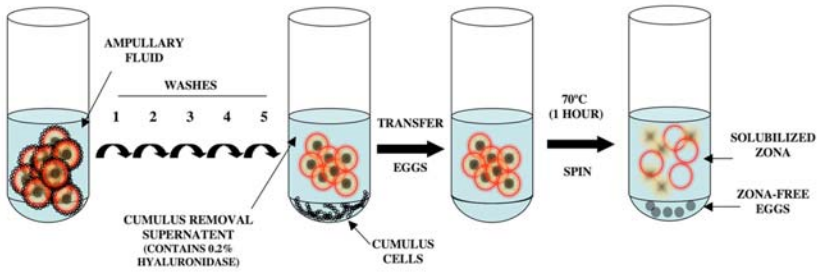
4.2.2 OGP shows distinct localizations within the cumulus-oocyte complex

Whereas the preceding results indicate specific localizations within the oviduct epithelium, we sought to determine whether OGP shows any distinct distribution within the cumulus-oocyte complex using fractionation procedures coupled with western blotting as well as indirect immunofluorescence. The ampullar contents were collected and fractionated as indicated in the Materials and Methods and as diagrammed in Figure 4-2 A.

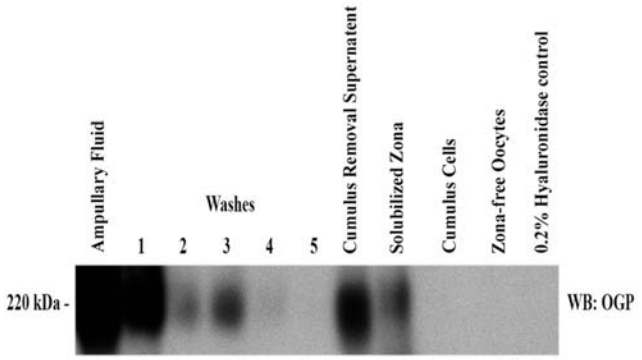
Western blot analysis of the SDS-PAGE resolved fractions illustrates the presence of two distinct pools of OGP; a freely soluble pool present in the ampullar fluid that can be removed from the cumulus-oocyte complex by washing, and a second pool associated with the cumulus-oocyte complex that is resistant to washing. Removal of cumulus cells by hyaluronidase treatment releases a portion of this cumulus-oocyte associated pool, while the remainder is released upon heat solubilization of the ZP. The cumulus cells themselves appear to be OGP-negative as are the ZP-free oocytes (Figure 4-2 B).

Figure 4-2. Secreted OGP within the ampullea environment of superovulated oviducts exists as distinct pools. A) Schematic of the fractionation protocol to separate the ampullar fluid, the cumulus cells and associated matrix and the ZP. After sequential washings to remove external proteins, 96 superovulated oviducts were submersed in a volume of PBS. The ampullea were pierced and the cumulus-oocyte complexes were removed and placed in fresh ice-cold PBS. The ampullea was then pressed to remove all luminal fluid. After all oviducts were processed, the buffer containing the luminal fluid was collected. Cumulus-oocytes were washed five times, and then treated with hyaluronidase to remove the cumulus cells. Cumulus-free ZP-intact eggs were washed and the ZP heat denatured for 1 hour at 65-70°C. All samples were collected and separated by 1D SDS-PAGE and probed for OGP. B) OGP western blot analysis of fractionated microenvironment of superovulated ampullea and ovulated oocytes. Results are representative of multiple experiments.

A.


AMPULLARY FLUID
WASHES
1 2 3 4 5
TRANSFER
EGGS
70°C (1 HOUR)
SPIN
SOLUBILIZED ZONA
ZONA-FREE EGGS
CUMULUS REMOVAL SUPERNATENT (CONTAINS 0.2% HYALURONIDASE)
CUMULUS CELLS

B.


Ampullary Fluid
Washes
1 2 3 4 5
Cumulus Removal Supernatent
Solubilized Zona
Cumulus Cells
Zona-free Oocytes
0.2% Hyaluronidase control
220 kDa -
WB: OGP

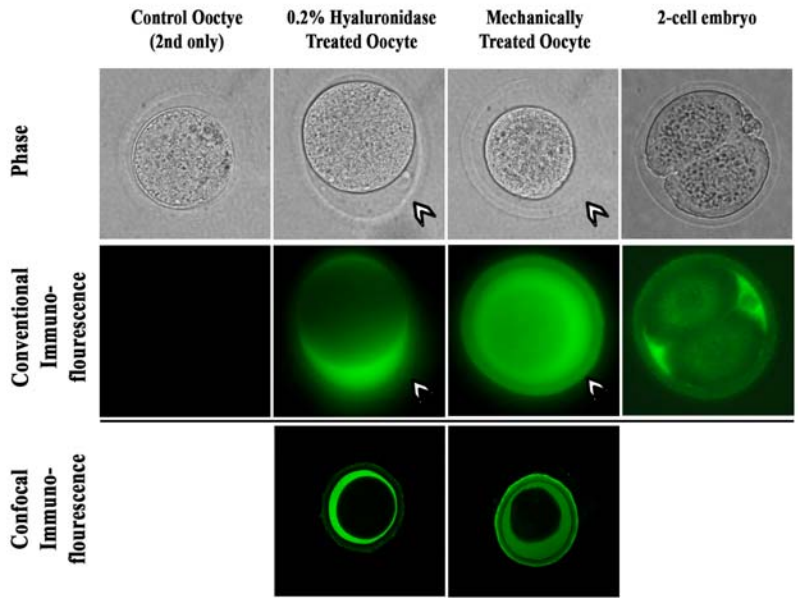
4.2.3 Immunostaining of ovulated oocytes reveals OGP localized to the ZP and the perivitelline space.

Consistent with our suggestion that OGP is an egg-associated ligand, we predict that OGP would be present on the surface of the ZP, thus correlating with the site of initial sperm-egg interaction. The OGP pool recovered following solubilization of the ZP could be associated with the ZP directly, as has been reported in other systems (McCauley et al., 2003; O'Day-Bowman et al., 2002), where it could function in sperm binding; or it could be released from the perivitelline space, where it may interact with sperm that have successfully penetrated through the ZP matrix. Consequently, we assessed OGP distribution within ovulated oocytes and 2-cell embryos by indirect immunofluorescence. Contrary to our initial expectations, OGP showed minimal localization to the ZP but strong localization to the perivitelline space (Figure 4-3). Interestingly, an earlier study reported that an unidentified 215 kDa glycoprotein, that we speculate may be OGP, is localized to the PVS in mouse oocytes (Kapur and Johnson, 1985),

The presence of OGP in the “cumulus removal supernatant” raised the possibility that OGP might be stripped from the ZP by hyaluronidase treatment and/or fixation methods. We therefore examined OGP distribution on oocytes whose cumulus cells were removed non-enzymatically (i.e., mechanically) by repetitively pipetting cumulus-oocyte complexes through small pore pipettes. Strong OGP immunoreactivity on the ZP was observed following mechanical removal of cumulus cells. Confocal image analysis of mechanically-treated oocytes shows clear surface staining with decreasing reactivity from the most exterior to the most interior regions of the ZP. In contrast, confocal imaging of

the ZP of hyaluronidase-treated oocytes shows greatly reduced OGP staining with punctate reactivity that is suggestive of a previously intact coating. Similar punctate OGP reactivity is observed on the ZP of 2-cell embryos.

