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Functional Transformations in the Sperm Glycome

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Neurosciences with a Specialization in Anthropogeny

by

Hector Sequoyah Reynoso

Committee in charge:

Professor Pascal Gagneux, Chair
Professor Heidi Cook-Andersen
Professor Maryann Martone
Professor Alysson Muotri
Professor Miles Wilkinson

2018

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2018

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ABSTRACT OF THE DISSERTATION

Functional Transformations in the Sperm Glycome

by

Hector Sequoyah Reynoso

Doctor of Philosophy in Neurosciences with a Specialization in Anthropogeny

University of California, San Diego, 2018

Professor Pascal Gagneux, Chair

Spermatozoa, or sperm for short, are the male gametes which must successfully navigate the female reproductive tract and fertilize an oocyte in natural conception. Sperm face a number of challenges in the completion of this task. This dissertation addresses two of these challenges, one immunological and one functional. First, the immunological challenge involves the fact that sperm must survive in a non-self body. The female immune system closely regulates the

environment of the female reproductive tract to protect against potentially pathogenic non-self organisms while tolerating symbiotic microbes. The endometrium initiates the leukocytic reaction in response to insemination, triggering a migration of various leukocytes to destroy threatening foreign cells. A successful sperm must evade destruction by these leukocytes. In chapter 2, I show that a subset of infertile human males exhibit a non-human cell-surface molecule, N-glycolylneuraminic acid (Neu5Gc), that is targeted by circulating antibodies within the female reproductive tract.

The second major challenge concerns the cellular functioning of the sperm. Sperm undergo a series of changes, collectively called capacitation, upon exposure to the female reproductive tract. The functional challenge is highlighted by the fact that a sperm cell must perform its various functions in the absence of gene expression and translation. Compared to somatic cells, the DNA in the mature sperm head is tightly packaged and inaccessible. Furthermore, sperm are virtually absent of ribosomes and thus are unable to mediate translation. Thus, sperm must survive and achieve fertilization using only post-translational modifications (PTMs). The PTMs examined in this dissertation include changes in the glycocalyx, phosphorylation of peptide residues, and a specific type of intracellular glycosylation of serine/threonine residues called O-GlcNAcylation. In chapter 3, I provide evidence that endogenous sialidases begin cleaving sialic acids from the sperm glycocalyx immediately upon exposure to capacitating conditions. Surprisingly, the lesser-sialylated capacitated sperm do not increase immune cell

activation *in vitro*. In chapter 4 I focus on O-GlcNAcylation, a lesser studied and often entirely overlooked PTM implicated in various cell processes that may compete with phosphorylating enzymes for certain peptide residues. Our data suggest that sperm exhibit capacitation-induced changes in O-GlcNAcylation.

CHAPTER 1:

INTRODUCTION

Sperm: the male gamete

The sperm cell is the male gamete. The sperm is composed of four major parts: the acrosome, the head, the midpiece, and the tail. The acrosome is a specialized vesicle capping the sperm head that contains various enzymes and covers the receptor necessary to fertilize an oocyte. The sperm head contains the nucleus with tightly compacted DNA, the genetic material that will be passed to the next generation. Next, the midpiece is the site connecting head to tail and where energy-producing mitochondria are segregated. Finally the tail is a long flagellum that gives sperm their tadpole-like shape. The tail is composed of both actin and tubulin filaments, formed by a core compartment called the axoneme that slides within an outer compartment called the fibrous sheath. Nine microtubule doublets form a circle around a central microtubule pair to form the axoneme. The axoneme slides inside of the fibrous sheath to create the flagellar sinusoidal movement that characterizes a motile sperm.

Mammalian reproduction is characterized by internal fertilization, whereby a sperm cell must physically enter into the female reproductive tract. In order to accomplish this task, sperm cells must be first produced in the testes. The prototypical tadpole-shape of a sperm cell actually represents a morphologically mature stage of the male gamete. All sperm begin as spermatogonial stem cells, a pool of self-renewing cells that line testis-specific structures called seminiferous

tubules. Imagining seminiferous tubules as a pipe, the primordial spermatogonial stem cells are positioned along the outer surface. When these cells divide, they give rise to daughter cells that are progressively pushed inwards towards the center of the pipe. A single diploid spermatogonial stem cell actually gives rise to four haploid daughter cells through two successive meiotic divisions. During these divisions, each developing sperm cell undergoes a recombination and halving of its total genetic material to result in a haploid cell with half the number of chromosomes as a somatic cell (i.e. from $2n$, or 46 chromosomes, to $1n$, or 23 chromosomes).

During this process, a number of events occur that are unique to sperm. First, the morphology of the sperm cell transforms from a round, symmetrical shape to the familiar elongated tadpole-like phenotype with a flattened head. The reduced genetic material remains in the nucleus, but instead of being wrapped around histones, the majority of DNA is further condensed along protamines and form donut-shaped regions called toroids. This is thought to allow for the reduction of physical space necessary to package the DNA, as well as provide stabilization and protection of the genome. However, this also results in the vast majority of the genome being inaccessible and thus protein synthesis is canonically thought to cease. At the same time, other cell organelles are reorganized and reduced as the majority of the cell's cytoplasm is purged from the cell. This includes the virtually complete loss of ribosomes, the organelles responsible for protein synthesis. Gene transcription and protein translation continue during the haploid phase of sperm development, but eventually wind down considerably with the loss of all cytoplasmic ribosomes prior to the sperm's exit from the testes (Christopher &

Christopher, 2017). As the cell now lacks both the machinery and the access to the instructions necessary for the translation of new protein, sperm are dogmatically termed translationally-silent. [Note: In contrast, researchers have shown the existence of translation in the sperm cell, accomplished via mitochondrial ribosomes (Bragg & Handel, 1979; Gur & Breitbart, 2006). Furthermore, these studies showed translation is essential for certain sperm functions. However, this aspect of sperm biology is relatively unstudied.]

Sperm that have finished morphologically maturing in the testis progress for further molecular maturation in the epididymis. Sperm are translocated via fluid motion, as they are still immotile at this stage. The epididymis contains three portions, the caput (head), corpus (body), and cauda (tail). Sperm first enter the caput and progress through the corpus until they reach the cauda. Whereas the journey through the testis takes roughly two months, passage through epididymis takes less than two weeks in most species. It is in the cauda epididymis that sperm are stored until ejaculation.

Interestingly, sperm stored in the cauda epididymis are not fully mature. During ejaculation, sperm are combined with other fluids from the seminal vesicles, prostate gland, and the bulbourethral (Cowper's) glands. The exposure of sperm to components of these other fluids triggers changes in the sperm cell. However, it is only after sperm arrive and spend a period of time in the female reproductive tract that they become fully functionally mature and capable of fertilizing an oocyte *in vivo*. The final changes during this maturation process are termed capacitation.

Capacitation

Capacitation involves several different physiological changes that prepare the sperm to fertilize the oocyte. Non-mammalian sperm do not require this additional step. Two groups independently reported capacitation in 1951 (Austin, 1951; Chang, 1951). Since then, researchers have shown that several signaling pathways are involved (Breitbart, 2003). In fact, the sperm cell undergoes so many changes during capacitation that using one umbrella term to describe all the different changes is misleading, as different components have been found to operate independently and at different rates. Thus, we must break down capacitation into several processes, some of which operate in serial while others run in parallel. These include the activation of adenylyl cyclases, cholesterol efflux, and phosphorylation. We will present them in a roughly chronological order.

It is possible to induce capacitation *in vitro* using a physiological buffer that includes a relatively high concentration of bicarbonate (25 mM) and albumin (10 mg/mL). Bicarbonate enters the cell using bicarbonate transporters (Liu, Wang, & Chen, 2012) and activates soluble adenylyl cyclases, creating cyclic adenosine monophosphate (cAMP) (Visconti et al., 1995a; Visconti et al., 1995b). The second messenger cAMP has many targets, but a hallmark of capacitation concerns the activation of protein kinase A (PKA). PKA phosphorylates serine and threonine residues in a rapid fashion following sperm exposure to bicarbonate (Visconti et al., 1995a). PKA is critically important for motility, and fittingly is located along the fibrous sheath along the length of the sperm tail. AKAP (PKA-anchoring protein) anchors PKA along the fibrous sheath (Christopher & Christopher, 2017).

Returning to the sperm head, an influx of bicarbonate is thought to have several roles besides activation of PKA. First, the bicarbonate triggers an initial lipid reorganization by activating a scramblase that flips phosphatidylserines (PS) and phosphatidylethanolamines (PE) from their normal position on the inner leaflet across to the outer leaflet (Flesch et al., 2001). This pushes cholesterol to the outer leaflet where cholesterol becomes available for acceptance by albumin, which allows for reorganization of lipid rafts and prepares the sperm for acrosome reaction. Follicular fluid albumin and serum albumin may provide this role *in vivo* (Langlais et al., 1988). A preliminary loose binding occurs between sperm and the zona pellucida (ZP) of the oocyte (Aitken, 1995; Bleil & Wassarman, 1980), a process that relies on the aggregation of lipid rafts (Buo Khalil et al., 2006), followed by a possibly species-specific tight binding. Sperm interaction with ZP induces acrosome reaction, the process of acrosomal exocytosis, during which the sperm spills out various enzymes that can etch through the protective vestments of the oocyte (Brucker & Lipford, 1995). Following ZP binding, membrane reorganization during acrosome reaction exposes the crucial receptor Izumo1 necessary for binding to the Juno protein on the underlying oocyte plasma membrane (Sebkova et al., 2014; Bianchi et al., 2014).

Additionally, bicarbonate is thought to facilitate the opening of voltage-gated calcium channels (Wennemuth, Westenbroek, Xu, Hille, & Babcock, 2000; Wennemuth, Carlson, Harper, & Babcock, 2003). Calcium can have a host of effects on cellular functioning through its actions as a second messenger (Clapham, 2007). Interestingly, it is not fully known whether the majority of the observed calcium

increase in sperm comes from internal stores or outside of the cell. However, buffers that induce capacitation *in vitro* include small concentrations of calcium (Will, Clark, & Swain, 2011).

Downstream of and perhaps in parallel to the bicarbonate-induced PKA activity, there is also an increase in tyrosine phosphorylation. Previous work has shown that several tyrosine kinases are involved, including Fer (Alvau et al., 2016), FAK (Roa-Espitia et al., 2016), Src (Mitchell, Nixon, Baker, & Aitken, 2008), and Lyn (Andrisani et al., 2015). However, the precise signaling streams which activate all the particular tyrosine kinases are not well understood, and the critical role of tyrosine phosphorylation is debated. For example, even though the increase in tyrosine phosphorylation is blocked via genetic inactivation of Fer, sperm from Fer KO mice are still fertile (Alvau et al., 2016). Additionally, the signaling events and enzymes involved can differ dramatically between species, as sperm cells are the targets of rapid evolution (Torgerson, Kulathinal, & Singh, 2002). Thus, it is important to make note of the particular species involved when interpreting the available evidence. In this dissertation, we will study human and mouse sperm exclusively.

Finally, capacitation involves a change in the motility characteristics of sperm. After ejaculation, sperm proceed in a symmetrical sinusoidal swimming pattern and proceed linearly in an aquatic medium. Over the course of several hours, they begin to adopt an asymmetrical flagellar beat, referred to as hypermotility or hyperactivation (Yanagimachi, 1969). The tail of hypermotile sperm traces a whipping motion, causing sperm to make rapid changes in direction.

Several theories account for this apparently paradoxical change in motility. First, when sperm are placed in a more biologically relevant viscous environment, as occurs within the female reproductive tract, this asymmetrical pattern actually aids in the sperm's linear progression (Suarez & Dai, 1992; Kirkman-Brown & Smith, 2011). Second, the rapid changes in direction may assist sperm in navigating the dead-end cervical crypts found within the oviduct. Third, the thrashing motion of hypermotility appears to assist in the freeing of sperm from an oviductal reservoir, discussed below, and aids in the penetration of the cumulus cells and zona pellucida surrounding the oocyte (Yanagimachi, 1970; Suarez, Dai, DeMott, Redfern, & Mirando, 1992; Suarez, 2008b; Suarez, Katz, Owen, Andrew, & Powell, 1991).

Correct timing of certain capacitation events appears to be crucial. For example, cyclic nucleotide receptor involved in sperm function (CRIS), a cAMP-binding protein, has been shown to control spermiogenesis and sperm motility, specifically the development of the flagellar bend (Krähling et al., 2013). *Cris*-deficient male mice are subfertile. Interestingly, the flagellar beating pattern in CRIS-deficient animals actually resembles the hyperactive phenotype. This is an example of how specific sperm phenotypes, here the development of hyperactivation, must be temporally controlled for successful sperm functioning. Apparently the early acquisition of hyperactivity is detrimental. The premature exocytosis of the acrosome, too, renders sperm incapable of fertilization (Florman and Ducibella, 2006).

A note on sperm energetics

The energy that powers the sperm's movement is derived from several energetic pathways. It is debated whether the bulk of the energy stems from glycolysis or oxidative phosphorylation (Ford, 2006; Turner, 2003). The mitochondria within the sperm cell are relegated to the midpiece. Some studies show that the ATP generated from the midpiece of the sperm cell is confined to only this compartment of the sperm cell. If this is true, the energy necessary for cellular processing along the remaining length of the sperm tail and the sperm head must be generated anaerobically. Indeed, glycolytic enzymes are bound along the length of the sperm tail, and hexokinase is found along the fibrous sheath separate from mitochondria (Storey & Kayne, 1975; Travis et al., 1998). Further evidence comes from experiments that genetically ablate the GAPDH enzyme, an enzyme important for glycolysis. GAPDH knockout (KO) animals have immotile sperm that are unable to fertilize (Miki et al., 2004). This suggests that glycolysis, and not oxidative phosphorylation in the midpiece, is critical for the energy demands of the sperm cell.

The glycocalyx and the function of sialic acids

Mature sperm are covered in a complex sugar coat called a glycocalyx (Schröter, Osterhoff, McArdle, & Ivell, 1999). The glycocalyx includes all the glycans and glycan-containing molecules (glycoconjugates) on a single cell. During epididymal transit, sperm gain and exchange various glycoconjugates secreted by the epithelial cells of the epididymis (Bendahmane & Abou-Haila, 1997). The process of glycosylation typically takes place inside the Golgi apparatus, but since

the Golgi has transformed into a portion of the sperm called the acrosome, sperm rely on the internal environment of the epididymis for changes in glycosylation. The glycocalyx represents the outermost layer of a cell, and thus is the first point of contact between anything the cell encounters externally (Schröter et al., 1999). The encounter may be another sperm cell, the lining of the epididymis where sperm are stored, bacteria, viruses, the cells lining the female reproductive tract, immune cells, or anything a sperm may bump into. The outermost surface is thus important for cell-cell communication.

An immune cell will examine the characteristics of the cells it encounters using receptors on the immune cell surface. Some characteristics will be indicative of a self-cell, meaning another cell of the same multicellular organism that shares the same DNA, and such molecular characteristics are classified as self-associated molecular patterns (SAMPs) (Varki, 2011). Though SAMPs can include any cellular characteristics including proteins, many SAMPs are glycans and glycoconjugates. Sialic acid, an abundant nine-carbon amino sugar found on the surface of sperm and all mammalian cells, can function as a SAMP. Sialic acids cap the terminal end of many glycoconjugates, and the masking properties of underlying immunogenic epitopes on sperm is well documented (Toshimori, Araki, & Oura, 1988; du Toit, Bornman, van Aswegen, & du Plessis, 1994). Sialidases, also known as neuraminidases, are enzymes that cleave sialic acids. Subnormal human semen contains reduced sialic acid (Warren, 1959), and immune cells phagocytose sialidase-treated sperm in greater numbers than fully sialylated sperm (Toshimori, Araki, Oura, & Eddy, 1991; Ma et al., 2016). The removal of sialic acid prevents

chicken sperm from ascending the female reproductive tract when deposited intravaginally, but has no effect when introduced into the uterus, indicating an initial sialic acid requirement in the early stages of the female reproductive tract (Steele & Wishart, 1996). In macaque sperm, the heavily-sialylated sperm coating protein DEFB126 appears to be critical for penetration of cervical mucus (Tollner, Yudin, Treece, Overstreet, & Cherr, 2008), and protects sperm from immune recognition (Yudin et al., 2005). Interestingly, when sperm sialic acids are blocked via lectins or cleaved via sialidases, binding of sperm to the zona pellucida is either reduced or increased, depending on the report (Velasquez et al., 2006; Lassalle & Testart, 1994; Ma et al., 2012). Additionally, sperm contain at least two endogenous sialidases and partially shed their sialic acid coat during capacitation (du Toit, Bornman, Fourie, Aneck-Hahn, & Pitout, 1998; Ma et al., 2012).

There are several proposed theories as to the function of the sialic acid loss in sperm. The first concerns the so-called sperm reservoir. Inseminated sperm can be stored within the female reproductive tract for a matter of days in most mammals, and up to years in certain organisms (Suarez, 2002). In mammals, sperm have been found within the cervical crypts and the oviduct (Topfer-Petersen et al., 2002). Sialic acids seem to play a role in the fixation of sperm to the oviductal walls (DeMott, Lefebvre, & Suarez, 1995). As stated above, sperm acquire a hypermotile phenotype during capacitation. The intense thrashing of the sperm flagellum creates a physical force that appears to aid in shearing of the sperm from the oviductal wall, and is absolutely essential for the sperm to penetrate the zona pellucida surrounding the oocyte to achieve fertilization (Suarez, 2008a). However, the

shedding of sialic acid may also allow the sperm to remove potential anchoring sites as it frees itself (Tecle & Gagneux, 2015).

A non-human sialic acid

Sialic acid comes in two major variations in mammals: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). The enzyme cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) catalyzes the conversion of the Neu5Ac type into Neu5Gc via the addition of a single hydroxyl group (Schauer, Schoop, & Faillard, 1968; Schoop, Schauer, & Faillard, 1969; Schauer, 1970). Humans lost the ability to make Neu5Gc roughly three million years ago via inactivation of the *CMAH* gene, roughly the same time as the origin of the genus *Homo* (Varki, 2010). Thus, the Neu5Gc variant is a non-human sialic acid.

Though humans lack the enzyme necessary to synthesize the Neu5Gc sugar endogenously, we still have the enzymes that attach Neu5Gc to our cells. This can and does occur if we acquire Neu5Gc through a diet of red meat or dairy (Tangvoranuntakul et al., 2003). Even though the two sugars differ by only a single oxygen atom, the Neu5Gc epitope is immunogenic in humans, and is linked to inflammation (Samraj et al., 2015). Furthermore, the presence of Neu5Gc on sperm can reduce fertility in mice when Neu5Gc+ males are bred with immunized Neu5Gc-females (Ghaderi et al., 2011). It is hypothesized that the loss of Neu5Gc contributed to reproductive incompatibility and speciation between ancestral hominins and the lineage leading to the genus *Homo*. However, whether the presence of diet-derived Neu5Gc on human sperm actually causes a loss of fertility remains to be seen.

Recent work has found that Neu5Gc can survive in three million year old fossils from east Africa, opening the possibility of determining the presence of Neu5Gc in hominin fossils (Bergeld et al., 2017).

Sperm survival in the female immune system

Sperm face the significant challenge of surviving in a non-self body. Deposition of semen in the female reproductive tract induces a significant immunological response (Schuberth et al., 2008). Depending on the species, the immune response is either triggered by the sperm themselves, as was originally found in humans (Pandya & Cohen, 1985), or by seminal plasma and not sperm, as was originally found in mice (Robertson, Mau, Tremellen, & Seamark, 1996). Subsequent work produced contradictory results as to whether sperm alone causes the immune reaction in humans, or whether sperm and semen both cause immune responses (Song et al., 2016). A host of immune cells is recruited to the site of insemination in what is termed the leukocytic reaction. This means that during a typical conception, the successful sperm has evaded a swarm of immune cells that outnumber sperm cells by roughly 100 to 1 (De, Choudhuri, & Wood, 1991). In humans, this includes a high number of neutrophils (Pandya & Cohen, 1985; Song et al., 2016).

It is not fully understood how some sperm survive inside the female reproductive tract. As mentioned above, immune cells depend in part on SAMPs for the detection of self-cells, and sialic acids can function as SAMPs. Thus, the heavily sialylated sperm glycocalyx may function as a “home-team jersey” with respect to

the female immune system. I sought to test this hypothesis by examining whether sperm become a greater target of neutrophils following capacitation. Since sperm shed their sialic acid coat at some point during capacitation, I hypothesized that the loss of the “home-team jersey” would trigger the activation of neutrophils. This hypothesis will be addressed in chapter 3.

O-linked N-acetylglucosamine (O-GlcNAc) as a post-translational modification

In the 1980's Carmen Rosa-Torres and Gerald Hart discovered a novel protein-glycan linkage called O-GlcNAc, a single N-acetyl-glucosamine sugar attached to a serine or threonine residue of intracellular proteins (Torres & Hart, 1984). Over the next several decades it became apparent that this post-translational single sugar modification was just as important in cell signaling as protein phosphorylation (Bond & Hanover, 2015; Mishra, Ande, & Salter, 2011). In fact, O-GlcNAc appears to compete for the same serine and threonine residues that phosphorylating enzymes act upon. O-GlcNAc appears to be important for various critical cell processes such as nutrient sensing (Hanover, Krause, & Love, 2012), the cell cycle (Drougat et al., 2012), apoptosis (Johnson, Opimba, & Bernier, 2014), gene regulation (Howerton, Morgan, Fischer, & Bale, 2013), development (Love, Krause, & Hanover, 2010), and as mentioned earlier, signal transduction (Ruan, Singh, Li, Wu, & Yang, 2013; Müller, Jenny, & Stanley, 2013).

