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Glycobiology of fertilization in the pig

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ABSTRACT By adopting internal fertilization, the meeting of both gametes - the sperm and the egg - and thus the highly coordinated sequence of interactions leading to fertilization, occur in the female reproductive tract. In mammals, the oviduct has been shown to translate the requirements of the female, coordinating sperm activation (capacitation) and sperm transport with the arrival of the ovulated egg. A hierarchy of carbohydrate-based interactions accompanies these events ranging from the binding of uncapacitated sperm to the oviductal epithelium (establishment of the female sperm reservoir), to the primary and secondary binding processes contributing to gamete recognition and sperm penetration of the oocyte zona pellucida. The current perspective will focus on the carbohydrate-recognition systems in the binding events during fertilization in the pig. The roles of the major carbohydrate-binding proteins, the spermadhesins and the acrosomal serine proteinase, pro/acrosin are discussed under consideration of recent structural data. The glycans and the glycoproteins of the porcine oviduct with a focus on the candidate sperm receptors as well as the zona pellucida N-glycans of prepuberal pigs have been characterized by a mass spectrometric approach. Furthermore, some preliminary data supporting the hypothesis that the zona pellucida has to undergo a maturation process during oocyte development are presented.

KEY WORDS: *gamete interaction, spermadhesin, acrosin, zona pellucida, glycoprotein, glycan*

A short review of mammalian fertilization

The fertilization of an egg by a sperm is the fundamental event in life, as it culminates in the creation of a new individual. In mammals, the meeting of the fertilizing competent sperm and the ovulated egg is the beginning of a highly coordinated sequence of cellular interactions between the haploid gametes, which leads to the formation of the diploid zygote and initiates embryonic development.

The first contact between sperm and egg takes place at the outer surface of the surrounding extracellular matrix of the egg, the zona pellucida (ZP). By binding to distinct oligosaccharides of the ZP glycoproteins, the fertilizing sperm recognizes the egg. Upon anchoring the sperm to the egg through the ZP carbohydrates the signaling cascade leading to acrosomal exocytosis of the sperm is activated allowing the sperm to pass through the zona pellucida. The acrosome-reacted sperm in the end interacts and fuses with the egg plasma membrane, which in turn enables the egg to complete meiosis, to initiate the mechanisms to prevent polyspermic fertilization and to start the embryonic developmental programs (reviewed by Yanagimachi, 1994).

By adopting internal fertilization in mammals the meeting point of both gametes, at which fertilization occurs, is postponed to the female reproductive tract (Fig. 1). One consequence of internal fertilization is the need for the sperm to acquire fertilizing capacity in the vicinity of the egg. Thus, in the oviduct the sperm undergoes a series of maturational changes - referred to as capacitation (reviewed by Salicioni *et al.,* 2007; Darson *et al.,* 2007) - preparing the fertilizing sperm for interaction with the oocyte in the appropriate manner and for undergoing the acrosome reaction in response to the zona pellucida. Since sperm reside for hours or even days in the female tract, the storage of sperm under conditions that maintain fertilizing competence and coordinate sperm function with ovulation is an important contribution to optimizing fertilization success. The oviduct has been shown to play a crucial role during the life span of sperm. It is the site in which sperm are stored to await the arrival of the egg, and in which capacitation and fertilization proceed. The oviduct is actively

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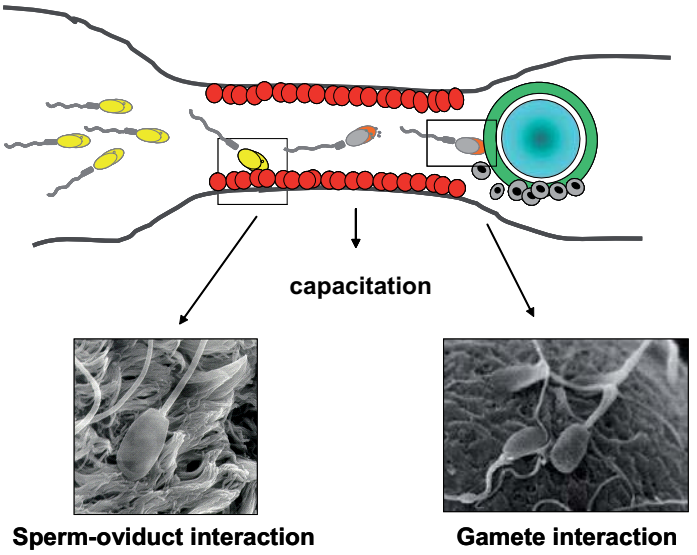


Fig. 1. Fertilization in the oviduct. *Sperm are trapped in the isthmic region of the oviduct by carbohydrate-mediated binding with the epithelium lining the oviduct and sperm are stored under protective conditions. Close to the time of ovulation the capacitation process is initiated and sperm dissociate from the epithelium and freely swim to the site of fertilization thereby completing capacitation. The capacitated sperm meets and recognizes the ovulated egg. Scanning electron micrographs show sperm binding to the oviduct and to the oocyte zona pellucida in the pig.*

involved in these events. It translates the requirements of the female and - for those sperm involved in fertilization - coordinates place and time of the ongoing capacitation process with ovulation (Hunter and Rodriguez-Martinez, 2004).

The events within the oviduct leading to fertilization involve a sequence of carbohydrate-mediated interactions. The adhesion of sperm to the oviductal epithelium establishing the sperm reservoir in the caudal portion of the isthmus has been shown for several mammalian species to involve carbohydrates (reviewed by Suarez, 2002, Töpfer-Petersen *et al.,* 2002). The initial binding and recognition process (primary binding) between both gametes, which starts the fertilization cascade as well as the transient binding of the acrosome-reacted sperm to the zona pellucida (secondary binding), which is an essential step in the penetration process operate by utilizing the information potential of carbohydrates (Töpfer-Petersen, 1999; Wassarman 2005; Wassarman *et al.,* 2005).

This review focuses on the molecules involved in the carbohydrate-mediated processes of the fertilization process in the pig.

Carbohydrate-binding proteins of boar spermatozoa

Since fucoidan has been found to effectively inhibit sperm-egg interaction in the pig (Töpfer-Petersen *et al.,* 1985), the identification of potential receptor molecules for the zona pellucida started with the search for fucoidan-binding proteins in boar sperm. Ultrastructural studies document that in freshly ejaculated sperm fucose-binding was particularly prominent at the apical region of the sperm head (Töpfer-Petersen *et al.,* 1985; Friess *et al.,* 1987a), whereas after induction of the acrosome reaction a dramatic exposure of binding sites was observed (Friess *et al.,* 1987b). After all, these fucoidan-binding components were identified as the major zona pellucida-binding proteins of boar sperm - the small surface-associated proteins of 12-16 kDa representing a novel class of proteins, the spermadhesins (Töpfer-Petersen *et al.,* 1998) and the long-known intra-acrosomal serine proteinase pro/acrosin having a molecular mass of 53-55 kDa (Töpfer-Petersen and Henschen, 1987). The localization of the zona-pellucida-binding proteins (spermadhesins and pro/acrosin) at different compartments of the sperm implicated that they may be involved in different steps of sperm-oocyte interaction. Indeed, spermadhesins and proacrosin have been shown to participate in the sequence of carbohydrate-mediated events proceeding in the porcine oviduct.

Another peripherally bound protein, P47 isolated by affinity chromatography on immobilized zona pellucida from the solubilized sperm membrane proteins was shown to be identical with the porcine milk fat globulin/lactadherin and is expressed in the testis and along the epididymis in all mammalian species studied so far (Ensslin *et al.,* 1998). It displays a mosaic structure organized into one N-terminal EGF-bimotif followed by two discoidin/F5/8 complement domains. Recently, the mouse homolog has found its way into literature as SED1 and has been implicated to participate in gamete adhesion in the mouse. Structure and function of P47/SED1 in mammalian fertilization have been recently reviewed by Shur and coworkers (2004).

Spermadhesins

Among the variety of sperm proteins known to display zona pellucida- and carbohydrate-binding capabilities, spermadhesins, a group of 110-133-residue polypeptides, occur as a novel protein family of animal lectins (Töpfer-Petersen *et al.,* 1999; Dostàlovà *et al.,* 1995a). Spermadhesins are male secretory proteins, which have so far been found in the seminal plasma, being peripherally associated with the sperm surface of ungulates (i.g. pig, bull, ram, stallion and buck). In the pig, the protein family exhibit the greatest diversity of members and contain five closely related genes encoding spermadhesins AQN-1, AQN-3, AWN, PSP-I, PSP-II and their glycosylated isoforms (Sanz *et al.,* 1991, 1992; Kwok *et al.,* 1993) that are located on SSC 14q28–q29 in a region syntenic to HSA 10q26 (Haase *et al.,* 2005). No spermadhesins have been found in human, canine, or murine seminal plasma. It seems clear that the spermadhesin gene has been functionally inactivated in the human by mutations that disrupt the coding sequence during evolution. Inactive copies of spermadhesin genes are also detectable in the chimp and dog genome while the entire region containing the hypothetical ancestral spermadhesin gene has been deleted from the genomes of mouse and rat (Leeb, 2007). Within the pig, the five spermadhesin genes contain both highly diverged and highly conserved regions.

Porcine spermadhesins are synthesized by the epididymis and accessory glands of the male genital tract. Interestingly, AWN is not only expressed in the male epididymis and seminal vesicle but also in the Fallopian tube of the female genital tract (Ekhlasi-Hundrieser *et al.,* 2002). In other species than pigs, the diversity and expression level of spermadhesin proteins is greatly reduced. In cattle, only two spermadhesins termed SPADH1 (aSFP) and SPADH2 (Z13) were identified (Wempe *et al.,* 1992; Tedeschi *et al.,* 2000). In the horse one spermadhesin (designated HSP-7)

homolog to boar spermadhesin AWN and in ram one spermadhesin (designated RSP) homolog to boar AQN-1 were reported (Reinert *et al.,* 1996; Bergeron *et al.,* 2005). Recently, Melo *et al.* (2007) have found a single member of the spermadhesin family homolog to boar AWN from buck seminal plasma termed BSFP. In animals outside the ungulate order, neither spermadhesin proteins nor spermadhesin-specific transcripts have been able to be detected so far.

The structure and function of spermadhesins have been most thoroughly investigated in the pig. Porcine spermadhesins, spanning 110-133 amino acids, comprise a single CUB domain. The CUB domain is stabilized by two conserved disulphide bonds between nearest neighbour cysteine residues. The crystal structures of two members of the spermadhesin family revealed for the first time insight into the architecture of a CUB domain. The overall structure of the subunit consists of a β-sandwich made up of two sheets. Each of the sheets contains four anti-parallel strands and one parallel β-strand (Romero *et al.,* 1997; Romao *et al.,* 1997; Varela *et al.,* 1997). CUB domains occur in diverse combinations in structurally and functionally unrelated mosaic proteins and serve as a structural scaffold, onto which different functionalities can be imprinted (Bork and Beckmann, 1993).

Spermadhesins are proteins with a vast variety of possible biochemical interactions. These proteins exhibit a range of ligand-binding capabilities to heparin, proteinase inhibitors, phospholipids and carbohydrates, which have been attributed to different biological activities. Sequence variation, glycosylation and the aggregation state of spermadheins modulate their specific functions (reviewed by Töpfer-Petersen *et al.,* 1998; Calvete and Sanz, 2007). For instance, AWN, AQN-1 and AQN-3 are sperm-binding proteins, which appear to be involved in the carbohydrate-mediated steps of fertilization. AQN-type proteins associate to the sperm surface at ejaculation predominantly at the acrosomal region of the sperm head and have been shown to contribute to the formation of the oviductal sperm reservoir by interacting with the glycoconjugates of the oviductal epithelium (Wagner *et al.,* 2002; Ekhlasi-Hundrieser *et al.,* 2005). AWN remains bound to the peri-acrosomal sperm plasma membrane after transport through the female genital tract and capacitation *in vivo* and may be implicated in gamete recognition (Rodriguez-Martinez *et al.,* 1998).

With regard to biochemical interactions, only non-aggregated AWN-1 and AQN-3 are able to interact with phosphorylethanolamine matrices, suggesting that this spermadhesin subpopulation can bind directly to the lipids of the sperm membrane (Dostàlovà *et al.,* 1995b). Phosphorylethanolamine is a major substituent of boar sperm membrane phospholipids (Watson, 1981). Aggregated spermadhesins could then become coated on top of this first layer, serving as stabilizing factors that protect the acrosome membrane from premature acrosome reaction. These spermadhesins are thought to stabilize the plasma membrane over the acrosomal vesicle and are mainly released from the spermatozoal surface during capacitation (Sanz *et al.,* 1993; Dostàlovà *et al.,* 1994; Calvete *et al.,* 1997). A phosphorylethanolamine–binding region has been mapped to a discontinuous region of AWN comprising residues 6-12 and 104-108 (Ensslin *et al.,* 1995), and a conformational heparin-binding surface, which partly overlaps with the phosphorylethanolamine–binding region, resides in an opposite location to the carbohydrate-binding region of the spermadhesin (Calvete *et al.,* 1996a).

AQN-1 binds to the sperm membrane by an indirect lipid binding mechanism. It forms a heteromer with the porcine Fn-2 type protein, pB1, which may associate to the sperm membrane via the phosphorylcholine-binding sites of pB1 (Calvete *et al.,* 1997; Ekhlasi-Hundrieser *et al.,* 2007)

PSP-I and PSP-II are the most abundant boar seminal plasma proteins and form a non-heparin binding heterodimer of glycosylated spermadhesins (Nimtz *et al.,* 1999). Recent studies support an immunosuppressive function for PSP-I and PSP-II glycoforms. They are immunostimulatory for lymphocyte activity *in vitro* (Nimtz *et al.,* 1999) and prevent possible infections of the lower reproductive tract and provide a foreign cell-free uterine environment for the descending early embryos (Assreuy *et al.,* 2003). The bovine spermadhesin aSFP binds only loosely to the surface of ejaculated bovine sperm and is quantitatively released during *in vitro* capacitation (Dostàlovà *et al.,* 1994). This protein appears to stimulate in a dose-dependent manner (in the ng/ml range) *in vitro* cell division of lymphocytes and progesterone secretion by bovine endometrium and ovarian granulosa cells (Einspanier *et al.,* 1991). This suggests a possible sperm protecting function for aSFP and a stimulative effect on ovulation upon insemination. Furthermore, aSFP possesses neither carbohydrate nor zona pellucida binding capabilities, strongly indicating that aSFP may not be involved in gamete interaction.

The remarkable feature of the porcine spermadhesins is their carbohydrate-binding property, which has been investigated most intensively. The underlying carbohydrate-recognition mechanisms have been found to differ in various species. The porcine spermadhesins recognize a broad spectrum of oligosaccharides, while the bovine member of spermadhesin family, aSFP exhibits no comparable carbohydrate-binding characteristics (Töpfer-Petersen *et al.,* 1998). Isolated porcine spermadhesins bind to their possible natural substrates – zona pellucida glycoproteins and oviductal glycoproteins – in a cation-independent manner with a Kd in a low micromolar range. AWN and AQN-3 show equivalent binding affinity for such glycoproteins that carry Galβ1-3GlcNAc and Galβ1-4GlcNAc sequences either in N- or O-linked oligosaccharides. AQN-3 and AWN differ from each other with respect to recognition of tri-/tetra-antennary N-glycans. Further, an interesting peculiarity is the modulation of binding after linkage of N-acetylneuraminic acid with a galactose residue, in which affinity of AWN is increased. Galβ1-3GalNAc sequences are about 50 times better recognized by AWN than N-acetyllactosamine residues in N-glycans (Dostàlovà *et al.,* 1995a; Calvete *et al.,* 1996b). AQN-1 shows a broad carbohydrate-binding pattern as it recognizes both α- and β-linked galactose as well as Man α1-3(Manα1-6)Man structures, whereas AWN bound only the galactose species (Ekhlasi-Hundrieser *et al.,* 2005).

Studies on ligand binding properties of spermadhesins revealed that N-glycosylation at the conserved asparagine residue (N_{50}) abolishes, or greatly impairs the carbohydrate-binding capability of the spermadhesins by steric hindrance of the sugar combining site. The heparin- and phospholipid-binding activity was however unaffected. The observation that carbohydrate-recognition ability could be reconstituted by cleavage of glycan residues leads to localizing one of the probable carbohydrate-recognition sites close to this asparagine residue (N_{50}). A stretch

of 11 amino acids including the glycosylation site has been found to be highly conserved in the members of the spermadhesin family. Site-directed mutagenesis of the solvent-exposed amino acids in this region abolishes the binding capacities to mannose. These findings strongly support the assumption that the sugar-combining amino acids are arranged around the Asn_{50} (Fig. 2) (Ekhlasi-Hundrieser *et al.,* 2008). The binding site and the respective amino acid sequences of the spermadhesins seem to be unique and do not resemble any other known carbohydrate recognition (Kaltner and Gabius, 2001). Further studies on the carbohydrate recognition sites of spermadhesins appear to be rewarding due to the remarkable variety/versatility for ligand binding and their biological activities in fertilization processes.

Proacrosin and acrosin

Acrosin (EC 3.4.21.10) is the major serine proteinase of mammalian sperm, which has been shown to be involved in the complex events of sperm-egg interaction. It is post-meiotically expressed in the testis (reviewed by Nayernia *et al.,* 1996) and becomes stored during differentiation in the acrosomal cap as its inactive form, proacrosin. DNA and amino acid sequences and its acrosomal localization are highly conserved throughout mammalian evolution (Klemm *et al.,* 1991).