Figure 4-3. OGP immunostaining of cumulus-free oocytes and 2-cell embryos reveal OGP localization to the zona pellucida and perivitelline space of oocytes. Oocytes and 2-cell embryos were collected from superovulated oviducts. Oocytes were washed five times in PBS to remove all loosely associated OGP. Cumulus cells were removed either by hyaluronidase treatment or mechanically by repetitive pipetting through a small pore pipette. Oocytes were fixed in 4% paraformaldehyde, blocked with 2% BSA, and stained with OGP. 2-cell embryos were washed three times in KBRT buffer. OGP was indirectly visualized with an anti-goat antibody-FITC secondary antibody. Oocytes and 2-cell embryos were processed for OGP indirect immunofluorescence and imaged by either conventional or confocal microscopy. Arrowheads indicates the position of the ZP


Control Ooctye (2nd only)
0.2% Hyaluronidase Treated Oocyte
Mechanically Treated Oocyte
2-cell embryo
Phase
Conventional Immuno-flourescence
Confocal Immuno-flourescence

4.2.4 Distinct OGP glycoforms exist with respect to localization, pI, and lectin reactivity

Two-dimensional gel electrophoresis of secreted oviductal proteins in porcine demonstrates high molecular weight proteins that range in pI from acidic to basic (Buhi et al., 2000). These protein species, although not identified, are assumed to be the porcine homologue of OGP. Consequently, we asked whether mouse OGP also exists as distinct isoforms and if any show restricted distributions. Similar to that reported in porcine, mouse OGP isoforms ranging from acidic to basic were observed within the ampullary fluid. However, the number of OGP isoforms decreases in the cumulus-oocyte associated pool, and reduces to a single, basic OGP species associated with the ZP (Figure 4-4A).

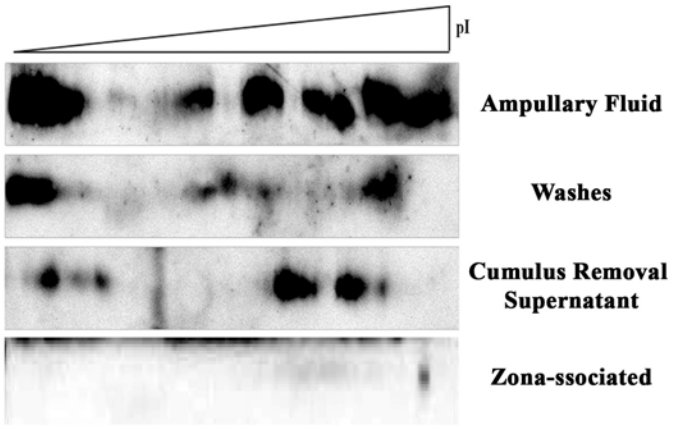
The OGP gene encodes a single, heavily glycosylated polypeptide species (Oliphant et al., 1984). It is therefore possible that the OGP isoforms result, at last in part, from differential glycosylation, resulting in varying pI. We therefore determined whether the distinct OGP pools identified above exhibit specific lectin reactivity. In an initial approach, we utilized lectin blot analysis of 1D SDS-PAGE separations of various OGP pools obtained from the fractionated ampullary environment. Lectin analysis demonstrated that the OGP pool associated with the ZP to be WGA and PNA, but not GS-1, Con A, RCA, or UEA-I reactive. The freely soluble pools of OGP in the ampullary fluid and washes, however, exhibited WGA, GS-1, ConA, RCA and PNA, but not UEA-I reactivity. Unfortunately, the presence of hyaluronidase adversely affected our ability to analyze lectin reactivity in the cumulus removal supernatent (data not shown).

Although the lectin blot data is informative, they are not conclusive due to the fact that lectin reactivity may be associated with non-OGP proteins of similar molecular

weight. In a second attempt to determine whether any of the specific OGP distributions and/or isoforms are associated with distinct OGP glycoforms, or differential OGP glycosylation, the various fractions were sequentially analyzed by sequential chromatography on immobilized GS-1, Con A, RCA-1, PNA, and WGA (Figure 4-4 B). The soluble fractions of OGP derived from the ampullar fluid and washes possess significant levels of GS-1, Con A, and RCA-1 binding glycoforms and minimal levels of a PNA-binding glycoform. This is distinct from the lectin-binding pattern of the "cumulus removal supernatant" and "zona-associated" fraction, which shows a single GS-1-binding and a single PNA-binding glycoform, respectively. The four lectin columns could account for all OGP glycoforms, since there was no residual OGP binding to WGA-agarose, which binds to all OGP forms.

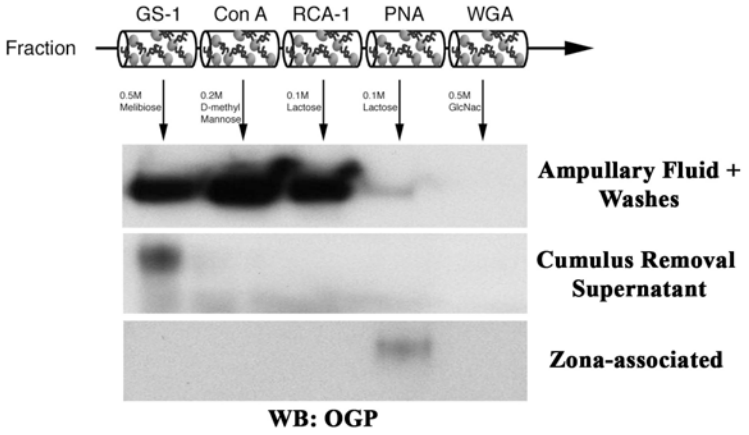
Figure 4-4. Distinct OGP glycoforms can be identified based on location, lectin reactivity, and pI. A) Fractions obtained as above resolved by 2-D gel analysis shows distinct OGP isoforms that range from acidic to basic pI. The number of isoforms decreases dramatically within the OGP pool associated with the cumulus-oocyte complex, such that only one basic isoform is found associated with the ZP. B) The distinct fractions were subjected to sequential lectin affinity chromatography, and the material eluted from each lectin column resolved by SDS-PAGE. OGP western blot analysis indicates that although the bulk of OGP remains in the ampullar fluid, distinct OGP glycoforms are found associated with the cumulus matrix and with the ZP. Western blots are representative of two experiments.

A.



WB: OGP

B.