Two enzymes control o-GlcNAc cycling. O-GlcNAc transferase (OGT) catalyzes the addition of the GlcNAc sugar from the nucleotide sugar donor uridine diphosphate GlcNAc (UDP-GlcNAc), while O-GlcNAcase (OGA) catalyzes the removal

of the GlcNAc sugar modification (Haltiwanger et al., 1992; Dong & Hart, 1994; Gao et al., 2001). OGT is an X-linked gene that resides on the Xq13.1 genomic locus (Nolte & Müller, 2002). OGT is alternatively spliced, with the largest sized variant residing in the nucleus and cytoplasm (115 kilodaltons (kD)), a medium length variant found within mitochondria (103 kD), and a short variant (75 kD) (Love et al., 2003; Hanover et al., 2003). There is lower expression of both OGT and O-GlcNAc in males (Howerton et al., 2013). Additionally, early prenatal stress is associated with reduced expression of the OGT gene, lower O-GlcNAcylation, and neurodevelopmental disease, such as cognitive impairments and stress dysregulation that can be transferred to the subsequent generation. Disturbed O-GlcNAcylation is associated with various pathologies, including cancer (Itkonen et al., 2013), diabetes (Ruan et al., 2012), and neurodegeneration (Gong, Liu, & Iqbal, 2012). OGT is overexpressed in many cancers, including lung, rectal (Mi et al., 2011), and breast cancers (Caldwell et al., 2010).

The O-GlcNAc modification is noteworthy in relation to sperm due to sperm's reliance on post-translational modifications. Without the ability to express genes or synthesize new proteins, a sperm must perform all of its necessary functions using only the available biological material and post-translational modifications. As stated above, previous studies in capacitation have already implicated serine/threonine and tyrosine phosphorylation as critical components of capacitation. Since O-GlcNAcylation influences and is influenced by phosphorylation, and many kinases appear to be at least partly regulated by O-GlcNAc modification (Dias, Cheung, & Hart, 2012), O-GlcNAc cycling may play a role in sperm. Furthermore, diabetes,

which involves disruption of O-GlcNAc cycling, affects male fertility (Ding et al., 2015; Agbaje et al., 2007; Bond & Hanover, 2013; Kilarkaje, Al-Hussaini, & Al-Bader, 2014).

Additionally, male fertility is also decreased when male rats are exposed to the diabetogenic agents alloxan and streptozotocin (Arikawe, Daramola, Odofin, & Obika, 2006; Paz, Homonnai, Harell, & Kraicer, 1978). Alloxan functions as both an OGT and OGA inhibitor, whereas streptozotocin acts solely as an OGA inhibitor (Konrad et al., 2002; Lee, Alborn, Knierman, & Konrad, 2006; Liu, Paterson, Chin, & Kudlow, 2000). While manipulating glucose metabolism, an important aspect of spermatogenesis, likely implicates a developmental disruption of the sperm cell, it remains to be seen whether O-GlcNAc cycling is critically involved in the final stages of sperm maturation following ejaculation.

Hypotheses

I hypothesize that changes in the sperm glycome play a critical role in sperm function, both in the immunological challenges that sperm face from the female immune system, and in the functional challenge of operating in the absence of gene expression and protein translation. This hypothesis is addressed by three specific aims:

Specific Aims

1. To determine whether Neu5Gc plays a role in human fertility by observing whether human sperm from fertile fathers carry the non-human sugar Neu5Gc.

2. To determine whether sialic acid changes during capacitation influence the activation of neutrophils, the most common type of immune cell in the female reproductive tract.

3. To determine whether O-GlcNAcylation changes during capacitation and/or influences three critical components of capacitation: tyrosine phosphorylation, motility, and acrosomal integrity.

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CHAPTER 2:
DETECTION OF THE DIETARY XENOGLYCAN N-GLYCOLYLNEURAMINIC ACID
(NEU5GC) AND ANTI-NEU5GC ANTIBODIES WITHIN REPRODUCTIVE TRACTS OF
MALE AND FEMALE INFERTILITY SUBJECTS

Abstract

Objective: To assess the frequency of dietary xenoglycan Neu5Gc and antibodies in males and females and its impact on fertility.

Design: Prospective study of semen, uterine lavage, and follicular fluid from subjects undergoing infertility evaluation or in vitro fertilization (IVF) and fertile controls.

Setting: University based infertility program.

Participants: Males (n=23) and females (n=27) undergoing semen analysis and saline infusion sonography as part of their diagnostic evaluation and 37 women undergoing IVF were compared to fertile male (n=15) and female (n=14) controls.

Intervention: Neu5Gc was measured by affinity purified antibody staining on Western blots, flow cytometry, and by high performance liquid chromatography. Anti-Neu5Gc antibodies were determined by ELISA.

Main parameters measured: Frequency and levels of Neu5Gc antigen within sperm and endometrial cells and antibodies in semen, uterine lavage, and follicular fluid. Semen quality and IVF outcomes were assessed between antigen and antibody positive and negative subjects.

Results: In infertile subjects, Neu5Gc was detected in 26% of sperm and 54% of endometrial cells, compared to 0% in male controls (female controls not measured). Anti-Neu5Gc antibodies were identified in 54% of seminal fluid, 41% in uterine lavage and 43% of follicular fluid samples. There were no differences in semen parameters, oocyte quality, and embryo development in the presence or absence of Neu5Gc antigen or antibody. However, clinical pregnancy rate was significantly lower in the presence of anti-Neu5Gc antibodies in uterine lavage (0% vs. 54.5.0%, $p < 0.05$).

Conclusions: Neu5Gc and directed antibodies are present in reproductive tracts of both male and female infertility subjects. Our results suggest their presence may interfere with fertility within the uterine environment.

Introduction

All cell surfaces are coated by a complex array of glycans. In mammals, the majority of these surface glycans are capped with sialic acids, nine carbon backbone, acidic amino sugars. By virtue of their position and high abundance at the outermost cell surface, sialic acids are involved in multiple functions including cellular recognition during development, infection and immune processes (Varki and Schauer, 2009). The role of sialic acids in reproduction is not fully understood, but acquisition of a sialylated glycocalyx is crucial to sperm function (Schröter *et al.*, 1999). Most recently, it has been shown that sperm sialidases are involved in desialylation during capacitation (Ma *et al.*, 2012) and that sialic acids of sialyl Lewis X glycans on human egg zona pellucida are essential for sperm binding (Pang *et al.*,

2011). Implantation also involves L-selectins on the blastocyst known to interact with sialic acid containing ligands on the endometrium (Genbacev *et al.*, 2003) (Margarit *et al.*, 2009). The two most common sialic acids in mammals are N-glycolylneuraminic acid (Neu5Gc) and N-acetylneuraminic acid (Neu5Ac) (Varki and Schauer, 2009). Most mammals have an abundance of each molecule in different ratios on various cell surfaces. In contrast, humans have lost the ability to convert Neu5Ac to Neu5Gc due to a loss-of-function mutation in the cytidine monophosphate N-acetylneuraminic acid hydroxylase (*CMAH*) gene encoding the CMAH enzyme (Chou *et al.*, 1998). As a result, humans lack glycans capped by endogenous Neu5Gc on their cell surfaces and have an excess of Neu5Ac (Chou *et al.*, 2002), a difference in hundreds of millions of molecules per cell. Despite the inability to endogenously produce Neu5Gc, dietary intake of red meat and dairy products, the richest sources of Neu5Gc, can result in accumulation and incorporation of trace amounts of Neu5Gc into the glycocalyx on human cells (Tangvoranuntakul *et al.*, 2003). This has been reported in both human tumors such as breast, colon, and skin cancers (Miyake *et al.*, 1988) and normal tissues including secretory epithelium and blood vessels (Tangvoranuntakul *et al.*, 2003). Moreover, most humans have circulating anti-Neu5Gc antibodies (Tangvoranuntakul *et al.*, 2003, Padler-Karavani *et al.*, 2008) targeting this xenoglycan in serum (Tangvoranuntakul *et al.*, 2003) (Nguyen *et al.*, 2005). Levels of anti-Neu5Gc antibodies vary among different individuals, but it has been suggested that chronic exposure to Neu5Gc and incorporation of this xenoglycan combined with circulating anti-Neu5Gc antibodies can promote chronic inflammatory states contributing to

various diseases (Varki and Schauer, 2009) including some of the diseases associated with Hanganutziu-Deicher antibodies, which are reactive against Neu5Gc (Miwa *et al.*, 2004, Varki, 2009). There is also experimental evidence for Neu5Gc acting as a xeno-auto antigen capable of exacerbating an inflammatory reaction within vasculature endothelium (Pham *et al.*, 2009). A recent study proved that the dietary xenoglycan Neu5Gc causes increased cancer via systemic inflammation in a mouse model (Samraj *et al.*, 2015). More sensitive detection methods have provided evidence for widespread anti-Neu5Gc antibodies in most humans tested, including IgM, IgA, and IgG antibodies (Padler-Karavani *et al.*, 2008). There is mounting evidence that the dietary bioaccumulation of xenoglycan and production of targeting antibodies might underlie various forms of xenosialitis (Varki *et al.*, 2011).

We are unaware of any information on the presence of the xenoglycan Neu5Gc and anti-Neu5Gc antibodies in the human reproductive tract or of their potential consequences on fertility. The female reproductive tract through which sperm must traverse and in which embryos must implant contains antibodies originating from plasma (IgG) and locally produced (IgA) antibodies (Kutteh *et al.*, 1988). Inflammation and the resulting abnormal milieu are a possible mechanism for impaired implantation as seen in certain disease states (i.e. endometriosis, autoimmune diseases, tubal infertility) (Dechaud *et al.*, 1998, Cervera and Balasch, 2008). It has recently been suggested that the loss of Neu5Gc may have caused reproductive incompatibility in ancestral hominin populations due to sexual selection by female immunity against the xenoglycan Neu5Gc on sperm or implanting blastocysts (Ghaderi *et al.*, 2011). In a murine model, *Cmah* null females

with antibodies against Neu5Gc have reduced fertility when mated with Neu5Gc positive wild-type males implicating that female immunity can result in reproductive incompatibility (Ghaderi *et al.*, 2011).

Thus, our objectives were 1) to determine if Neu5Gc antigen and targeting antibodies are present within the human reproductive tracts of male and females; 2) to compare levels between patients seeking infertility evaluation and therapy and fertile controls; 3) to assess whether antigen or antibody presence in the reproductive tract had any impact on semen quality and ART outcomes, and 4) to investigate if they were associated with dietary intake of foods containing the antigenic Neu5Gc xenoglycan.

Materials and Methods:

This study was reviewed and approved by the Christ Hospital Institutional Review Board (Cincinnati, OH) and the study was in compliance with privacy-act guidelines.

Patient Population

From July 2010 to August 2014, subjects were recruited to participate in this prospective clinical study. Subjects enrolled were 1) those undergoing diagnostic infertility testing including a semen analysis (n=23) and saline infusion sonography (n=27); 2) patients undergoing in vitro fertilization (IVF) cycles (n=37, 20 of which underwent a uterine lavage after a saline infusion sonography) and 3) fertile male (n=15) and female oocyte donors (n=14). Written informed consent was obtained

from each subject candidate. Men between the ages of 18–50 years without previous vasectomy were eligible to participate.

Subjects meeting inclusion criteria were given a dietary questionnaire specifically designed for this study (Figure 1) including frequency of meat, dairy, and protein supplement intake, as well as formula use as an infant, all of which contain the xenoglycan Neu5Gc. Xenoglycan load based on the questionnaire were calculated by using values from Tangvoranuntakul *et al.* 2003 (Tangvoranuntakul *et al.*, 2003).

Sample acquisition

Seminal fluid and sperm samples

Semen was collected on site in a sterile plastic specimen cup after a recommended period of abstinence of 2-5 days. The specimens were allowed to liquefy at 37°C for 30 minutes and analyzed within one hour of collection time for semen quality parameters and motion characteristics. Semen samples were then centrifuged twice for 8 minutes each time at 300 x g (1200 RPM), and the supernatant (seminal fluid) was isolated and frozen at -20°C before sample analysis. The remaining sperm pellet was reconstituted with 1-2 mL of phosphate buffered saline (PBS), then frozen at -20°C for later analysis.

Uterine samples

Saline infusion sonography was performed either during the follicular phase of a spontaneous menstrual cycle or following a progestin withdrawal bleed.

Following speculum examination and cervical cleansing, a 2 lumen 5 French balloon

catheter (Cooper Surgical Inc. Trumbull, CT) flushed with sterile saline was placed into the lower uterine segment followed by instillation of sterile saline through the catheter until an adequate evaluation of the uterine cavity was obtained. At completion of the saline infusion sonography, fluid was aspirated collecting both endometrial cells and saline (mean volume ~ 2ml) for uterine lavage specimens. Uterine specimen samples were then centrifuged 15 minutes at 300 x g (1200 RPM), and the supernatant (UL) was isolated from the remaining pellet (EC). Both samples were frozen at -20°C until sample analysis.

Assisted Reproduction

Ovarian Stimulation-Oocyte Retrieval

Prior to ovulation induction for IVF, all female patients had normal uterine cavities as demonstrated on saline infusion sonography and operative hysteroscopy to correct cavitary pathology.

Subjects undergoing IVF used a GnRH antagonist protocol. Briefly, ovarian stimulation was started on cycle day 3 following an oral contraceptive pill withdrawal bleed using daily injections of 150 to 300 IU of recombinant follicle stimulating hormone (rFSH) [Follistim[®] AQ Cartridge; Merck & Co., Inc., Whitehouse Station, NJ, USA]. All cycles were monitored by serum estradiol levels and transvaginal ultrasound and the dose was adjusted according to response. GnRH-antagonist (Ganirelix, 0.25 mg, subcutaneously daily [Merck & Co., Inc., Whitehouse Station, NJ, USA]) was begun when lead follicles were 12-14 mm. When >3 follicles reached 18-20 mm, urinary hCG 10,000 IU was given and was followed by transvaginal oocyte aspiration 36 hours later.

Follicular Fluid and Serum Samples

Follicular fluid from the first dominant follicle was collected for evaluation. FF samples were centrifuged similar to uterine lavage specimens. Granulosa cells were discarded and follicular fluid was stored at -20°C. Serum was collected around the time of hCG administration, and samples were centrifuged (same as uterine lavage), red blood cells discarded, and serum was similarly stored at -20°C until processing.

IVF-ICSI-Embryo Quality Grading, Culture, and Embryo Transfer

Fertilization was performed on the day of retrieval with sperm from a partner or donor using conventional IVF or intracytoplasmic sperm injection (ICSI). Three to four hours after oocyte retrieval, fertilization was performed in IVC-1 media by either conventional insemination or by ICSI in the presence of male factor infertility. The following day, fertilization was confirmed by the presence of two pronuclei. All normally fertilized embryos were cultured sequentially in Vitrolife's G1.3 and G2.3 media, supplemented with 5% protein. An embryo transfer (ET) was performed either three or five days after oocyte retrieval under ultrasound guidance. At least 4 good quality cleavage stage embryos were required to continue culture to the blastocyst stage. Day-3 embryos were evaluated according to cell number and were graded according to blastomere uniformity and the degree of fragmentation. Day-3 embryo quality was ranked as good if >6 cells, and at least grade 2 (slight to moderately uneven cells and slight to moderate fragmentation). Two to three embryos with the highest cell number and best morphology were selected for embryo transfer. Surplus embryos were then transferred to IVC-3

blastocyst media, which were re-evaluated on days 5 and/or 6 for development into suitable blastocysts for cryopreservation (Veeck, 1999).

Pregnancy Confirmation

Pregnancy was confirmed by serial serum β -hCG levels 7 to 10 days following ET. Clinical pregnancies were confirmed with transvaginal ultrasonography by a gestational sac at 6 to 7 weeks. Hormonal supplementation was continued until 10 weeks gestation or until a negative serum β -hCG.

Neu5Gc Antigen Detection

Neu5Gc was detected using an affinity purified polyclonal IgY antibody raised in chickens (Diaz et al., 2009). Frozen sperm were thawed and washed 3x with PBS. For flow cytometry, thawed sperm were first blocked with 0.5% fish gelatin in PBS (PBSfg) for 30 min at RT, then incubated with anti-Neu5Gc IgY at 1:1000 in PBSfg for 30 min on ice, washed 3 times with PBSfg, incubated with secondary donkey-anti chicken Dy488 at 1:1000 in PBSfg for 30 min on ice, washed 3X with PBSfg and measured on a FACScalibur Flow cytometer. Controls included: mild periodate treatment (2mM NaIO₄), which truncates the side chain of sialic acid and confirms sialic acid dependence of Ab staining; inhibition of the Ab with 10 nM polyNeu5Gc, and non-specific isotype control.

Human Sperm Neu5GC Detection via Western Blot

Frozen sperm samples from control fertile males were thawed and centrifuged (500g, 5 minutes). The supernatant, containing seminal fluid, was aspirated, followed by two washing cycles characterized by addition of 1 mL of phosphate buffered saline (PBS), centrifugation (500g, 5 minutes), and aspiration of

the supernatant. The remaining pellet was resuspended in 100 uL of PBS. Protein concentration was determined by BCA, then an equal amount of protein (18 or 30 ug per lane) was loaded on a 1.5 mm 7% SDS-PAGE gel and run until the dye front almost ran off the gel (20 minutes at 20 mAmps per gel, 60-80 minutes at 40 mAmps per gel). Gels were washed with milliQ water, then transferred onto a polyvinylidene fluoride (PVDF) membrane using a Bio-Rad SemiDry Transfer Cell for 1 hour (100 mAmps per membrane). Membranes were washed (5 minutes) in PBS-T (0.1% Tween), blocked for one hour at room temperature in 0.5% fish gelatin PBS-T. Membranes were then incubated in PBS-T with 1:10,000 anti-Neu5GC chicken IgY overnight at 4°C. Membranes were washed four times (7 minutes in PBS-T), followed by a secondary incubation in PBS-T with 1:10,000 LiCOR donkey a-chicken 800 antibody for 45 minutes at room temperature. Membranes were washed four more times (7 minutes in PBS-T) before being imaged on a LiCOR Imaging System.

For western blots, thawed sperm membrane proteins were extracted with lysis buffer and loaded on denaturing SDS PAGE. The gels were transferred to membranes and blocked with PBSfg overnight at 4°C. Blots were incubated in 1:50,000 affinity purified chicken IgY in TBST, 0.5% fish gelatin (Sigma) and 2 mg/ml of purified bovine serum albumin (BSA). Blots were washed and incubated with secondary HRP-conjugated donkey anti-chicken Ab for 1 h at RT. Blots were developed using luminescent substrate (Pierce, Thermo Fisher Scientific, Rockford IL).

Shed endometrial cells were harvested from the pellet of the centrifuged thawed uterine flush samples. The cells were washed 3X with ddH₂O and then

incubated in 2M acetic acid at 80°C for 3 hrs for total sialic acid release. Released sialic acids were analyzed by high performance liquid chromatography (HPLC) of 1,2-diamino-4,5-methylenedeoxybenzene (DMB) derivatized sialic acid extracts obtained from shed epithelial cells. Released sialic acids were filtered through microcon 10 columns, MW 10 KD (Amicon Millipore, Billerica, MA) by centrifuging at 13,000g for 20 min, treated with mild base and derivatized in DMB for 2.5 h at 50°C in the dark prior to HPLC analysis. HPLC was performed over Varian C18 reverse phase column, under isocratic conditions in 83% water, 7% methanol and 8% acetonitrile at a flow rate of 0.9 ml/min over 50 min on a Hitachi HPLC (Manzi et al., 1990, Lewis et al., 2004). Sialic acid standards were from bovine submaxillary mucin as well as commercially available Neu5Ac (Nacalai, San Diego, CA) and Neu5Gc (Inalco, San Luis Obispo, CA).

Anti-Neu5Gc Antibody Detection

Antibody titers in seminal fluid, uterine lavage, follicular fluid, and serum were determined by ELISA using Neu5Gc- and Neu5Ac-polyacrylamide beads as plated targets (Padler-Karavani *et al.*, 2008). Sialic acid dependence of antibody binding was confirmed by mild periodate treatment to destroy the side chain of sialic acids and controlled for by mock treatment without the premixed periodate and sodium borohydride quenching agent.

Additional control samples were tested for anti-Neu5Gc antibodies by plating pooled WT mouse sera free of any anti human IgG and IgA. Human IgG or IgA were coated at various concentrations for standards (Padler-Karavani *et al.*, 2013). All samples were diluted in 1XPBS/chicken albumin/pooled CMAH KO mouse sera (free

of anti-human IgG and IgA). All samples were diluted 100 fold, except for uterine samples which were diluted 10-fold. The secondary antibody used was AP goat anti human IgG or AP goat anti human IgA .