Porcine proacrosin (P08001) is a single chain molecule of 55/53 kDa, which shows about 50% sequence similarity with other serine proteinase in the catalytic domain including the catalytic triade (H_{70}, D_{124}, S_{222}). A feature not observed in other serine proteinases is the 125 amino acid C-terminal extension, including a stretch of 23 consecutive proline residues. The biological role of this proline-rich domain is not yet clear. It has been thought to be responsible for the strong binding of proacrosin to biological membranes and possibly to the sperm inner acrosomal membrane (Zelezna *et al.,* 1989). Autocatalytic cleavage at the proteolytic clip between the Arg_{23}-Val_{24} peptide bond leads to the formation of an active two-chain molecule of the same molecular mass, in which the 23 amino acid long L-chain (light chain) remains linked by two disulfide bonds to the A-chain (heavy chain) (Fock-Nüzel *et al.,* 1984; Cechova *et al.,* 1988). Also unusual for a serine proteinase is that in addition to the N-terminal processing (activation), sequential C-terminal processing releases the last 77 amino acids including the polyproline motif giving rise to the mature 38-kDa β-acrosin (Baba *et al.,* 1989). Acrosin contains 12 cysteine residues, of which eight cysteines involved in four intrachain bonds within the heavy chain are conserved among the serine proteinase family. The remaining two cysteines cross-link the light chain to the heavy chain (Töpfer-Petersen *et al.,* 1990). Both N-glycosylation sites, N_3 in the light and N_{10} in the heavy chain are glycosylated (Cechova *et al.,* 1988; Töpfer-Petersen *et al.,* 1990a).

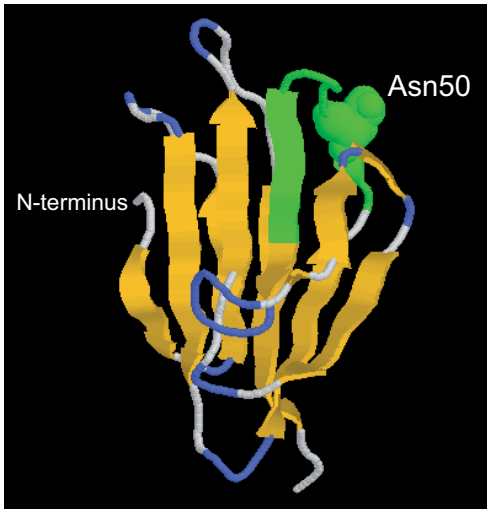


Fig. 2. Structure of the carbohydrate-binding proteins AQN-1. *The model of AQN-1, based on the crystallographic data of the PSP-1 (1spp chain A; http://swissmodel.expasy.org) (Romero* et al., *1997). The conserved 11-mer peptide, which is part of the carbohydrate-recognition domain (CRD) is labelled in green. Asn50, which can be glycosylated thereby abolishing the carbohydrate-binding affinity is shown in spacefill.*

Pro/acrosin is characterized by its high affinity binding activity ($K_d \sim 10^{-8}$) to sulfated oligosaccharide chains such as fucoidan and the ZP-glycoproteins, a property, which is not correlated with the proteolytic activity (Töpfer-Petersen and Henschen, 1987, 1988; Jones, 1991, Urch and Patel, 1991). Autoproteolysis of acrosin *in vitro* generates a N-terminal 15-kDa fragment of the heavy chain with ZP-binding capacity (Töpfer-Petersen *et al.,* 1990b) containing at least one of two clusters of basic amino acids (H_{47}, R_{50}, R_{51} and R_{250}, K_{252} and R_{253}), which has been shown to be involved in sulfate-binding (Richardson and O´Rand, 1996; Jansen *et al.,* 1998, Jansen *et al.,* 2001). Crystallographic analysis of boar β-acrosin has demonstrated the localization of those positively charged patches within the surface-oriented loops peripheral to either side of the active site (Tranter *et al.,* 2000).

Although the step-wise conversion of proacrosin to the mature β-acrosin occurs autocatalytically at an elevated pH, polysulfated structures, namely fucoidan, heparin, and the ZP glycoproteins are able to accelerate the autoactivation process at low concentrations and to inhibit acrosin amidase activity at high concentration - a feature which could not be shown for e.g. the activation of trypsinogen to trypsin (Töpfer-Petersen and Cechova, 1990). A basic 18-amino acid peptide Ile_{43}-Leu_{60} containing one polysulfate-binding subsite has been found to mediate the activation process (Moreno and Barros, 2000), indicating that the zona pellucida as the natural target is able to finely regulate the enzyme activation and activity by sulfate-recognition mechanisms. Recent studies have revealed the relevance of the stereo-specific presentation of the sulfate groups involved in proacrosin binding and activation (Gaboriau *et al.,* 2007). High affinity binding, proacrosin-to-acrosin conversion and enzyme activity control seem to be dependent on the spatial proximity of the active site and the polysulfate-binding sites, in that upon binding the zona pellucida is fixed close to the active site of the enzyme allowing subsequent steps to take place (Tranter *et al.,* 2000).

The features basically unusual for the serine proteinase family may point to a multifunctional role of proacrosin/acrosin in mammalian fertilization.

The female sperm reservoir in the pig

The small sperm population, which is able to survive the passage through the uterus and surmount the barrier of the uterotubal junction, is stored in the isthmic portion of the porcine oviduct under protective conditions to span the time between insemination and ovulation. Numerous studies on the pig and

other species (reviewed by Töpfer-Petersen *et al.,* 2002; Suarez and Pacey, 2006) have demonstrated that the sperm to be stored in the oviduct must fulfill several criteria. In the pig, these criteria have been found to be: maturity (Petrunkina *et al.,* 2001a), morphological intactness (Waberski *et al.,* 2006), viability and an uncapacitated status (Fazeli *et al.,* 1999; Petrunkina *et al.,* 2001b). Recently, this list has been extended with the stability of chromatin structure (Ardon *et al.,* 2008) and good osmoregulatory abilities (Petrunkina *et al.,* 2007), indicating that strict selection mechanisms take place in the oviduct to ensure that only sperm with a high fertilization potential will be able to reach and fertilize the oocyte. Upon binding to the epithelial cells typical capacitation-related changes such as the uptake of extracellular calcium ions and tyrosine phosphorylation of sperm proteins are suppressed (Petrunkina *et al.,* 2001b). As capacitation can be understood as a process of controlled destabilisation (Harrison, 1996) reducing the life span of capacitating sperm, protective mechanisms prolonging viability during storage must affect the sequence of capacitational changes at an early stage (Töpfer-Petersen *et al.,* 2002). However, this also implies that the initiation and completion of capacitation must be synchronized with ovulation. Before ovulation most viable sperm found *in vivo* in the storage site in the pig are uncapacitated (Tienthai *et al.,* 2004). Around the time of ovulation the inhibitory influence of the oviduct switches to an activating influence on sperm function allowing the sperm to be released from the surface, to progress towards the site of fertilization and complete capacitation after ovulation. The sequence of events in the oviduct *in vivo* is under hormonal control of the ovaries and may be characterized in the pig by (1) the storage of sperm in the pre-ovulatory phase, (2) the peri-ovulatory release of a discrete sperm population from the storage site by developing a hyperactivated motility and (3) completion of capacitation in response to post-ovulatory signals (Hunter and Rodriguez-Martinez, 2004; Tienthai *et al.,* 2004; Hunter, 2008). However, there is growing evidence that the dialogue between oviduct and sperm is not a one-way road. In response to its recognition and binding in the storage site, the fertilization-competent sperm modulates oviductal gene expression and thus the secretory proteome in the oviduct (Fazeli *et al.,* 2004; Georgiou *et al.,* 2005). The regulatory fine-tuning of the oviductal functions by the sperm may contribute to the creation of a favorable microenvironment in which sperm storage, sperm release and capacitation can occur. This network of interactions between the sperm, the ovulating oocyte, the oviduct and the ovary in the end ensure successful fertilization *in vivo*.

Although *in vitro* methods lack the regulatory function of the ovaries on the oviductal epithelium, they allow at least molecular studies of basic principles of sperm storage in the duct e.g. the recognition system involved in sperm-oviduct binding and the selection parameters. In the pig, oviductal explants were obtained shortly after slaughtering the animals, thus creating conditions that are close to the *in vivo* situation. The binding capacity is expressed as Binding Index (BI= sperm cells/0.01 mm^2) showing a linear relationship with the number of sperm added (for details, see Petrunkina *et al.,* 2001a). No differences were found in the initial sperm binding between the isthmic and ampullary explants and there was no effect of the stage of the oestrus cycle or the reproductive status of the female donor (Petrunkina *et al.,* 2001a). To mimic the natural situation as best as possible only explants of the isthmic region of oviducts from prepuberal animals were used for further experiments.

Carbohydrates are involved in the establishment of the sperm reservoir

The intimate contact between the sperm and the epithelium lining the duct may be one of the prerequisites for the cross-talk between gamete and somatic cells, which enables the oviduct to fulfill the various functions of the sperm reservoir. Cumulating evidence implicates that the binding events involve carbohydrate-recognition mechanisms. Competitive inhibition studies *in vitro* were used to determine the key sugar residues in various mammalian species indicating that the sugar signals vary among species (e.g. terminal sialic acid in hamster and rat; fucose in the bovine and galactose in horse) (reviewed by Suarez, 2001, 2002).

In the pig, mannosyl glycoconjugates were found to mediate binding of sperm to the oviduct (Wagner *et al.,* 2002). From a set glycoproteins, e.g. fetuin, asialofetuin, ovalbumin representing common glycan structures with different terminal sugar residues (sialic acid, galactose and mannose) only ovalbumin was able to effectively inhibit the binding *in vitro* (70%) in a low micromolar concentration (3 μM). Fetuin carrying terminal sialic acids on the glycans showed no effect and asialofetuin had a small but significant inhibitory effect. In order to take into account the differing glycosylation sites of the glycoproteins, the IC_{50} values were determined on the basis of the number of glycan chains. The inhibitory activity (IC_{50} ~18 μM) of asialofetuin (6 mole glycan/per mole proteins; Pisano *et al.,* 1996) carrying galactose residues at the non-reducing end of the complex N-glycans and O-glycans was significantly lower as compared to ovalbumin with a single carbohydrate chain (IC_{50} ~ 1.3 μM). Ovalbumin carries a collection of high-mannose type and hybrid type N-glycans. Isolation of the glycopeptides by ConA-affinity chromatography and HPLC following tryptic digestion and the release of the glycans by N-glycosidase F treatment allowed the structural elucidation of glycan structures used in the experiment. MALDI-ToF-MS demonstrates the preferential occurrence of the oligomannosyl glycans ($Man_{5,6}HexNAc_2$) and, to a minor degree, the hybrid N-glycans, ($Man_{3\text{-}6}GlcNAc_{3\text{-}4}$). As the glycoprotein, the glycopeptides and the released glycans effectively inhibited the sperm binding to the explants (~ 80-84% at 3 μM ovalbumin equivalents). The deglycosylated peptide had no effect. The contribution of N-acetylglucosamine residues of the hybrid type glycans seems to be negligible because the inhibitory activity of mannopentaose, having the same structure as oligomannose sequence of $Man_5GlcNAc_2$, was found comparable to that of the glycopeptides and the released glycans (Wagner *et al.,* 2002). These data strongly indicate the carbohydrate-based nature of the sperm-oviduct interaction in the pig and the cooperative interaction of oligomannose high affinity sites, and possibly low affinity sites as galactose in glycan chains in the creation of the sperm reservoir.

The glycoproteome of the porcine oviduct

Both counterparts, the sperm and the oviduct, are equipped with the molecules that may participate in the sperm-oviduct binding (Walter and Bavdek, 1997, Ekhlasi-Hundrieser *et al.,* 2005; Töpfer-Petersen *et al.,* 1998; Biermann *et al.,* 1997; Runnebaum *et al.,* 1994). Current understanding (Revah *et al.,* 2000; Ignotz *et al.,* 2001; Ekhlasi-Hundrieser *et al.,* 2005), however, supports the

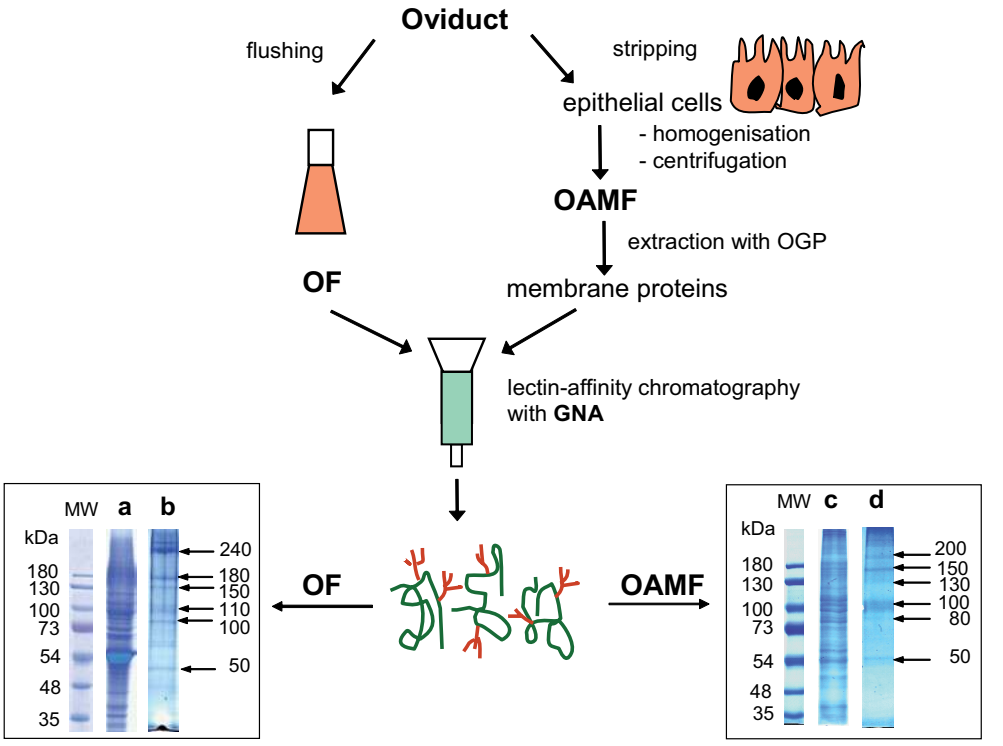


Fig. (3A). Strategy for isolation of the mannose-rich glycoproteins of the porcine oviduct. *The oviductal fluid (OF) was isolated by flushing the oviducts with 0.15 M NaCl (Buhi* et al., *1990), followed by centrifugation to separate cell debris and dialysis against water. The oviductal apical membrane fraction (OAMF) was isolated according to the method of Fazeli* et al., *2003. Transmission electron microscopical examination verified the preferential occurrence of plasma membrane vesicles. The OAMF was solubilized in 1% OGP (octyl glucopyranoside) in phosphate-buffered saline pH 7.4 containing protease inhibitors. Affinity chromatography was performed on AffiSep-GNA-columns (0.75ml, Galab Technologies, Germany), which were run in PBS. GNA-binding proteins were released with 200mM α-methyl-mannopyranoside in PBS. Insets: SDS-PAGE of OF and OAMF. Aliquots (5-20 μg) were subjected on 8% gels. Proteins were visualized with Coomassie-Brilliant Blue-staining.*

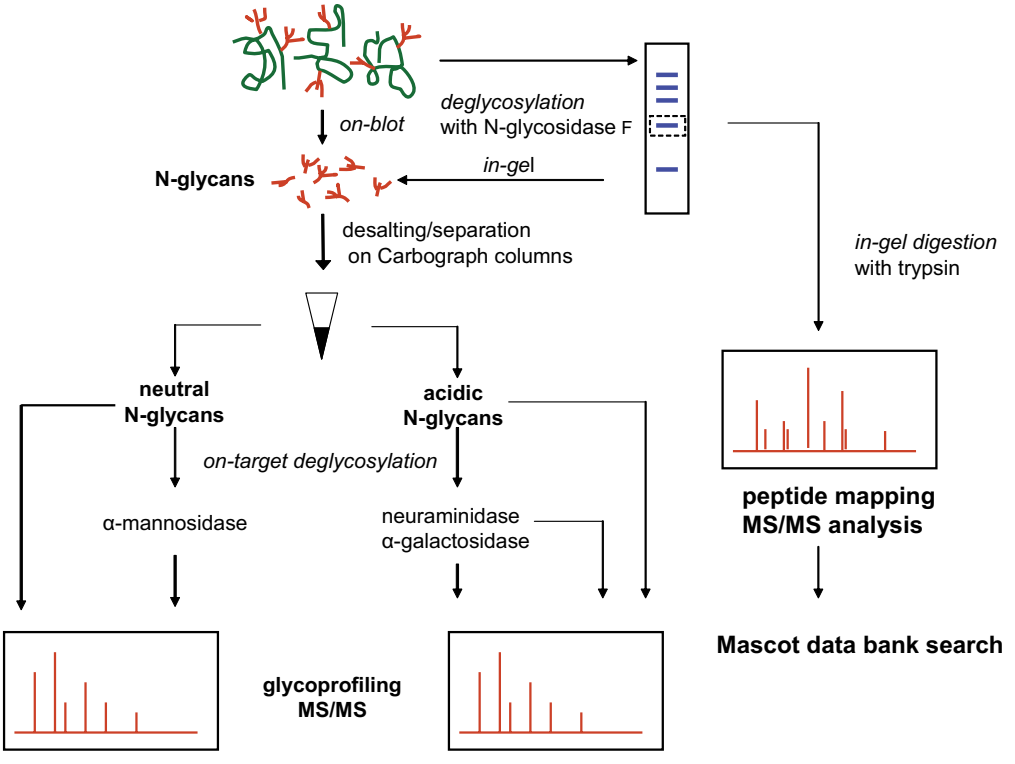


Fig. (3B). Strategy for the structural elucidation of oviductal N-glycans. *For on blot deglycosylation (Zhou* et al., *2004), aliquots of the OF and OAMF fractions were blotted onto PVDF membranes. Alternatively, protein bands were excised and prepared for in-gel digestion (Küster* et al., *1997). The proteins were sequentially reduced with (2.8 mM DTT for 30 min, 60 min), alkylated (6 mM iodacetamide, 30 min, 25°C) and deglycosylated with N-glycosidase F (15 U/ml overnight at 37°C). Glycans were released by extraction with water. The extracted glycans were desalted on carbograph mini column by eluting the column with water. Neutral and acidic glycans were separated by eluting the column with 25% acetonitril and then 25% acetonitril/0.05% TFA (Packer et al., 1998; Wheeler and Harvey, 2001) On-target deglycosylation was performed as described by Geyer et al., 1999. Glycan profiles were recorded with a Kompact MALDI-2 PE (V 5.2, Kratos Analytical, Manchester, England) in the positive mode (neutral glycans) and in the negative mode (acidic glycans). Additionally MS and MS/MS spectra of the glycans were recorded on an Ultraflex-MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany).*

idea that the main road of interactions involves the recognition of the flexible oviductal oligosaccharides by the entering sperm. By a histochemical approach using a set of lectins the distribution of carbohydrate moieties within the porcine oviduct has been studied demonstrating that cytoplasm, secretory granules, Golgi regions and the apical surface coat of the oviductal epithelium cells express a variety of glycan structures including those containing α-mannosyl residues (Walter and Bavdek, 1997) or oligomannosyl sequences (Ekhlasi-Hundrieser *et al.,* 2005). These findings prompted us to investigate the mannose-rich glycans and glycoproteins of the porcine oviduct.