WB: OGP

4.2.5 Enrichment of distinct OGP glycoforms reveals glycoform-specific bioactivity and gamete binding.

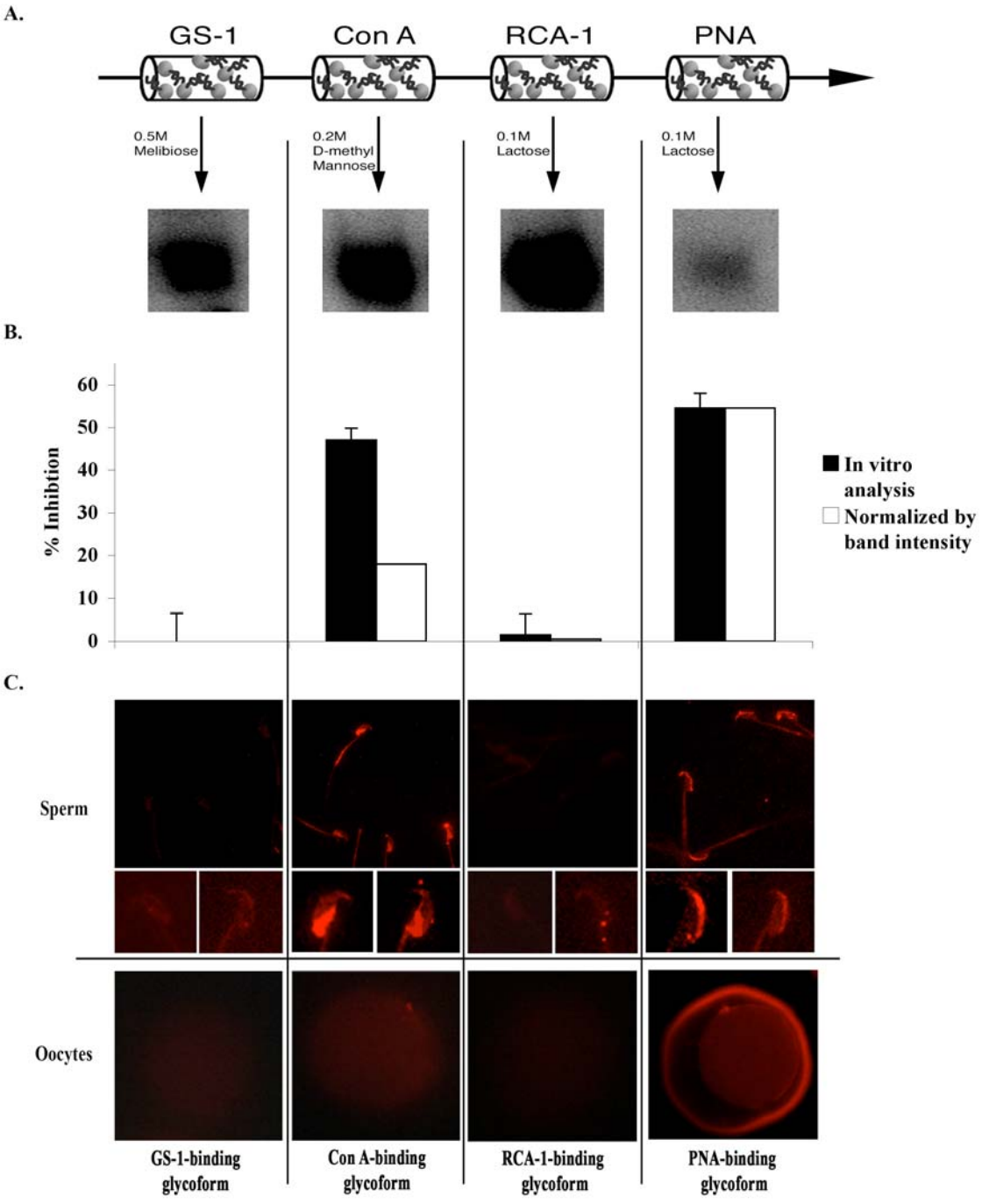
The realization that OGP distribution is correlated with distinct OGP glycoforms raised the possibility that the sperm-binding activity characterized in this report is actually associated with only a subset of the OGP glycoforms. To assess this, the biological activity (i.e., sperm-binding) of distinct OGP glycoforms was analyzed. Native OGP was purified from oviduct lysates as above, and sequentially applied to GS-1, Con A, RCA-1, and PNA lectin columns. OGP purified from oviduct lysates produced an array of glycoforms similar to that identified in ampullar exudates, i.e., significant amount of OGP bound to GS-1, Con A and RCA-1-agarose columns, with a lesser amount bound to PNA-agarose (Figure 4-5 A).

While it might appear ideal to normalize the bioactivity of the distinct OGP glycoforms to their protein concentration, this was not practical due to the small amount of recoverable PNA-binding OGP. Instead, bioactivity was normalized to relative OGP concentration as determined by western blot analysis. All OGP glycoforms were concentrated to equal volumes, OGP resolved by SDS-PAGE and western blotting, and the band intensities quantified using spot densitometer software. Only the Con A- and PNA-binding glycoforms showed any sperm-binding activity, i.e., competitive inhibition of sperm-ZP binding (Figure 4-5 B). Normalized to OGP levels, the PNA-binding glycoform was 2.5-fold more bioactive than the Con A-binding glycoform. The remaining two glycoforms, GS-1- and RCA-1-binding, did not compete for sperm-ZP binding, despite the present of significant amounts of OGP (Figure 4-5 B).

Additionally, we investigated the ability of the OGP glycoforms to bind to intact

sperm as well as the ZP. Consistent with the finding that GS-1- and RCA-1-binding OGP glycoforms did not inhibit sperm-ZP binding, neither of the glycoforms demonstrated an interaction with sperm or oocytes (Figure 4-5 C). Interestingly, the Con A-binding OGP glycoform, which modestly inhibits sperm-ZP binding, shows a strong interaction with the equatorial region of the sperm head, but no interaction with the ZP. Localization to the equatorial segment suggests a role for Con A-binding OGP other than during initial sperm-egg binding. In marked contrast to all other glycoforms, the PNA-binding glycoform demonstrates distinct binding to the acrosomal cap of capacitated sperm, as well as the ZP of hyaluronidase-treated oocytes.

Figure 4-5. Distinct OGP glycoforms exhibit differential ability to interact with gametes and competitively inhibit sperm-ZP binding. A) The spectrum of OGP glycoforms present in 48 superovulated oviducts was analyzed by 1D SDS-PAGE and OGP western blot, and B) sperm-binding activity was assayed. The low level of some OGP glycoforms, i.e., PNA, precluded normalization by protein concentration, and consequently, the all column eluents were concentrated to equal volumes and their bioactivity assayed (■) and normalized to OGP concentration (□) as judged by densitometric analysis of OGP western blots. C) Each OGP glycoforms was biotinylated to enable their identification without obstruction by endogenous OGP. Biotinylated glycoforms were incubated with hyaluronidase-treated oocytes or capacitated sperm. Gametes were washed, fixed and stained with streptavidin-Texas Red. Fluorescence microscopy demonstrates GS-1- and RCA-1-reactive OGP glycoforms do not associated with either gamete. Con A-reactive OGP associated with the equatorial region of sperm heads, but not oocytes. Representative high magnifications are included for each sperm sample. PNA-reactive OGP interacted with the sperm head over the acrosome vesicle as well as the oocyte ZP.