Clinical Outcomes in Fertility Patients

Outcomes measured included 1) The frequency of Neu5Gc Antigen in semen samples and EC and Neu5Gc antibody in seminal fluid, uterine lavage and follicular fluid; 2) the correlation of Neu5Gc antigen or anti-Neu5Gc antibodies on semen parameters (semen quality including volume, concentration, motility, and strict morphology) and ART outcomes (i.e. number of retrieved oocytes, fertilized embryos, embryo quality, rates of blastulation, pregnancy rate (PR) and clinical pregnancy rate (CPR). Analyses were also performed to correlate Neu5Gc antigen or anti-Neu5Gc antibody presence in reproductive tissue with dietary intake.

Statistics

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 18; SPSS, Inc., Chicago, IL). Student's t-test was utilized to compare means for parametric data between Neu5Gc Ag and Ab positive and negative subjects. Chi-square was used for categorical comparisons. Pearson correlation was used for correlation analysis. Significance was assessed at $p < 0.05$. Means expressed as $\pm$ SEM.

Results

Detection of Neu5Gc Antigen and Anti-Neu5Gc Antibodies

Semen and Sperm Samples

Neu5Gc was identified in 26% (6/23) of sperm samples from males undergoing fertility evaluation compared to 0/15 fertile male controls, $p < 0.05$. AntiNeu5Gc antibodies in males undergoing fertility evaluation were detected in 54% of seminal fluid (SF) (8/13; insufficient sample in 10 patients. Specifically, 54% had IgA Abs detected, 38% had IgGs, and 38% had IgMs. Antibodies were not measured in fertile male controls. (Figure 2 and Table 1).

Uterine Lavage, Follicular Fluid, and Serum

The presence of Neu5Gc in endometrial cells from female infertility patients was detected in 54% (9/17; insufficient sample in 10 patients). Neu5Gc antigen was not measured in fertile female controls. Anti-Neu5GC antibodies were detected in 41% of uterine lavages (11/27) from female infertility patients (specifically, 37% IgA, 27% IgG, and 0% IgM) compared to none of the female controls (0%, 0/14, $p < 0.05$). Anti-Neu5Gc antibodies were detected in 43% of follicular fluid (16/37), and 44% of serum ([14/32]; insufficient sample in 5 patients) (Table 2). Of the follicular fluid samples, 22% had IgA, 32% IgG, and 19% IgM while in serum samples, 33% had IgA, 60% IgG, and 0% IgM, respectively. Neu-5Gc antibodies in follicular fluid and serum were not measured in fertile female controls (Table 2).

Demographics

Male Subjects

Demographics including age (30.5 ± 3.5 years vs 32.2 ± 5.8 years) or BMI (27.3 ± 5.7 kg/m² vs 28.7 ± 6.5 kg/m², p -NS) were similar between patients that were positive or negative for Neu5Gc antigen on sperm or targeting antibodies within SF and controls (31.4 ± 8.5 years and 25.3 ± 4.7 kg/m²) (Table 3).

Female Subjects

Age (36.8 ± 6.9 years vs 33.0 ± 5.7 years), BMI (29.1 ± 5.7 kg/m² vs 26.5 ± 8.3 kg/m², p-NS) or primary infertility diagnosis (data not shown) was not different between patients that were positive or negative for anti-Neu5Gc antibodies in uterine or ovarian environments. While fertile controls were significantly younger (27.7 ± 2.9 years, $p < 0.05$), BMI was similar (25.2 ± 4.7 kg/m²) to fertility clinic patients. Subjects who were positive for any antibody type (IgA or IgG) within the uterine lavage had a lower gravidity (1.5 ± 1.7 vs 2.9 ± 1.8 , $p < 0.05$) and parity (0.2 ± 0.4 vs 1.3 ± 1.5 , $p < 0.05$) when compared to antibody negative subjects and controls (1.4 ± 0.4 and 1.3 ± 0.4 , p-NS. No difference was seen in any demographic parameter and antibody presence within follicular fluid subjects undergoing IVF cycles.

Diagnostic Testing and Clinical Outcomes

Semen Analysis and Saline Infusion Sonography

Overall, the presence of Neu5Gc antigen or anti-Neu5Gc antibodies did not correlate with differences in semen quality or the presence of uterine pathology. Specifically, semen volume (3.0 mL vs 2.7 mL), total sperm concentration (55.7×10^6 vs. 42.1×10^6), motility (54.2% vs. 57.9%) and morphology (5.5% vs 6.4%), and intrauterine pathology identified on saline infusion sonography (18% vs. 38%) were similar regardless of Neu5Gc antigen or anti-Neu5Gc antibody status (Table 3).

Follicular Fluid, Uterine Lavage and IVF Outcomes

In those undergoing IVF, the total number of retrieved oocytes (10.7 ± 5.5 vs 11 ± 5.9 , p-NS) and Metaphase II oocytes (8.1 ± 4.2 vs 8.1 ± 4.0 , p-NS) were similar

regardless of detectable anti-Neu5Gc antibodies within follicular fluid of fertility patients. There were no adverse effects of anti-Neu5Gc antibodies on high quality embryos and rates of blastulation (data not shown). Pregnancy rate (PR) and clinical pregnancy rate (CPR) of these 37 subjects were similar in follicular fluid anti-Neu5Gc antibody positive or negative patients, respectively (25.0% vs 23.8%, p-NS) and CPR (18.8% vs 23.8%, p-NS).

Analysis was done on 20 of 37 IVF subjects who had uterine lavage with known Neu5Gc antigen and antibody status, The CPR per patient was significantly lower in those who were positive for any antibody (0% vs. 54.5%, $p<0.05$) and specifically for IgG antibody (0% vs. 50%, $p<0.05$), though not different in the presence or absence of IgA antibody (0% vs. 42.8%, $p=0.08$). CPR per cycle (fresh and frozen cycles, $n=27$) was lower in the presence of any antibody (any antibody - 0% vs. 50.0%, $p<0.01$, IgA antibody, IgA - 0% vs. 31.4%, $p<0.01$); and IgG antibody IgG 0% vs. 46.1%; $p<0.05$) when compared to antibody negative subjects (Figure 4).

Dietary Intake and Correlation between Serum and Reproductive Fluids

No positive correlation was noted between dietary intake of red meat, dairy products nor infant formula use and Neu5Gc or anti-Neu5Gc antibody status in any reproductive fluid or serum. The presence of any serum anti-Neu5Gc antibody positively correlated to antibody presence within follicular fluid ($r=0.58$, $p<0.001$).

Discussion

To our knowledge this study is the first to explore the frequency of incorporation of non-human xenoglycan Neu5Gc sialic acid and the potential role of

Neu5Gc and targeting antibody in male and female infertility subjects. Our data demonstrate that Neu5Gc and targeting antibody are abundantly expressed within both the male and female reproductive tracts of infertility subjects. Our preliminary results also suggest that the presence of the Neu5Gc xenoantigen or anti-Neu5Gc antibodies do not adversely impact sperm parameters or ovarian function including oocyte development and embryo quality. However, the presence within the uterine environment may possibly impact implantation and pregnancy outcomes.

The presence of Neu5Gc antigen or anti-Neu5Gc antibodies is not surprising as other investigators have reported the presence of this xenoglycan and associated antibodies in both normal and diseased human tissues (Tangvoranuntakul *et al.*, 2003, Miyake *et al.*, 1988). With respect to fertility, it has been suggested that antibodies within the uterine environment may impair fertility by two potential mechanisms including sperm inactivation and embryo rejection or failed implantation secondary to incompatibility between the blastocyst and receptive uterus (Ghaderi *et al.*, 2011). Our results reveal a lower CPR in the presence of anti-Neu5Gc antibodies within the uterine environment. Moreover, none of the women positive for anti-Neu5Gc IgG antibodies achieved pregnancy even with repeat embryo transfer. In light of our study subjects undergoing IVF and the production of HQ embryos, it would suggest that implantation may be negatively affected in the presence of anti-Neu5Gc antibodies, contributing to infertility in these subjects.

The unique human sialic acid biology has brought about a situation where a dietary molecule can get incorporated into healthy human tissues and become a xeno-auto-antigen. The presence of the xeno-auto-antigen Neu5Gc in human

reproductive tracts, cells, and secretions could contribute to anti-glycan immune related impaired fertility (xenosialitis affecting fertility). The presence of a non-self glycan (Neu5Gc) at the molecular frontier of the embryo, combined with antibodies targeting this molecule, is consistent with our observed adverse effects on implantation success.

The fact that we found no consistent correlations between the dietary factors and antigen or antibody status is not surprising. The accumulation of xenoglycan Neu5Gc is highly variable between individuals and between tissues within individuals (Tangvoranuntakul *et al.*, 2003). Additionally, dietary intake over a lifetime is challenging to assess accurately and would require very strong associations to detect significant correlations with our current intake assessment methodology. For future studies to better address the relationship between dietary load of xenoglycans and the biological consequences, extensive resources will need to be devoted to either collecting dietary intake prospectively over long periods of time or developing detailed recall questionnaires specifically targeting dietary sources of Neu5Gc.

Our study demonstrates that Neu5Gc and targeting Ab are abundant within the human reproductive tract. To our knowledge, this is the first study to evaluate the role of this xenoglycan and targeting antibodies within human fertility. However, there are a number of limitations including: 1) small sample size which was not powered to detect clinical outcomes; 2) fluids collected were not all from same cohort (semen-UL-FF-serum); and 3) the precise localization of Neu5Gc

antigen in the uterus could not be established beyond its presence on shed epithelial cells recovered from the UL.

Future studies will focus on elucidating the potential functional deficits of sperm bearing Neu5Gc and targeting antibodies and evaluating the male and female systems within the same couple in order to better understand the role of Neu5Gc and targeting antibodies in sub or infertile couples. Such investigations will reveal to what extent xenosialitis, the contribution of the xenoglycan Neu5Gc to heightened or chronic inflammation, also affects human reproduction (Varki *et al.*, 2011) and will point to potential mechanisms involved.

Appendix

Abbreviations: 1,2-diamino-4,5-methylenedioxybenzene (DMB), assisted reproductive technologies (ART), clinical pregnancy rate (CPR), cytidine monophosphate N-Acetylneuraminic acid hydroxylase (*CMAH*), embryo transfer (ET), high performance liquid chromatography (HPLC), *in vitro* fertilization (IVF), N-glycolylneuraminic Acid (Neu5Gc).

**Xenosialic Acid Neu5Gc in
helpings of sialic acid rich foods**

	Eggs	2 eggs	no Neu5Gc
	Bacon	3 strips	2.6 mg
	Breakfast Sausage	2 links	3.0 mg
	Hot dog or Sausage	1 (2oz)	1.68 mg
	Lunch meats (roast beef, ham, corn beef, salami)	2 slices	1.68 mg
	Beef, Pork, Ham, or lamb (size of a deck of cards)	4 oz	3.36 mg
	Ground meat (hamburger, meatloaf)	1 med. patty (3oz)	2.52 mg
	Milk	1 cup	1.92 mg
	Milk on cereal	1/2 cup	0.96 mg
	Cheese	1 slice, 1/2 cup shredded	0.48 mg
	Yogurt	1 cup	1.92 mg
	Ice cream	1 cup	1.92 mg
	Protein supplement (whey)	20-30 g	0.3 mg

How Often Do You Eat These Foods?

	Serving size	NEVER	Or	< 1 per month	1 per month	2-3 per month	1 per week	2-6 per week	1 per day	2+ per day
Bacon	3 strips	☐	☐	☐	☐	☐	☐	☐	☐	☐
Breakfast Sausage	2 links	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hot dog or Sausage	1 (2oz)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lunch meats (roast beef, ham, corn beef, salami)	2 slices	☐	☐	☐	☐	☐	☐	☐	☐	☐
Beef, Pork, Ham, or lamb	4 oz (size of a deck of cards)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Ground meat (hamburger, meatloaf)	1 med. patty (3oz)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Milk	1 cup	☐	☐	☐	☐	☐	☐	☐	☐	☐
Milk on cereal	1/2 cup	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cheese	1 slice, 1/2 cup shredded	☐	☐	☐	☐	☐	☐	☐	☐	☐
Yogurt	1 cup	☐	☐	☐	☐	☐	☐	☐	☐	☐
Ice cream	1 cup	☐	☐	☐	☐	☐	☐	☐	☐	☐
Protein supplement (whey)	20-30 g	☐	☐	☐	☐	☐	☐	☐	☐	☐

Figure 2.1: Dietary Questionnaire.

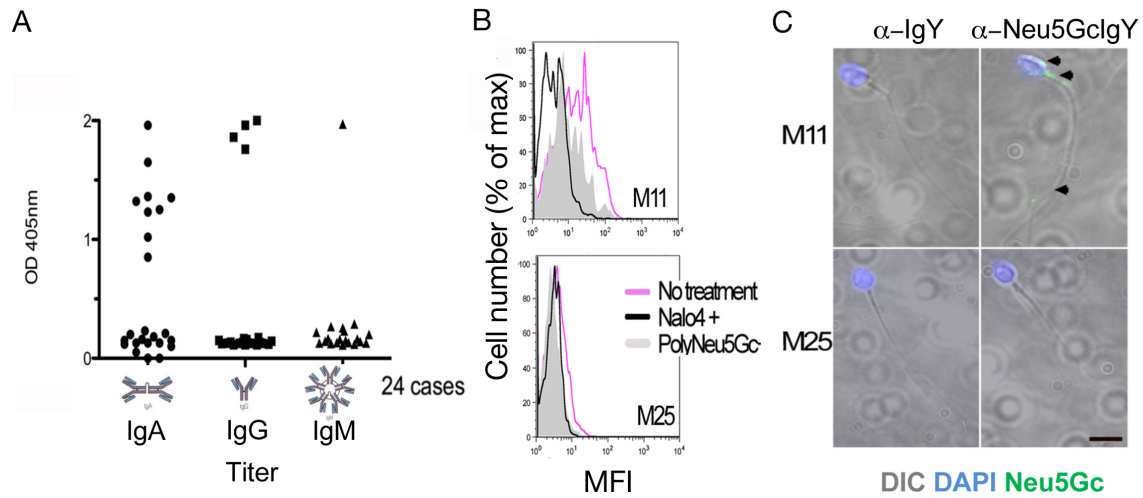
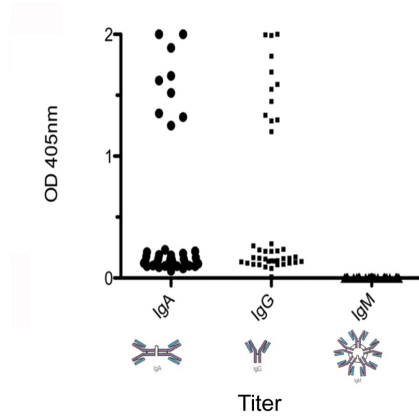


Figure 2.2. ELISA for Anti-Neu5Gc antibodies, flow cytometry and fluorescent microphotograph of sperm. A) ELISA data for anti-Neu5Gc antibodies in seminal fluid from infertility patients. B) Flow cytometry on sperm cells from infertility patients showing Neu5Gc positive (M11) and negative individual (M25), treatment by sodium periodate abrogates the side chain of sialic acid and reduces the signal and so does blocking with poly Neu5Gc. C) fluorescent microphotograph of sperm positive and negative for Neu5Gc (green fluorescence for anti-Neu5Gc antibody indicated by black arrowheads, sperm nuclei stained blue with DAPI).

A



B

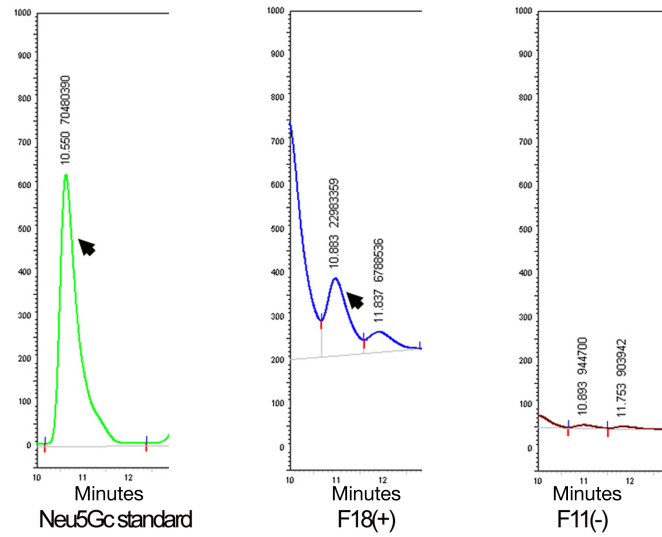
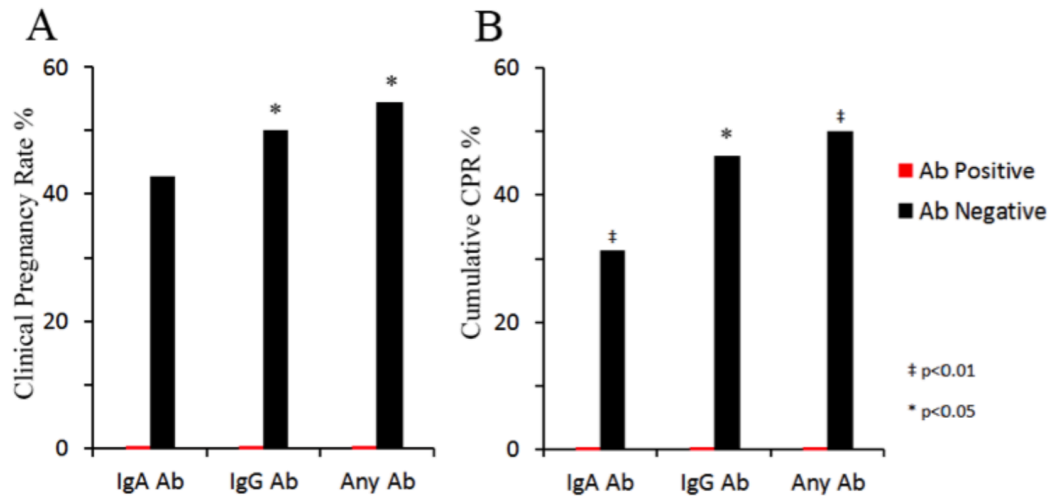


Figure 2.3: Uterine lavage anti-Neu5Gc antibodies by ELISA. A) Measurements of anti-Neu5Gc antibodies by ELISA in uterine lavage of infertility patients. B) Detection of Neu5Gc in uterine lavage (shed endometrial cells) by HPLC, standard, positive sample and negative sample.



*p<0.05; †p<0.01.

Figure 2.4: Pregnancy and clinical pregnancy rates in IVF subjects positive and negative for anti-Neu5Gc antibodies (Ab) in uterine lavage.

Table 2.1: Prevalence of Neu5Gc antigen and Anti-Neu5Gc antibody in semen of male subjects undergoing fertility evaluation compared to controls.

	Neu5 Gc Antigen in Sperm Samples	Neu5 Gc Antibody in Seminal Fluid
Males Undergoing Fertility Evaluation	26% (6/23)*	54% (8/13)†
Male Controls	0% (0/15)*	Not measured

*p<0.05

†Insufficient seminal fluid sample in 10 patients

Table 2.2: Prevalence of Neu5Gc antigen and Anti-Neu5Gc antibodies in uterine lavage, follicular fluid, and serum compared to controls.

	Uterine Lavage Neu5 Gc Antigen	Uterine Lavage Neu5 Gc Antibody	Follicular Fluid Neu5 Gc Antibody	Serum Neu5 Gc Antibody
Females Undergoing Fertility Evaluation	54% (9/17)*	41% (11/27)†	43% (16/37)	44% (14/32)‡
Female Controls	Not measured	0% (0/14)†	Not measured	Not measured

*Insufficient follicular fluid sample in 10 patients

†p<0.05

‡ Insufficient follicular fluid sample in 5 patients

Table 2.3: Comparison of demographics in Neu5Gc antigen positive and negative semen and endometrial cells subjects.