To analyze the membrane-bound glycoproteins, the apical membrane fraction of the oviduct (OAMF) was generated. In order to estimate the occurrence of membrane-associated glycoproteins also the oviductal fluid (OF) was isolated (for methods, see Fig. 3A). The solubilized fractions were subjected to affinity chromatography with Galanthus nivalis agglutinin (GNA) recognizing specifically mannose residues in α1- 3 - and α1-6 - linkages (Shibuya *et al.,* 1988). The GNA-binding fractions of both the OF and OAMF were separated by 1-dimensional SDS-PAGE (Fig. 3, insets) under conditions allowing further analysis of the glycans as well as the proteins. Although both fractions, the OAMF and the OF contain numerous proteins over the whole mass range (Fig. 3A insets, lanes a and c), preferentially the high molecular mass glycoproteins between >200 – 80 kDa carry mannose-rich N-glycans and were retained by the GNA-column (Fig. 3A, insets, lanes b and d). The strategy, which was used to analyze the glycans and the glycoproteins in both fractions is outlined in Fig. 3B.

The glycans

The major neutral N-glycans of the GNA-binding proteins of the membrane fraction are high-mannose type glycans with 5-9 mannosyl residues, $Man_{5-9}GlcNAc_2$ (Fig. 4A). Neutral complex N-glycans are minor components. However, the signal intensities significantly increase after desialylation indicating their predominant existence in the sialylated forms. By tandem mass spectrometry (MALDI-MS/MS (CID)) of selected species and on-target deglycosylation the structures of the N-glycans were verified. The resulting data are summarized in Table 1. The fragmentation pattern of the oligomannose glycans in the collision induced dissociation (CID) experiments are characterized by the appearance of the typical series of B-ions, e.g. the series $Hex_{1-8}GlcNAc_1$ and Hex_{2-8} for the mother ion 1742.5, corresponding to $Hex_8GlcNAc_2$ (as evaluated by the GlycoPeakfinder tool). On target deglycosylation with α-mannosidase confirms their identity as belonging to the high mannose type N-glycans. The molecular masses are reduced by up to five α-mannosyl residues leading to the incompletely digested pentasaccharide core (Man_3GlcNA_2 and the hexasaccharide (Man_4GlcNA_2) (Fig. 4B). The biantennary complex N-glycans with two terminal hexose residues ($Hex_2HexNAc_2Fuc_1$+$Man_3GlcNAc_2$) and with one additional hexose residue ($Hex_3HexNAc_2Fuc_1$+$Man_3GlcNAc_2$) are the dominant species in the desialylated glycan pool. As minor components tri-and tetra-antennary glycans with and without additional hexose residues are observed. MS/MS analysis confirms the occurrence of terminal Hex-Hex linkages in selected spectra. Deglycosylation with α-galactosidase specifically hydrolyzing Gal(α1-3)-linkages, however, causes a shift of 162 Da and 324 Da of the respective signals (before/after treatment: m/z 1971.4, 2133.1/1810.2; 2336.1, 2497.3/2175,5; 2700.7, 2862.1/2540.4 $[M+Na]^+$) demonstrating the existence of com-

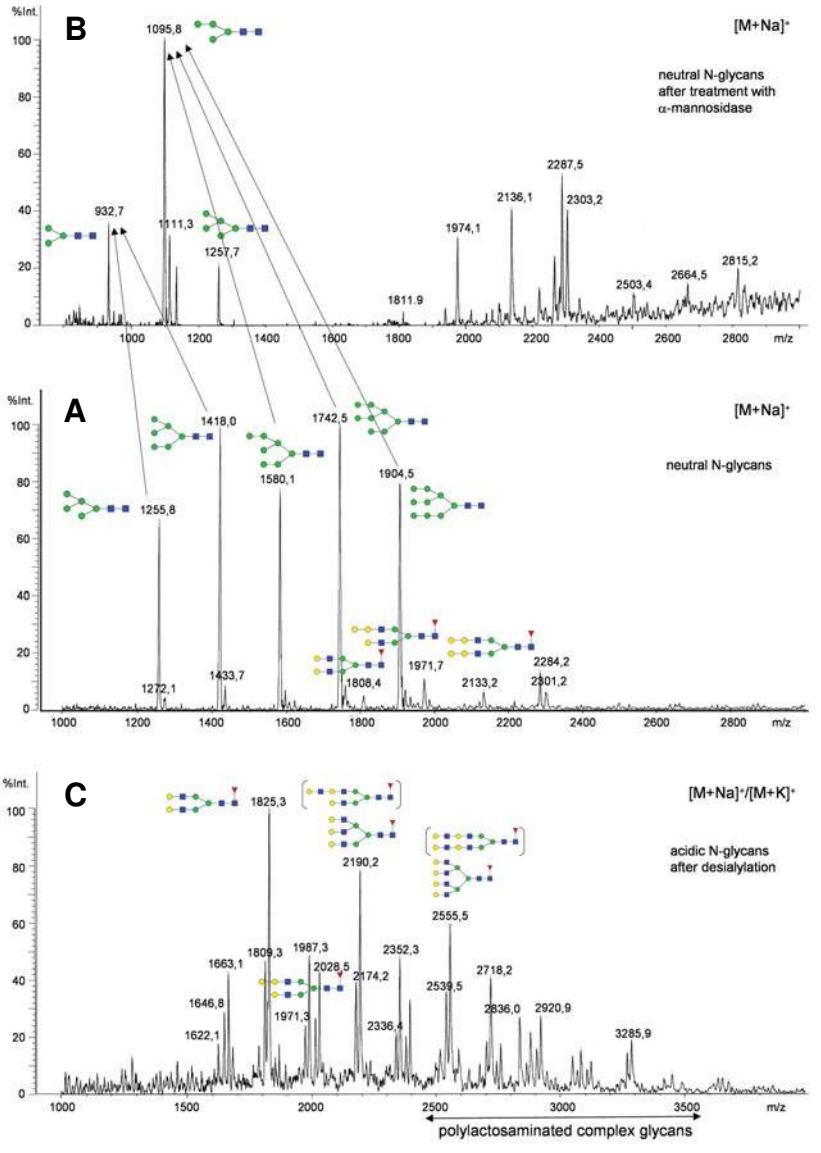


Fig. 4. Glycan profiles of the oviductal apical membrane. (A) *Neutral fraction showing predominantly high-mannose type glycans.* **(B)** *Glycan profile after on-target treatment with α-mannosidase.* **(C)** *Glycan profile of the acidic fraction after on-target desialylation demonstrating the occurrence of polylactosaminylated N-glycans. On-target deglycosylation was performed according to the protocol of Geyer* et al. *(1999). Glycan profiles were recorded with a Kompact MALDI-2 PE (V 5.2, Kratos Analytical, Manchester, England) in the positive mode.*

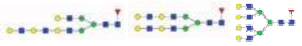
TABLE 1

N-GLYCANS OF THE OVIDUCT APICAL MEMBRANE FRACTION (OAMF)

NEUTRAL N-GLYCANS

m/z_{exp} $[M+Na]^+$	m/z_{calc} $[M+Na]^+$	SI nativ	SI α-mann	GLYCAN STRUCTURE	
932,7	933,3	-	++	$Man_3GlcNAc_2$	
1095,8	1095,4	-	+++++	$Hex_1+Man_3GlcNAc_2$	
1255,8	1257,4	++++	+	$Hex_2+Man_3GlcNAc_2$	
1418,0	1419,5	+++++	-	$Hex_3+Man_3GlcNAc_2$	
1580,1	1581,5	++++	-	$Hex_4+Man_3GlcNAc_2$	
1606,0	1606,6	(+)	-	$Hex_2HexNAc_1Fuc_1+Man_3GlcNAc_2$	
1620,8	1622,6	(+)	-	$Hex_3HexNAc_1+Man_3GlcNAc_2$	
1742,5	1743,6	+++++	-	$Hex_5+Man_3GlcNAc_2$	
1808,4	1809,6	(+)	(+)	$Hex_2HexNAc_2Fuc_1+Man_3GlcNAc_2$	
1904,5	1905,6	++++	-	$Hex_6+Man_3GlcNAc_2$	
1971,7	1971,7	+	++	$Hex_3HexNAc_2Fuc_1+Man_3GlcNAc_2$	
2133,2	2133,7	+	++	$Hex_4HexNAc_2Fuc_1+Man_3GlcNAc_2$	

ACIDIC N-GLYCANS AFTER DESIALYLATION

m/z_{exp} $[M+Na]^+$ ($[M+K]^+$)	m/z_{calc} $[M+Na]^+$ ($[M+K]^+$)	SI	GLYCAN STRUCTURE	
1646,8	1647,6	++	$Hex_1HexNac_2Fuc_1+Man_3GlcNac_2$	
(1663,1)	(1663,6)	++		
1809,3	1809,6	+++	$Hex_2HexNac_2Fuc_1+Man_3GlcNac_2$	
(1825,3)	(1825,6)	+++++		
1971,3	1971,7	++	$Hex_3HexNac_2Fuc_1+Man_3GlcNac_2$	
(1987,3)	(1987,7)	+++		
2012,3	2012,7	++	$Hex_2HexNac_3Fuc_1+Man_3GlcNac_2$	
(2028,5)	(2028,7)	+++		
2174,2	2174,8	+++	$Hex_3HexNac_3Fuc_1+Man_3GlcNac_2$	
(2190,2)	(2190,7)	++++		
2336,4	2336,8	+	$Hex_4HexNac_3Fuc_1+Man_3GlcNac_2$	
(2352,3)	(2352,8)	+++		
2498,2	2498,9	(+)	$Hex_5HexNac_3Fuc_1+Man_3GlcNac_2$	
(2515,2)	(2514,8)	+		
2539,5	2539,9	++	$Hex_4HexNac_4Fuc_1+Man_3GlcNac_2$	
(2555,5)	(2555,9)	+++		
2701,1	2701,9	+	$Hex_5HexNac_4Fuc_1+Man_3GlcNac_2$	
(2718,2)	(2717,9)	+++		
2742,0	2743,0	(+)	$Hex_4HexNac_5Fuc_1+Man_3GlcNac_2$	
(2758,0)	(2759,0)	+		
2862,9	2864,0	(+)	$Hex_6HexNac_4Fuc_1+Man_3GlcNac_2$	
(2879,1)	(2880,0)	+		
2905,0	2905,0	+	$Hex_5HexNac_5Fuc_1+Man_3GlcNac_2$	
(2920,9)	(2921,0)	++		
3067,7	3067,1	(+)	$Hex_6HexNac_5Fuc_1+Man_3GlcNac_2$	
(3083,5)	(3083,1)	+		
3268,6	3270,1	+	$Hex_6HexNac_6Fuc_1+Man_3GlcNac_2$	
(3285,9)	(3286,1)	+		

Neutral glycans without enzymatic treatment (nativ) and neutral glycans after treatment with α-mannosidase (α-mann). The relative appearance of the species is roughly estimated from the respective signal intensities (SI). The glycan structures were calculated with ExpasyGlycoMod-Tool (Cooper *et al.,* 2001), GlycoPeakfinder (EuroCarbDB, http://www.eurocarbdb.org) and GlycosciencesDB (http://www.glycosciences.de) and drawn with GlycoWorkbench (EuroCarbDB).

(SI) - signal intensity: (+) - 0-5%, + - 5-20%, ++ - 20-40%, +++ - 40-60%, ++++ - 60-80%, +++++ - 80-100%. Key symbols for constituent monosaccharides: ● - mannose, ■ - N-acetylglucosamin, ● - galactose, ▲ - fucose. The sodium (or potassium) adducts are estimated as monoisotopic masses.

plex N-glycans with one or two terminal α1-3-linked galactose residues in the oviductal membrane fraction (Fig. 4A). The dominant acidic species are biantennary complex N-glycans in their mono- and di-sialylated forms with N-acetylneuraminic and N-glycolylneuraminic acid. On-target desialylation resulted in the appearance of signals corresponding to polylactosaminylated structures e.g. $Hex_{5,6}HexNAc_{5,6}Fuc_1+Man_3GlcNAc_2$ indicating the occurrence of highly sialylated polylactosaminoglycans in the porcine oviduct (Fig. 4C and Table 1).

The membrane fraction of the oviductal epithelium appears to contain several glycoproteins expressing besides mostly acidic complex N-glycans also the oligomannosyl ligands, which can be recognized by the sperm.

The glycoproteins

MS-based proteomics were used to study the major GNA-binding proteins of the oviduct. N-terminal sequencing and sequence data bank searching of the CID spectra obtained from tryptic fragments after in gel digestion of the electrophoretically separated protein bands led to the identification of three proteins, which constitute the major GNA-binding proteins in the membrane fraction. The band at 150 kDa corresponds to the porcine homolog of the human oxygen-regulated protein (ORP150) and the band at 100 kDa is a mixture of the porcine lysosome-associated membrane protein 1 (LAMP-1) and the homolog to the human LAMP-2 (Table 2). Databank searches of peptide sequences obtained from the minor bands 200, 130, 80 and 50 kDa of OAMF (Fig. 3A, inset) do not lead to adequate protein identification. Interestingly, from the oviductal fluid about 0.2% of the total protein content was retained at the GNA-column. Under these proteins soluble forms of the ORP150, LAMP-1 and aminopeptidase N (APN) and small amounts of the oviduct specific glycoprotein (OGP or oviductin), were identified (Table 3). Aminopeptidase N (APN) is a multifunctional enzyme, which is expressed in various cell types. It exists in a membrane-bound as well as a soluble form exerting a variety of functions e.g. on cell proliferation and antigen presentation (reviewed by Luan and Xu, 2007). In the human oviduct APN was shown to participate in the regulation of the IL-8 system (Palter *et al.,* 2001). It has been

TABLE 2

***GALANTHUS NIVALIS* AGGLUTININ-BINDING GLYCOPROTEINS OF THE OVIDUCT APICAL MEMBRANE FRACTION**

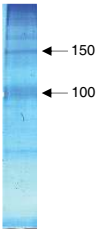
Swiss-Prot Acc. Nr.	Protein	MW, kDa	N-terminal sequence	8% SDS-PAGE
Q9Y4L1 H. sapiens	ORP150 (150kDa oxygen-regulated glycoprotein)	150	LAVMSVDLGSE 33-43	
Q5K4G2 S. scrofa	LAMP-1 (lysosome-associated membrane glycoprotein 1)	100	VFVVSDGNGTA 28-38	
Q99534 H. sapiens	LAMP-2 (lysosome-associated membrane glycoprotein 2)	100	LELNLTDSENAT 29-40	

TABLE 3

***GALANTHUS NIVALIS* AGGLUTININ-BINDING GLYCOPROTEINS OF THE OVIDUCTAL FLUID**

Swiss-Prot Acc. Nr.	Protein	MW, kDa	Identified peptide squences/position	Mascot-score	Function
Q6R6N0 S. scrofa	α-2-macroglobulin	240	KYSNPSTCFGGESQATCEK 65-83 XSGELNSQGCFSQSVETK 84-101 RQEFEMK 107-113 IKEEGTEVELT 120-132	80	inhibition of metaloproteases
Q6GTS2 M. musculus	angiotensin-converting enzyme, somatic isoform	180	ENYNQEWWSLR 1089-1099 GPQFGSEVELR 1300-1310	89	regulation of electrolytic homeostasis, proliferation and differentiation of the epithelial cells in the oviduct
Q29242 S. scrofa	aminopeptidase N	150	SDQIALPDFNAGAM 336-358 SALACSNEVMLLNR 827-840 YLGYTLNPDLIR 841-852 FSSEFELQQLEQFK 906-920	227	production of bioactive peptides, antigen presentation, control of the cell proliferation
Q9Y4L1 H. sapiens	ORP150 (150kDa oxygen-regulated glycoprotein)	150	FPEHELGFDPQR 120-131 DAVIYPILVEFTR 439-451	121	protein folding, protection from hypoxia-mediated apoptosis
Q28990 S. scrofa	oviduct-specific glycoprotein	110	DESIFYPEFNQLK 70-85 FTTMLSTFTNR 107-117 SSAYTMNYWR 238-247 LLMGFPTYGR 256-265	89	stimulation of sperm capacitation and motility, increase of penetration rates of the ZP, reduction of polyspermy, stimulation of early cleavages of the blastocyst
Q5K4G2 S. scrofa	LAMP-1 (lysosome-associated membrane glycoprotein 1)	100	TTFGGSCSAQLVTLELQGESLR 259-280 LQVTEAFALNVFQVR 340-354	68	cell interactions and cell adhesion, protective function
O02705 S. scrofa	heat shock protein HSP90-alpha	100	ELTSNSSDALDKIR 47-60 TLTIVDTGIGMTKADLINNLGTIAK 88-112 GVVDSEDLPLNISR 388-401	209	protein folding, regulation of some protein kinases and transcription factors, blocking of steroid receptors