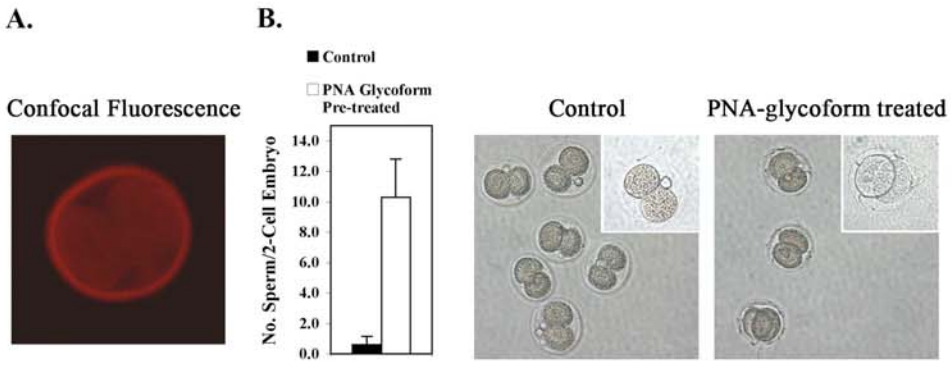

A.
GS-1
Con A
RCA-1
PNA
0.5M Melibiose
0.2M D-methyl Mannose
0.1M Lactose
0.1M Lactose
B.
% Inhibtion
60
50
40
30
20
10
0
In vitro analysis
Normalized by band intensity
C.
Sperm
Oocytes
GS-1-binding glycoform
Con A-binding glycoform
RCA-1-binding glycoform
PNA-binding glycoform

4.2.6 PNA-reactive OGP is able to induce sperm binding to 2-cell embryos.

Following fertilization the block to polyspermy is mediated by the release of enzymes from the egg cortical granules, which modify the egg coat to prevent additional sperm binding and/or penetration. We asked if the ZP of fertilized eggs retain the ability to interact with the PNA-reactive OGP by incubating 2-cell embryos PNA-reactive glycoform. Confocal immunofluorescence reveals a specific localization of the exogenous PNA-reactive OGP to the ZP of 2-cell embryos (Figure 4-6 A).

Furthermore, we predicted the addition of the PNA-reactive glycoform to 2-cell embryos, which no longer bind sperm nor express OGP on their ZP, would induce sperm binding. This prediction was verified as shown in Figure 4-6 B, in which 2-cell embryos treated with the PNA-reactive OGP bound sperm, whereas control 2-cell embryos do not.

Figure 4-6. Exogenous PNA-reactive OGP binds to the zona pellucida of 2-cell embryos and supports sperm-ZP binding. A) PNA-reactive OGP binds to the ZP of 2-cell embryos, which leads to B) an increase in sperm binding, relative to control 2-cell embryos that do not normally bind sperm. Error bars = s.d.


A.
B.
Confocal Fluorescence
Control
PNA Glycoform
Pre-treated
No. Sperm/2-Cell Embryo
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Control
PNA-glycoform treated

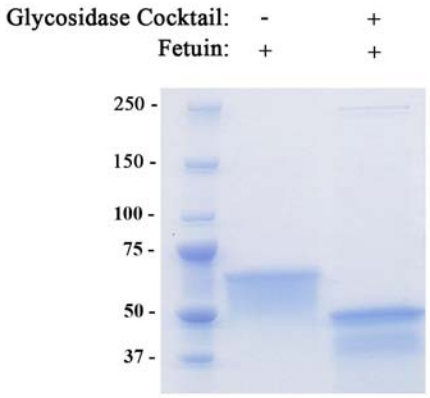
4.2.7 Bioactivity of the PNA-reactive OGP glycoform is carbohydrate-dependent

The ability of the LE 200-250 kDa OGP-enriched fraction to competitively inhibit sperm-ZP binding following excision from SDS-polyacrylamide gels suggests that the bioactivity is not dependent upon protein tertiary structure. We therefore directly tested whether PNA-binding OGP inhibited sperm-egg binding in a carbohydrate-dependent manner by heat denaturation and enzymatic deglycosylation using a cocktail of glycosidases that recognize both *N*- and *O*-glycans. We reasoned that if bioactivity is dependent on OGP carbohydrate structures, heat denaturation should have no affect on sperm-egg binding, but deglycosylation would ablate bioactivity. As before, the limited amount of the PNA glycoform precluded the ability to demonstrate efficient deglycosylation before and after deglycosylation. Consequently, validation of the glycosidase digestion was completed on a surrogate glycoprotein, bovine serum fetuin, which has both *N*- and *O*-glycans. Glycosidase treatment of fetuin results in an electrophoretic migration shift reflecting the deglycosylated polypeptide, and demonstrates the effectiveness of the treatment procedure (Figure 4-7 A).

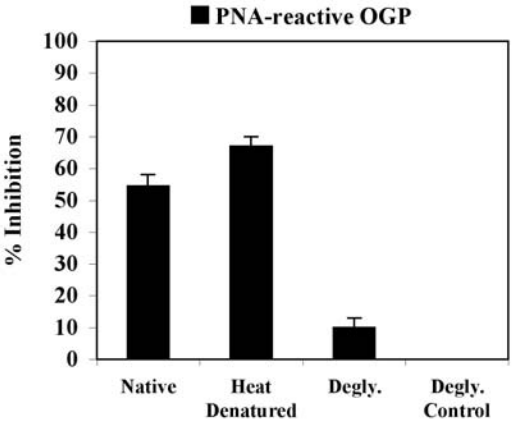
Heat denaturation did not eliminate the bioactivity of the PNA-reactive OGP glycoform, and in fact it produced a slight increase (~10%) in activity over the native glycoform. Deglycosylation, on the other hand, reduced the bioactivity of the PNA-reactive OGP glycoform to near background levels. This loss of bioactivity is not due to the presence of the denatured glycosidases, since the glycosidase control did not affect the number of sperm bound per egg (Figure 4-7 B).

Figure 4-7. Inhibition of sperm-ZP binding by the PNA-reactive OGP glycoform is carbohydrate-dependent. A) The small amount of PNA-reactive OGP glycoform available precluded confirmation that the protein was deglycosylated by SDS-PAGE and protein staining. Validation of the deglycosylation was completed on fetuin, a surrogate glycoprotein. B) When treated under identical conditions, the heat-denatured deglycosylated ("Degly.") PNA-reactive OGP glycoform lost sperm-binding activity. Controls included a sample of glycosidase cocktail without added OGP ("Degly. Control"), as well as heat-denatured OGP not treated with any glycosidases ("Heat Denatured"). Bioactivity assessment of heat denatured and deglycosylated PNA-reactive OGP glycoform demonstrated that the ability to inhibit sperm-ZP binding was carbohydrate dependent. Error bars = s.d.

A.


Glycosidase Cocktail: - +
Fetuin: + +
250 -
150 -
100 -
75 -
50 -
37 -

B.


PNA-reactive OGP
% Inhibition
100
90
80
70
60
50
40
30
20
10
0
Native
Heat Denatured
Degly.
Degly. Control

Chapter 5: Conclusions and Future Directions

5.1 Conclusions

5.1.1 Oviduct-specific glycoprotein is a ZP associated sperm-adhesion ligand

The inability to ablate fertility through single gene deletions, as well as the identification of numerous molecular players in gamete recognition, supports the "multiple mechanism" model of gamete adhesion. Although several sperm-associated proteins (i.e. GalT-1, SED1, FUT5, arylsulfatase-A, etc.) have been shown to participate in sperm-ZP binding, only recently has a second egg coat-associated ligand, in addition to ZP3, been identified in mouse. Originally characterized as a peripherally-associated ligand on ovulated eggs, but not found on ovarian eggs, we hypothesized that the ZP3-independent ligand is expressed by the oviduct and absorbs to the ZP upon ovulation where it facilities initial sperm adhesion.