	Sperm Neu5Gc		p-value
	+	-	
Subjects	n=6	n=17	
Age (years)	30.5 ± 3.5	32.2 ± 5.8	NS
BMI (kg/m²)	27.3 ± 5.7	28.7 ± 6.5	NS
Semen Volume (ml)	3 ± 1.6	2.7 ± 1.4	NS
Sperm Conc. (million)	55.7 ± 62.7	42.1 ± 31.1	NS
Total Motility (%)	54.2 ± 20.1	57.9 ± 12.3	NS
Normal Morphology (%)	5.5 ± 6.1	6.4 ± 15.1	NS
	Endometrial Cells Neu5Gc		
	+	-	
Subjects	n=9	n=8	
Age (years)	36.8 ± 6.9	33 ± 5.7	NS
BMI (kg/m²)	29.1 ± 5.7	26.5 ± 8.3	NS
Gravidity	1.4 ± 1.6	1.5 ± .6	NS
Parity	1 ± 1.7	0.3 ± .5	NS
Pathology on SIS (%)	18	38	NS

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CHAPTER 3:

MULTIPLE INNATE SELF-SENSING IMMUNE RECEPTORS (SIGLECS) IN THE FEMALE REPRODUCTIVE TRACT

Abstract

A sperm that fertilizes an egg has successfully survived multiple checkpoints within the female reproductive tract, termed pre-fertilization events. The leukocytic response is a pre-fertilization event in which sperm trigger an immunological response that promotes homing of circulating leukocytes to the uterine lumen to destroy the majority of sperm. Like in all mammalian cells, various glycoconjugates decorate the sperm surface. Sialic acid is very abundant at the sperm surface as it caps the majority of glycan chains and has documented roles in regulating migration through cervical mucus, formation of the sperm oviductal reservoir, and its role in sperm capacitation. However, the role of sperm sialic acid in the leukocytic reaction remains unknown. Sialic acids and their cognate ligands Siglecs (sialic acid-binding immunoglobulin-like lectins) play a pivotal role in regulating immune responses in many cellular and disease states. We sought to investigate the role of sperm sialic acid for inhibition of neutrophil activation, as neutrophils are one of the major immune cells of the leukocytic reaction. In addition, we sought to determine if Siglecs are expressed by human and mouse endometrium, as the endometrium has an established role in initiating the leukocytic reaction. Surprisingly, capacitated, less sialylated sperm did not increase neutrophil activation *in vitro*. In addition, we describe the expression of Siglecs on the endometrium, their regulation by estrogen,

and their interaction with sperm. Our data indicate that sperm sialic acid may interact with endometrial Siglecs to promote sperm survival during the leukocytic reaction.

Introduction

Like all living cells, sperm are covered in a glycocalyx, a complex “sugar coat” consisting of diverse glycan chains conjugated to cell surface proteins and lipids (Figure 1A). Unlike most cells, mammalian sperm are introduced into the reproductive tract of another individual where they have to survive and remain functional until they fertilize an egg in the ampulla of the fallopian tubes (Tecle and Gagneux, 2015). This feat is made more difficult because of the presence of large numbers of immune cells and secretions in the female reproductive tract (Brandtzaeg, 1997). The female tract is well equipped with innate immune receptors including several Toll-like receptors (Zandieh et al., 2015) that target potentially harmful, non-self molecules such as pathogen-associated molecular patterns. In contrast, previous work has shown that components of the glycocalyx can function as self-associated molecular patterns (SAMPs) by interacting with inhibitory receptors on immune cells (Varki, 2011). Thus, sperm glycans may contribute to successful fertilization by binding to glycan-recognizing receptors along the female reproductive tract, allowing sperm to “blend in” with local female cells and evade destruction by the immune system (Springer and Gagneux, 2013).

An abundant component within the sperm glycocalyx is the terminal monosaccharide sialic acid. Sialic acid is a nine carbon, amino sugar located at the terminal end of gangliosides, N-glycans and O-glycans (Varki et al., 2015). Sialic acids can modulate immune response by binding to sialic acid-binding immunoglobulin-like lectins (Siglecs) (Crocker et al., 2007, Varki and Gagneux, 2012), (Figure 1B). All Siglecs contain one or more Ig domains (C2-set) and a N-terminal sialic acid-binding domain (V-set). Inhibitory Siglecs contain cytosolic ITIM (immunoreceptor tyrosine-based inhibition motif) and ITIM-like domains (ITIM-L) (Varki and Angata, 2006). In contrast, activating Siglecs contain a transmembrane ITAM (immunoreceptor tyrosine-based activation motif) domain. Via the ITAM, activating Siglecs bind to DAP12 which is phosphorylated by a Src family tyrosine kinase allowing for recruitment of SYK resulting in a signaling cascade that promotes activation of an immune response (Crocker and Redelinghuys, 2008).

It is unknown whether Siglecs play a role in the modulation of the immune system during insemination. Sperm arriving in the uterus trigger the leukocytic reaction, an immunological response that promotes homing of circulating leukocytes to the uterine lumen. This results in the active destruction of the majority of sperm. It is not fully understood how some sperm survive the leukocytic reaction. The female reproductive tract has been previously shown to have immune surveillance that is hormonally regulated to promote fertility while still providing antimicrobial resistance (Wira et al., 2011). However, whether Siglecs are present or under hormonal control within the female reproductive tract remains to be seen.

On the other hand, the presence of sialic acid appears to be important for sperm function. For instance, sialic acid on highly sialylated beta-defensin 126 is absolutely required for human sperm to penetrate cervical mucus and for sperm to adhere to the oviductal epithelium before capacitation occurs (Tollner et al., 2012). Capacitation refers to several cellular processes that occur in mammalian sperm before they are competent to fertilize an egg, including an increase in mitochondrial activity to sustain vigorous motility (Srivastava and Abou-Issa, 1977, Ma et al., 2012, Huo et al., 2002). Interestingly, previous reports indicate that mammalian sperm shed sialic acids during capacitation (Ma et al., 2012). Though sialic acids are known to be critical for sperm migration, it is unclear whether they also facilitate sperm survival within the female reproductive tract.

We hypothesize that sperm sialic acid and female-expressed Siglecs may modulate the leukocytic reaction and promote sperm survival within the female reproductive tract. Indeed, a recent report has shown that sialic acid on sperm is protective against phagocytosis by immune cells, *ex vivo* (Ma et al., 2016). To begin to address this, we investigated interactions between sperm and isolated leukocytes *in vitro*. Neutrophils were chosen because they represent the most abundant immune cell in the human leukocytic reaction following insemination (Thompson et al., 1992) and have documented expression of inhibitory Siglecs 5 and 9 (Cornish et al., 1998, Ali et al., 2014). As capacitation is associated with the loss of sialic acid, which are required to engage inhibitory Siglecs, we investigated via several measures of neutrophil reactivity whether capacitated (C) sperm invoke greater reactivity than non-capacitated (NC) sperm. Within the female reproductive tract,

the endometrium has an established role in responding to and promoting the destruction of pathogens and/or sperm in the uterus (Nasu and Narahara, 2010). However, at least some sperm must survive within the female reproductive tract during the fertile window in order to achieve fertilization. Therefore we examined the expression of Siglecs in the human and mouse endometrium, as well as whether Siglec expression is estrogen-dependent. Finally, we tested sialic acid-dependent binding of sperm by Siglecs using recombinant chimeric Siglec Fc proteins.

Results

Sperm exposed to capacitating conditions immediately begin shedding sialic acids.

We measured the temporal dynamics of sialic acid loss in capacitating sperm using the reporter molecule 4-MU-NANA. Cleavage of the sialic acid analog NANA results in the emission of fluorescence from the separated 4-MU molecule. Sperm were incubated together with 4-MU-NANA in either non-capacitating or capacitating buffers, and the resulting changes in fluorescence were measured via plate reader. Sperm show an increase in fluorescence immediately upon exposure to capacitating conditions, indicative of sialidase activity. Non-capacitated sperm do not show any increase in fluorescence. The percentage of capacitated sperm following *in vitro* capacitation was measured via flow cytometry by probing for an increase in mitochondrial activity using the mitochondrial membrane potential probe JC-1. The majority of C sperm showed a robust increase in JC-1 staining compared to NC sperm (~81% vs ~10%) (Figure 2).

Capacitated sperm do not increase neutrophil activation in vitro.

We investigated the impact of sperm exposure on neutrophil activation *in vitro* using four different measures. First, activation of human neutrophils was determined by shedding of the quiescence marker L-selectin after exposure to capacitated (C) and non-capacitated (NC) human sperm. The amount of L-selectin present on neutrophils after incubation *in vitro* was probed using L-selectin antibodies and analyzed via flow cytometry. In one trial exposure to both C and NC sperm preserved L-selectin as compared to untreated neutrophil controls (Figure 3a), however this effect was not consistent (Supplemental Figure 1). We next determined the reactivity state of neutrophils exposed to C and NC sperm by assessing levels of the activation marker CD11b. Anti-CD11b reactivity was measured via flow cytometry. C sperm did not generate activation of neutrophils *in vitro*, except for a small subpopulation in 1 out of 3 experiments (Supplemental Figure 2). In 1 out of 3 replicates, C sperm generated the weakest activation response in neutrophils as compared to that of NC sperm and untreated neutrophils (Figure 3b). Neutrophils can perform their microbicidal function via the creation of reactive oxygen species (ROS) following endocytosis of foreign microbes (Lamb et al., 2012). Thus, we investigated the generation of reactive oxygen species in neutrophils after exposure to C and NC sperm as an alternate measure of neutrophil activation. ROS production was probed using OxyBURST and measured via flow cytometry. C sperm did not generate strong increases in ROS production by neutrophils (19.2% OxyBURST positive) compared to that of NC sperm (28.2%) and

untreated neutrophils (25.8%, Figure 3c). Neutrophils can immobilize and destroy pathogenic threats via the creation of neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). Neutrophils triggered by phorbol 12-myristate 13-acetate (PMA) displayed a robust increase in NET formation that immobilized both NC and C sperm (Figure 3d, Supplemental Video 1). Neutrophils that were not exposed to PMA and briefly exposed to NC or C sperm remained membrane intact and did not capture NC or C sperm.

The human endometrium expresses Siglecs and downstream signaling proteins.

In light of our surprising results regarding sperm sialic acid and neutrophils, we decided to investigate the expression of Siglecs in the female reproductive tract. As heavily sialylated NC sperm directly interact with the endometrium, we hypothesized that the endometrium may express Siglecs. We probed for the expression of various Siglecs in human post-mortem uterine samples and found that Siglec-10 was robustly expressed (n=7) (Figure 4). We also screened two human endometrial cell lines (Ishikawa and HEC-1B) for cell surface SIGLEC expression by flow cytometry analysis (Table 1, Figure 5). These analyses revealed that both cell lines express two inhibitory Siglecs (Siglec-10 and Siglec-11), as well as activating Siglecs (Siglec-16). Importantly, both SHP-1, the downstream signaling molecule of immune-inhibitory Siglec-10 and Siglec-11, and DAP12, the downstream signaling molecule of immune-activating Siglec-16, are expressed in the two endometrial cell lines (Figure 5).

SIGLEC expression in the human endometrium is sensitive to 17-beta- estradiol.

Next, we investigated if the expression of SIGLEC-10, 11 and 16 in the endometrium was under hormonal control. Both Ishikawa and HEC-1B cell lines have been used extensively to investigate hormonal responses of various genes or in the context of infection (Tamm-Rosenstein et al., 2013, Yang et al., 2012, Boehme et al., 2009, Guseva et al., 2005). While progesterone treatment did not significantly alter the expression of any Siglecs in either cell line, the expression of three Siglecs increased in both cell lines, although with different dynamics. In HEC-1B (Figure 6a), all tested concentrations of 17-beta estradiol increased the expression all three Siglecs at various time points. Conversely, Siglec-10 expression in Ishikawa cells (Figure 6b) increased the most at 1 hour post dosing with 1uM 17-beta-estradiol treatment.

Human sperm bind select Siglecs in a sialic acid dependent way.

To investigate the possible role of Siglecs in the leukocytic reaction, we assessed the binding of Siglec-FC fusion proteins of Siglecs expressed by the endometrium to human sperm. Siglec-FC fusion proteins contain the V-set and one or more underlying C-set of a given Siglecs in frame to the human IgG FC region. We tested the binding of Siglec10,11 and 16 FC fusion proteins to sperm. Sperm did not bind Siglec-11 or Siglec-16 FC proteins (Figure 7a and 7b). However, sperm robustly bound the Siglec-10 FC fusion protein. In addition, this binding was sialic acid dependent as no binding was observed using a Siglec-10 mutant protein with a R120K mutation, which abrogates its sialic acid binding ability (Figure 7c).

Furthermore, we found by FACS analysis using an anti-CD24 antibody, that a subset (approximately 25%) of human sperm expresses CD24, a known ligand for Siglec-10 (Figure 7d).

Mouse endometrium also expresses Siglecs.

We initially focused on the Siglec-G, the mouse homologue of Siglec-10 that was expressed in the human post-mortem endometrium and human endometrial cell lines. However, both IHC and FACS analyses revealed an absence of Siglec-G expression in the mouse endometrium. However, we were able to identify the expression of Siglec-3 in the mouse uterus by qPCR (Figure 8a) and specifically in the endometrium by IHC (Figure 8b). Both the IHC and qPCR results indicate that Siglec-3 expression may be under hormonal control as it is significantly higher in pro-estrus in both cases (Figure 8a and 8c).

Discussion

As sialic acid and Siglecs have a well-established role in modulation of immune responses, we hypothesized that sperm sialic acid would interact with various inhibitory Siglecs expressed by neutrophils to inhibit their activation. In contrast to our hypothesis, co-incubation of neutrophils with C sperm did not result in additional neutrophil reactivity compared to when neutrophils were co-incubated with NC sperm. Though C sperm immediately begin to shed a portion of their sialic acids during capacitation, they still retain residual sialic acid within their glycocalyx

and would still be able to interact with inhibitory Siglecs. Co-incubation of neutrophils with C sperm resulted in similar levels of neutrophil reactivity when measured via CD11b reactivity, reactive oxygen species production, and in the shedding of L-selectin, a leukocyte quiescence marker (Lizcano et al., 2017). Furthermore, neutrophils are capable of capturing both NC and C sperm via the formation of NETs *in vitro*.

This discrepancy and departure from our hypothesis may be due to the fragile nature of sperm cells and the difficulty in keeping sperm alive in a non-capacitated state *in vitro*. Though efforts are made to keep the number of live sperm cells equal between conditions, non-capacitated sperm have a higher rate of cell death than capacitated sperm, as evidenced by a higher proportion of propidium-iodide positive cells (see Supplemental Figure 3). The high rate of apoptosis in non-capacitated sperm likely results in the presentation of danger-associated molecular patterns (DAMPs) that have been shown to contribute to the overall reactivity of nearby immune cells (Miller et al., 2011). Others have previously suggested that a primary purpose of the leukocytic reaction is to remove dead and/or dying sperm (Aitken et al., 2015). If the non-capacitated sperm samples contain a higher percentage of such cells, the resultant DAMPs may generate a danger signal that overpowers the reduction of immunoreactivity imparted by the sperm sialic acid-inhibitory Siglec interaction. While all efforts were taken to reduce the number of dead or moribund sperm in all conditions, slight differences in viability remain. Controlling for a higher level of dead or moribund sperm in the non-capacitated condition is challenging because viability-enhancing components such as albumin

also function as capacitation factors. While future experiments might inspect neutrophil reactivity to increasing proportions of dead sperm, the current data must be interpreted with the caveat that the observed neutrophil reactivity towards non-capacitated and capacitated sperms is confounded with roughly 20% difference in sperm viability.

Nevertheless, we have documented the expression of Siglecs-10, 11 and 16 in human endometrial cell lines. Endometrial cells also express SHP-1 and DAP12, the respective downstream signaling components of inhibitory and activating Siglecs. In addition, we show that the expression of human Siglec-10, -11 and -16 is sensitive to exogenous 17-beta estradiol treatment. Ishikawa cells express estrogen receptor alpha (ERa) and beta (ERb) while HEC-1B cells only weakly express ERb (Guseva et al., 2005). Preliminary bioinformatic analysis indicates that estrogen response elements are in the genomic sequences flanking Siglec-11 and Siglec-16.

Transcription-binding sites for one of more transcription factors (AP-1, SP-1, NFkB and CREB1) known to act in the tethering pathway for estrogen receptors have been identified in the genomic sequences flanking all three Siglecs by bioinformatics analysis. The robust transcriptional response seen in Ishikawa cells at one hour after treating with estrogen indicates that Siglecs-10, -11 and -16 may be regulated by the cell surface estrogen receptor GPER, which is only expressed in Ishikawa cells, not HEC-1B (Skrzypczak et al., 2013).

We have shown Siglec-10 expression in post-mortem human endometrium sections and have established that human sperm bind Siglec-10 in a sialic acid-dependent manner. We also report that a subset of sperm bears CD24, a sialic acid

containing glycoprotein that is an established activator of Siglec-10 signaling. Although a recent paper has identified the presence of Siglec-10 in first trimester placental cells (Rumer et al., 2013), the majority of work on Siglec-10, and its mouse homologue Siglec-G, concerns its role in the immune system. Generally, Siglec-10/G is an inhibitor of immune responses in various cellular contexts. For example, mouse Siglec-G functions in B1a-cells to inhibit their proliferation and induce their tolerance to self antigens (Nitschke, 2015). Consequently, genetic ablation of Siglec-G (in select generic backgrounds) results in an age-dependent autoimmunity phenotype and increases the severity of collagen-induced arthritis in an autoimmune prone mouse model (Bökers et al., 2014). CD24 is a ligand for Siglec-G and Siglec-10 in mouse and human, respectively. *In vivo*, Siglec-G and CD24 are required to inhibit the production of inflammatory cytokines in an acetaminophen induced liver necrosis model and graft versus host disease model (Chen et al., 2009). Our results indicate that sialic acid-containing ligands, including those carried by CD24, on human sperm may interact with endometrial expressed Siglec-10 during the leukocytic reaction (Figure 9). *In vivo*, this interaction could inhibit the release of cytokines by the endometrium, decrease the recruitment of leukocytes to the uterine lumen during the leukocytic reaction, and result in increased sperm survival. Future experiments exposing live sperm to cultured female tract cell lines should be very informative in this regard.

The reasons why strong binding were only seen with the Siglec-10-FC construct could include one or more of the following. While Siglecs are defined as sialic acid binding, the precise specificity for sialylated glycan patterns of each Siglec

remain poorly defined. In addition, Siglecs can themselves aggregate on the cell surface and such aggregation strongly influences their binding to cis or trans ligands (Razi and Varki, 1999). The use of soluble Siglec-FC constructs as molecular probes do not allow to fully mimic these cell-surface mechanisms.

To investigate the role of Siglec-10 in the leukocytic reaction, we sought to determine the expression of SiglecG, the mouse homologue of Siglec-10, in the mouse endometrium. We were surprised to find that SiglecG was not expressed in the mouse endometrium. We did, however, determine that another CD33-related SIGLEC, Siglec-3, is expressed in the mouse endometrium and may be under hormonal control. Although no reproductive problems have been reported within Siglec-3 KO mice, it would be very interesting to determine if these female mutants show increased infiltration of leukocytes into the uterine lumen after mating.

In conclusion, we provide evidence for the presence of Siglec innate immune receptors in the female reproductive tract and their capacity to recognize sialylated glycans on sperm in humans and mice. Our results suggest that sperm engage female innate immune receptors en route to fertilization. The female immune system may spare some non-self sperm cells in part because sperm are coated with highly sialylated glycan patterns shared between sperm and local female cells. Altogether, our results suggests that the challenge of mammalian insemination may in part be accomplished via the sensing of shared, species-specific glycan patterns on sperm by innate immune-modulating receptors expressed along the endometrium.

Materials and Methods

In vitro interactions between human sperm and activated neutrophils

Human sperm were collected from volunteers via masturbation under UCSD human subjects protocol # 16027, liquefied at room temperature for 30 minutes, and washed with TYH media before being exposed to non-capacitating (NC) or capacitating conditions (C) for 4 hours, as described previously (Munne and Estop, 1993). Blood was collected in heparin-coated tubes from human donors using UCSD human subjects protocol # 170921X. Red blood cells (RBCs) were removed from whole blood using an Easysep magnetic separation kit (Stemcell Technologies) with anti-glycophorin A-conjugated magnetic beads. The remaining plasma and leukocytes were co-incubated for 30 minutes with either NC sperm, C sperm, or sperm-free capacitation media (C buffer alone), fixed in 10% PFA on ice for 20 minutes and stained with: FITC mouse anti-CD62L IgG1 (Biolegend, 304802) or mouse IgG1, k isotype control (BD Biosciences, 349041), FITC rat anti-CD11b (Tonbo Biosciences, 70-0112-OWL-A11503) or Rat IgG2b, k isotype control (BD Biosciences, 554688). Phagosomal reactive oxygen species (ROS) production was analyzed using Fc-OxyBURST Green Assay Reagent (Thermofisher, F2902) for 15 minutes then fixed. The stained sperm and leukocytes were submitted to flow cytometry on a BD FACSCalibur. The population was gated based on forward and side scatter to analyze a minimum of 10,000 neutrophils for each experiment. Data were analyzed using FlowJo. NET formation was visualized using the live cell-impermeable DNA stain SYTOX green (Thermofisher, S7020).

Sialidase activity and confirmation of capacitation

Swim-up sperm were incubated with 100 pM 2'-(4-Methylumbelliferyl)- α -D-N-acetylneuraminic-acid sodium salt hydrate (4-MU-NANA; Sigma-Aldrich, M8639) and exposed to an equal volume of either NC or C buffer. Control samples contained the NC or C buffer alone. Samples were read on a Molecular Devices SpectraMAX M3 plate reader using excitation and emission values of 365 and 450 nm, respectively. Confirmation of sperm capacitation was performed after one hour of capacitation with the MitoProbe JC-1 Assay Kit for Flow Cytometry (Molecular Probes, MP34142) following the manufacturer's recommendations.