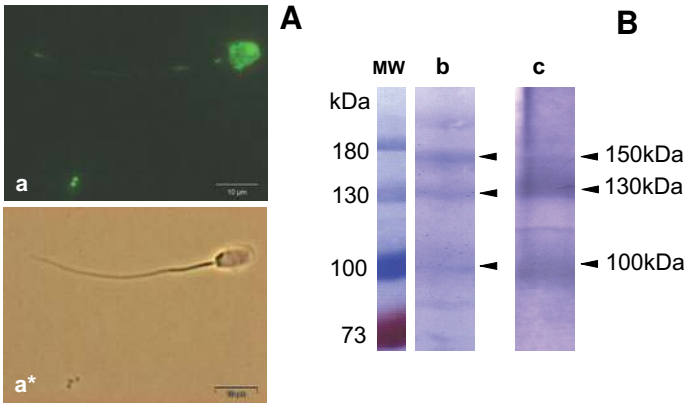


Fig. 5. Localization of oviduct apical membrane fraction (OAMF)-protein binding sites at sperm and identification of sperm-binding proteins. (A) *Detection of the binding of OAMF-proteins on the periacrosomal sperm surface by fluorescence microscopy. Fresh ejaculated sperm (3x 10^6 cells) were incubated with about 25 μg biotin-labelled OAMF-proteins (GNA-binding fraction) in Androhep-buffer for 10min at 37°C. After washing sperm smears were made and the bound OAMF-proteins were detected with streptavidin-FITC: (a) - green fluorescence, (a*) - bright field image of (a). Bar, 10 μm.* **(B)** *Detection of the binding of OAMF-proteins on the sperm surface by electrophoresis and blotting. After the incubation the sperm were detergent-extracted, subjected on 8% SDS-PAGE and transferred on PVDF-membrane. The bound biotinylated OAMF-proteins were visualized with streptavidin-alkaline phosphatase: (b) - native GNA-binding fraction of the OAMF (8% SDS-PAGE, Coomassie stain), (c) - biotin-labelled OAMF-proteins bound on the sperm surface. The proteins with MW 100kDa (LAMP-1/2) and 130kDa (not identified) accumulate predominantly at the sperm surface. The 150kDa-protein (homologue of human ORP150) was also detected.*

recently identified as a component of apical membranes of the porcine oviductal epithelium. The global profiling of oviductal surface membrane proteome by an MS-based approach has led to the identification of more than 270 proteins including APN and various members of the heat-shock protein family (Sostaric *et al.,* 2006). The major GNA-binding proteins of the OAMF, LAMP-1 and 2 as well as ORP150, however, are not listed. LAMP-1 and LAMP-2 constitute the main glycoprotein components of the lysosomal membranes (Fukuda *et al.,* 1991) and have also been detected at the extracellular surface of various cells (Chang *et al.,* 2004; Krishnan *et al.,* 2005) and as a soluble form of LAMP-1 (Chang *et al.,* 2004). LAMP-1 found in the oviductal fluid may therefore also present the soluble form, which has lost the membrane anchor and the small cytoplasmatic domain. ORP150 belongs to the family of molecular chaperones, which are expressed by cellular stress (Ikeda *et al.,* 1997). As the glucose-regulated proteins GRPs, the ORP-family may be involved in cell protection mechanisms by facilitating protein transport and maturation (Sidrauski *et al.,* 1998, Bando *et al.,* 2000). OGP is the major secretory protein in the mammalian oviduct, which has been shown to execute important functions during fertilization and development (for review, Buhi, 2002). OGP has a highly O-glycosylated mucin-like domain and contains three N-glycosylation sites in the porcine proteins, which are occupied preferentially by complex N-glycans (Tsolova M. and Töpfer-Petersen E., unpublished observations).The small amount found in the GNA-binding fraction may be therefore due to co-elution with other proteins.

The sequences of the GNA-binding proteins of the OAMF contain several putative N-glycosylation sites or have been already shown to be N-glycosylated. Human LAMP-1 and LAMP-2 are highly O- and N-glycosylated with up to 18 N-glycosylation sites partially occupied with acidic polylactosaminylated complex N-glycans (Carlsson and Fukuda, 1990). Also for the porcine LAMP-1 several N-glycosylation sites are predicted. Human ORP150 has nine N-glycosylation sequons, NXT/S, of which at least seven asparagine residues have been shown to be glycosylated (Zhang *et al.,* 2003). The glycan profiles of the electrophoretically separated bands after treatment with N-glycosidase F demonstrate the occurrence of high mannose N-glycans in each fraction. High mannose type glycans with 5-9 mannosyl residues are the dominant neutral species of the membrane-bound ORP150 and the LAMP-1/2 mixture. Minor signals are in agreement with hybrid and complex N-glycans with and without terminal α-1-3-linked galactose residues. The results obtained by the structural analysis of the glycan pool suggest that the bulk of complex glycans exists in their sialylated forms. The sialylated polylactosaminylated structures detected in the glycan pool may origin from LAMP-1/2 (Carlsson and Fukuda, 1990).

Candidate molecules involved in sperm-oviduct interactions

Current evidence implies that molecules associating to the sperm surface at ejaculation may promote sperm binding to the oviductal epithelial cells. In the pig and also in cattle, ejaculated sperm showed a significantly higher binding capacity to oviductal explants then epididymal sperm from the same animals (Petrunkina *et al.,* 2001a; Gwathmey *et al.,* 2003). Seminal plasma components do not enter the oviduct, unless they are tightly bound to the sperm surface and are transported with the sperm through the female reproductive tract. Therefore, the putative receptor molecules must be multifunctional inasmuch as they should express both sperm-binding and carbohydrate-binding abilities. The major seminal plasma proteins of pig and cattle, the porcine spermadhesins and the Fn-2 type proteins belonging to the bovine BSP-family, respectively, fulfill these conditions. The bovine oviduct-binding sperm protein has been identified as PDC-109, which is characterized by its ability to interact with phosphorylcholine-containing lipids of the outer leaflet of membranes (Müller *et al.,* 1998; Müller *et al.,* 2002, Manjunath *et al.,* 2002). The spermadhesins, AWN, AQN-3, and AQN-1 tightly attach to the sperm surface presumably via a direct or indirect phospholipid-binding mechanism (Dostàlovà *et al.,* 1995b, Ensslin *et al.,* 1995). Thus, porcine spermadhesins (Ekhlasi-Hundrieser *et al.,* 2005) and bovine Fn-2 type proteins as shown for PDC-109 (Gwathmey *et al.,* 2003) possess both sperm- and carbohydrate-binding abilities and may represent functional equivalents.

Some properties argue for AQN-1 as the most likely spermadhesin contributing to sperm-oviduct binding. In contrast to AWN and AQN-3, AQN-1 is distinguished by its ability to recognize a broad spectrum of carbohydrates including terminal galactose and oligomannose structures. The observation that AQN-1 but not AWN inhibits *in vitro* binding to oviductal explants in a concentration-dependent manner (IC_{50} ~ 4.5 μM) is in line with the prospective role of AQN-1 as the carbohydrate receptor for the oviductal glycoconjugates. During the early phase of *in vitro* capacitation AQN-1 is released from the surface and this loss is accompanied by a reduction in mannose-binding sites on viable sperm and a significant decrease in the ability of sperm to

bind to the oviductal explants (Ekhlasi-Hundrieser *et al.,* 2005). The percentage of acrosome reaction have been found to be low (<2%) indicating that indeed coating substances and AQN-1 are lost from the surface, thereby facilitating the release of sperm from the epithelium. Under the same conditions only small, but stable amounts of sperm-bound AWN are detected (Ekhlasi-Hundrieser *et al.,* 2005), pointing to different functions of distinct spermadhesins in the fertilization process. The role of AQN-1 as the sperm-associated receptor for oviductal glycoconjugates, thereby contributing to the formation of the oviductal sperm reservoir, is strongly supported by the finding that AQN-1 binds to the oviductal epithelium by way of a mannan-sensitive mechanism (Liberda *et al.,* 2006).

Mannose-binding sites (Wagner *et al.,* 2002) and AQN-1 molecules (Jonakova *et al.,* 1998) are localized over the acosomal region of the sperm. GNA-binding proteins of the oviductal membrane bind to the same region of the sperm surface when tested by fluorescence microscopy after treatment of a sperm suspension with the biotinylated protein fraction (Fig. 5). About 40-50% of sperm in a population containing 60% motile sperm are found to express a bright fluorescence over the acrosomes. Cells, which have lost their acrosomes, are never stained. The bound glycoproteins were identified by SDS-PAGE, probing the protein blot of the treated sperm population with the biotin/avidin system. Enrichment of bands corresponding to LAMP-1/2 and the unidentified 130 kDa band is observed. As a minor component also ORP150 appears to bind to the sperm surface.

LAMP-1 and LAMP-2 have been localized to some extent at the plasma membrane of various cells (Fukuda, 1991). Cell surface expression of LAMPs has been linked to platelet activation (McKenzie *et al.,* 2003), the adhesion of peripheral blood mononuclear cells to the vascular endothelium (Kannan *et al.,* 1996) and the adhesive processes of tumor invasion and metastasis (Sarafian *et al.,* 1998; Ochwat *et al.,* 2004). The precondition for oviductal glycoproteins, e.g. LAMP-1/2, the unidentified 130 kDa protein and others in the pig to participate in sperm-oviduct binding is the presentation of the carbohydrate ligands at the right position to be recognized by the entering sperm. The large extracellular domain, highly decorated with N-glycans, predestinates the LAMPs to play a role in those adhesion processes.

The LAMPs appearing at the mucosal surface of the oviductal epithelium and the still unidentified 130 kDa protein may trap the entering sperm by exposing their high-mannose glycans ($Man_{5\text{-}9}GlcNAc_2$) that are recognized by the sperm mannose-binding proteins as AQN-1. As at an early stage of capacitation these proteins begin to detach from the sperm surface, the sperm loses its contact to the epithelium and is able to swim freely to the site of fertilization.

Recently, in the bovine system another group of proteins, the annexins, has been identified as the candidate oviductal receptors interacting with the short Fn-2 proteins associated with the sperm (Ignotz *et al.,* 2007), pointing to a species-specific mechanism. However, although the key sugar signals and the complementary receptor molecules of sperm and oviduct vary among species, sperm storage and activation successfully work in heterologous systems (Petrunkina *et al.,* 2003a, 2004). This may imply that the flexible oligosaccharide chains presented by the oviduct rather function as a "catcher" of the entering sperm and locate the sperm head in the vicinity of mucosal surface than being actively involved in the subsequently following down stream events.

Gamete interaction in the pig

The fundamental mechanism of gamete recognition appears to be conserved throughout evolution from marine vertebrates to eutherian mammals in that the sperm interaction with the egg involves receptors associated with the sperm surface that recognize oligosaccharide ligands of the envelope glycoproteins.

The vertebrate egg coat - the vitelline envelope (VE) and the mammalian zona pellucida (ZP) - are composed of three to eight related glycoproteins, which assemble into a three-dimensional matrix surrounding the egg. The ZP-glycoproteins named for the mammalian zona pellucida are encoded by about seven ZP-gene families, which appear to origin from an ancestral gene by a gene duplication event occurring early during vertebrate evolution (this topic is reviewed by Spargo *et al.,* 2003; Hughes, 2007). They all share a common ZP domain spanning about 260 amino acids (Bork and Sander, 1992), which has been shown to play a general role in the polymerization of the ZP-glycoproteins into filaments forming the protective egg coat (reviewed by Litscher and Wassarman, 2007, and references therein). In mammals, beside

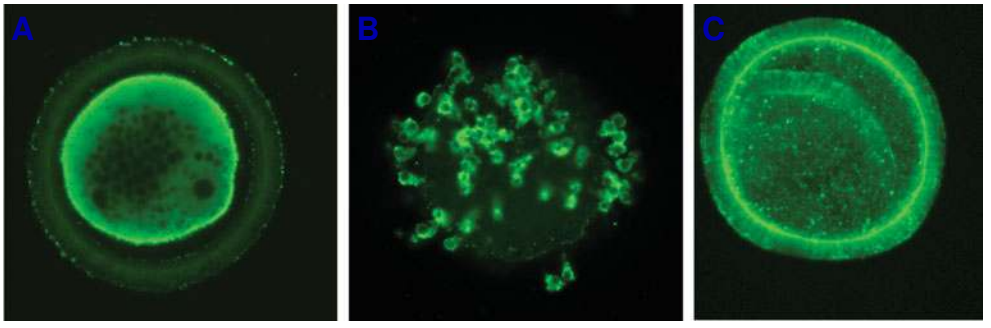


Fig. 6. Laser scanning confocal microscopy of immature oocytes of prepuberal pigs. *Oocytes were permeabilized during fixation in 3.7 % formaledehyde in phosphate- buffered saline, pH 7.2/ 1% polyvinylpyrrolidone-40 with 2% Triton X-100 for 15 min at room temperature (RT). The specimens were than sequentially incubated with ZPA-20, directed against the internal peptide sequence of ZPA, DPNIKLVLDDCWAT in rabbit (the antibody was a gift of Dr. Elvira Hinsch, University of Giessen, Germany) for 60 min at RT and with anti-rabbit IgG- FITC (150ng/ml) for further 60 min at RT (Hettel C., Rath D., Töpfer-Petersen E., unpublished data). Positive staining was observed in about 80% of immature oocytes and within the zona pellucida* **(A)** *and in the surrounding follicle cells* **(B)**. *Isolated zonae pellucidae stained for free thiol groups* **(C)**. *The ZPs were incubated with 0.02 mM 5-iodoacetamidefluorescein (5-IAF) in 50 mM Bis-TRIS pH 6.5 for 30 min at RT. The specificity of the reaction was controlled by blocking the thiol groups with iodacetamide before incubation with 5-IAF (Moreira AC and Töpfer-Petersen E; unpublished data). Free thiol groups are distributed through the zona pelluicda matrix. The specimens were monitored by a LSCM (microsocope model LSM510, Carl Zeiss GmbH, Jena, Germany) equipped with an Argon laser.*

its protective role the ZP serves important functions during fertilization by modulating sperm function and participating in mechanisms preventing polyspermic fertilization.

The zona pellucida glycoproteins

In the pig as in related domestic animals, the zona pellucida is built up by three glycoproteins, which are the products of the ZP-gene subfamilies ZPA, ZPB and ZPC (Harris *et al.,* 1994). Exemplary for the glycoproteins ZPB and ZPC their biosynthesis was traced by immunhistochemical and *in situ* hybridization studies indicating the contribution of both, the oocyte and the surrounding follicle cells in the assembling of the matrix. During the early phase of follicle development (primordial and primary follicles) the proteins are produced by the developing oocyte. During later stages the contribution of the follicles cells increases, whereas the synthesis rate in the oocyte continuously decreases (reviewed by Sinowatz *et al.,* 2001). Immature oocytes collected from tertiary follicles (3-5mm) are commonly used after *in vitro* maturation for IVF experiments in the pig. In these oocytes the secretion rate of ZPA appears to be delayed. Confocal fluorescence microscopy of immature oocytes demonstrates the compelling occurrence of ZPA within the oocyte cytoplasm (Fig. 6A), whereas ZPC and ZPB are rarely detected in the oocyte, but are recovered throughout the zona pellucida matrix. The surrounding follicle cells produce all three ZP-glycoproteins (Fig. 6B). These observations may suggest that the spatio-temporal expression/secretion of the three glycoproteins in the pig is differently regulated during folliculogenesis.

ZPA proteins participate in late fertilization events leading to zona hardening and the prevention of polyspermic fertilization. The mature ZPA is the largest glycoprotein of the ZP and is N-terminally elongated from the ZP-domain by a 330-amino acid polypeptide. Processing at the conserved proteolytic clip at A_{168} and D_{169} contributes or even induces the changes of ZP structure resulting in zona hardening. For the bovine, Iwamoto and coworkers (1999) have shown that fertilization is accompanied by the formation of intra-/intermolecular disulfide bonds. This also applies for the pig. Laser scanning confocal microscopy following fluorescence labeling of the free thiol groups demonstrated the occurrence of cysteine residues not engaged in disulfide bridges throughout the intact zona pellucida of immature and mature oocytes (Fig. 6C). During *in vitro* fertilization the fluorescence intensities continuously decreases until equal-zero. The decrease in the number of free thiol groups can be interpreted as meaning that novel disulfide bonds were formed (Moreira A.C., Rath D., and Töpfer-Petersen E., unpublished data) probably stabilizing the altered protein conformation, thus contributing to the hardening of the zona pellucida.