Data presented here demonstrates that the oviduct to be a rich source of a ZP3-independent ligand. A high molecular weight, WGA-reactive glycoprotein was identified in hormonally-primed oviduct lysates that binds to sperm surfaces and reduces the ability of sperm to bind to intact ZP of oocytes in vitro. The purified protein was identified as Oviduct-Specific Glycoprotein (OGP). Targeted purification of native OGP and specific immuno-depletion studies validate OGP as a sperm-binding ligand capable of competitively inhibiting sperm-ZP interactions in vitro. Contrary to earlier reports (Kapur and Johnson, 1986), OGP is found associated with the mouse ZP of the ovulated oocyte, as well as within the perivitelline space as previously reported. Furthermore, OGP is

removed from the ZP after fertilization, potentially representing an additional block to polyspermy.

Importantly, fractionation of native OGP through sequential GS-1, Con A, RCA-1, and PNA chromatography reveals distinct glycoforms that show specific localizations and biological activities. In this regard, sperm-binding activity is specifically attributed to a PNA-reactive glycoform that localizes to the ZP and perivitelline space, whereas glycoforms bound by the other matrices remain in the ampullar fluid and fail to interact with sperm or eggs in a manner that suggests a role in initial sperm-ZP binding. The role of these additional glycoforms remains unknown. Overall, OGP function during initial sperm-ZP binding represents a previously unappreciated example of carbohydrate-dependent cell adhesion.

Identification of the ZP3-independent ligand as OGP was unexpected for two reasons: 1) GalT 1-null sperm bind OGP-null eggs in vitro, and 2) ZP3-independent bioactivity could be extracted from egg coats of OGP-null oocytes (Rodeheffer and Shur, 2004). The possibility that two ZP3-independent ligands exist on the egg coat is plausible, and is supported by the fact that WGA depletion of egg coat associated proteins did not completely remove all biological activity (Rodeheffer and Shur, 2004).

OGP is composed of two major domains: a catalytically inactive chitinase domain and a C-terminal *O*-glycosylation (mucin) domain (Buhi et al., 1996; Takahashi et al., 2000; Watanabe et al., 1993). An additional consensus heparin-binding domain has been observed in porcine OGP (Buhi et al., 1996). The chitinase and mucin domains are hypothesized to facilitate OGP interactions through substrate (polymers of GlcNAc) binding in the absence of enzymatic function and carbohydrate moieties respectively

(Buhi, 2002). The mouse *O*-glycosylation domain demonstrates strong mucin properties, containing 21 mucin-like tandem repeat sequences and 67 predicted *O*-glycosylation sites. Also, seven putative *N*-glycosylation sites have been predicted (Buhi, 2002).

OGP is expressed in the oviduct of numerous mammals in response to estrogen stimulation, including pig (Buhi et al., 1990), hamster (Robitaille et al., 1988), bovine (Boice et al., 1990), mice (Kapur and Johnson, 1988), and humans (Verhage et al., 1988). Co-incubation of OGP, or media conditioned with OGP (i.e. oviductal fluid), with sperm, oocytes, or embryos promotes fertilization and enhances early embryo development in numerous model systems (Killian, 2004). It is generally accepted that OGP is hormonally regulated and secreted from non-ciliated "peg" cells of the oviduct epithelium (Oliphant et al., 1984). Differences in the expression of OGP within distinct regions of various mammalian oviducts have been validated by immunohistochemistry and in situ hybridization (Gandolfi et al., 1991; Kapur and Johnson, 1988). It has been hypothesized that the region-specific expression of OGP suggests location specific function (Buhi, 2002).

Our western blot and immunohistochemical data supports the notion of hormonal stimulation and regulated expression in the mouse oviduct. Western blot analysis demonstrated significant up-regulation of OGP in superovulated oviducts. Although our findings provide no insight into OGP mRNA levels, up-regulation of the 220 kDa OGP glycoprotein was observed 48 hours after pregnant mare serum (PMS), a surrogate for follicle stimulating hormone (FSH), injection. OGP levels did not change in response to human chorionic gonadotropin (hCG), a surrogate for luteinizing hormone (LH). However, preliminary data suggests OGP glycosylation states vary in response to hCG

(data not shown). It is important to note that PMS/FSH is responsible for stimulating the development of the egg and the granulosa (cumulus) cells, suggesting an elegant association of OGP production with egg maturation. FSH stimulates estrogen synthesis in the maturing follicle, which is most likely directly responsible for inducing OGP expression in the oviduct. Such regulation is supported by several findings, including genetic analysis of the 5' flanking region of mouse and human OGP gene, which reveals several half-estrogen response elements (ERE) and one imperfect ERE (Takahashi et al., 2000). Furthermore, in humans, OGP mRNA and protein levels increase dramatically with estrous, but decrease in diestrous and luteal phase (Arias et al., 1994). Additionally, cultured human oviductal cells demonstrate an estrogen-dependent increase in OGP mRNA and protein levels in vitro. However, researchers have begun to challenge the estrogen model of regulation in larger mammals, as they discovered a role of LH/hCG receptors within the oviduct of these animals (Shemesh, 2001). Direct stimulation of OGP mRNA and protein by hCG and hCG mimetics, but not estrogen, in cultured oviductal cells from pigs, bovine and humans has been observed. Moreover, in bovine, LH/hCG acts to stabilize the mRNA of OGP and extend its half-life (Sun et al., 1997).

In our studies, non-stimulated oviducts showed a low level of sperm-binding activity, which most likely reflects the residual OGP present in these preparations, since random cycling females were used for the non-stimulated controls, one or more of which may be ovulating or in late follicular phase. However, this bioactivity might also result from the acquisition of cellular forms of OGP (Kadam et al., 2007) released by tissue homogenization and/or reflect a different sperm-binding activity.

Previous studies by Kapur and Johnson have demonstrated localization of a GP 215 glycoprotein, presumed to be OGP, to the ampullea and infundibulum, but not the isthmus of the mouse oviduct (Kapur and Johnson, 1988). Reminiscent to that reported for the GP 215 glycoprotein, immuno-localization studies utilizing anti-OGP antibodies showed OGP present in the ampullea and infundibulum, but not in the isthmus of mouse. Higher magnification reveals OGP reactivity in distinct cells that are reminiscent of secretory “peg” cells. Logically, oviduct-derived, egg-associated sperm-binding ligands should demonstrate expression in the early portions of the oviduct, since ubiquitous expression and secretion of a sperm-binding species throughout the oviduct would presumably lead to an inhibition of sperm-egg binding. As a result, regional regulation of OGP expression, secretion, and localization suggest the contribution of OGP to the oviductal milieu, and fertilization, is coordinated and controlled.