Endometrial Cell Line maintenance and flow cytometry experiments

Ishikawa cells and HEC-1B cells were purchased from Sigma (99040201) or ATTC (HTB-113), respectively. Both cell lines were maintained in RPMI with phenol red and supplemented with 10%FBS, 1% penicillin/streptomycin and 1% L-glutamate. For the flow cytometry based screen for Siglec expression, cells were fixed for 10 minutes in 10% PFA. 10^6 cells were submitted to antibody staining for each experiment. The following primary antibodies were used: APC mouse anti-human Siglec-3 IgG1 (BD, 551378), PE mouse anti-human CD170 (Biolegend, cat # 352004), APC mouse anti-human Siglec-7 IgG1(R&D, FAB1138), mouse anti-human Siglec-9 (R&D Systems, AF1139), APC mouse anti-human Siglec-10 (Biolegend, 347606), mouse anti-human Siglec-11 (R&D Systems, MAB3258), mouse anti-

human Siglec-11 (R&D Systems, MAB3258). All unconjugated antibodies were stained with Alexa Fluor 647 goat anti-mouse IgG. Siglec staining was determined by flow cytometry on a BD FACSCalibur. A minimum of 10,000 events were counted for each experiment. Data were analyzed using FlowJo.

17-beta estradiol exposure of Endometrial Cell lines.

For estrogen dosing, experiments 1×10^6 cells were plated and allowed to adhere overnight in RMPI with phenol red. The following day, cells were washed 3 times in PBS and RMPI minus phenol red, with 10%FBS, 1% penicillin/streptomycin and 1% L-glutamate was used. After two days, cells were exposed to 17-beta estradiol at 10^{-6} M, 10^{-8} M and 10^{-10} M concentrations and mock ethanol control. All 17-beta estradiol dilutions were made in absolute ethanol and for all concentrations cells were exposed to 1% ethanol. The cell lines were incubated with 17-beta estradiol for various time-points after which total mRNA was isolated using the Qiagen RNA mini kit and converted to cDNA using the BioRad iScript kit. qPCR was performed using gene specific primers, the Invitrogen Power Sybr green master mix and the BioRad CFX96 Real-time system. Gene specific primers for Siglec-10, Siglec-11 and Siglec-16 were designed using NCBI Primer Blast. The following are the primer sequences used.

Siglec-10 Forward: ACTCAGACAGAAACCCCGAG

Siglec-10 Reverse: GGACTGTTTGGTGTGGCTTT

Siglec-11 Forward: AGGGTGAAGATCTGCAGGAA

Siglec-11 Reverse: GAGGGAGGCATAGTGGAGC

Siglec-16 Forward: GTTCAAATGGAGCACGAAGGAG

Siglec-16 Reverse: GATCTTCATAGCCACCTCCCC

qPCR was performed on three biological replicates in triplicate. Ct values for each Siglec after 17-beta estradiol were normalized the Ct values of the same Siglec in mock treat controls.

Siglec-FC fusion protein purification

Purification of human Siglecs was performed as previously described (Angata and Varki, 2000). Briefly, 18ug of each human Siglec-FC plasmid were transfected into 2×10^7 HEK293A cells. Transfected cells were grown in 50% DMEM, 50% RMPI supplemented with 2mM L-glutamine, 1mM sodium pyruvate and 1% Nutridoma for 4 days. Secreted Siglec-FC proteins were purified from conditioned media using protein-A sepharose beads and treated with AUS to remove any possible interaction with sialic acid containing secreted proteins. All Siglec-FC encoding plasmids were kindly provided by Dr. Ajit Varki.

Human sperm isolation and binding to Siglec-FC by flow cytometry analysis

Human ejaculate was donated by volunteers recruited under UCSD IRB protocol # 160274. To remove seminal proteins and any other contaminants, donated ejaculate was washed three times with 10 times volume of PBS and filtered through glass wool. The isolated sperm were then fixed in 2% PFA for 10 minutes at room temperature. 1×10^6 sperm cells in suspension were incubated with 1ug of Siglec-Fc for 30 minutes followed by a 30 minute incubation with R-Phycoerythrin

AffiniPure F(ab')₂ Fragment Goat Anti-Human IgG, Fc γ Fragment Specific (Jackson ImmunoResearch, 109-116-170). For CD24 staining, 10⁶ sperm cells were incubated with Alexa Fluor 647 mouse anti-human CD24 (Biolegend, 311109) . Binding of the Siglec-FC and CD24 expression was determined by flow cytometry on a BD FACSCalibur. A minimum of 10,000 events were counted per experiment. Data were analyzed using FlowJo.

Mouse estrus tracking and uterine qPCR

C57/B6 mice were kept under UCSD IACUC protocol #S16223. The estrus cycle was tracked by vaginal cytology for a minimum to two weeks before mice were sacrificed. Briefly, the vagina of mice was washed with ~100ul of PBS and the isolated cells were analyzed under a dissecting microscope. The stage in the estrus cycle was determined by the proportion of cornified epithelial cells, nucleated epithelial cells and leukocytes as described in Caligioni 2009 (Caligioni, 2009). 3 to 4 month old female mice were humanely euthanized on select stages of the estrus cycle. Harvested uterine horns were homogenized. Total mRNA was isolated using the Qiagen RNA mini kit and converted to cDNA using the BioRad iScript kit. qPCR was performed using gene specific primers, the Invitrogen Power Sybr green master mix (4367659) and the BioRad CFX96 Real-time system. Sequence for the Siglec-3 primers (ID: 10946590a1P) used were taken from Primerbank (Spandidos et al., 2010). qPCR was performed on two biological replicates in triplicate. Ct values for Siglec-3 were normalized the Ct values TBP. Significance was calculated using the Student's t- test. * indicates a p-value less than 0.05.

Immunofluorescence staining of human and mouse uterus

Frozen post-mortem human uteri were kindly provided by Dr. Nissi Varki. Mouse uterine horns at pro-estrus and estrus were harvested from 3-4-month-old mice. Both human and mouse tissues were flash frozen in optimal cutting temperature (OCT ®) and sectioned into 7µm slices. Human endometrial samples were stained with goat anti-human Siglec-10 (Santa Cruz, sc-240882) or mouse anti-human Siglec11 (R&D Systems, MAB3258) and visualized with Cy3 bovine anti-goat IgG (Jackson ImmunoResearch, 705-165-147) or Cy3 donkey anti mouse IgG (Jackson ImmunoResearch, 715-165-150), respectively. Mouse uteri were stained rabbit anti-mouse Siglec-3 (Santa Cruz, sc-28810) and visualized with Alexa Fluor 488 goat anti rabbit IgG (A11008). All images were taken using Zeiss AXIO Observer D1 Inverted Disc Fluorescence Microscope. For image quantification, exposure time was the same and the fluorescence intensity for a given antibody stain was quantified using Image J. Five images from two different mice were used for quantification. Significance was calculated using the Student's t- test. * indicates a p-value less than 0.05.

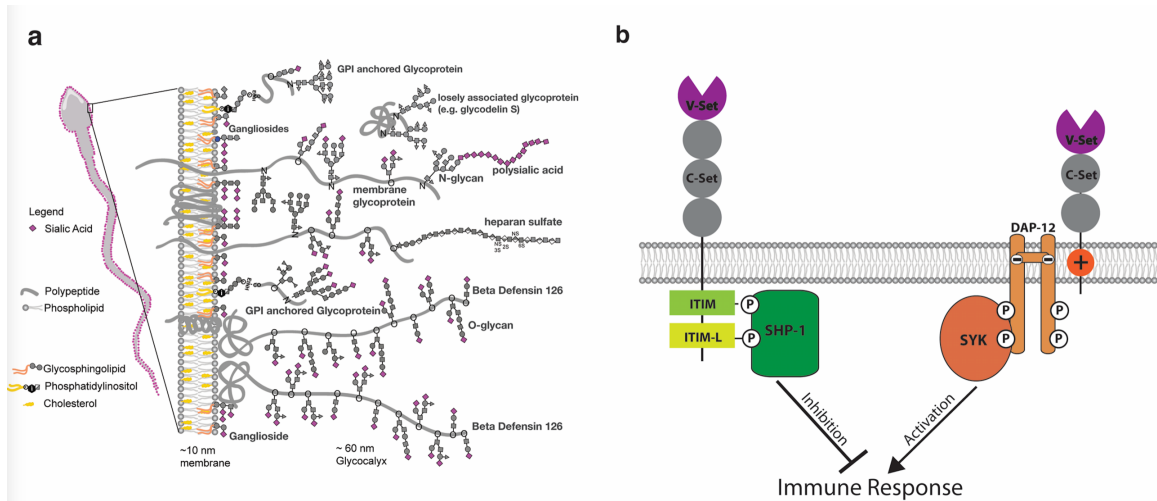


Figure 3.1: The Sperm glycoalyx and Siglec Signaling. **a)** A cartoon of the glycoalyx of mammalian ejaculated sperm. Sialic acid is presented as purple diamonds. Modified from Tecle and Gagneux, 2015. **b)** Siglecs and downstream Signaling. A schematic of an inhibitory Siglec (left) and activating Siglec (right). After binding a sialic acid ligand, via the V set domain, Siglecs can modulate an immune response by downstream signaling pathways. Phosphorylation the ITIM and ITIM-L domains of inhibitory Siglecs by SHP-1 initiates a signaling cascade resulting in immune inhibition. Activating Siglecs bind to DAP-12 via their transmembrane ITAM domains initiating a signaling cascade resulting in immune activation.

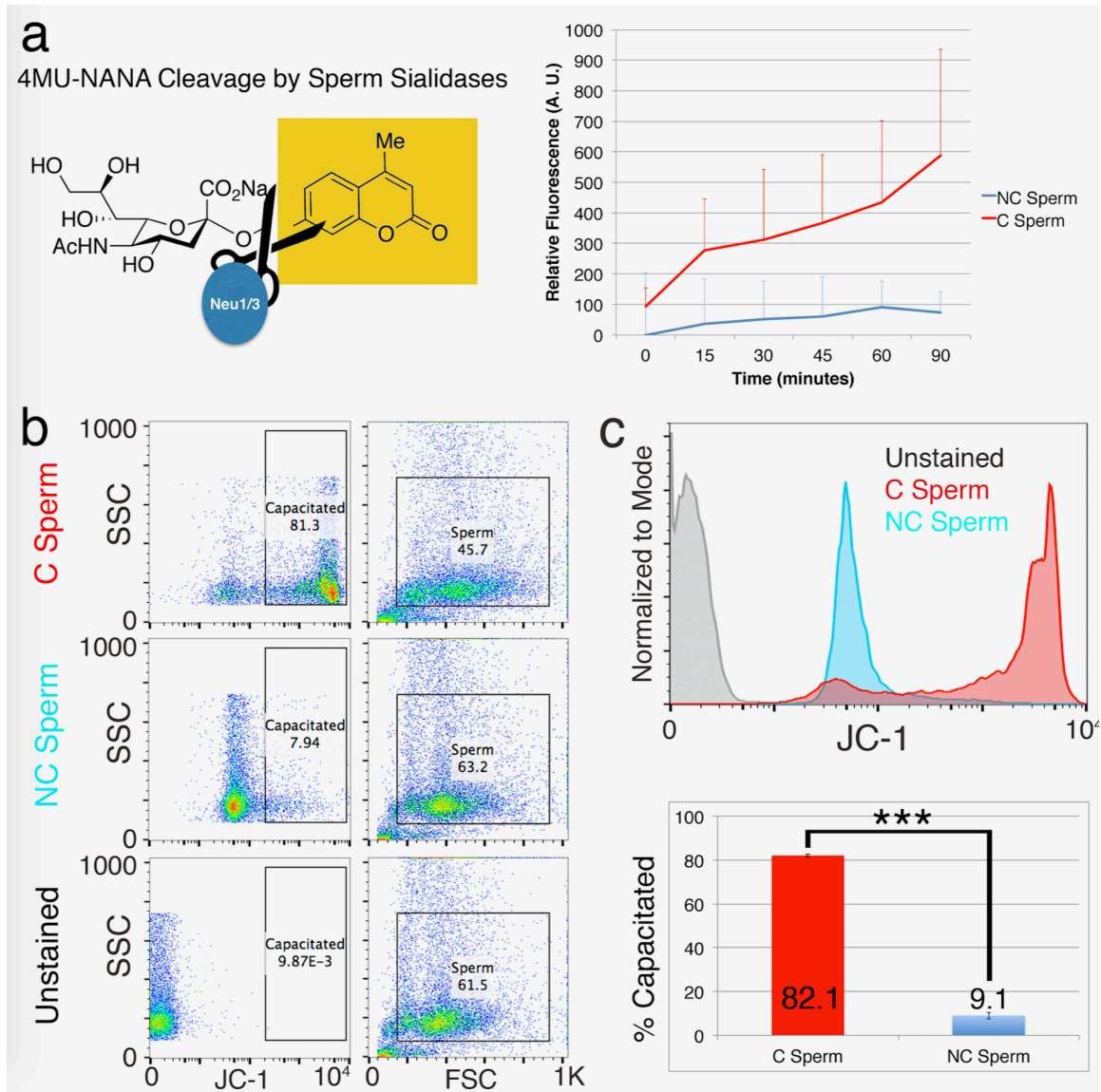


Figure 3.2: Confirmation of sperm capacitation and sialic acid shedding. Sperm co-incubated with the fluorogenic sialic acid analog 4-MU-NANA were exposed to a non-capacitating or capacitating buffer. Only sperm in the capacitating buffer show an immediate increase in fluorescence (**a**), indicative of rapidly activated sperm sialidases. Sperm capacitation was confirmed by using the fluorescent molecule JC-1 to probe for increased mitochondrial activity. The majority of sperm incubated under capacitating conditions show an increase in JC-1 staining (**b**), compared to sperm in non-capacitating conditions (~81% vs ~10%, respectively).

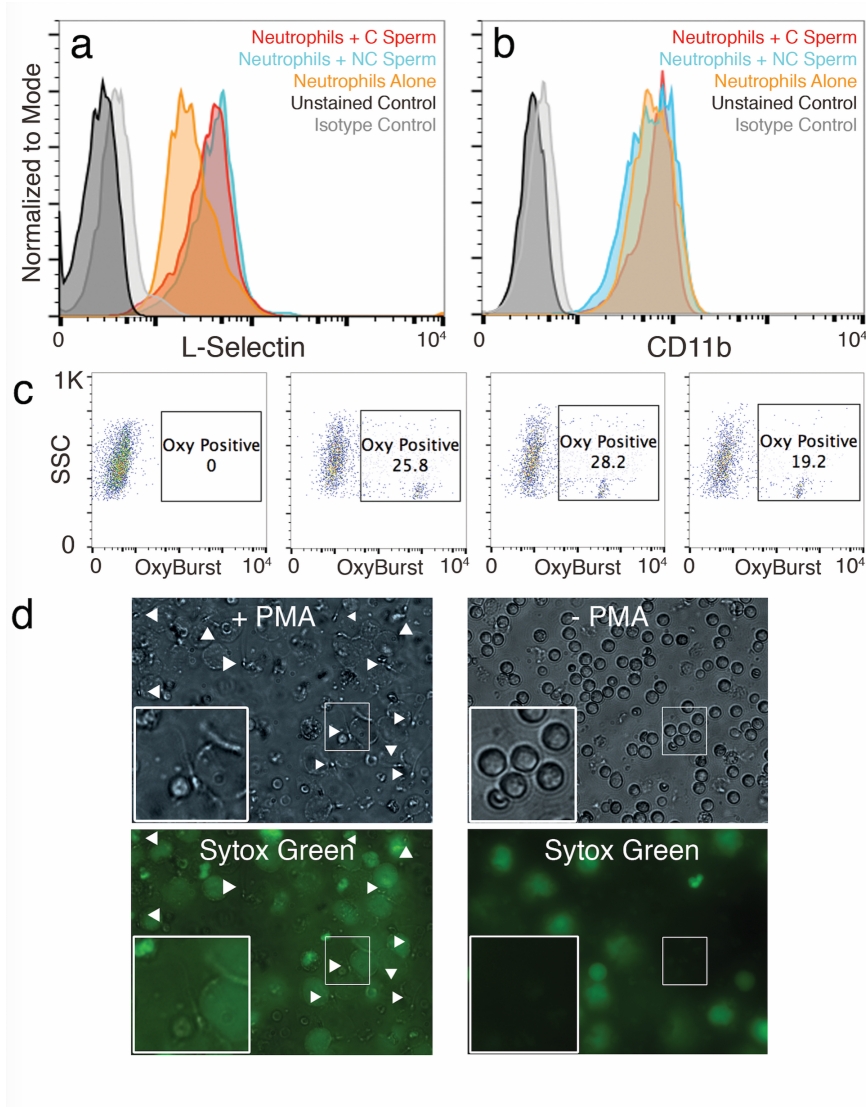


Figure 3.3: Impact of sperm on neutrophil activation. Flow cytometry analysis of the expression of several neutrophil reactivity markers (a-c). Neutrophils co-incubated with NC or C sperm were stained with antibodies targeting several markers of neutrophil activation and measured using flow cytometry. Co-incubation of isolated human leukocytes with NC or C sperm causes no additional shedding of the neutrophil quiescence marker L-selection when compared to leukocytes alone (a). Isolated human neutrophils co-incubated with C or NC sperm show similar CD11b expression compared to isolated leukocytes alone (b). Isolated human neutrophils co-incubated with C sperm show a non-significant decrease in the percentage of OxyBURST-positive (positivity indicates an increase in intracellular reactive oxygen species) cells compared to isolated neutrophils alone or neutrophils co-incubated with NC sperm (c). PMA-induced isolated human neutrophils trigger NET formation and capture live NC and C sperm (d). Captured sperm indicated by white arrowheads.

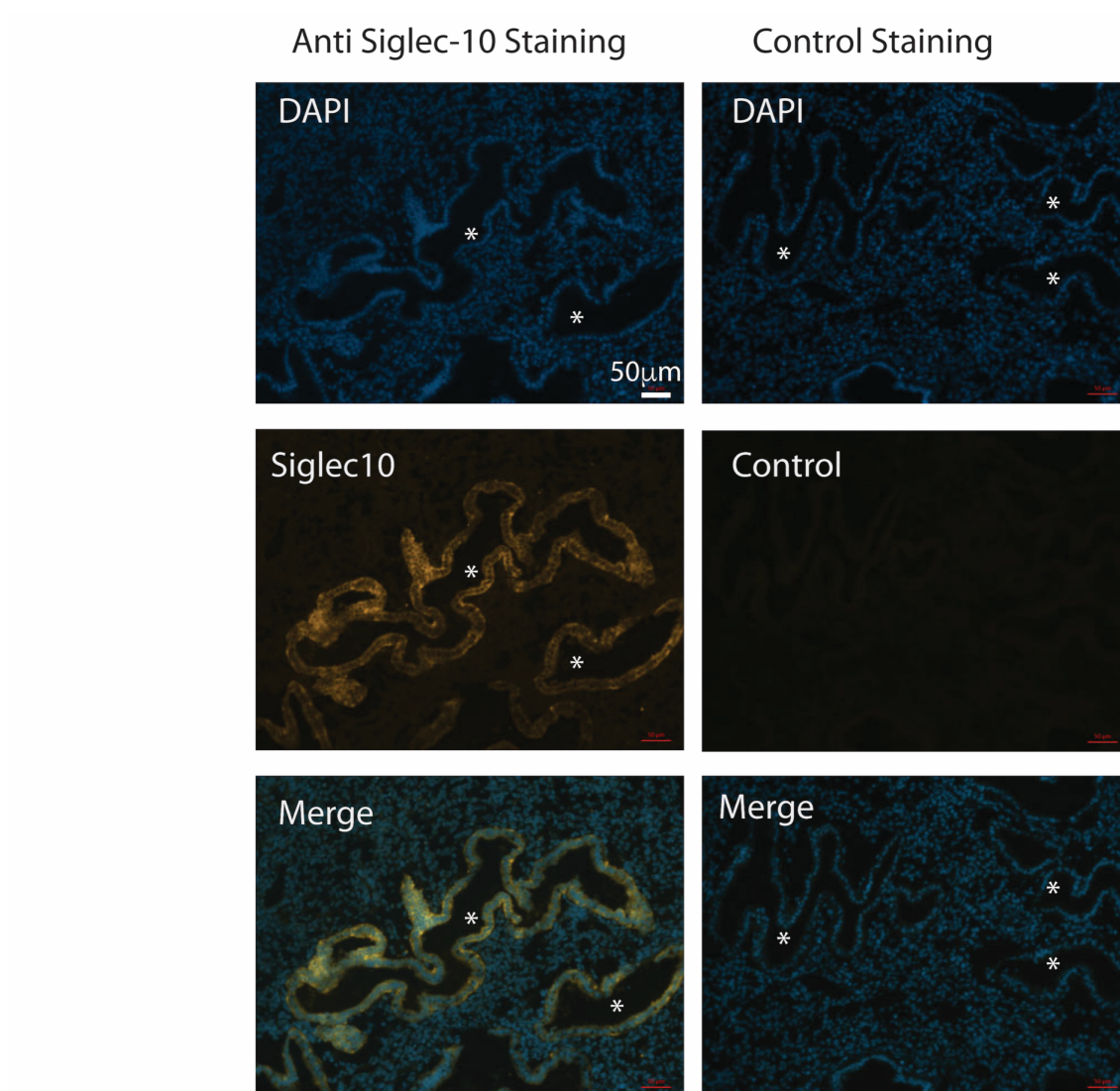


Figure 3.4: Siglec10 is expressed in the human endometrium. Immunofluorescence staining of Siglec 10 (orange) and all nuclei (DAPI). Siglec10 expression is observed exclusively in the endometrium and not in the underlying stroma. Asterisks indicate the uterine lumen. Images are representative for a sample of n=7.