ZPB and ZPC glycoproteins undergo post-translational processing, which leads to their mature forms of 330 and 326 amino acids, respectively. ZPB contains an additional trefoil-domain next to the ZP-module, which may increase the resistance to proteolytic degradation (Bork, 1993). The ZPB glycoprotein has been identified as the sperm receptor inasmuch as it carries the biologically active carbohydrate chains (Kudo *et al.,* 1998). However, maximal sperm binding is achieved until ZPB forms heteromultimeric complexes with ZPC (Yurewicz *et al.,* 1998), which may induce conformational changes of the ZPB molecules to maximally expose the sperm binding sites. This finding also highlights the relevance of a critical density of the carbohydrate ligands presented within the supramolecular architecture to achieve high-affinity binding between sperm and egg.

The glycans

Porcine ZP glycoproteins are highly glycosylated with neutral and acidic N- and O-linked oligosaccharides, both carrying tandemly arranged N-acetyllactosamine repeats. The enormous heterogeneity is due to varying degrees of sialylation and sulfation. The sulfate group is only linked to the C-6 position of GlcNAc residues (SO_3-6GlcNAc) of the N-acetyllactosamine repeating units, but has also been detected at the non-repeated antennae of N-glycans (Noguchi and Nakano, 1992; Noguchi *et al.,* 1992; Mori *et al.,* 1998). The major O-linked chains are shown to be core-1 type (Galβ1-3GalNAc) and as minor components also core-3 type glycans (GlcNAcβ1-3GalNAc) have been identified (Hokke *et al.,* 1994). N-linked glycans belong preferentially to the complex type containing bi, tri and tetra

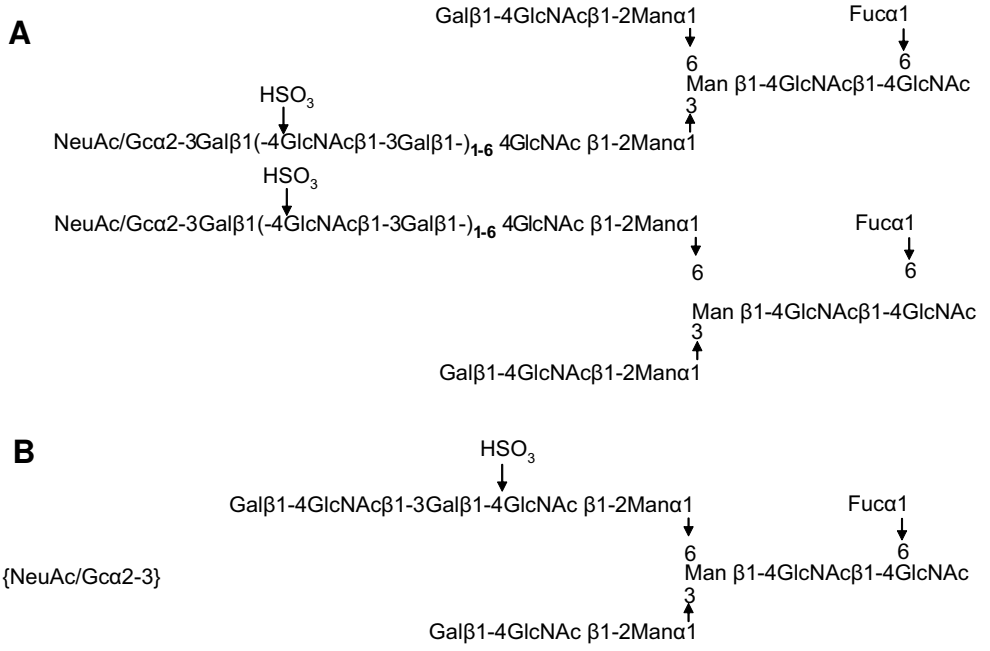


Fig. 7. Structures of the dominant sulfated N-glycans of the porcine zona pellucida. (A) *The dominant bi-antennary acidic N-glycans of the isolated PZP3 family (ZPB/ZPC) according to Noguchi and Nakano (1992).* **(B)** *The major acidic N-glycan of the ZP glycoproteins of prepuberal pigs. To a minor extent this structure also exists in the sialylated form.*

antennae, which are all α1,6-fucosylated at the innermost N-acetylglucosamine residue (Noguchi and Nakano, 1992; Noguchi *et al.,* 1992, von Witzendorff *et al.,* 2005). N-glycans released from the ZP-glycoprotein mixture has been shown to be composed of neutral and acidic structures in a proximate molar ration of 1:2. The major neutral structure has been reported to belong to the bi-antennary complex type N-glycans with a fucosylated trimannosyl core (reviewed by Takasaki *et al.,* 1999). Analysis of the acidic fragments released by endo-β-galactosidase digestion of the ZPB/ZPC complex has shown that the bulk of acidic N-glycans are elongated by up to six sulfated N-acetyllactosamine repeats carrying N-acetyl- or N-glycolylneuraminic acid at the non-reducing ends (Fig. 7A; Noguchi and Nakano, 1992). Additionally, sulfation at the non-repeated antennae at C-6 position and to a minor extent at the C-3 position of the innermost N-acetylglucosamine residues has been described (Mori *et al.,* 1998).

Since in the pig prepuberal animals are generally the source of the oocytes used for *in vitro* fertilization techniques, the ZP N-glycans of immature oocytes collected from the ovaries of prepuberal gilts (80-120 kg) were analyzed by a mass spectrometrical approach. On-blot deglycosylation of total ZP, glycoproteins with N-glycosidase F allowed the structural characterization of the neutral and acidic N-glycans with MALDI-ToF mass spectrometry. Interestingly, the dominant neutral species were shown to be a high-mannose type glycan with five mannosyl residues and the core-fucosylated bi-antennary N-glycans with one and two terminal galactose residues (Fig. 8A). The glycan profiles of the electrophoretically isolated ZPA and ZPB/ZPC fraction showed that the high-mannose type glycan is exclusively linked to ZPA (Fig. 8 B,C). The glycan profile of the acidic glycan fraction and CID experiments of selected signals demonstrated the considerable occurrence of N-glycans with a single sulfate group located at the C-6 position of the non-repeated GlcNAc residues (Fig. 7B). There was no evidence of sulfation of the repeated N-acetylglucosamine or the innermost residues. The differences between the formerly reported structures (Noguchi *et al.,* 1992; Noguchi and Nakano, 1992; Takasaki *et al.,* 1999) and the structures obtained from strongly selected prepuberal animals (Töpfer-Petersen *et al.,* in preparation) may implicate that the glycans seem to vary dependent on the developmental stage of the oocytes, on the age of the pigs at death and possibly on race and pig diet.

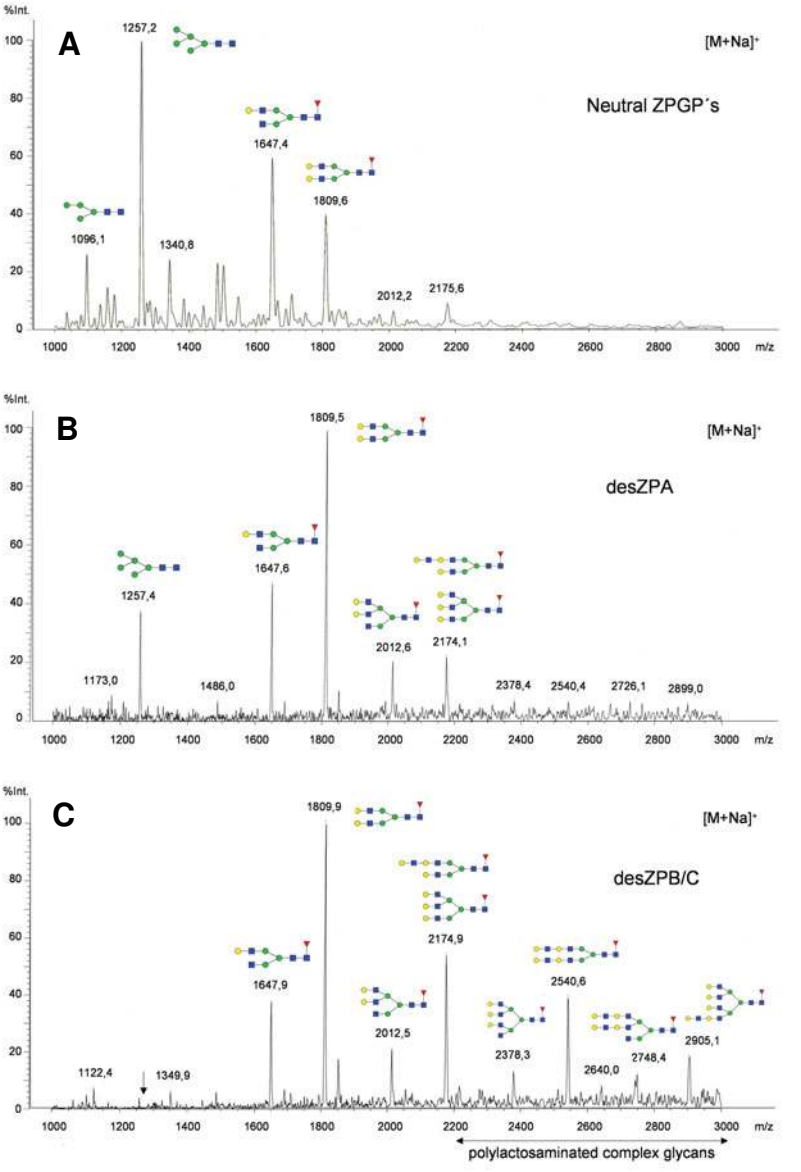


Fig. 8. Glycan profiles of the zona pellucida of prepuberal pigs. (A) *Neutral glycan fraction showing the predominant occurrence of a pentamannosyl glycans. Neutral glycan profiles after enzymatic desialylation and electrophoretic separation of ZPA* **(B)** *and ZPB/ZPC* **(C)**. *The glycans were released by in gel- digestion with N-glycosidase F. Polylactosaminylated N-glycans are identified by treatment with endo-β-galactosidase (von Witzendorff* et al., *2005). Glycan profiles were recorded with a Kompact MALDI-2 PE (V 5.2, Kratos Analytical, Manchester, England) in the positive mode.*

The ZP glycoproteins carry different numbers of potential N-glycosylation sites (Fig. 9). The three sequons within each, the ZPB and ZPC sequences predicting N-glycosylation, are all occupied and are located within the ZP-domain (ZPB: N_{202}, N_{220}, N_{333}; ZPC: N_{124}, N_{146}, N_{271}), thereby only the asparagine residues N_{220} in ZPB and N_{271} in ZPC carry tri-, and tetra-antennary complex N-glycans (Kudo *et al.,* 1998; Yonezawa *et al.,* 1999). Five of six glycosylation sites predicted for ZPA are glycosylated. Cleavage of ZPA at the proteolytic clip (N_{168}/D_{169}) divided the molecule into the N-terminal peptide of 25 kDa and the 65 kDa component bridged by disulfide bonds. The potential glycosylation sites at N_{84} and N_{94} are located in the N-terminal 25 kDa peptide, thereby only N_{84} has been found to be glycosylated, carrying predominantly tri-and tetra-antennary glycans. The other glycosylated sites N_{268}, N_{316} and N_{323} are located in the 65 kDa peptide N-terminally to the ZP-domain and N_{530} is located in the ZP-domain occupied with bi-antennary chains. ZPA possesses, beside the set of complex chains at

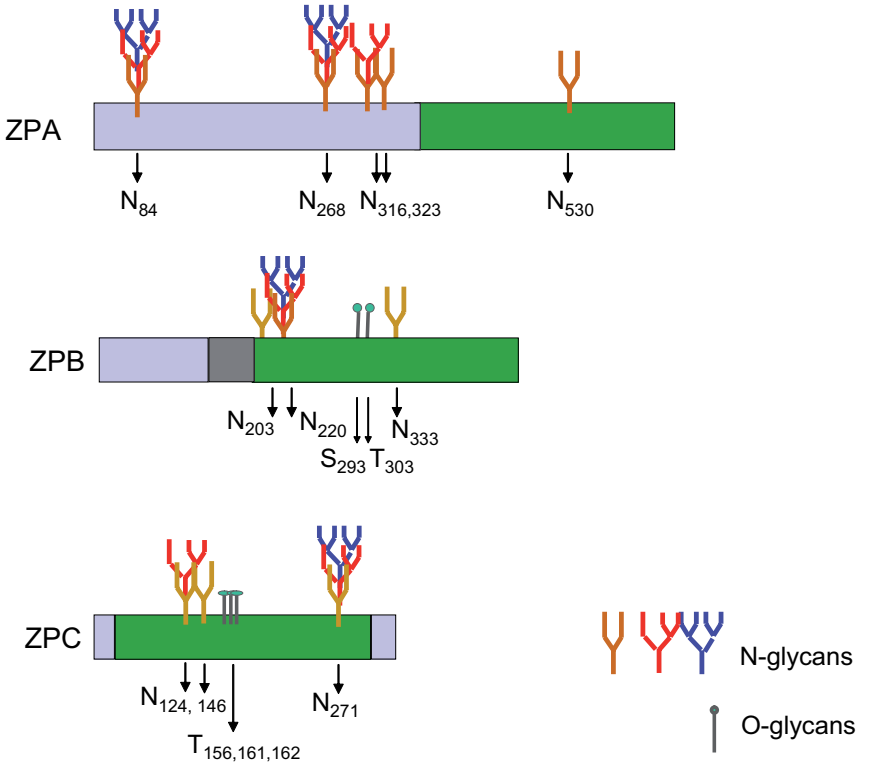


Fig. 9. The glycosylation sites of the porcine zona pellucida (ZP) glycoproteins. *The glycosylation sites are taken for ZPA from von Witzendorff* et al. *(2005); for ZPB from Kudo* et al. *(1998) and for ZPC from Yonezawa* et al. *(1999). The high mannose type N-glycan is linked to* N_{268} *of ZPA of prepuberal animals.*

N_{268}, also the pentamannosyl glycans, Man_5GlcNA_2 (von Witzendorff *et al.,* 2005).

Receptor-ligand systems involved in primary binding

The sperm-binding activity has been mapped to the neutral tri- and tetra-antennary complex N-glycans of ZPB expressing non-reducing terminal β-galactosyl residues (Kudo *et al.,* 1998; Yonezawa *et al.,* 2005). The request for polyvalency to achieve stable binding may be the reason that tri-and tetra-antennary glycans are shown to possess higher binding activity than the bi-antennary chains. Properly arranged at the surface of the zona pellucida, these carbohydrate ligands are then recognized by the surface-associated carbohydrate-binding proteins of the fertilizing sperm. Fractionation by affinity chromatography of the solubilized plasma membrane proteins on immobilized ZP glycoproteins, identified the spermadhesins AWN and AQN-3 as well as P47 (lactadherin, SED-1) and the short Fn-2 type protein pB1 (also pAIF) as the major zona pellucida-binding proteins (Ensslin *et al.,* 1995), indicating the contribution of multiple sperm proteins in the recognition and binding events. Very recently these results have been corroborated by using a similar approach identifying AQN-3 as the main ZP-binding protein, but also P47 (lactadherin, SED1) beside two other persistently bound protein (fertilin β and peroxiredoxin 5; van Gestel *et al.,* 2007). Among these proteins only AWN and AQN-3 have known carbohydrate-binding properties being both able to recognize the terminal non-reducing β-galactosyl residues in the exposed tri- and tetra-antennary chains of the zona pellucida. Small amounts of the sperm-binding spermadhesins - as demonstrated for AWN - were found on capacitated sperm, which are able to bind to and to penetrate the oocyte zona pellucida *in vitro* (Dostàlovà *et al.,* 1995a) and *in vivo* indicating that sperm-bound AWN molecules survive sperm passage through the female tract after mating (Rodriguez-Martinez *et al.,* 1998). Taken together, AWN and AQN-3 fulfill the requirement to act as primary zona pellucida binding molecules. They are the main zona pellucida–binding proteins, they are located at the right position to bind to the oocyte zona pellucida (the plasma membrane overlying the acrosome) and they are actually present on the fertilizing sperm that meet the ovulated oocyte at the time of fertilization *in vivo* (although the latter remains to be established for AQN-3).

P47/lactadherin is another attractive candidate, which may be involved in the sperm-egg binding events in the pig. Following capacitation it is exposed at the sperm surface overlying the acrosome, namely by unmasking (Petrunkina *et al.,* 2003b) and has been shown to be also present at sperm that bind to the oocyte zona pellucida *in vitro*, (Ensslin *et al.,* 1998). Its role in mammalian gamete interaction has been demonstrated in the mouse model (reviewed by Shur *et al.,* 2004). In mice, SED1 effectively inhibits sperm-zona binding. Furthermore, sperm null for SED1 are infertile and their sperm fail to bind to the zona pellucida *in vitro* pointing to an essential role of SED1 in sperm-egg adhesion. The binding abilities, both to interact with the lipid layer of the sperm membrane and with the zona pellucida have been attributed to the discoidin F6/8 domains (Shur *et al.,* 2004). However, the nature of the interaction of porcine P47 or murine SED1 with the zona pellucida (carbohydrate-protein or protein-protein interactions) remains to be proven. The contribution of P47/SED1 in sperm-zona binding in mouse and pig may implicate a general role in mammalian sperm-egg adhesion events.

These data support the current concept of a multimeric receptor involved in primary binding, thereby the contributing proteins may act sequentially or synergistically.