The oviduct has long been known to create a microenvironment suitable for fertilization through the expression and secretion of numerous glycoproteins (Killian, 2004). Several of these glycoproteins, including OGP, demonstrate specific associations with the ZP or sperm in several model systems (McCauley et al., 2003; O'Day-Bowman et al., 2002; Oliphant et al., 1984). The potential biological roles of OGP, however, appear to vary between species. Pre-treatment of sperm with OGP has been shown to increase sperm mobility, capacitation, and ability to fertilize in bovine (Abe et al., 1995; King et al., 1994; Martus et al., 1998); increased sperm viability, ZP penetration, and block polyspermy in porcine (McCauley et al., 2003); and increased sperm-ZP binding and penetration in hamsters and humans (Boatman and Magnoni, 1995; O'Day-Bowman et al., 2002). Furthermore, pre-treatment of ovarian eggs with OGP increases sperm

binding, penetration, and overall fertilization in bovine and porcine (Martus et al., 1998; McCauley et al., 2003). These differences may represent model system specific uses for OGP, or may be the result of variation in OGP isolation procedures and in vitro assays.

Collectively, these findings have led many to suggest a role for OGP in initial sperm-ZP binding in most model systems, with the exception being mouse. Immunolocalization studies by Kapur and Johnson utilizing antibodies against GP 215, which is generally assumed to be OGP, showed that this protein is not associated with the ZP, but rather, is localized in the perivitelline space (Kapur and Johnson, 1985; Kapur and Johnson, 1986). Furthermore, female mice bearing targeted deletions in OGP remain fertile, and OGP-null eggs bind sperm in vitro (Araki et al., 2003). Although there is some indication that sperm-ZP binding in vitro may be compromised when assaying sperm numbers closer to that in vivo (Araki et al., 2003), it has been assumed that OGP does not have a role in initial mouse sperm-ZP binding, or moreover, a role in mouse fertilization. Our demonstration that OGP is present on the surface of the ZP, and that a selective subpopulation of OGP, a PNA-reactive glycoform, interacts directly with gametes and inhibits sperm-ZP binding, indicates a need to re-examine the role of OGP in mouse fertilization using more rigorous in vitro and in vivo experimental protocols.

Several considerations may explain inconsistencies between earlier studies and results reported here. First, Kapur and Johnson completed GP 215 immunolocalization studies on hyaluronidase-treated oocytes and on fixed cumulus-oocyte complexes (Kapur and Johnson, 1985; Kapur and Johnson, 1986). Our data clearly demonstrate that OGP is removed during hyaluronidase treatment, and therefore, it is not surprising that GP 215 was not detected on the hyaluronidase-treated ZP. Furthermore, immunolocalization of

GP 215 in cumulus-oocyte complexes required reduced fixation protocols, as traditional methods were detrimental to the ZP matrix, raising the possibility that GP 215 (OGP) peripherally associated with the ZP surface was lost during these fixation procedures (Kapur and Johnson, 1986). Furthermore, GP 215 was detected using either WGA or antibodies raised against purified GP 215, and not with antibodies to a specific OGP peptide sequence. Whether the anti-GP 215 antibodies used would detect all forms of GP 215 (OGP), or specifically the PNA-reactive ZP-associated form, remains unknown. Finally, it is not surprising that OGP-null females remain fertile, since targeted deletions of numerous molecular players in sperm-ZP interactions have failed to ablate fertility, reinforcing the concept of redundant binding mechanisms.

5.1.2 The sperm-binding activity of OGP is attributed to a minor PNA-reactive glycoform.

Several lines of evidence suggest that only a minor OGP glycoform functions in sperm-ZP binding, including 1) the vast majority of OGP in the ampullea is not associated with the cumulus-oocyte complex, although there is a cumulus-oocyte associated pool of OGP that can not be dissociated with repeated washing; 2) proteins collected from the ampullar fluid are not sufficient to provide bioactivity despite an abundance of OGP; 3) numerous OGP isoforms exists with regards to pI and lectin reactivity, however, only a single basic, PNA-reactive OGP is associated with the ZP; 4) purification of these OGP glycoforms by lectin affinity chromatography of enriched superovulated oviduct lysates demonstrated Con A and PNA, but not the GS-1 and RCA-1, OGP glycoforms to be competitive inhibitors of sperm-ZP binding; 5) the association

of the PNA-reactive OGP glycoform with both the ZP and the acrosomal cap of the sperm plasma membrane reinforces its role as a sperm-binding ligand; and 6) PNA-reactive OGP pretreatment of 2-cell embryos, which are normally refractory to sperm interactions, results in a dramatic increase in sperm binding.

The identification of a minor, PNA-reactive OGP glycoform that accounts for sperm-binding activity is not surprising. First, the freely soluble OGP within the ampullary lumen exhibits significant levels of GS-1, Con A, and RCA-1 reactive OGP, but only a barely detectable amount of PNA reactive OGP. If all OGP contained the recognition signal, the freely soluble OGP portion would undoubtedly restrict or prevent fertilization. Second, the ZP-associated OGP was shown to be PNA reactive. The comparatively high level of PNA-reactive OGP in the cumulus-oocyte complex, relative to its level in the freely soluble fraction reinforces the hypothesized role of this glycoform. Furthermore, the presence of PNA-binding OGP within the perivitelline space may also serve as a sperm-binding component, but in this context, it may function to aggregate excess sperm in the perivitelline space to prevent their contribution to polyspermy, as has been suggested in other species (McCauley et al., 2003).

The discovery that PNA-reactive, but not GS-1-reactive, OGP is biologically active, however, does present a conundrum. The PNA-reactive OGP glycoform was observed in the zona-associated fraction of hyaluronidase-treated oocytes. However, immunolocalization studies clearly show that the majority of OGP in hyaluronidase-treated oocytes arises from the perivitelline space, with much lesser amounts on the surface of the ZP. In contrast, the pool of OGP released by hyaluronidase treatment of the cumulus-oocyte complex, and which presumably contains a potential cumulus matrix-

associated OGP as well as OGP removed from the ZP surface, is GS-1- and not PNA-reactive. This implies that the PNA-reactive OGP on the ZP surface is either below the limit of detection in the total "cumulus removal supernatant," or is somehow destroyed or converted to GS-1-reactive OGP by during hyaluronidase removal of cumulus cells. Consistent with this, high levels of PNA-reactive OGP are observed on the surface of the ZP following mechanical removal of the cumulus cells. Furthermore, no GS-1-reactive OGP was detected when cumulus-oocytes complexes were heat denatured, rather than hyaluronidase treated (unpublished data).

The localization of the PNA-reactive OGP to the basic region of a 2D SDS-PAGE separation was also worthy of note because glycoproteins generally exhibit acidic characteristics. Utilizing ExPASy, the theoretical pI of the mouse OGP protein sequence was determined to be 9.19. The range of OGP isoforms, from the basic to the acidic, most likely results from variable glycosylation. One potential carbohydrate modification that could be responsible for the creation of various isoforms is sialic acid. In bovine, purified OGP exhibits a pI range of 3.0 to 6.5. Interestingly, removal of sialic acids, by neuraminidase, shifts its pI to 9.3. Furthermore, neuranminidase-treated, but not untreated, bovine OGP binds strongly to PNA-agarose columns (Satoh et al., 1995).

On the other hand, the inhibition of sperm-ZP binding by the Con A-reactive OGP glycoform is interesting. Although this glycoform only weakly inhibits sperm-egg binding, relative to the PNA-reactive glycoform, a high level of Con A-reactive OGP is found in the ampullary fluid. Results show that this glycoform does not interact with oocytes, and instead of associating with the plasma membrane over the acrosome, the Con A-reactive OGP associates with the equatorial segment of sperm. Since the

equatorial segment is exposed after the acrosome reaction, we believe this interaction does not play a role in initial sperm-egg binding, but rather reflects a novel, uncharacterized function, possibly to bind and remove acrosome-reacted sperm from the population arriving at the oocyte.