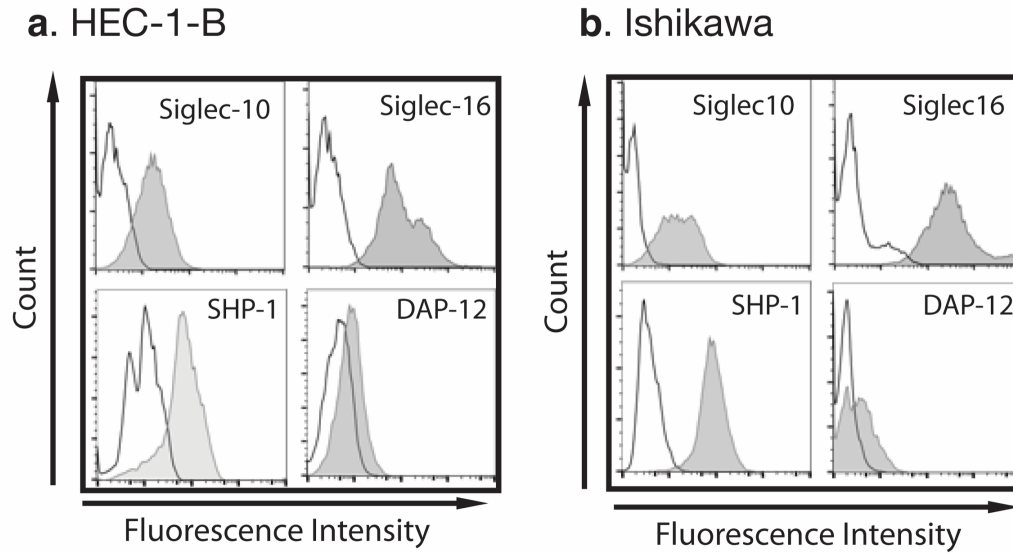


Figure 3.5: Siglecs and their downstream Signaling proteins of Siglecs are present in Endometrial Cancer cell Lines. Flow cytometry analysis of the expression of select Siglec and their Signaling molecules in endometrial cell lines. Both HEC-1B (**a**) and Ishikawa (**b**) cell lines express Siglec-10 and Siglec-16. Both cell lines also express SHP-1 and DAP-12, the downstream signaling molecules of Siglec-10 and Siglec-16, respectively.

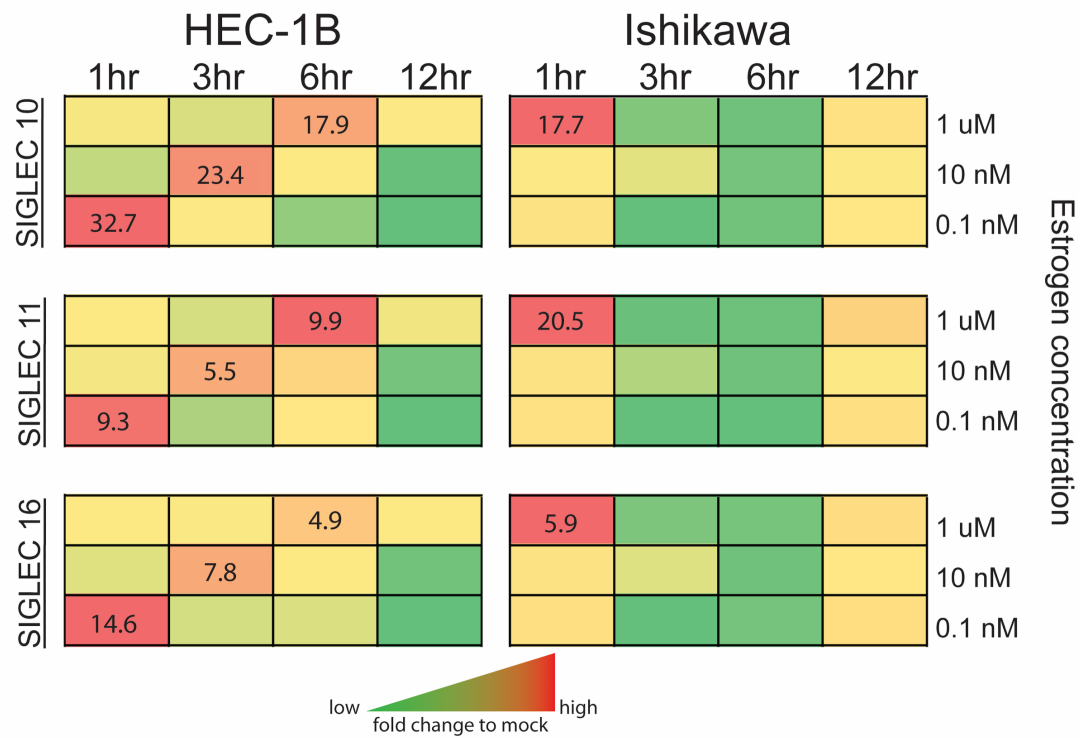


Figure 3.6: Expression of Siglec-10, Siglec-11 and Siglec-16 in Endometrial cancer cell lines is sensitive to exogenous 17-beta-estradiol treatment. mRNA levels, as determined by qPCR analysis of Siglec-10, Siglec-11 and Siglec-16 increase in dosage and time dependent manner in both HEC-1B (**a**) and Ishikawa (**b**) cell lines. For each estrogen dose and time-point, Ct values for each Siglec were normalized to the Ct value of mock treated cells for the same time-point. The highest fold change for each gene is indicated.

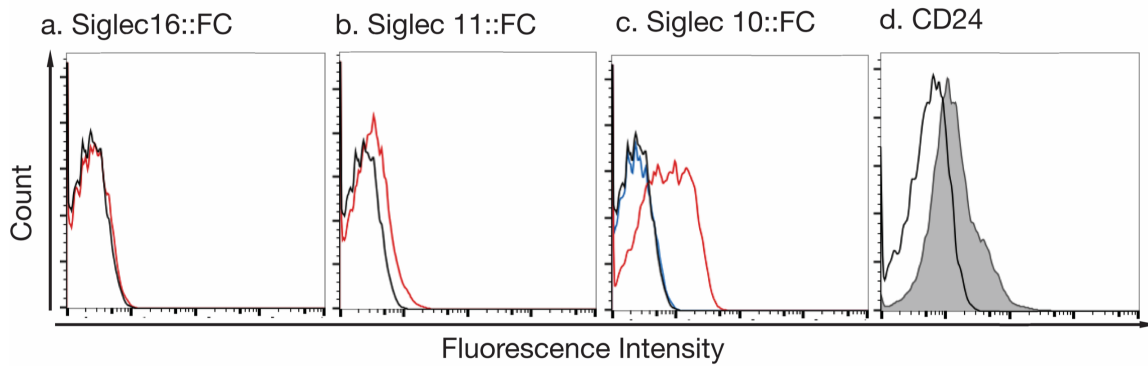


Figure 3.7: Human spermatozoa carry sialylated ligands for Siglec-10. FACS analysis indicates that human sperm do not bind Siglec-11::FC (**a**) and Siglec-16::FC (**b**) (black line is control and red is the Siglec::FC fusion protein.) Human sperm do bind Siglec-10::FC **c**) in a sialic acid dependent manner (black is control, red is wildtype Siglec-10::FC, blue is Siglec-10(R120K)::FC). A subset of sperm also express CD24 (**d**), a known sialylated ligand of Siglec10.

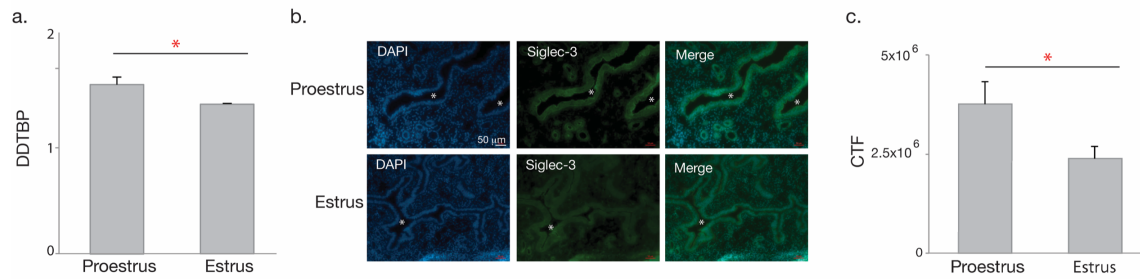


Figure 3.8: Siglec-3 is expressed in the mouse endometrium in an estrus stage dependent manner. qPCR analysis (a) of the whole mouse uteri indicates that Siglec-3 is expressed at statistically significantly higher levels in proestrus than estrus. Immunofluorescence staining for Siglec-3 (b) indicates Siglec-3 expression is enriched in the endometrium and endometrial glands as compared to the underlying myometrium in proestrus and estrus (Asterisks indicate the uterine lumen). (c) Quantification of Siglec-3 antibody staining shows that the Siglec-3 protein levels are higher in proestrus than estrus.

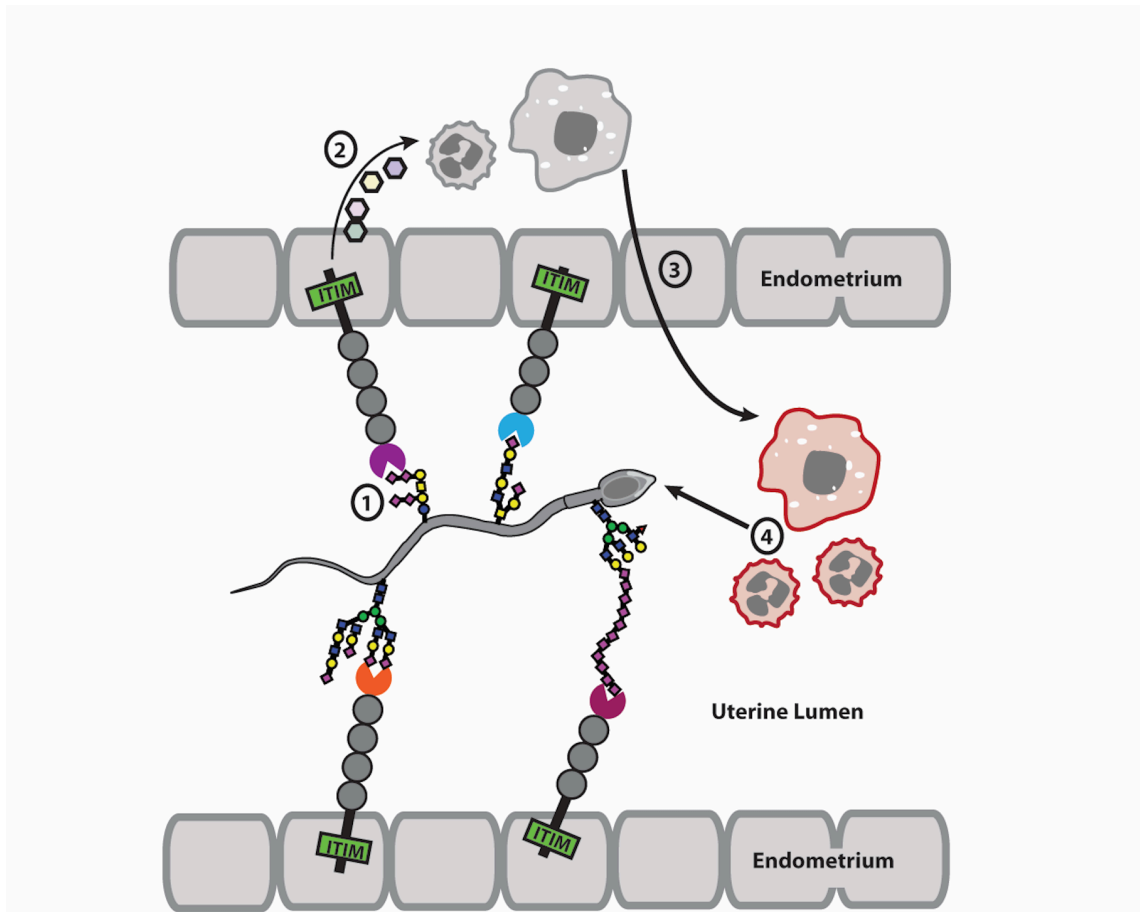


Figure 3.9: Sperm sialic acid and endometrial Siglecs may interact to modulate the female immune response to sperm. Both human and the mouse endometrium express inhibitory Siglecs (Siglec-10 and Siglec-3, respectively). When bound by sialic acid on sperm, the expressed inhibitory Siglecs may inhibit the immune response of the endometrium by inhibiting the release of cytokines, complement or other pro-inflammatory proteins via SHP-1 signaling. Sperm may further directly interact with neutrophils and macrophages and inhibit their immune response.

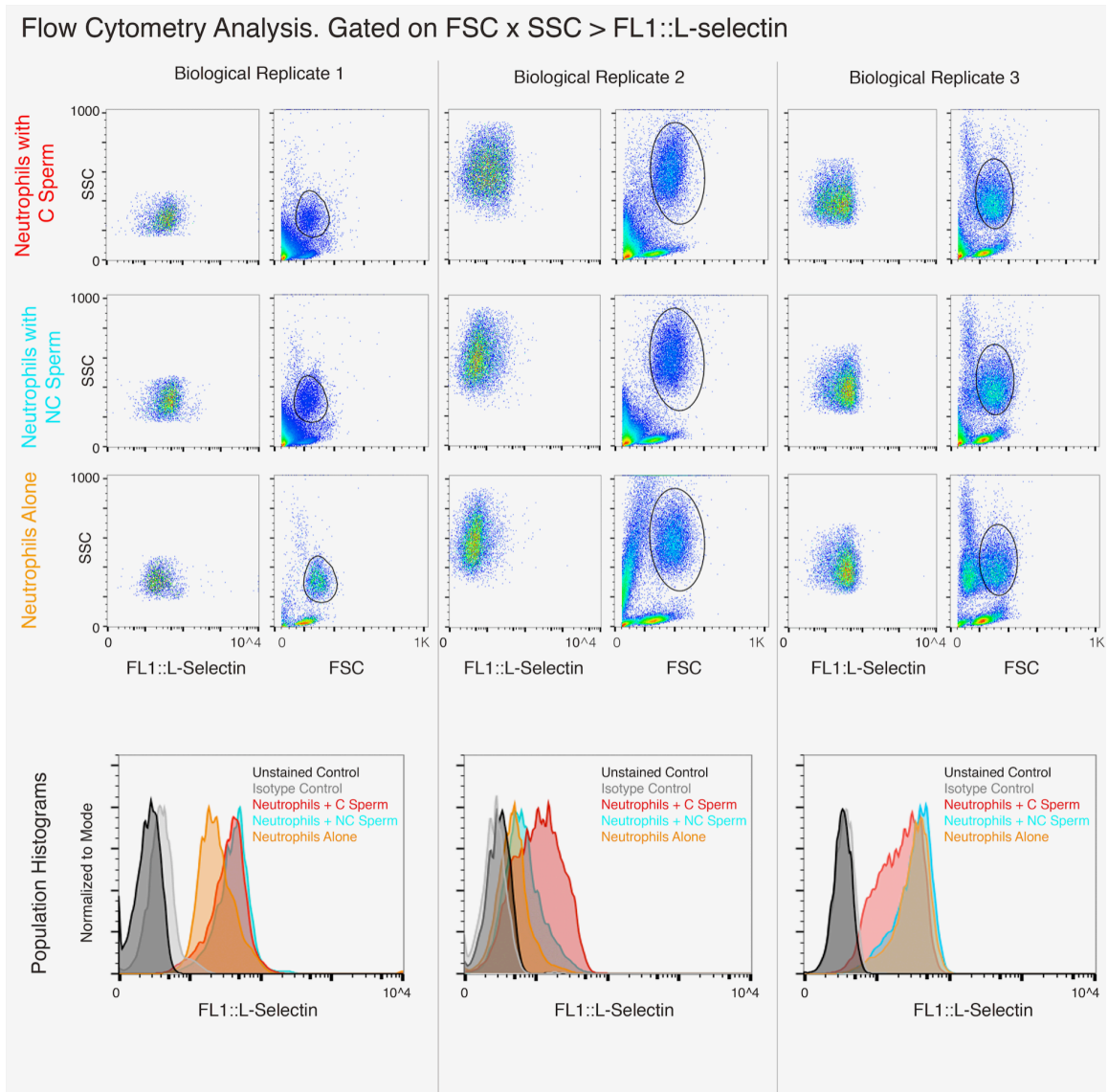


Figure S3.1: Flow cytometry analysis of replicates of human neutrophil L-selectin staining following exposure to NC vs C sperm. Neutrophils were gated by FSC x SSC > FL1::L-selectin. Three biological replicates displaying the variance in L-selectin staining are shown. In two cases, exposure to C sperm caused greater expression of L-selectin compared to neutrophils incubated alone (Replicates 1 and 2), while a third participant showed reduced expression of L-selectin after exposure to C sperm (Replicate 3).

Flow Cytometry Analysis. Gated on FSC x SSC > FL1::CD11b

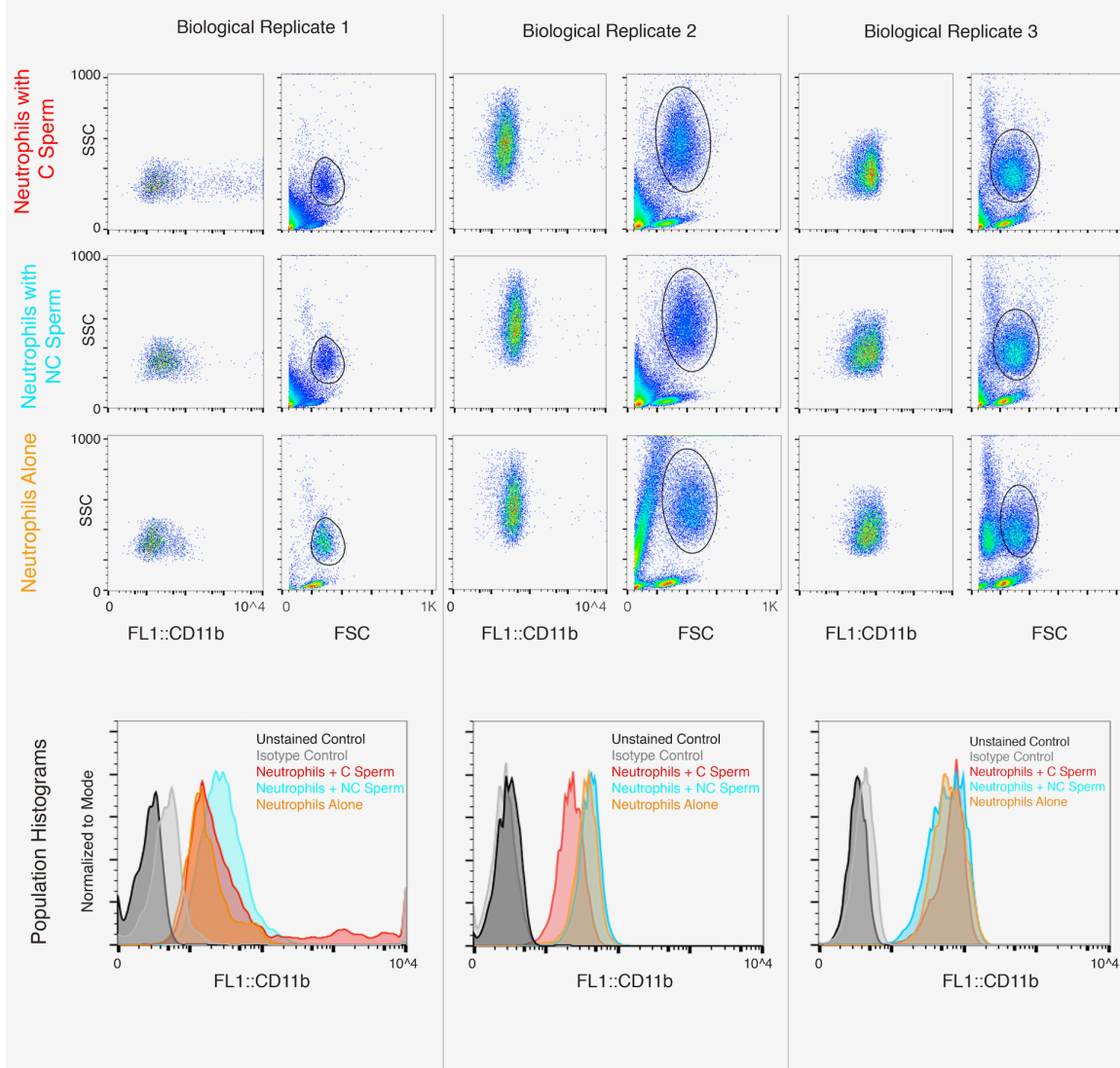


Figure S3.2: Flow cytometry analysis of replicates of human neutrophil CD11b staining following exposure to NC vs C sperm. Neutrophils were gated by FSC x SSC > FL1::CD11b. Three replicates displaying the variance in CD11b staining are shown. In one case, exposure to C sperm reduced neutrophil expression of CD11b compared to when neutrophils were incubated alone (Replicate 2), while in another case, exposure of neutrophils to C sperm had no effect (Replicate 3).