Species-specificity of gamete-recognition

The receptor-ligand systems mediating gamete recognition within vertebrates vary among species. Complex N-glycans with terminal fucose and GlcNAc residues have been shown to be essential in sperm binding to the egg envelope in *Xenopus laevis* (Vo and Hedrick, 2000). Further, O-linked oligosaccharides located at defined regions of the ZP-glycoproteins play a role in the mouse (reviewed by Wassarman *et al.,* 2005), and mannose-binding has been proposed to determine human oocyte recognition (Rosano *et al.,* 2007). The variety of sperm proteins interacting with the egg envelopes, the diversity of the biologically active oligosaccharides and ZP-glycoproteins among species may implicate that the barrier to interspecies fertilization is controlled at the level of the zona pellucida (Wassarman, 2005). However, the

concept of strict species-specificity of the sperm-zona pellucida recognition does not hold for domestic animals. Gamete interaction in domestic animals has been best studied in pig and cattle. The polyvalent presentation of terminal α-mannosyl residues in high-mannose N-glycans has been found to determine bovine sperm-egg binding (Amari *et al.,* 2001), whereas sperm recognition in the pig is mediated by terminal β-galactosyl residues in complex N-glycans (Yonezawa *et al.,* 2005). In both species, the biologically active carbohydrates are linked to the ZPB glycoprotein. The heteromultimeric complex of recombinant porcine ZP-glycoproteins expressed in baculovirus-Sf9 insect cells carrying pauci- and high-mannose type glycans binds bovine but not porcine sperm (Yonezawa *et al.,* 2005), indicating that in fact the binding event is dependent on the expression of specific carbohydrates. Despite the use of differing receptor-ligand systems, sperm are able to interact with the heterologous zona pellucida *in vitro*. Electron microscopic studies demonstrated that under *in vitro* conditions porcine and equine sperm firmly bind to bovine zona pellucida and undergo the acrosome reaction. Equine sperm are even able to penetrate the zona pellucida and to enter the bovine oocyte (Sinowatz *et al.,* 2003). The expression of structurally similar glycans, the eventually broad carbohydrate specificity and multiplicity of the zona pellucida binding proteins, may account for heterologous recognition and binding *in vitro*. The barrier to interspecies fertilization in distantly related species with internal fertilization may be the result of other mechanisms (e.g. behaviour, anatomy, sperm survival in the female tract etc) or may be controlled by post-fertilization events.

Pro/acrosin and secondary binding

When the fertilizing sperm has bound to the oocyte zona pellucida, the signal cascade leading to the exocytosis of the sperm acrosome is initiated (reviewed by Jungnickel *et al.,* 2001), and the acrosome-reacted sperm begins to penetrate the zona pellucida by using a combination of forces produced by the hyperactivated sperm and enzymatic digestion (Green, 1987). In 1986 O´Rand and co-workers proposed a still actual model of the penetration process, in which alternating cycles of binding of the acrosome-reacted sperm to the zona pellucida (secondary binding), limited proteolysis of the matrix and release of the sperm from the zona pellucida together with the sperm forward motility is required to achieve penetration (O´Rand *et al.,* 1986). Pro/acrosin may be instrumental in all three stages: the binding, digestion and release reactions. Thereby, the zona pellucida seems to control or finely tune these processes via the sulfate-recognition mechanisms.

The biological role of pro/acrosin is still controversially discussed. The scepticism came from the observation that mice sperm null for proacrosin were able to penetrate the zona pellucida and to fertilize the egg (reviewed by Honda *et al.,* 2002). However, Acr- sperm showed delayed fertilization and the fertilization rate is affected by the thickness of the zona pellucida (Adham *et al.,* 1997; Nayernia *et al.,* 2002). Thus, sperm lacking acrosin have selective disadvantage in competition with wild type sperm. As it is found for the primary binding events, secondary binding to and penetration through the zona may also involve multiple factors, enzymes and binding proteins, which may act synergistically to achieve maximal fertilization. In pig a 38-kDa zona pellucida binding protein, sp38 has been identified, which interacts with the zona pellucida presumably by a comparable sulfate-binding mechanism (Mori *et al.,* 1995). In mice at least two other serine proteinases have been detected (Honda *et al.,* 2002), which are able to compensate at least partly for the function of acrosin. In domestic animals such as the pig, having oocytes surrounded by a thick zona pellucida (16-20μm), acrosin seems to essentially contribute to the secondary binding interaction and sperm penetration through the zona pellucida. Old and new data support the role of acrosin in mammalian fertilization and give insight into the underlying mechanisms, although species-specific differences should not be ignored.

Does the zona pellucida undergo a maturation process?

Porcine *in vitro* fertilization (IVF) techniques have been improved in recent years however the selective competence of *in vitro* matured oocyte is still low and can result in polyspermic fertilization (Niemann and Rath, 2001).

Recent scanning electron microscopical studies have shown that the ZP of the immature oocyte (generally oocytes collected from 3-5 mm follicles of prepuberal animals of 80-120 kg were used for *in vitro* maturation protocols; Niemann and Rath, 2001) displays a spongy appearance with numerous, regularly arranged small pores, which might be resulted by the cytoplasmatic filaments from the corona radiata cells penetrating the zona pellucida during oogenesis. In contrast, the majority of the zonae pellucidae of *in vitro* and *in vivo* matured oocytes showed a smoother surface (Rath *et al.,* 2005, 2006; Michelmann *et al.,* 2007). The degree of oocyte maturation does not only influence the morphology but also the integrity and functionality of the ZP. A flow cytometric approach using double staining with fluorescein isothiocyanate-conjugated Peanut agglutinin and propidium iodide was developed to evaluate the acrosome reaction of the viable sperm population (for methodical details see Petrunkina *et al.,* 2005). The ZP of *in vitro* matured oocytes was shown to induce the acrosome reaction of sperm at two-fold higher rates as compared to the ZP of immature oocytes when ejaculated precapacitated sperm ($0.5\text{-}1x10^6$ cells/ml in Tyrode´s medium) were co-cultured with solubilized ZP glycoproteins (10 ZP equivalents/ml) evaluating the viable sperm population over a time period of 60 min. A similar result was obtained when the kinetic of acrosome reaction of sperm that bind to the intact zonae pellucidae, was determined using the PNA-FITC/PI double staining in combination with laser scanning confocal microscopy (Rath *et al.,* 2006).

These data suggest that not only the nucleus and cytoplasm of the oocyte require adequate maturation to become fully competent, but also the zona pellucida has to undergo a maturation process. This suggestion is underlined by additional although preliminary observations. As stated before immature oocytes of prepuberal animals showed a retarded secretion of ZPA from the oocyte (Fig. 6A). After *in vitro* maturation still about 50% of the oocytes showed a positive ZPA signal in the cytoplasm, but the number of positively stained oocytes is equal-zero when *in vivo* matured oocytes (collected from follicles after hormonal stimulation, Niemann and Rath, 2001) were studied (Hettel C., Rath D., Töpfer-Petersen E., unpublished data). The incomplete secretion of ZPA under *in vitro* maturation conditions may influence the three-dimensional architecture of the zona pellucida and may be

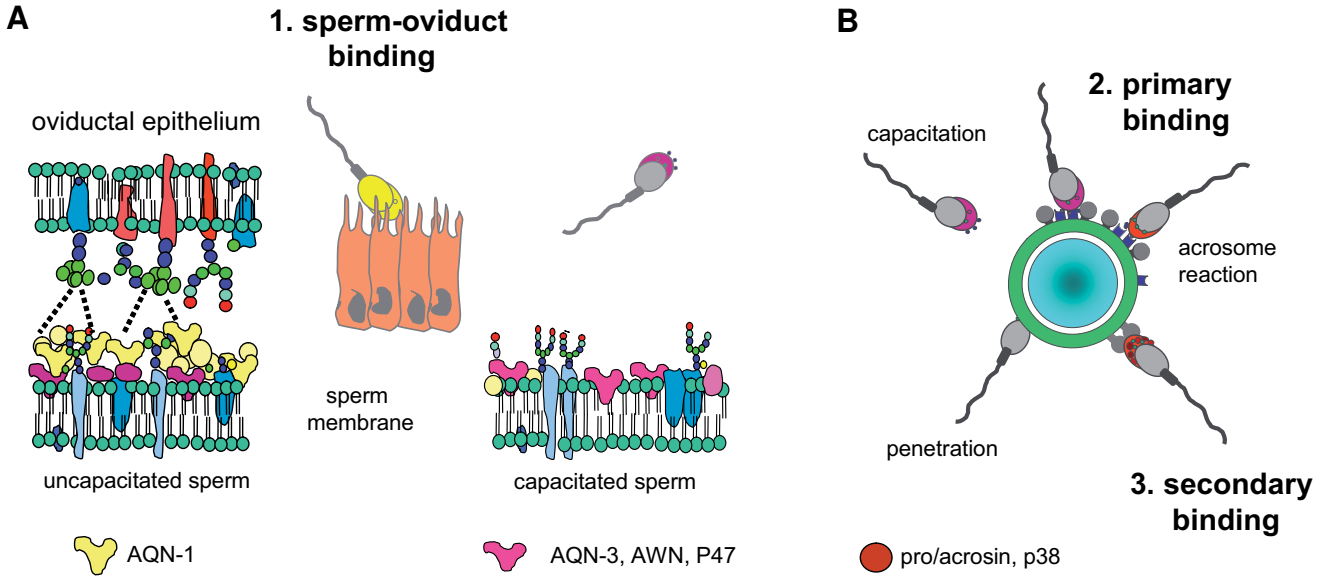


Fig. 10. Carbohydrate-mediated events during fertilization in the pig. (A) *Sperm-associated carbohydrate-binding proteins such as AQN-1 bind to the exposed high-mannose type N-glycans of oviductal membrane glycoproteins such as LAMP-1/2 and others (1. sperm-oviduct binding). When capacitation is initiated the coating proteins dissociate from the surface exposing proteins of the multimeric receptor (AWN, AQN-3, P47 and others) in the capacitated sperm.* **(B)** *The capacitated sperm binds to the zona pellucida by recognizing a set of neutral complex N-glycans (2. primary binding). Upon binding the acrosome reaction is induced and the acrosome reacted sperm binds to polysulfated glycan structures of the zona pellucida via pro/acrosin, p38 and others (3. secondary binding).*

thus responsible for the higher incidence of polyspermic fertilization after IVF.

Furthermore, studies point to the contribution of the carbohydrate side chains in the maturation process of the zona pellucida (Rath *et al.,* 2005). Two-dimensional electrophoresis of the solubilized ZP-glycoproteins from prepuberal animals resolved over a wide pI range as the known spot-like pattern of heterogenous charge isomer families as described elsewhere (von Witzendorff *et al.,* 2005). The electrophoretic pattern of adult animals varied from that of prepuberal animals by showing an acidic shift of the ZPB/ZPC band of about 1.5 pI units (prepuberal pI 3-7 vs adult pI 3-5.5) and ZPA of about 1 pI unit (prepuberal pI 3.5-6.5 vs adult pI 3.5-5.5). After *in vitro* maturation a comparable shift was observed (Rath *et al.,* 2005). As shown in Fig. 7 the degree of sulfation of the ZP glycans of a strictly selected prepuberal animal group is particularly low in comparison with the highly sulfated N-glycan structures reported in former studies (Noguchi and Nakano, 1992; Mori *et al.,* 1998). The combined data provide some evidence that in the pig an increase of sulfated N-acetyllactosamine repeats may be part of the maturation process of the zona pellucida. In the light of what is known on the mechanisms of secondary binding, it could be suggested that the increased presentation of polysulfate structures during oocyte development and maturation may have an impact on the penetration process of sperm through the zona pellucida.

Conclusion

The events in the oviduct leading to fertilization involve a sequence of carbohydrate recognition mechanisms. For the pig, based on the current knowledge, a model of these interactions is presented in Fig. 10. Uncapacitated sperm are trapped in the sperm reservoir of the oviduct by binding to the flexible high-mannose type glycans exposed by oviductal glycoproteins such as LAMP-1/2 and the unknown 130 kDa protein, which are recognized by the sperm surface-associated spermadhesin AQN-1. At an early step of capacitation these proteins are shed from the surface thus unmasking the proteins of the multimeric receptor complex such as AWN, AQN-3 and P47/SED at the sperm surface. During swimming to the site of fertilization, the sperm completes capacitation and binds to the zona pellucida of the ovulated oocyte by recognizing properly arranged neutral complex N-glycans of the ZP glycoproteins. Upon binding, the acrosome reaction is induced by which the intraacrosomal pro/acrosin is exposed and activated. Pro/acrosin binds to the polysulfated glycans of the zona pellucida and mediates the secondary binding of the acrosome-reacted sperm to the matrix. Acrosin can be instrumental in the alternating cycles proposed for the penetration process (O'Rand *et al.,* 1986): the secondary binding event, the limited proteolysis of the zona pellucida and the release of sperm from the matrix (Fig. 10a, b).

Furthermore, some preliminary data, presented here, support the idea that also the zona pellucida has to undergo a maturation process during oocyte development to achieve full fertilization competence.

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References

ADHAM, I.M., NAYERNIA, K., ENGEL, W. (1997). Spermatozoa lacking acrosin protein show delayed fertilization. *Mol Reprod Dev* 46:370-376.

AMARI, S., YONEZAWA, N., MITSUI, S., KATSMATA, T., HAMANO, S., KUWYAMA, M., HASIMOTO, Y., SUZUKI, A., TAKEDA, Y., NAKANO, M. (2001). Essential role of the nonreducing terminal α-mannosyl residues of the N-linked carbohydrate chain of bovine zona pellucida glycoproteins in sperm-egg binding. *Mol Reprod Dev* 59:221-226.

ARDON, F., HELMS, D., SAHIN, E., BOLLWEIN, H., TÖPFER-PETERSEN, E. and WABERSKI, D. (2008). Chromatin-unstabile boar spermatozoa have little chance of reaching oocytes *in vivo*. *Reproduction*, 135: 461-470.

ASSREUY, A.M.S., ALENCAR, N.M.N., CAVADA, B.S., ROCHA-FILHO, D.R., FEITOSA, R.F.G., CUNHA, F.Q., CALVETE, J.J., RIBEIRO, R.A. (2003). Porcine spermadhesin PSP-I/PSP-II stimulates macrophages to release a neutrophil chemotactic substance: modulation by mast cells. *Biol Reprod* 68:1836-1841.

BABA, T., KASHIWABARA, S., WATANABE, K., ITOH, H., MICHIKAWA, Y., KIMURA, K., TAKADA, M., FUKAMIZU, A., ARAI, Y. (1989). Activation and maturation mechanisms of boar acrosin zymogen based on the deduced primary structure. *J Biol Chem* 264(20):11920-11927.

BANDO, Y., OGAWA, S., YAMACHI, A., KUWABAR, K., OZAWA, K., HORI, O., YABAGI, H., TAMATANI, M., TOHYAMA, M. (2000). 150-kDa oxygen-regulated protein (ORP150) functions a novel molecular chaperone in MDCK cells. *Am J Physiol Cell Physiol* 278:1172-1182.

BERGERON, A., VILLEMURE, M., LAZURE, C., MANJUNATH, P. (2005). Isolation and characterization of the major proteins of ram seminal plasma. *Mol Reprod Dev* 71(4):461-470.

BIERMANN, L., GABIUS, H.J., DENKER, H.W. (1997). Neoglycoprotein-binding sites (endogenous lectins) in the Fallopian tube, uterus and blastocyst of the rabbit during the preimplantation phase and implantation. *Acta Anat* 160:159-171.

BORK, P. and SANDER, C. (1992). A large domain common to sperm receptors (Zp2 and Zp3) and TGF-beta type III receptor. *FEBS Lett* 300:237-240.

Bork, P. (1993). A trefoil domain in the rabbit zona pellucida protein. *Protein Sci* 2: 669-670

BORK, P. and BECKMANN, G. (1993). The CUB domain. A widespread module in developmentally regulated proteins. *J Mol Biol* 231(2):539-545.

BUHI, W.C., ALVAREZ, I.M., SUDHIPONG, V., DONES-SMITH, M.M. (1990). Identification and characterization of de novo-synthesized oviductal secretory proteins. *Biol Reprod* 43:929-938.

BUHI, W.C. (2002). Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoproteins. *Reproduction* 123:355-362.

CALVETE, J.J., DOSTÀLOVÀ, Z., SANZ, L., ADERMANN, K., THOLE, H.H., TÖPFER-PETERSEN, E. (1996a). Mapping the heparin-binding domain of boar spermadhesins. FEBS Lett 379(3):207-211.

CALVETE, J.J., CARRERA, E., SANZ, L., TÖPFER-PETERSEN, E. (1996b) Boar spermadhesins AQN-1 and AQN-3: oligosaccharide and zona pellucida binding characteristics. Biol Chem 377:521-527.

CALVETE, J.J., RAIDA, M., GENTZEL, M., URBANKE, C., SANZ, L., TÖPFER-PETERSEN, E. (1997). Isolation and characterization of heparin- and phosphorylcholine-binding proteins of boar and stallion seminal plasma. Primary structure of porcine pB1. *FEBS Lett* 407(2):201-206.

CALVETE, J.J. and SANZ, L. (2007). Insights into structure-function correlations of ungulate seminal plasma proteins. *Soc Reprod Fertil* 65: 201-216.

CARLSSON, S.R. and FUKUDA, M. (1990). The polylactosaminoglycans of human lysosmal membrane glycoproteins Lamp-1 and Lamp-2. *J Biol Chem* 265:20488-20496.

CHANG, M.H., ISAAC, E.L., LITJEN, T., HODGE, G., KARAGEORGOS, L.E., MEIKLE, P.J. (2004). Transthyretin interacts with the lysosome-associated membrane protein (LAMP-1) in circulation. *Biochem J* 382: 481-489.

CECHOVA, D., TÖPFER-PETERSEN, E., HENSCHEN, E. (1988). Boar proacrosin is a single chain molecule which ahs the N-terminus of the acrosin-A chain (light chain). *FEBS Lett* 241:136-140.

COOPER, C.A., GASTEIGER, E., PACKER, N.H. (2001). GlycoMod— a software tool for determining glycosylation compositions from mass spectrometric data. *Proteomics* 1:340-349.