To our knowledge, this is the first time OGP functionality has been associated with individual isoforms. Previous studies have analyzed the total OGP product, which could produce misleading results. For example, assays using total unfractionated OGP may fail to identify a potential role in fertilization if the bioactive glycoform is present at very low concentrations. It must also be noted that no conclusions can be made regarding the functional of the other glycoforms if the assay being employed is not aligned appropriately with a putative function being analyzed.

The fact only a specific glycoform of OGP possesses sperm-binding activity suggests that functionality is derived from the associated carbohydrate structures. Indeed, deglycosylation, but not heat denaturation, of the bioactive OGP glycoform significantly reduced bioactivity. As a result, OGP not only represents a previously unappreciated means of adhesion, it also represents a new pool of carbohydrate structures that could be recognized by sperm. Analysis of mouse OGP saccharide composition has not been documented, however, six monosaccharides including GalNAc, GlcNAc, mannose, fucose, galactose, and sialic acids (NANA and NGNA) were detected on bovine OGP at molar ratios of 15:4:2:1:17:16 respectively (Satoh et al., 1995). Terminal versions of these sugars, as well as, the disaccharide Gal-GalNAc have been documented in sheep, bovine, and porcine (Buhi et al., 1996; DeSouza and Murray, 1995; Satoh et al., 1995). Although we cannot make any conclusions about the functional carbohydrate ligand on

OGP, the potential exists that bioactive OGP presents sugar structures that are known to inhibit sperm-ZP binding, but that are not found on ZP3 (Aviles et al., 1999; Aviles et al., 2000a; Aviles et al., 2000b). Such possibilities require additional study, but do provide an attractive opportunity to reconcile previous results.

5.1.3 The role of GalT 1 in OGP function remains unclear.

The original identification of a ZP3-independent ligand on ovulated mouse oocytes was facilitated by the development of GalT 1-null mice, sperm from which no longer bind to soluble ZP3 but retain binding to the intact ZP. Thus, studies of the GalT 1-null mouse dissected sperm-egg binding into at least two distinct steps: a GalT 1/ZP3-independent adhesion followed by a GalT 1/ZP3-dependent binding that facilitates acrosomal exocytosis. The original characterization of the ZP3-independent ligand considered OGP as a likely candidate. However, OGP was eliminated as a candidate based upon the ability of GalT 1-null sperm to bind OGP-null eggs. Nevertheless, these studies did not address the sperm-binding activity of OGP itself. In this regard, it is of interest that in the present study, denatured OGP inhibited the binding of both wild-type and GalT 1-null sperm with similar efficacy, but native OGP showed reduced bioactivity against GalT 1-null sperm. Since the sperm-binding activity of OGP appears to lie within its glycan chains, it is likely that denaturing the polypeptide backbone relaxes any conformational specificity restricting glycan presentation to its receptor. In this context, denatured OGP would present the glycan epitopes with limited specificity, similar to the reduced affinity seen when glycosyltransferase substrates are removed form their native polypeptide backbone (Baranski et al., 1990). Limited specificity could result in greater

association with the sperm surface and greater inhibition. Furthermore, the reduced activity of native OGP towards GalT 1-null sperm suggests that the loss of GalT influences OGP binding to sperm, not unlike the reduced binding of ZP3 to GalT 1-null sperm (Lu and Shur, 1997).

These issues are of interest in light of the suggestion that the egg binding machinery (EBM) is organized into lipid rafts on the sperm plasma membrane. Several studies have reported alterations in lipid raft composition during sperm capacitation, which are thought to be a prerequisite for sperm binding to the egg coat (Bou Khalil et al., 2006; Cross, 2004). GalT 1 has been shown to locate to lipid rafts in somatic cells (Hathaway et al., 2003), although it is still unclear whether GalT 1 is present within lipid rafts on sperm as well. If so, then the loss of GalT 1 may disrupt or alter the presentation of the EBM components within the lipid raft, leading to reduced affinity for egg coat ligands, including ZP3 and OGP.

5.2 Future Directions

5.2.1 Elucidate the carbohydrate structure responsible for the sperm-binding activity of the PNA-binding OGP

The role of carbohydrates in sperm-egg binding has long been theorized. However, the search for the sperm-binding ligand has been marred by the fact that i) numerous carbohydrate structures have been shown to affect binding, ii) sugars not found on the ZP can inhibit binding in vitro, and iii) elimination of these structures genetically does not affect overall fertility. Many investigations and models, however, have been

skewed towards ZP3 as being the only structure capable of facilitating adhesion. The PNA-binding OGP glycoform offers an additional means of carbohydrate dependent sperm-ZP binding. Elucidation of the carbohydrate structures on this glycoform that are bound by sperm will expand our overall knowledge of fertilization. Three experimental approaches could be selected to determine the sugar structures responsible for sperm-adhesion: 1) monosaccarhide or oligosaccarhide competitive inhibition of sperm-OGP binding, 2) specific glycosidase treatment of OGP to assess effects on binding to sperm, and 3) isolate and characterize the glycan structures of the PNA-binding OGP, which can then be screened for sperm-binding activity.

First, based on our deglycosylation results, we concluded that sperm bind specific sugar structures on OGP. Accordingly, the specific sugar responsible for this interaction could be elucidated by the ability of free monosaccarhides, oligosaccaridhes or neoglycoprotiens (Loeser and Tulsiani, 1999) to competitively inhibit the PNA-binding OGP-sperm association. The PNA-reactive OGP-sperm interaction may be monitored in the presence of free sugar(s) over a range of concentrations through indirect fluorescence, as demonstrated in Chapter 4. Perturbations by a free sugar(s) would imply a role in sperm-OGP interaction. Second, functional domain analysis of the PNA-binding OGP utilized a glycosidase cocktail active against *N*- and *O*-glycans. As a result the specific glycan or sugar(s) involved remain unknown. Initially, the bioactive glycan could be established by pre-treatment with *N*- and *O*- directed glycosidases. Based on the fact that PNA-binding OGP did not bind to Con A, which recognizes structures associated with *N*-glycans, we predict *O*-glycosidase, and not *N*-glycosidase, treatment will ablate the sperm-egg binding inhibitory capabilities of this glycoform. Once the glycan chain is

established, specific endoglycosidases may be selected to systematically deglycosylate OGP, providing a direct comparison of the removal of a carbohydrate moiety to inhibition of sperm-egg binding. Finally, the technology is now available to isolate and characterize the glycan structures of the PNA-reactive OGP, which can then be screened for sperm-binding activity, or alternatively, be printed onto slides than can be subsequently incubated with labeled sperm to assess binding to distinct carbohydrate structures.

Included within the scope of these investigations would be determining the mechanisms supporting OGP-ZP binding. Similar studies into the effect of heat denaturation and deglycosylation would implicate either the inactive chitinase or the *O*-glycosylation domain. Furthermore, interaction studies with the ZP proteins would identify the recognition signal on the ZP, whether it is protein, carbohydrate, or supramolecular structure based. Caution should be exercised during these studies because the OGP-ZP interaction may not be direct, and thus facilitated by a ZP-associated intermediate.