Supplemental Figure 3

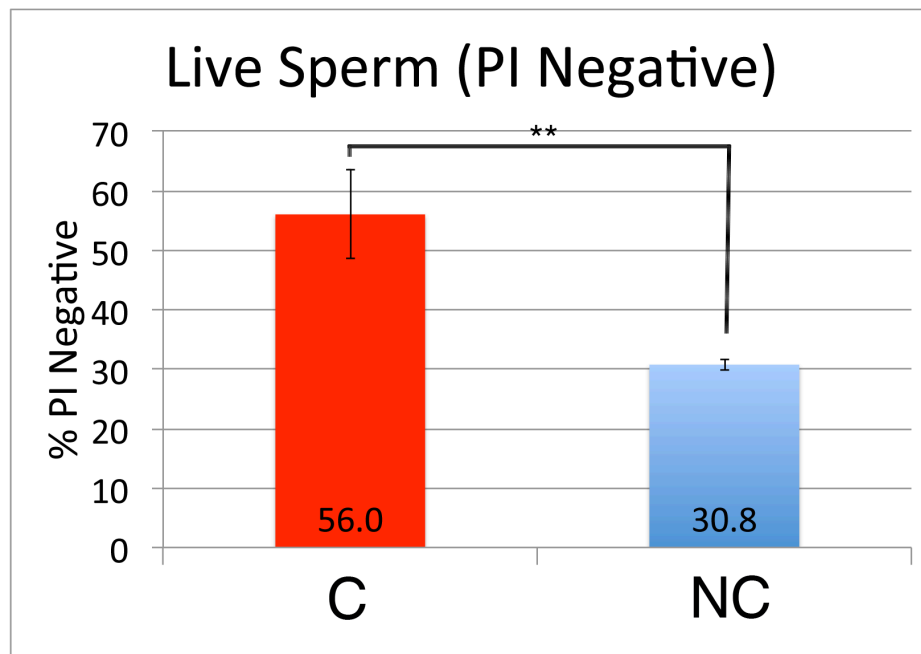


Figure S3.3: Capacitating buffer increases sperm viability. Sperm were incubated in a capacitating (C) or non-capacitating (NC) buffer for 1 hour and then stained with the membrane-impermeable DNA dye propidium iodide (PI). A higher percentage of C sperm were alive (PI negative) compared to NC sperm (56% vs 31%, respectively).

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Chapter 3, in full, is currently in review in the journal Molecular Reproduction and Development. Tecle, Eillen; Reynoso, Hector Sequoyah; Wang, Ruixuan; Kwong, Kelly; Gagneux, Pascal. The dissertation author was a primary investigator and co-author of this material.

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CHAPTER 4:
SPERM MATURATION AND CAPACITATION IS LINKED TO DYNAMIC CHANGES IN
OGT LOCALIZATION AND O-GLCNACYLATION

Abstract

Mammalian sperm must undergo capacitation before they are competent to fertilize an oocyte. The cellular changes associated with capacitation consist entirely of posttranslational mechanisms, since sperm lack gene expression or protein synthesis. During capacitation, sperm are primed to undergo the acrosome reaction in a temporally controlled manner. Capacitation is known to involve changes in protein phosphorylation and lipid remodeling, however, associated glycan changes remain poorly understood. Here we show that protein O-GlcNAcylation, an intracellular glycan modification, plays a role in spermatogenesis and changes during sperm capacitation. The enzyme O-GlcNAc transferase (OGT) adds N-acetylglucosamine (GlcNAc) to serine or threonine residues of nuclear and cytoplasmic proteins. The O-GlcNAc modification is removed by a single enzyme, O-GlcNAcase (OGA). Dynamic O-GlcNAcylation is implicated in many cellular processes, including neurodevelopment and neurodegeneration, and exhibits a complex interplay with O-phosphorylation. We find that both human and mouse sperm contain the enzyme OGT, which relocates from the post-acrosomal region of the sperm head to the acrosomal cap in mice. The levels of O-GlcNAcylation increase during capacitation in both human and mouse sperm. Inhibition of O-GlcNAc cycling with the OGT/OGA inhibitor alloxan results in aberrant tyrosine phosphorylation and reduces sperm

motility. Additionally, alloxan, streptozotocin (an OGlcNAcase inhibitor) and more specific inhibitors of O-GlcNAc cycling (OSMI-1, and thiamet G) reduce the percentage of acrosome-intact sperm without affecting viability. Consistent with these findings, *Oga*-null mice showed reduced testes size, altered spermatogenesis, and reduced sperm motility. This data suggest that O-GlcNAc cycling plays a significant role in spermatogenesis and functions as a dynamic signaling event in the capacitation of mammalian sperm.

Introduction

Mammalian sperm must undergo capacitation before they are able to fertilize an egg. Capacitation is a progression of cellular changes that activate sperm cells for fertilization. Sperm are translationally-silent. As they do not express genes or synthesize proteins, cellular signaling in sperm must occur by non-translational mechanisms. Increased phosphorylation of serine/threonine residues via PKA is known to be a necessary step in capacitation, as the PKA-inhibitor H89 blocks capacitation and prevents fertilization (Visconti et al., 1995). Capacitation is also associated with a number of changes in signaling and cell physiology including increased tyrosine phosphorylation of a number of proteins (Visconti et al., 1995). Capacitation renders sperm capable of the acrosome reaction (Zaneveld et al., 1991) and is also correlated with a change in sperm motility from symmetrical swimming to whip-like hypermotility (Ho and Suarez, 2001). The acrosome reaction is a temporally-controlled event that must occur before the sperm is able to penetrate

the zona pellucida surrounding the oocyte (Hirohashi et al., 2015). Though it is no longer considered crucial for the acrosome reaction to occur only after contact with the zona pellucida (Jin et al., 2011), the timing of acrosome reaction is still considered crucial due to the immunogenicity of inner-acrosomal proteins, such as Izumo and SP-10 (Inoue et al., 2005; Hamatani et al., 2000). Antibodies targeting Izumo or SP-10 prevent sperm:egg fusion between human sperm and zona-free hamster eggs.

The O-linked monosaccharide N-acetylglucosamine (O-GlcNAc) epitope is a post-translational protein modification defined by the attachment of a single GlcNAc sugar to a serine/threonine residue. The enzyme O-GlcNAc transferase (OGT) catalyzes GlcNAc attachment from its nucleotide sugar donor UDP-GlcNAc, and O-GlcNAcase (OGA) catalyzes the modification's removal (Haltiwanger et al., 1992, Dong and Hart, 1994, Gao et al., 2001)). The functional consequences of O-GlcNAc attachment are broad, but not fully understood. O-GlcNAcylation is implicated in numerous biological processes. For instance, O-GlcNAc functions as a nutrient sensor (Banerjee et al., 2016, Eustice et al., 2017), appears to be involved in neurodevelopment (Howerton et al., 2013, Olivier-Van Stichelen et al., 2017), and is found on many transcription factors (Ozcan et al., 2010). Additionally, aberrant O-GlcNAcylation is associated with pathologies such as Alzheimer's (Liu et al., 2004), cardiovascular disease (Wright et al., 2017), diabetes (Huang et al., 2017, Ida et al., 2017, Gurel and Sheibani, 2018), cancer (Harosh-Davidovich and Khalaila, 2018, Banerjee et al., 2016), and lupus (Hewagama et al., 2013, Olivier-Van Stichelen and

Hanover, 2015), among others. The human *OGT* gene, residing on the Xq13.1 genomic locus, is alternatively spliced into nucleocytoplasmic (~115 kD), mitochondrial (~103 kD), and short (~75 kD) variants (Love et al., 2003, Hanover et al., 2003). These differentially-targeted variants may perform different functions in the cytoplasm, nucleus and mitochondrion.

At the molecular level, O-GlcNAc may interact with or compete with phosphorylation, since the O-GlcNAc modification is found on many of the same serine/threonine residues that are phosphorylated by serine/threonine kinases, including protein kinase A (PKA). Several pharmacological compounds (alloxan, streptozotocin, OSMI-1, and thiamet G) act as inhibitors of OGT, OGA, or both. Alloxan was the first chemical agent found to inhibit OGT and later studies indicated it inhibited OGA as well (Konrad et al., 2002, Lee et al., 2006). Unlike alloxan, which requires millimolar concentrations for enzyme inhibition, micromolar concentrations are required for OGT inhibition by OSMI-1 and OGA inhibition by streptozotocin and thiamet G (Roos et al., 1998). Inhibition of either OGA or OGT in cultured cells disrupts cellular signaling and, in some cases, results in cell death (Liu et al., 2017, Konrad and Kudlow, 2002). Indeed, a systemic *Ogt*^{-/-} mutation is embryonic lethal in mammals (Shafi et al., 2000; O'Donnell et al., 2004; Hart & Akimoto, 2009) supporting that inhibition of these enzymes must be considered carefully. Interestingly, however, it has been recently shown that OGT inhibition can block neural tube defects in diabetic embryopathy in mice (Kim et al., 2017). In addition, the OGA inhibitor thiamet G is currently in clinical trials as a treatment for

Alzheimer's, which is associated with hypo-O-GlcNAcylation of tau protein (Abdel-Magid, 2014).

O-GlcNAcylation may be another component of capacitation since it shows a complex interaction with protein phosphorylation and may compete for the same serine and threonine residues. Many signaling pathways are involved in sperm maturation and capacitation including GSK-3, AKT, and PKA and O-GlcNAcylation has been shown to alter each of these signaling pathways (Refs for sperm and include the refs at right) In addition, other glycan-modifications have been implicated in capacitation. For example, both human and mouse sperm shed sialic acids via the action of two sperm sialidase enzymes during capacitation (Ma et al., 2012). Here, we ask whether O-GlcNAcylation is associated with spermatogenesis and the cellular process of capacitation. First, we test for the presence of OGT enzyme in the translationally silent sperm cell. Then we probe for a change in levels of O-GlcNAc modification during the capacitation of human and mouse sperm. Additionally, the inhibition of OGT and/or OGA via streptozotocin, alloxan, OSMI-1, and thiamet G is tested to assess impact on several known aspects of capacitation: tyrosine phosphorylation, motility, and maintenance of acrosomal integrity. Finally, we examined sperm maturation in OGA knockout mice.

Methods

Materials

Human sperm samples were donated by volunteers recruited under UCSD IRB protocol # 160274. In brief, whole ejaculates are allowed to liquefy for 30 minutes before they are layered underneath a physiological buffer (modified TYH media) and sperm allowed to swim up into the TYH for 90 minutes. Sperm from male mice are obtained via bilateral removal of the cauda epididymides, whereby incisions are made into the epididymis and motile sperm are able to swim out into TYH buffer for 15 minutes at 37°C. Mouse sperm from C57 Black 6 mice (Jackson Labs) and B6D2F1-Tg(CAG/su9-DsRed2,Acr3-EGFP)RBGSoo2Osb (RIKEN) were used. Motile sperm are then transferred into new Eppendorf tubes, introduced to a capacitating or a non-capacitating buffer (C vs NC buffer), and allowed to capacitate for 2-4 hours at 37°C prior to further analysis.

Knockout Mice and Spermatogenesis

Knockout mice obtained using MMTV-Cre to generate germline deletion of *Oga* (*Mgea5*) as previously described (Keembiyehetty et al., 2015). Genotyping was performed as previously described (Keembiyehetty et al., 2015). Testis were dissected and measured to determine the mass. Sectioning of the testis and Hematoxylin-Eosin staining was used to visualize seminiferous tubules. Epididymal sperm motility was monitored by video microscopy using the Worm tracking acquisition and software package (MBF Bioscience).

SDS-PAGE and immunoblot

Samples with 10-30 ug of protein were run on 7% polyacrylamide gels at constant amperage 20 mAmps for 20 minutes, followed by 40 mAmps for roughly 60-80 minutes until running front approaches edge of the gel. Gels were transferred to polyvinylidene difluoride (PVDF) membrane activated by methanol at constant voltage (15V) for 40 minutes. Membranes were briefly washed in PBS-Tween (0.1%) before blocking using 50/50 PBS-T/Odyssey blocking buffer (LI-COR, 927-40100) for 1 hour at room temperature. Primary antibody staining was done using mouse anti-O-GlcNAc (1:1K, clone RL2, BioLegend, Cat# 677902), rabbit anti-OGT (1:2K, Abcam, CAT# EPR12713), and mouse α -phosphotyrosine (1:10K, clone 4G10, Millipore, Cat# 05-321) at 4°C overnight on a rocker. After three washes in PBS-T at room temperature, blots were incubated with species-appropriate 1:10K secondary IR-labeled antibodies for 45 minutes, (Goat anti-rabbit IRDYE 800CW, 926-32211; Goat anti-rabbit IRDYE 680RD, 925-68071; Goat anti-mouse IRDYE 800CW, 827-08364; Goat anti-mouse IRDYE 680RD, 925-68070). After a second set of three washes, blots were imaged on LI-COR Odyssey (ODY-3043). Actin was used as a loading control and stained with rabbit or mouse anti-beta-actin (Cell Signaling Technologies, Cat# 4970S; SIGMA-ALDRICH, A2228).

Immunofluorescence

Sperm were allowed to adhere to poly-lysine-coated glass coverslips (VWR, Cat# 48366-251) and washed thrice with PBS. Coverslips were placed in 3% PFA for

20 minutes, washed thrice with PBS, and then placed in a blocking and permeabilizing solution (1% BSA, 0.1% Triton-X100, 0.3M glycine in PBS) for 10 minutes. Adherent sperm were washed with PBS and then incubated with rabbit monoclonal anti-OGT IgG (Abcam, EPR12713), mouse monoclonal anti-O-GlcNAc IgG1 (RL2 - Biolegend Cat# 677902), or mouse monoclonal anti-O-GlcNAc (Cell Signaling Technology, CTD110.6; graciously donated by Lance Wells) at the manufacturers' recommendation for 1 hour. Coverslips were washed thrice in PBS and incubated in goat anti-rabbit FITC (Jackson, Cat # 111-094-141) or goat anti-mouse Alexa488 (Life Technologies, Cat # A11001) in the dark for 30 minutes. Coverslips are washed thrice and mounted on glass slides with a drop of VectaMount AQ (Vector, H-5501). Slides were examined on a Zeiss Observer D1 and processed using Carl Zeiss Zen 2012 (Blue Edition) software.

Mass-spectrometry

Proteins from mouse epididymal swim-out sperm were resolved by SDS-PAGE on a 7% polyacrylamide gel. Individual gel sections were isolated and subjected in in-gel trypsin digestion. Peptides were identified by FT-ICR/Orbitrap MS Scan Mode, MS/MS Scan Mode: Linear Ion Trap using ion source ESI (nano-spray) with high energy CID fragmentation mode. Extraction of the peaks from the generated mass spectra were matched using the PEAKS search engine, with a De novo score (ALC%) threshold of 15. The peptide hit threshold ($-10\log P$) was 30.0. The HexNac post-translational modification was defined as the addition of mass 203.08 on a serine or threonine (ST) residue.

OGT and/or OGA inhibition

Following sperm swim-out (mouse) or swim-up (human), sperm are incubated with 1.25 mM Alloxan (SIGMA-ALDRICH, A7413), 50 μ M OSMI-1 (SIGMA-ALDRICH, SML1621), 5 μ M streptozotocin (Enzo Life Sciences, 380-010-M100), or 2 μ M thiamet G (Abcam, ab146193) for 30 minutes before exposure to non-capacitating or capacitating buffer for 1 hour (mouse) or 4 hours (human). Concentrations were optimized based on prior studies and the published half maximal inhibitory concentrations (IC 50). A control group was incubated with 1% v/v DMSO alone.

Statistics

Statistics were performed using Excel and the StatPlus.mac LE software. Significance was determined by one way analysis of variance (ANOVA) with follow-up pairwise comparisons using Student's T test.

Results

The OGT enzyme is present in mouse and human sperm.

Mouse sperm were examined for OGT protein via immunofluorescence and western blot. Mouse sperm showed a persistent non-specific staining along the midpiece (Figure 1a). When incubated with anti-OGT, both non-capacitated and capacitated mouse sperm showed staining consistent with specific labeling of the

OGT enzyme. Non-capacitated sperm stained mostly in the post-acrosomal region of the sperm head with a small number showing slight staining in the acrosomal cap region. Capacitated sperm showed staining in both the post acrosomal region and the acrosomal cap. When resolved by SDS-PAGE and transferred to PVDF, both mouse and human sperm proteins included an OGT variant at a molecular weight close to 105 kilodaltons (kD) (Figure 1b-c). Further, both NC and C sperm had the 105 kD OGT variant as well as additional immunoreactive bands at lower weight, whose identity was inconclusive. Yellow boxes highlight the position of OGT.

Location and level of O-GlcNAc modification is increased upon capacitation in mouse and human sperm.

Mouse sperm were examined for the presence of O-GlcNAc via immunofluorescence and western blot. When using the RL2 O-GlcNAc antibody, fixed mouse sperm showed an increase in staining in capacitated compared to non-capacitated cells (Figure 2a). This increase was limited to the acrosomal cap. An alternate O-GlcNAc specific antibody (110.6) stained the post-acrosomal region in non-capacitated sperm and confirmed the localization of staining to the acrosomal cap in capacitated sperm (Figure 2b). Western blot staining with RL2 was inconsistent but on several blots indicated an increase in O-GlcNAc expression in capacitated sperm (Figure 2c). In contrast, human sperm also showed an increase in RL2 staining in capacitated sperm compared to non-capacitated sperm, but staining was limited to the connecting piece between sperm head and midpiece (Figure 2d).

Alloxan induces a partial capacitation-like phosphotyrosine phenotype.

Sperm in either a non-capacitating or capacitating buffer were incubated with DMSO (control), alloxan, OSMI-1, streptozotocin, or thiamet G before being subjected to SDS-PAGE and western blot analysis. Capacitated sperm show an expected increase in phosphotyrosine staining when probed with a general anti-phosphotyrosine antibody, pY (Figure 3). Sperm incubated with 1.25 mM Alloxan induces a partial capacitation-like phenotype in non-capacitated sperm (yellow asterisk). Incubation of non-capacitated or capacitated sperm with OSMI-1, streptozotocin, or thiamet G had no effect on phosphotyrosine staining.

Alloxan induces a capacitation-associated O-GlcNAc staining of the acrosomal cap in non-capacitated sperm.

Mouse and human sperm in either a non-capacitating or capacitating buffer were incubated with DMSO (control), alloxan, OSMI-1, or streptozotocin before being fixed and stained with the O-GlcNAc antibody RL2. Non-capacitated mouse sperm incubated with alloxan presented the same acrosomal cap staining restricted to capacitated sperm in the control DMSO treatment (Figure 4a). Human sperm treated with streptozotocin and OSMI-1 had no effect on RL2 staining, but alloxan treatment induced an acrosomal cap staining in both non-capacitated and capacitated sperm previously only seen in mouse sperm (Figure 4b).

Alloxan reduces motility of mouse sperm without affecting viability.

Non-capacitated sperm were incubated with DMSO, alloxan, OSMI-1, or streptozotocin, and analyzed for motility characteristics on a Computer Assisted Semen Analysis (CASA) machine. Incubation with alloxan reduced motility in several categories. The percentage of motile sperm was reduced in the alloxan condition compared to DMSO alone, as were the percentage of sperm moving in a linearly progressive fashion (% Progressive), the percentage of sperm rated in the fastest-moving category (% Rapid), and the average curvilinear (VCL) and straight-line (VSL) velocity (Figure 5a-e). Sperm incubated with OSMI-1 showed a non-significant trend for reductions in the % Motile ($p = .08$) and % Rapid ($p = .08$) motility categories. Incubation with streptozotocin did not significantly affect the five reported motility categories.

Following CASA analysis, sperm were exposed to the membrane-impermeable propidium iodide dye and measured via flow cytometry to detect the percentage of live sperm (Figure 5f). All treatments resulted in 40-60% of live sperm (propidium-iodide negative). There were no significant differences between any treatment group. Sperm cells were first gated based on forward and side scatter (FSC x SSC; Figure 4g) and then on absence of propidium iodide to select for live sperm (Figure 5h). Example gating dot plots contain flow cytometry data from one characteristic DMSO-treated sperm sample.

Alloxan, OSMI-1, streptozotocin, thiamet G increase spontaneous acrosome reaction.