DARSON, A., TREVINO, C.L., WOOD, C., GALINDO, B., RODRIGUEZ-MIRANDA, E., ACEVEDO, J.J., HERNANDEZ-GONZALEZ, E.O., BELTRAN, C., MARTINEZ-LOPEZ, P., NISHIGAKI, T. (2007). Ion channels in sperm motility and capacitation. *Soc Reprod Fertil Suppl* 65: 229-244.

DOSTÀLOVÀ, Z., CALVETE, J.J., SANZ, L., HETTEL, C., RIEDEL, D., SCHÖNECK, C., EINSPANIER, R., TÖPFER-PETERSEN, E. (1994). Immunolocalization and quantitation of acidic seminal fluid protein (aSFP) in ejaculated, swim-up, and capacitated bull spermatozoa. *Biol Chem Hoppe Seyler* 375(7):457-461.

DOSTÀLOVÀ, Z., CALVETE, J.J., SANZ, L., TÖPFER-PETERSEN, E. (1995a). Boar spermadhesin AWN-1: Oligosaccharide and zona pellucida binding characteristics. *Eur J Biochem* 230:329-336.

DOSTÀLOVÀ, Z., CALVETE, J.J., TÖPFER-PETERSEN, E. (1995b). Interaction of non-aggregated boar AWN-1 and AQN-3 with phospholipid matrices. A model for coating of spermadhesins to the sperm surface. *Biol Chem Hoppe Seyler* 376:237-242.

EINSPANIER, R., EINSPANIER, A., WEMPE, F., SCHEIT, K.H. (1991). Characterization of a new bioactive protein from bovine seminal fluid. *Biochem Biophys Res Commun* 179:1006-1010.

EKHLASI-HUNDRIESER, M., SINOWATZ, F., GREISER DE WILKE, I., WABERSKI, D., TÖPFER-PETERSEN, E. (2002). Expression of spermadhesin genes in porcine male and female reproductive tracts. *Mol Reprod Dev* 61:32-41.

EKHLASI-HUNDRIESER, M., GOHR, K., WAGNER, A., TSOLOVA, M., PETRUNKINA, A., TÖPFER-PETERSEN, E. (2005). Spermadhesin AQN1 is a candidate receptor molecule involved in the formation of the oviductal sperm reservoir in the pig. *Biol Reprod* 73: 536-545.

EKHLASI-HUNDRIESER, M., SCHÄFER, B., PHILIPP, U., KUIPER, H., LEEB, T., MEHTA, M., KIRCHHOFF, C., TÖPFER-PETERSEN, E. (2007). Sperm-binding fibronectin type II-module proteins are genetically linked and functionally related. *Gene* 392(1-2):253-265.

EKHLASI-HUNDRIESER, M., CALVETE, J. J., VON RAD, B., HETTEL, C., NIMTZ, M. and TÖPFER-PETERSEN, E. (2008) Point mutations abolishing the mannose-binding capability of boar spermadhesin AQN-1. *Biochim Biophys Acta,* 1784: 856-862.

ENSSLIN, M., VOGEL, T., CALVETE, J.J., THOLE, H.H., SCHMIDTKE, J., MATSUDA, T., TÖPFER-PETERSEN, E. (1998) Molecular cloning an characterization of P47: a novel boar sperm-associated zona pellucida-bninding protein homologous to a family of mammalian secretory proteins. *Biol Reprod* 58:1057-1064.

FAZELI, A., DUNCAN, A.E., WATSON, P.F., HOLT, W.V. (1999). Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biol Reprod* 60:879-886.

FAZELI, A., ELLIOTT, R.M.A., DUNCAN, A.E., MOORE, A., WATSON, P.F., HOLT, W.V. (2003). *In vitro* maintenance of boar sperm viability by solubile fractions obtained from apical plasma preparations. *Repoduction* 125, 509-517

FAZELI, A., AFFARA, N.A., HUBANK, M., HOLT, W.V. (2004). Sperm-induced modification of the oviductal gene expression profile after natural insemination in mice. *Biol Reprod* 71:60-65

FOCK-NÜZEL, R., LOTTSPEICH, F., HENSCHEN, A., MÜLLER-ESTERL, W. (1984). Boar acrosin is a two-chain molecule. Isolation and primary structure of the light chain: Homology with the pro-part of other serine proteinases. *Eur J Biochem* 141:441-446.

FRIESS, A.E., TÖPFER-PETERSEN, E., NGUYEN, H., SCHILL, W.B. (1987a). Electron micorscopic localization of a fucose-binding protein in acrosome-reacted boar spermatozoa by the fucosyl-peroxidase-gold method. *Histochemistry* 86:297-303.

FRIESS, A.E., TÖPFER-PETERSEN, E., SCHILL, W.B. (1987b). Fracture-labelling of boar spermatozoa for fucose-bindng protein (FBP) *Histochemistry* 87:181-183.

FUKUDA, M. (1991). Lysosomal membrane glycoproteins. *J Biol Chem* 266: 21327-21330.

GABORIAU, D., HOWES, E.A., CLARK, J., JONES, R. (2007). Binding of sperm proacrosin/β-acrosin to zona pelluicda glycoproteins is sulfate and stereodependent. Synthesis of a novel fertilization inhibitor. *Dev Biol* 306: 646-657.

GEORGIOU, A.S., SOSTARIC, E., WONG, C.W., SNIDJERS, A.P.L., WRIGHT, P.C., MOORE, H.D., FAZELI, A. (2005). Gametes alter the oviductal secretory proteome. *Mol Cell Proteomics* 411:1785-1796

GEYER, H., SCHMITT, S., WUHRER, M., GEYER, R. (1999). Structural analysis

of glycoconjugates by on-target enzymatic digestion and MALDI-TOF-MS. *Anal Chem* 71:476-482

GREEN, D.P.L. (1987). Mammalian sperm can not penetrate the zona pellcuida soley by force. *Exp Cell Res* 169:31-38

GWATHMEY, T.M., IGNOTZ, G.G., SUAREZ, S.S. (2003). PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium *in vitro* and may be involved in forming the oviductal sperm reservoir. *Biol Reprod* 69:809-815

HAASE, B., SCHLÖTTERER, C., EKHLASI-HUNDRIESER, M., KUIPER, H., DISTL, O., TÖPFER-PETERSEN, E., LEEB, T. (2005). Evolution of the spermadhesin gene family. *Gene* 352:20-29.

HARRIS, J.D., HIBLER, D.W., FONTENOT, G.K., HSU, K.T., YUREWICZ, E.C., SACCO, A.G. (1994). Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: the ZPA, ZPB and ZPC gene families. *DNA Seq* 4:361-393.

HARRISON, R.A.P. (1996). Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. *Reprod Fertil Dev* 8:581-594

HOKKE, C.H., DAMM, J.B., PENNINKHOF, B., AITKEN, R.J., KAMERLING, J.P., VLIEGENTHART, J.F. (1994). Structure of the O-linked carbohydrate chains of porcine zona pellucida glycoproteins. *Eur J Biochem* 221:491-512.

HONDA, A., SIRUNTAWINWTI, J., BABA, T. (2002). Role of acrosomal matrix proteinases in sperm-zona pellucida interactiosn. *Hum Reprod Update* 5:405-412.

HUGHES, D.C. (2007). ZP genes in the avian species illustrates the dynamic evolution of the vertebrate egg envelope. *Cytogenet Genome Res* 117:86-91.

HUNTER, R.H., RODRIGUEZ-MARTINEZ, H. (2004). Capacitation of mammalian spermatozoa *in vivo*, with a specific focus on events in the Fallopian tubes. *Mol Reprod Dev* 67: 243-250.

HUNTER, R.H. (2002). Vital aspects of Fallopian tube physiology in pigs. *Reprod Domest Anim* 37:186-190

HUNTER, R.H.F., RODRIGUEZ-MARTINEZ, H. (2004). Capacitation of mammalian spermatozoa *in vivo*, with specific focus on the Fallopian tubes. *Mol Repod Dev* 67:242-250.

HUNTER, R.H. (2008). Sperm release from oviduct epithelial binding is controlled hormonally by peri-ovulatory graafian follicles. *Mol Repod Dev* 75: 167-174.

IGNOTZ, G.G., LO, M.C., PEREZ, C.L., GWATHMEY, T.M., SUAREZ, S.S. (2001). Characterization of a fucose-binding protein from bull sperm and seminal plasma that may be responsible for formation of the oviductal sperm reservoir. *Biol Reprod* 64:1806-1811.

IGNOTZ, G.G., CHO, M.Y., SUAREZ. S.S. (2007). Annexins are candidate oviductal receptors for bovie sperm surface proteins and thus may serve to hold bovine sperm in c ovidcutal reservoir. *Biol Reprod* 77:906-13.

IKEDA, L.J., KANEDA, S., KUWABARA, K., OGAWA, S., KOBAYASHI, T., MATSUMOTO, M., YURA, T., YANAGI, H. (1997). Cloning and expression of cDNA encoding the human 150 kDa oxygen-regulated protein, ORP 150. *Biochem Biophys Res Commun* 230:94-99.

IWAMOTO, K., IKEDA, K., YONEZAWA, N., NOGUCHI, S., KUDO, K., HAMANO, S., KUWAYAMA, M., NAKANO, M. (1999). Disulfide formation in bovine zona pellucida glycoproteins during fertilization: evidence for the involvement of cystine cross-linkages in hardening of the zona pellucida. *J Reprod Fertil* 117:395-402.

JANSEN, S., JONES, R., JENNECKEN, I. (1998). Site directed mutagensis of boar proacrosin reveals residues involved in binding to zona pellucida glycoproteins. *Mol Reprod Dev* 51:184-192.

JANSEN, S., EKHLASI-HUNDRIESER, M., TÖPFER-PETERSEN, E. (2001). Sperm adhesion molecules: structure and function. *Cells Tissues Organs* 168(1-2):82-92. Review.

JONAKOVA, V., KRAUS, M., VESELSKY, L., CECHOVA, D., BEZOUSKA, K., TICHA, M. (1998). Spermadhesins of the AQN and AWN families, DQH sperm surface protein and HNK protein in the heparin-binding fraction of boar seminal plasma. *J Reprod Fertil* 114:25-34.

JONES, R. (1991). Interaction of zona pellcuida glycoproteins, sulphated carbohydrates and synthetic polymers with proacrosin, the putative egg-binding molecule from mammalian spermatozoa. *Development* 111:1155-1163.

JUNGNICKEL, M.K., MARRERO, H., BIRNBAUMER, L., LEMOS, J.R., FLORMAN, H.M. (2001). Trp2 regulates entry of ca2+ into mouse sperm triggered by egg ZP3. *Nat Cell V Biol* 3:499-502.

KALTNER, H. and GABIUS, H.J. (2001). Animal lectins: from initial description to elaborated structural and functional classification. *Adv Exp Med Biol* 491:79-94.

KANNAN, K., STEWART, R.M., BOUNDS, W., CARLSON, S.R., FUKUDA, M., BETZING, K.W., HOLCOMBE, R.F. (1996). Lysosome-associated membrane proteins h-LAMP1 (CD107a) and h-LAMP2 (CD107b) are activation-dependent cell surface glycoproteins in human peripheral blood mononuclear cell which mediate cell adhesion to vascular endotehlim. *Cellular Immunology* 171:10-19.

KATSUMATA, T., NOGUCHI, S., YONEZAWA, N., TANOKURA, M., NAKANO, M. (1996). Structural characterization of the N-linked carbohydrate chains of the zona pellucida glycoproteins from bovine ovarian and fertilized eggs. *Eur J Biochem* 240:448-453.

KLEMM, U., MÜLLER-ESTERL, W., ENGLE, W. (1991). Acrosin, the peculiar sperm-specific serine protease. *Human genetics* 87:635-641.

KRISHNAN, V., BANE, S.M., KAWLE, P.D., NARESH, K.N., KALRAIYA, R.D. (2005). Altered melanoma cell surface glycosylation mediates organ specific adhesion and metastasis via lectin receptors on the lung vascular endothelium. *Clin Exp Metastasis* 22(1):11-24.

KUDO, K., YONEZAWY, N., KATSUMATA, T., AOKI, H., NAKANO, M. (1998). Localization of carbohydrate chains of pig sperm ligand in the glycoprotein ZPB of egg zona pellucida *Eur J Biol* 252:492-499.

KÜSTER, B., WHEELER, S.F., HUNTER, A.P., DWEK, R.A., HARVEY, D.J. (1997). Sequencing of N-linked oligosaccharides directly from protein gels: in-gel deglycosylation followed by matrix-assisted laser desorption/ionization mass spectrometry and normal-phase high-performance liquid chromatography. *Anal Biochem* 250(1):82-101

KWOK, S.C., YANG, D., DAI, G., SOARES, M.J., CHEN, S., MCMURTRY, J.P. (1993). Molecular cloning and sequence analysis of two porcine seminal proteins, PSP-I and PSP-II: new members of the spermadhesin family. *DNA Cell* Biol 12(7):605-10.

LEEB, T. (2007). The horse genome project- sequence based insights into male reproductive mechanisms. *Reprod Dom Anim* 42 Suppl. 2: 45-50.

LIBERDA, J., MANSKOVA, P., PERELOVSKA, L., TICHAI, M., JONAKOVA, V. (2006). Saccharide-mediated interactions of boar sperm surface proteins with components of the porcine oviduct. *J Reprod Immunol* 71:112-125.

LUAN, Y. and XU, W. (2007). The structure and main function of aminopeptidase *N. Curr Med Chem* 14: 639-647.

MANJUNATH, P. and THERIEN, I. (2002). Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capcitation. *J Reprod Immun* 53:109-119.

MCKENZIE, M.E., MALININ, A.I.V., BELL, C.R., DZHANASHVILI, A., HOROWITZ, E.D., OSHRINE, B.R., ATAR, D., SEREBRUANY, V.L. (2003). Aspirin inhibts surface glycoprtein IIb/IIa, P-selectin, CD63 and CD107a recepror expression on human platelets. *Bloos Coagul Fibrinolysis* 14: 249-253.

MELO, L.M., TEIXEIRA, D.I., HAVT, A., DA CUNHA, R.M., MARTINS, D.B., CASTELLETTI, C.H., DE SOUZA, P.R., FILHO, J.L., FREITAS, V.J., CAVADA, B.S., RÁDIS-BAPTISTA, G. (2008). Buck (Capra hircus) genes encode new members of the spermadhesin family. *Mol Reprod Dev* 75(1):8-16.

MICHELMANN, H.W., RATH, D., TÖPFER-PETERSEN, E., SCHWARTZ, P. (2007). Structural and functional events on the porcine zona pellucida during maturation, fertilization and embryonic development: a scanning electron microscopy analysis. *Reprod Domest Anim* 42(6):594-602.

MORENO, R.D. and BARROS, C. (2000). A basic 18-amino acid peptide contains the polysulfate-binding domain responsible for activation of the boar proacrosin/acrosin system. *Biol Reprod* 62:1536-1542.

MORI, E., KASHIWABARA, S., BABA, T., INAGAKI, A.Y., MORI, T. (1995). Amino acid sequences of porcine Sp38 and proacrosin required for binding to the zona pellucida. *Dev Biol* 168: 575-593.

MORI, E., HEDRICK, J.L., WARDRIP, N.J., MORI, T., TAKASAKI, S. (1998). Occurrence of reducing terminal N-acetylglucosamine 3-sulfate and fucosylated outer chains in acidic N-glycans of porcine zona pellucida glycoproteins. *Glycoconj J* 15:447-456.

MÜLLER, P., ERLEMANN, K.R., MÜLLER, K., CALVETE, J.J., TÖPFER-PETERSEN, E., MARIENFELD, K., HERRMANN, A. (1998) Biophysical characterization of the interaction of bovine seminal plasma protein PDC-109 with phospholipid vesicles. *Eur Biophys J* 27:33-41.

MÜLLER, P., GREUBE, A., TÖPFER-PETERSEN, E., HERRMANN, A. (2002). Influence of the bovine seminal plasma protein PDC-109 on cholesterol in the presence of phospholipids. *Eur Biophys J* 31:438-447.

NAYERNIA, K., ADHAM, I.M., KREMLING, H., REIM, K., SCHLICKER, M., SCHLÜTER, G., ENGEL, W. (1996). Stage and developmental specific gene expression during mammalian spermatogenesis. *Int J Dev Biol* 40:379-383.

NAYERNIA, K., ADHAM, I.M., SHAMSADIN, R., MÜLLER, C., SANCKEN, U., ENGEL, W. (2002). Proacrosin-deficient mice and zona pellucida modifications in an experimental model of multifactorial infertility. *Mol Hum Reprod* 8:434-440.

NIEMANN, H. and RATH, D. (2001). Progress in reproductive biotechnology in swine. *Theriogenology* 56:1291-1304.

NIMTZ, M., GRABENHORST, E., CONRADT, H.S., SANZ, L., CALVETE, J.J. (1999). Structural characterization of the oligosaccharide chains of native and crystallized boar seminal plasma spermadhesin PSP-I and PSP-II glycoforms. *Eur. J Biochem* 265:703-718.

NOGUCHI, S. and NAKANO, M. (1992). Structure of the acidic N-linked carbohydrate chains of the 55-kDa glycoprotein family (PZP3) from porcine zona pellucida. *Eur J Biochem* 209:883-894

NOGUCHI, S., HATANAKA, Y., TOBITA, T., NAKANO, M. (1992). Structural analysis of the N-linked carbohydrate chains of the 55-kDa glycoprotein family (PZP3) from porcine zona pellucida. *Eur J Biochem* 207:1130.

O'RAND, M., WELCH, J.E., FISHER, S.J. (1986). Sperm membrane and zona pellucida interaction during fertilization. In Molecular and Cellular Aspects of Reproduction (eds. Dhindsa, D.S., Bahl, O.) New York, Plenum Press 131-144.