5.2.2 Identifying the sperm receptor for PNA-binding OGP

Several experimental approaches are available to identify the OGP receptor on sperm. First, the PNA-binding OGP could be immobilized on beads and used as an affinity matrix to “pull-down” the sperm receptor from sperm lysates, similar to the methodology utilized to identify p47, an SED1 homologue, in boar sperm (Ensslin et al., 1998). Alternatively, sperm lysates could be resolved by 1D or 2D SDS-PAGE and transferred to membranes for an overlay blot with PNA-binding OGP, followed by

immuno-detection of OGP to identify potential specific bands that interact with OGP. Any reactive bands or regions could be extracted from similar membranes or gels and sequenced. As an alternative to methods in which the sperm is disrupted, PNA-binding OGP could be cross-linked to its receptor as it associates with intact sperm that have been previously biotinylated (Benashski and King, 2000). After cross-linking, the biotinylated sperm receptor bound to OGP could be isolated by immunoprecipitation, the cross-linker reduced and resolved by 1D SDS-PAGE. After transfer to membranes, the biotinylated sperm protein can be identified and selected for microsequencing. Similarly, biotin transfer technology, available commercially as Sulfo-SBED Biotin Label Transfer Reagent by Pierce Biotechnology, could be employed. This reactive chemistry allows one to tag a known protein or "bait" with the biotin-containing chain. The "bait" is then placed in contact with the "prey," in this case the intact sperm, in the dark. After free "bait" is removed, the sperm are exposed to light, which causes a catalytic transfer of the biotin group from the "bait" to the closest interacting protein. The interacting protein may then be enriched by streptavidin-bead precipitation and extracted for microsequencing.

5.2.3 Investigating the role and function of OGP localized to the perivitelline space

Our data suggests the PVS-associated OGP has similar glycosylation states as the zona-associated form that facilitates initial binding, thus we suggest PVS-associated OGP also has sperm-interacting capabilities. Since OGP has been shown to decrease polyspermy in other model systems (McCauley et al., 2003), we suggest the PVS OGP might serve a similar role. However, an alternative function for OGP in the PVS has been

proposed by Natraj and colleagues, who have reported that OGP interacts with non-muscle myosin IIA (Kadam et al., 2006). They suggest the fertilized embryo presents myosin on the cell surface at the cleavage furrow during cytokinesis, and extracellular PVS OGP assists in this process. In any event, a greater understanding of the role and function of PVS-associated OGP is needed.

An initial series of experiments could be completed to approach the role of PVS-associated OGP. First, an analysis of the frequency of polyspermy in OGP-null females (Araki et al., 2003) should be performed to identify any potential phenotype. Second, work completed here demonstrates an interaction of the PNA-binding OGP, presumed to the same glycoform as in the PVS, with capacitated, acrosome-intact sperm. Similar studies need to be completed with acrosome-reacted sperm. To account for any false results due to this in vitro approach, ex vivo studies should be completed in which sperm within the PVS are imaged for OGP binding. An extension of such experiments would determine if *N*-acetylglucosaminidase modulates the bioactivity of the PVS-associated OGP. *N*-acetylglucosaminidase is released from egg cortical granules and facilitates the block to polyspermy, presumably by cleaving hexosamine residues on ZP3 (Miller et al., 1993). *N*-acetylglucosaminidase may have a similar effect on OGP, such that OGP facilitates binding until fertilization, at which time it is cleaved to prevent polyspermy. Such models, however, remain conjecture.

5.2.4 Characterization of additional OGP glycoforms

Based on the hypothesis that differential OGP glycosylation profiles directly correlate with distinct localization and function, we would predict the additional OGP

glycoforms have specific roles as well. Our preliminary data demonstrating an association with the Con A-binding OGP glycoform, found in the ampullar environment, with the equatorial region of sperm supports this possibility. OGP has been attributed to numerous functions other than initial sperm-egg binding, including increased sperm viability, mobility, capacitation and acrosome reaction, as well as enhancing survival and cleavage rates of embryos. These studies, however, have been completed in other model systems and were conducted using the total OGP fraction. Again, we believe the ability to enrich and purify distinct OGP glycoforms provides a unique opportunity to dissect the role of each glycoform that may have been overlooked when assaying unfractionated OGP.

Potential assays include, but are not limited to, OGP glycoform binding to acrosome intact and acrosome reacted sperm, or capacitated and non-capacitated sperm; computer assisted sperm analysis (CASA) of sperm motility; sperm viability assessment, status of capacitation assessed by i) CTC staining, ii) tyrosine phosphorylation of sperm proteins, iii) cAMP levels, and/or iv) the ability to fertilize eggs; and studies of sperm penetration rates, polyspermy, embryo viability and cleavage parameters, as well as uterine implantation rates.

5.2.5 Identifying the non-OGP, ZP3-independent ligand

The overall goal of this project was to identify a ZP3-independent ligand, initially believed not to be OGP due to the fact a ZP3-independent ligand could be purified from OGP-null egg coats. The discovery and characterization of OGP as a ZP3-independent

ligand associated with the egg coat suggests that there remains another ZP3-independent sperm-binding ligand.

Since the superovulated oviduct proves to be a rich source of ZP3-independent ligands, it could be used as a starting point to identify a second ZP3-independent ligand. In this regard, it is interesting that data presented here did show bioactivity at molecular weights higher than the OGP range of 200-250 kDa. These bioactivities were not pursued because their lectin depletion profiles did not match the originally characterized ZP3-independent ligand. These high molecular weight bioactivities represent an immediate approach to identify additional sperm-binding ligands. However, they may be an artifact of OGP dimerization and differential OGP glycosylation. Utilizing OGP-null oviducts may represent an easier path to avoid issues of contaminating OGP. Second, a 2D SDS-PAGE profile of the ovulated egg coat was attempted using heat denatured, biotinylated proteins from hyaluronidase-treated oocytes, and several basic proteins were identified. Wild-type and GalT 1-null sperm pull-down studies using this fraction, followed by 1D or 2D SDS-PAGE, revealed two proteins “pulled-down” at roughly 150 and 65 kDa (data not shown). Unfortunately, the possibility that these bands represent carry over from the starting material could not be eliminated, and therefore, these results require further study.

5.3 Conclusion

Reproductive management has become a worldwide issue as overpopulation has become a concern for developing nations, while developed countries are experiencing a

decrease in fertility due to environmental and societal factors. Technological advancement requires a greater fundamental knowledge of fertilization and reproduction.

A clear understanding of the mechanisms supporting fertilization, specifically sperm-zona binding, has been complicated by the existence of many molecular players and conflicting results. The demonstration that oviduct-specific glycoprotein (OGP) is a oviduct secreted, zona pellucida-associated glycoprotein capable of facilitating initial sperm binding has advanced the field in three significant ways: 1) it represents the first identified non-zona matrix based sperm-binding ligand, 2) it supports a carbohydrate-mediated model of sperm-egg binding, and 3) it demonstrates multiple functionality of a single protein through distinct glycosylation states. These findings also challenge the field by demonstrating that the in vitro assays and protein isolation procedures used can influence our understanding of function. Overall, OGP provides new directions for fundamental research into reproductive biology and development of new technologies to assess and regulate fertility.

Chapter 6: References

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