OCHWAT, D., HOJA-LUKOWICZ, D., LITYNSKA, A. (2004). N-glycoprotein bearing beta1-6 branched oligosaccharides from A375 human melanoma cell line analysed by tandem mass spectromety. *Melanoma Res* 14:479-485.

PACKER, N.H., LAWSON, M.A., JARDINE, D.R., REDMOND, J.W. (1998). A general approach to desalting oligosaccharides released from glycoproteins. *Glycoconj J* 15:737-747.

PALTER, S.F., MULAYIM, N., SENTURK, L., ARICI, A. (2001). Interleukin-8 in the humans fallopian tube. *J Clin Endocrinol Metab* 86:2660-2667.

PETRUNKINA, A., GELHAAR, R., DROMMER, W., WABERSKI, D., TÖPFER-PETERSEN, E. (2001a). Selective sperm binding to pig oviductal epithelium *in vitro*. *Reproduction* 121:889-896.

PETRUNKINA, A.M., FRIEDRICH, J., DROMMER, W., BICKER, G., WABERSKI, D., TÖPFER-PETERSEN, E. (2001b). Kinetic characterization of the changes in protein tyrosine phosphorylation of membranes, cytosolic Ca^{2+} concentration and viability in boar sperm populations selected by binding to oviductal epithelial cells. *Reproduction* 122:469-480.

PETRUNKINA, A.M., SIMON, K., GÜNZEL-APEL, A.R., TÖPFER-PETERSEN, E. (2003a). Regulation of capacitation of canine spermatozoa during co-culture with heterologous oviductal epithelial cells. *Reprod Dom Anim* 38:455-463.

PETRUNKINA, A.M., LÄKAMP, A., GENTZEL, M., EKHLASI-HUNDRIESER, M., TÖPFER-PETERSEN, E. (2003b). Fate of lactadherin P47 during post-testicular maturation and capacitation of boar spermatozoa. *Reproduction* 125:3777-3387.

PETRUNKINA, A.M., SIMON, K., GÜNZEL-APEL, A.R., TÖPFER-PETERSEN, E. (2004). Kinetics of protein tyrosine phosphorylation in sperm selected by binding to homologous and heterologous oviductal explants: how specific is the regulation by the oviduct? *Theriogenology* 61:1617-1634.

PETRUNKINA, A.M., VOLKER, G., BRANDT, H., TÖPFER-PETERSEN, E., WABERSKI, D. (2005). Functional significance of responsiveness to capacitating conditions in boar spermatozoa. *Theriogenology* 64:1766-1782.

PETRUNKINA, A.M., WABERSKI, D., GÜNZEL-APEL, A.R., TÖPFER-PETERSEN, E. (2007). Determinants of sperm quality and fertility in domestic animals. *Reproduction* 134:3-17.

PISANO A, JARDINE DR, PACKER NH, FARNSWORTH V, CARSON W, CARTIER P, REDMOND JW, WILLIAMS KL, GOOLEY UAA (1996): Carbohydrate linkage sites. In: R. R. Townsend u. AT Hotchkiss Jr (Hrsg.): Techniques in Glycobiology. Marcel Dekker, New York, S. 299-320

RATH, D., TÖPFER-PETERSEN, E., MICHELMANN, H.W., SCHWARTZ, P., EBELING, S. (2005). Zona pellucida characteristics and sperm binding patterns of *in vivo* and *in vitro* produced porcine oocytes inseminated with differently prepared spermatozoa. *Theriogenology* 63:532-362.

RATH, D., TÖPFER-PETERSEN, E., MICHELMANN, H.W., SCHWARTZ, P., VON WITZENDORFF, D., EBELING, S., EKHLASI-HUNDRIESER, M., PIEHLER, E., PETRUNKINA, A., ROMAR, R. (2006). Structural, biochemical and functional aspects of sperm-oocyte interactions in pigs. *Soc Reprod Fertil Suppl*. 62:317-330.

REINERT, M., CALVETE, J.J., SANZ, L., MANN, K., TÖPFER-PETERSEN, E. (1996). Primary structure of stallion seminal plasma protein HSP-7, a zona-pellucida-binding protein of the spermadhesin family. *Eur J Biochem* 242(3):636-40.

REVAH, I., GADELLA, B.M., FLESCH, F.M., COLENBRANDER, B., SUAREZ, S.S. (2000). Physiological state of bull sperm affects fucose- and mannose-binding properties. *Biol Reprod* 62:1010-1015.

RICHARDSON, R.T. and O'RAND, M. (1996). Site directed mutagenesis of rabbit proacrosin. *J Biol Chem* 39:688-701.

RODRIGUEZ-MARTINEZ, H., IBORRA, A., MARTINEZ, P., CALVETE, J.J. (1998). Immunoelectronmicroscopic imaging of spermadhesin AWN epitopes on boar spermatozoa bound *in vivo* to the zona pellucida. *Reprod Fertil Dev* 10:491-497.

ROSANO, G., CAILLE, A.M., GALLARDO-RIOS, M.N., MUNUCE. M.J. (2007). D-mannose binding sites are putative sperm determinants of human oocyte recognition and fertilization. *Reprod Biomed online* 15:182-190.

ROMÃO, M.J., KÖLLN, I., DIAS, J.M., CARVALHO, A.L., ROMERO, A., VARELA, P.F., SANZ, L., TÖPFER-PETERSEN, E., CALVETE, J.J. (1997). Crystal structure of acidic seminal fluid protein (aSFP) at 1.9 Å resolution: a bovine polypeptide of the spermadhesin family. *J. Mol. Biol.* 274: 650-660.

ROMERO, A., ROMÃO, M.J., VARELA, P.F., KÖLLN, I., DIAS, J.M., CARVALHO, A.L., SANZ, L., TÖPFER-PETERSEN, E., CALVETE, J.J. (1997). The crystal structures of two spermadhesins reveal the CUB domain fold. *Nature Struct Biol* 4: 783-788.

RUNNEBAUM, I., SCHILL, W.B., TÖPFER-PETERSEN, E. (1994). ConA-binding proteins of sperm surface are conserved through evolution and in sperm maturation. *Andrologia* 27: 81-90.

SALICIONI, A.M., PLATT, M.D., WERTHEIMER, E.V., ARCELAY, E., ALLAIRE, A., SOSNIK, A., VISCONTI, P.E. (2007). Signaling pathways involved in capacitation. *Soc Reprod Fertil Suppl* 65:245-59

SANZ, L., CALVETE, J.J., MANN, K., SCHÄFER, W., SCHMID, E.R., TÖPFER-PETERSEN, E. (1991). The amino acid sequence of AQN-3, a carbohydrate-binding protein isolated from boar sperm. Location of disulphide bridges. *FEBS Lett* 291(1):33-36.

SANZ, L., CALVETE, J.J., SCHÄFER, W., MANN, K., TÖPFER-PETERSEN, E. (1992). Isolation and biochemical characterization of two isoforms of a boar sperm zona pellucida-binding protein. *Biochim Biophys Acta* 1119:127-132

SANZ, L., CALVETE, J.J., MANN, K., GABIUS, H.J., TÖPFER-PETERSEN, E. (1993). Isolation and biochemical of heparin-binding proteins from boar seminal plasma: a dual role for spermadhesins in fertilization. *Mol Reprod Dev* 35: 37-43.

SARAFIAN, V., JADOT, M., FOIDART, J.M., LETESSON, J.J., VAN DEN BRAULE, F., CASTRONOVO, V., WATTIAUX, R., CONICK, S.W. (1998). Expression of LAMP-1 and Lamp-2 and their interaction with galectin-3 in human tumor cells. *Int J Cancer* 75:105-111.

SHIBUYA, N., GOLDSTEIN, I.J., VAN DAMME, E.J., PEUMANS, W.J. (1988). Binding properties of a mannose-specific lectin from the snowdrop (galanthus nivalis) bulb. *J Biol Chem* 263:728-734.

SHUR, B.D., ENSSLIN, M., RODEHEFFER, C. (2004). SED1 function during mammalian sperm-egg adhesion. *Current opinion in cell biology* 16:477-485.

SIDRAUSKI, C., CHAPMAN, R., WALTER, R. (1998). The unfold protein response: an intracellular signalling pathway with many surprising features. *Trends Cell Biol* 8:245-249.

SINOWATZ, F., KÖLLE, S., TÖPFER-PETERSEN, E. (2001). Biosynthesis and expression of zona pellcucida glycoproteins in mammals. *Cell Tissues Organs* 168:24-35.

SINOWATZ, F., WESSA, E., NEUMÜLLER, C, PALMA, G. (2003). On the species specificity of sperm binding and sperm penetration of the zona pellucida. *Reprod Domest Anim* 38:141-146.

SOSTARIC, E., GEORGIOU, A.S., WONG, C.H., WATSON, P.F., HOLT, W.V., FAZELI, A. (2006). Global profiling of surface plasma membrane proteome of oviductal epithelial cells. *J Proteome Res* 5:3029-3037.

SPARGO, S.C. and HOPE, R.M. (2003). Evolution and nomenclature of the zona

pellucida gene family. *Biol Reprod* 68:358-362.

SUAREZ, S.S. (2001). Carbohydrate-mediated formation of the oviductal sperm reservoir in mammals. *Cells Tissues Organs* 168:105-112.

SUAREZ, S.S. (2002). Formation of a reservoir of sperm in the oviduct. *Reprod Domest Anim* 37:140-143.

SUAREZ, S.S. and PACEY, A.A. (2006). Sperm transport in the female reproductive tract. *Human Reproduction Update* 12: 23-37.

TEDESCHI, G., OUNGRE, E., MORTARINO, M., NEGRI, A., MAFFEO, G., RONCHI, S. (2000). Purification and primary structure of a new bovine spermadhesin. *Eur J Biochem* 267(20):6175-6179.

TAKASAKI, S., MORI, E., MORI, T. (1999). Structures of sugar chains included in mammalian zona pellucida glycoproteins and their potential roles in sperm-egg interaction. *Biochim Biophys Acta* 1473:206-215.

TIENTHAI, P., JOHANNISSON, A., RODRIGUEZ-MARTINEZ, H. (2004). Sperm capacitation in the porcine oviduct. *Anim Reprod Sci* 80:131-146.

TÖPFER-PETERSEN, E., FRIESS, A.E., NGUYEN, H., SCHILL, W.B. (1985). Evidence for a fucose-bindng protein in boar spermatozoa. *Histochemistry* 83:139-145.

TÖPFER-PETERSEN, E. and HENSCHEN, A. (1987). Acrosin shows zona and fucose- binding, novel properties for a serine proteinase. *FEBS Lett* 226:38-42.

TÖPFER-PETERSEN, E. and HENSCHEN, A. (1988). Zona pellucida-binding and fucose-binding of boar sperm acrosin is not correlated with proteolytic activity. *Biol Chem Hoppe Seyler* 369:69-76.

TÖPFER-PETERSEN, E. and CECHOVA, D. (1990). Zona pelluciuda induces conversion of proacrosin to acrosin. *Int J Androl* 13:190-196.

TÖPFER-PETERSEN, E., CALVETE, J.J., SCHÄFER, W., HENSCHEN, A. (1990a). Complete localization of disulfide bridges and glycosylation sites in boar sperm acrosin. *FEBS Lett* 275:139-142.

TÖPFER-PETERSEN, E., STEINBERGER, M., VON ESCHENBACH, C. E. and ZUCKER, A. (1990b) Zona pellucida-binding of boar sperm acrosin is associated with the N-terminal peptide of the acrosin B-chain (heavy chain). *FEBS Lett,* 265: 51-4.

TÖPFER-PETERSEN, E., ROMERO, A., VARELA, P.F., EKHLASI-HUNDRIESER, M., DOSTAOVA, Z., SANZ, L., CALVETE, J.J. (1998). Spermadhesins: a new protein family. Facts, hypotheses and perspectives. *Andrologia* 30:217-224.

TÖPFER-PETERSEN, E. (1999). Carbohydrate-based interactions on the route of a spermatozoon to fertilization. *Hum Reprod Update* 5:314-329.

TÖPFER-PETERSEN, E., WAGNER, A., FRIEDRICH, J., PETRUNKINA, A., EKHLASI-HUNDRIESER, M., WABERSKI, D., DROMMER, W. (2002). Function of the mammalian oviductal sperm reservoir. *J Exp Zool* 292:210-215.

TRANTER, R., READ, J.A., JONES, R., BRADY, R.L. (2000). Effector sites in the three-dimensional structure of mammalian sperm beta- acrosin. *Structure* 8:1179-1188.

URCH, U.A., PATEL, H. (1991). The interaction of boar sperm proacrosin with its natural substrate, the zona pellucida, and with polysulphated polysacchrides. *Development* 111:1165-1171.

VARELA, P.F., ROMERO, A., SANZ, L., ROMÃO, M.J., TÖPFER-PETERSEN, E., CALVETE, J.J. (1997). The 2.4 Å resolution crystal structure of boar seminal plasma PSP-I/PSP-II:a zona pellucida-binding glycoprotein heterodimer of the spermadhesin family built by a CUB domain architecture. *J Mol Biol* 274: 635-649.

VAN GESTEL, R.A., BREWIS, I.A., ASHTON, P.R., BROWERS, J.F., GADELLA, B. (2007). Multiple proteins present in purified porcine sperm apical plasma membranes interact with the zona pellucida of oocyte. *Mol Hum Reprod* 13:445-454.

VON WITZENDORFF, D., EKHLASI-HUNDRIESER, M., DOSTALOVA, Z., RESCH, M., RATH, D., MICHELMANN, H.W., TÖPFER-PETERSEN, E. (2005). Analysis of N-linked glycans of porcine zona pellucida glycoprotein, ZPA by MALDI-TOF MS: a contribution to understanding zona pellucida structure. *Glycobiology* 15:475-488.

VO, L.H. and HEDRICK, J.L. (2000). Independent and hetero-oligomeric-dependent sperm binding to egg envelope glycoprotein ZPC of Xenopus laevis. *Biol Reprod* 62:766-774.

WABERSKI, D., MAGNUS, F., ARDON, F., PETRUNKINA, A.M., WEITZE, K.F., TÖPFER-PETERSEN, E. (2006). Binding of boar spermatozoa to oviductal epithelium *in vitro* in relation to sperm morphology and storage time. *Reproduction* 131(2):311-318.

WAGNER, A., EKHLASI-HUNDRIESER, M., HETTEL, C., PETRUNKINA, A., WABERSKI, D., NIMTZ, M., TÖPFER-PETERSEN, E. (2002). Crabohadrate-based interactiosn of oviducvtal sperm reservoir formation – studies in the pig. *Mol Reprod Dev* 61:249-257.

WALTER, I., BAVDEK, S. (1997). Lectin binding pattern of porcine mucosa and endometrium during oestrous cycle. *J Anat* 190:299-307.

WASSARMAN, P.M. (2005). Contribution of egg zona pellucida glycoproteins to gamete recognition during fertilization. *J Cell Physiol* 204:388-391.

WASSARMAN, P.M., JOVINE, L., QI, H., WILIAMS, Z., DARIE, C., LITSCHER, E.S. (2005). Recent aspects of mammlian fertilization research. *Mol Cell Endocrinol* 29:95-103.

WATSON, P.F. (1981). The roles of lipid and protein in the protection of ram spermatozoa at 5 degrees C by egg-yolk lipoprotein. *J Reprod Fertil* 62(2):483-492.

WEMPE, F., EINSPANIER, R., SCHEIT, K.H. (1992). Characterization by cDNA cloning of the mRNA of a new growth factor from bovine seminal plasma: acidic seminal fluid protein. *Biochem Biophys Res Commun* 183(1):232-237.

WHEELER, S.F. and HARVEY, D.J. (2001). Extension of the in-gel release method for structural analysis of neutral ans sialylated N-linked glycans to the analysis of sulfated glycans from bovine thyroid-stimulating hormone. *Anal Biochem* 296:92-100.

YANAGIMACHI, R. (1994). Mammalian fertilization In The Physiology of Reproduction (eds Knobil, E., Neill, J.D.) 2nd ed, 1 Raven Press New York, USA pp189-317.

YONEZAWA, N., FUKUI, N., KUDO, K., NAKANO, M. (1999). Localization of neutral N-linked carbohydrate chains in pig zona pellucida glycoprotein ZPC. *Eur J Biochem* 260, 57-63.

YONEZAWA, N., AMARI, S., TAKAHASHI, K., IKEDA, K., IMAI, F.L., KANAI, S., KIKUCHI, K., NAKANO, M. (2005). Participation of the non-reducing terminal β-galactosyl residues of the neutral N-linked carbohydrate chains of porcine zona pellucida glycoproteins in sperm-egg binding. *Mol Reprod Dev* 70:222-227.

YUREWICZ, E.C., SACCO, A.G., GHUPTA, S.K., XU, N., GAGE, D.A. (1998). Hetero-oligomerisation-dependent binding of pig oocyte zona pellucida glycoproteins ZPB and ZPC to boar sperm membrane vesicles. *J Biol Chem* 273:7488-7494.

ZELEZNA, B., CECHOVA, D., HENSCHEN, A. (1989). Isolation of boar sperm acrosin peptide released during conversion of α-form to β-form. Biol Chem Hoppe Seyler 370, 323-327.

ZHANG, H., LI, X.J., MARTIN, D.B., AEBERSOLD, R. (2003). Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labelling and mass spectrometry. *Nat Biotechnol* 21:660-666.

ZHOU, Q., PARK, S.H., BOUCHER, S., HIGGINS, E., LEE, K., EDMUNDS, T. (2004). N-linked oligosaccharide analysis of glycoprotein bands from isoelectric focusing gels. *Anal Biochem* 335:10-16