Sperm from the transgenic mouse line B6D2F1-Tg(CAG/su9-DsRed2,Acr3-EGFP)RBGSoo20sb present red fluorescent protein (DsRed) within the midpiece

and green fluorescent protein (GFP) within the intact acrosome. Sperm from these transgenic mice were incubated with DMSO, alloxan, OSMI-1, streptozotocin, or thiamet G before being exposed to a capacitating buffer for 1.5 hours and analyzed for spontaneous acrosome reaction. Sperm were considered “acrosome intact” if they retained endogenous GFP-acrosin or “acrosome reacted” if they lost GFP expression (Figure 6a). Sperm were first gated for absence of propidium iodide to select for live sperm, and then for presence of GFP to select for acrosome intact (Figure 6b). The percentage of acrosome intact sperm was significantly lower for sperm treated with alloxan (51.4%), OSMI-1 (81.7%), streptozotocin (82.1%), and thiamet G (81.6%) compared to DMSO alone (89.8%), (Figure 6c, $p < 0.05$). The number of live sperm in each condition was not significantly affected by any treatment (Figure 6d).

Mass spectrometry identifies two O-HexNAcylated proteins.

Isolated mouse epididymal sperm swim-out protein lysates were resolved by SDS-PAGE and entire lanes were excised and analyzed via mass spectrometry to probe for characteristic signatures of the O-GlcNAc modification. The method used determined whether there was positive evidence for a N-acetylated hexose (HexNAc) modification, as the GlcNAc and N-acetyl-galactosamine (GalNAc) are stereoisomers sharing identical masses (203.08 amu). Our analyses revealed several proteins with a mass increase corresponding to the addition of a HexNAc moiety to a serine or threonine residue. Subsequent analysis of individual scans revealed only two proteins that exhibited the presence of oxonium ion fragmentation, which is

strongly indicative of O-HexNAc. These were B1AWG4 Leucine-rich protein Protein LRRC37A and the Lysosomal Pro-x carboxypeptidase PCP, previously found in the sperm proteome (Wang et al., 2013).

Spermatogenesis and sperm motility are altered in an O-GlcNAcase mouse model.

The in vitro findings above suggested that O-GlcNAc cycling may play a role in sperm maturation, motility, and capacitation. Analysis of an *Ogt*-null mutation in mature males is difficult since this knockout is embryonic lethal. To evaluate this in a whole animal context, we used a recently developed mouse line in which the *Oga* (*Mgea5*) gene was deleted in the germline (Keembiyehetty et al., 2015). These mice exhibit a high penetrance of perinatal lethality, but some males develop to adulthood. These animals are very rare and so only a limited number of these animals could be analyzed. We noted that in these KO animals, the testis were greatly reduced in size compared to their WT and heterozygous littermates (Figure 7). Histochemical analysis of the testis revealed that the seminiferous tubules were greatly altered in structure with very few mature spermatozoa visible. (Figure 8). We prepared epididymal sperm preparations of wild type (WT), Heterozygous and KO males and examined the numbers and mobility of these sperm. In three independent experiments, the KO animals showed a greatly reduced sperm count with diminished motility (Supplemental movie 1). These data strongly suggest that O-GlcNAc cycling plays a role in spermatogenesis.

Discussion

Despite a lack of gene transcription and translation, mammalian sperm undergo many physiological changes during their final maturation process of capacitation. While several components of capacitation are well-characterized, glycan modifications have been less studied (Teclé and Gagneux, 2015). Here, we find evidence that proteins are O-GlcNAc modified in sperm and that the level of the modification correlates with capacitation state. Interruption of endogenous O-GlcNAc cycling via OGT and OGA inhibitors reduces motility and interferes with acrosomal integrity. Furthermore, the pharmacological agent alloxan produces a partial capacitation-like tyrosine phosphorylation phenotype in non-capacitated sperm.

OGT, the enzyme that catalyzes the addition of O-GlcNAc to intracellular proteins, is present in both mouse and human sperm (Figures 1 and 2). Based on molecular weight, both mouse and human sperm contain an OGT variant whose molecular weight is consistent with either the ~103 kD mitochondrial splice variant or the ~115 kD nucleocytoplasmic OGT variant (Figure 1b and 1c). Sperm from both sample sets have immunoreactive bands of smaller molecular weights, which may represent additional uncharacterized splice variants, cleavage products resulting from degradation by proteases during sample preparation, or unknown, nonspecific immunoreactive bands. Intriguingly in mouse sperm, OGT is localized to the sperm head, specifically in the post-acrosomal region near the midpiece, and appears to relocate to the acrosomal cap region during capacitation (Figure 1a).

In addition, the O-GlcNAc modification was identified via two monoclonal antibodies (RL2 and CTD110.6). In mouse sperm, the O-GlcNAc modification

appears to parallel the relocalization of OGT enzyme, moving from the post-acrosomal region to the acrosomal cap (Figure 2a-b). While the post-acrosomal staining is only seen with the CTD110.6 antibody in non-capacitated sperm, both antibodies indicate an increase in acrosomal cap staining in capacitated mouse sperm. In contrast, capacitated human sperm exhibit an increase in O-GlcNAcylation at the connecting piece of the sperm between the head and the midpiece. O-GlcNAc is absent from the acrosomal cap region in human sperm (Figure 2d). This discrepancy in localization between human and mouse sperm is not too surprising, as mammalian sperm proteins are rapidly evolving (Torgerson et al. , 2002).

The addition of a single monosaccharide posttranslational modification to proteins in human sperm was explored by mass spectrometry. Two peptides, corresponding to B1AWG4 and PCP, had diagnostic HexNAc mass shifts as defined by signature oxonium ion fragmentation (Carr et al., 1993). B1AWG4, or Protein LRRC37A (leucine rich repeat containing 37A) is an uncharacterized transmembrane protein. PCP (lysosomal Pro-x carboxypeptidase) is a protease that is active at acidic pH and inactivates angiotensin II. This is noteworthy because angiotensin II has been shown to stimulate sperm motility in rat and human sperm and acrosome reaction in bovine sperm (Vinson et al., 1995, Gur et al., 1998), and we observed associated phenotypes utilizing O-GlcNAc cycling inhibitors. While we were surprised to identify only two glycosylated proteins, we expect that additional experiments using enrichment strategies might yield additional proteins given that we show multiple RL2-positive bands by western blot (Figure 2c). Further, although there have been many advances in identifying O-GlcNAcylated proteins,

there are limitations to the methods we used (Hart and Akimoto, 2009) and alternatives would be advantageous (Zhao et al., 2011).

While several inhibitors of OGT and OGA have been identified, each has advantages and drawbacks. Alloxan was the first published OGT inhibitor, but several publications have demonstrated that alloxan has off-target effects (Lenzen and Panten, 1988, Zhang et al., 1992). Furthermore, alloxan or streptozotocin can both be used to induce a diabetic state in a mouse model of diabetes due to a selective destruction of pancreatic beta cells (Dunn, Sheehan, and McLethie 1943; (Rossini et al., 1977). It is debated whether the cytotoxic effect is independent of OGT inhibition. Competing theories regarding both of the inhibitors' method of action include oxidative stress due to the creation of reactive oxygen species, changes to cellular metabolism, and disruption of mitochondrial function (Szkudelski, 2001, Nahdi et al., 2017). In addition to inhibiting OGA, streptozotocin has been shown in some cases to decrease OGT activity and decrease O-GlcNAcylation and OGA levels (Ramirez-Correa et al., 2015), while in other cases to increase protein levels of OGT and O-GlcNAc levels (Akimoto et al., 2000) in rats. The interpretation of the effects of alloxan and streptozotocin on sperm function should consider these off-target effects.

Sperm treatment with different OGT and OGA inhibitors yielded disparate results depending upon the specificity of the inhibitor used. Alloxan treatment led to a partial capacitation-like phenotype. Whereas RL2 reactivity increased in the acrosomal region of mouse sperm only after capacitation (Figure 2a), RL2 reactivity increased in the acrosomal region of both NC and C human and mouse sperm

following alloxan treatment (Figure 4a-b). This was surprising, as neither human NC or C sperm showed RL2 staining in the acrosomal cap under control conditions (Figure 2d). Alloxan may have a pronounced effect on the acrosomal cap region due to the presence of GLUT2 in this region (Angulo et al., 1998), as alloxan has been shown to depend on the glucose transporter GLUT2 for uptake (Elsner et al., 2002). In addition, alloxan treatment reduced the percentage of motile, progressive (moving in a straight path), and rapidly moving sperm, as well as straight-line and curvilinear velocity (Figure 5a-e). Our results are consistent with a reduction in sperm motility seen after rats were made diabetic via injection of alloxan (Arikawe et al., 2006). Alloxan treatment also induced a partial capacitation-like increase in phosphotyrosine in NC mouse sperm (Figure 3, yellow asterisk). Finally, alloxan triggered a 40% reduction in acrosome-intact NC sperm (Figure 6c). Altogether, these results suggest that alloxan disrupts the normal cellular signaling events that take place during capacitation.

Since alloxan functions as both an OGT and OGA inhibitor with known side effects, we sought to discern whether these findings would hold with more specific OGT or OGA inhibitors. In contrast to alloxan and streptozotocin, the commercially available OSMI-1 and thiamet G inhibitors are more specific in their respective targeting of OGT or OGA, respectively (Ortiz-Meoz et al., 2015, Yuzwa et al., 2008). Neither OSMI-1 or streptozotocin treatment fully recapitulated the effects triggered by alloxan treatment. Though OSMI-1-treated sperm did show a trend towards moving slower in all motility categories (Figure 5a-e), there was no significant difference. Like alloxan, OSMI-1 has been shown to have off target effects that

reduce cell viability (Ortiz-Meoz et al., 2015), but sperm viability was unaffected by any of the inhibitors used in this study (Figures 5f, 6d). With respect to spontaneous acrosome reaction, there was a similar reduction in the percentage of acrosome intact cells regardless of treatment with the more specific OSMI-1 or thiamet G inhibitors versus the less specific streptozotocin-treated sperm (Figure 6c). Alloxan-treated cells had the lowest percentage of acrosome-intact cells, perhaps due to other off-target effects besides OGT inhibition (Boquist & Nelson, 1982). The maintenance of the acrosome is a critical component of sperm fertility, as exposure of the inner acrosomal membrane renders sperm susceptible to detection by antibodies that prevent sperm:egg binding (Inoue et al., 2005; Hamatani et al., 2000). These data suggest disruption of endogenous O-GlcNAcylation dynamics may disrupt acrosomal maintenance and reduce fertility.

To avoid the use of pharmacological agents we attempted to test the impact of loss of O-GlcNAc cycling on spermatogenesis, sperm maturation and motility. Mice lacking *O*-GlcNAcase as a result of targeted mutation of the *Oga* gene in the germline showed an interesting spermatogenesis deficit compared to wild-type animals. Testes from these mice are smaller and the seminiferous tubules of these animals exhibit a disorganized structure. The epididymal sperm number and motility were greatly reduced in these *O*-GlcNAcase-deficient mice suggesting that O-GlcNAc cycling plays a role in the process of spermatogenesis. Future studies will be required to determine the downstream effects of these alterations in sperm maturation and capacitation.

The results presented herein suggest that O-GlcNAc cycling may play a key role in spermatogenesis and sperm maturation. Our findings suggest that O-GlcNAcylation may influence three critical aspects of sperm capacitation state: tyrosine phosphorylation, motility, and the maintenance of acrosomal integrity. O-GlcNAcylation has been shown to alter many key pathways associated with sperm maturation and capacitation, including interactions with GSK-3 (Somanath et al., 2004), Akt (Gallardo Bolaños et al., 2014), and PKA (Hanover et al., 2010; Visconti et al., 1995; Bond & Hanover, 2015). However, OGT and OGA cannot be definitively implicated due to the potential off-target effects of the chemical inhibitors used. Other OGT or OGA inhibitors exist, such as GlcNAcstatin, which exhibits picomolar selectivity for OGA (Dorfmueller et al., 2006). The newest inhibitors were not tested in this study, in part because several were not available commercially at the time of this study. In contrast, our genetic findings in O-GlcNAcase-deficient mice support a role for O-GlcNAc cycling in spermatogenesis, sperm maturation and motility. It would be interesting to compare the effects of other inhibitors as they become available in the future. Overall, our findings reveal that a common form of intracellular glycosylation, O-GlcNAcylation, is a strong candidate to serve as a dynamic signaling event critical for the capacitation of translationally silent mammalian sperm.

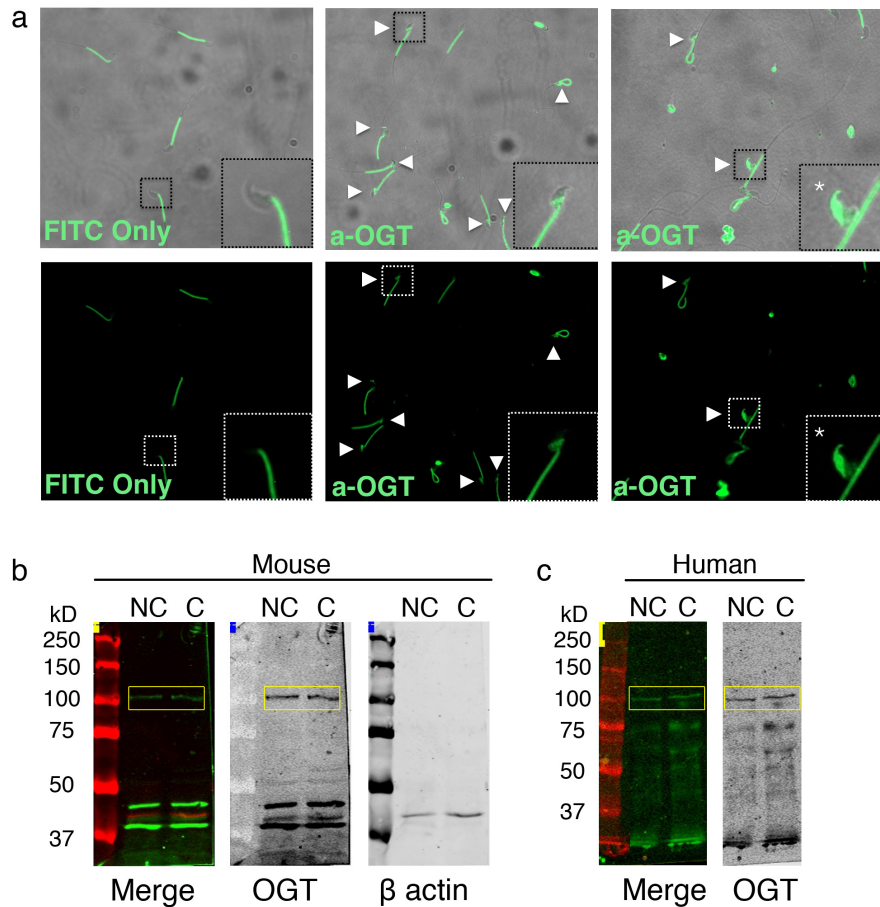


Figure 4.1: OGT is present in mouse and human sperm.

Sperm were probed via immunofluorescence and western blot for the presence of the OGT enzyme. Fixed non-capacitated mouse sperm were stained in the post acrosomal region of the sperm head (1a, middle panels). This staining migrated to the acrosomal cap region (right panels, asterisk). White arrowheads indicate sperm showing positive staining. The midpiece region was stained nonspecifically. Western blot staining with anti-OGT revealed several bands in both mouse and human sperm (1b-c). Yellow boxes indicate band of similar size as mitochondrial OGT splice variant.

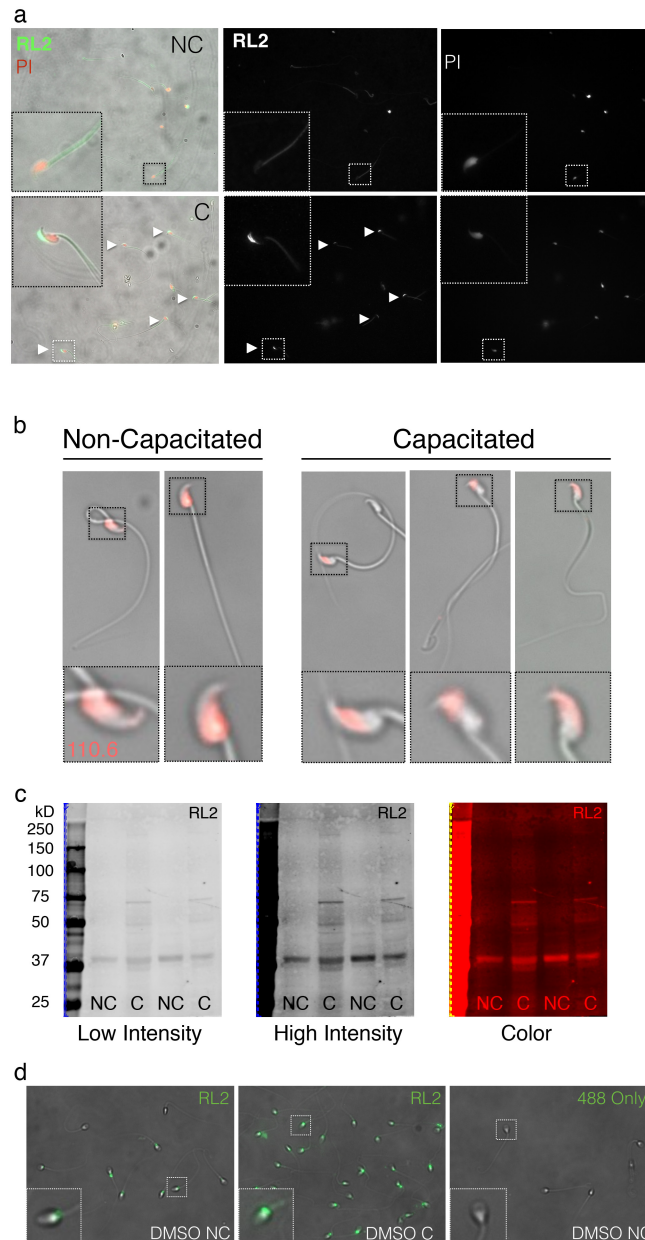


Figure 4.2: O-GlcNAc expression during capacitation. Mouse and human sperm were probed via immunofluorescence and western blot for the presence of O-GlcNAc modification with two α -O-GlcNAc antibodies, RL2 and CTD110.6, in non-capacitated (NC) and capacitated (C) sperm. Propidium iodide (PI) was used to label the nucleus. C mouse sperm show an increase in O-GlcNAc staining in the acrosomal cap region of the sperm head (2a). The O-GlcNAc appears to migrate from the post acrosomal region in NC sperm (2b, left panels) to the acrosomal cap in C sperm (2b, right panels). Western blot staining of mouse sperm using RL2 reveals an increase in O-GlcNAc staining in a range of bands only in C sperm (2c). A ~40 kD band is seen in both NC and C sperm. Human sperm show an increase in O-GlcNAc staining in C compared to NC sperm (2d).

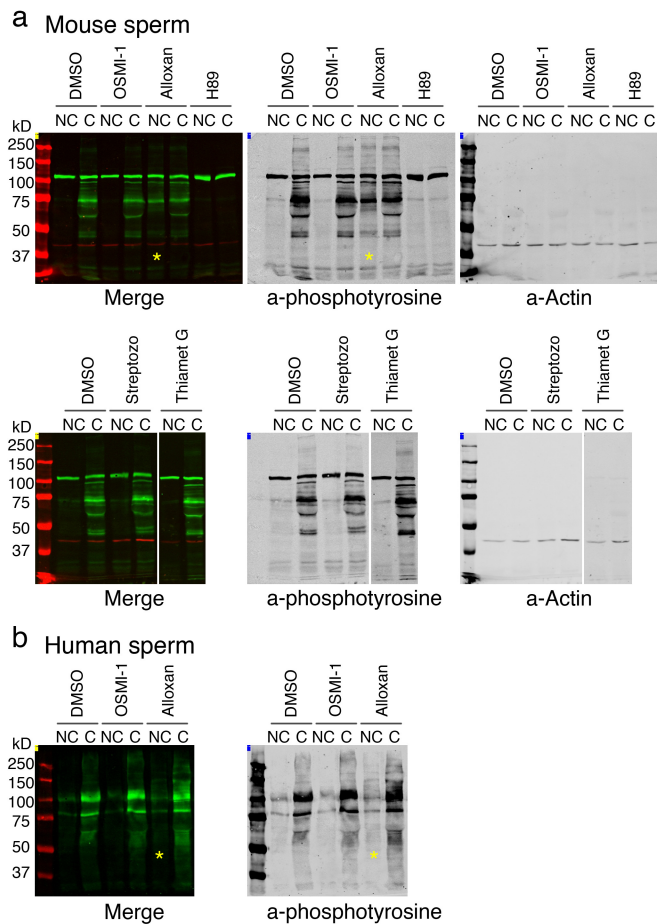


Figure 4.3: Phosphotyrosine expression after OGA/OGT inhibition. Sperm from human and mouse were treated with OGT and OGA inhibitors before exposure to a non-capacitating or a capacitating buffer. Western blots of whole cell lysates were stained with an anti-phosphotyrosine (pY) antibody. Mouse sperm showed an expected increase of α -pY staining in C compared to NC sperm, which was blocked when incubated with the PKA-inhibitor H89 (3a). Alloxan caused a partial capacitation-like pY pattern in NC sperm from mouse and human (3a-b, yellow asterisk). OSMI-1, streptozotocin, and thiamet G had no effect on pY.

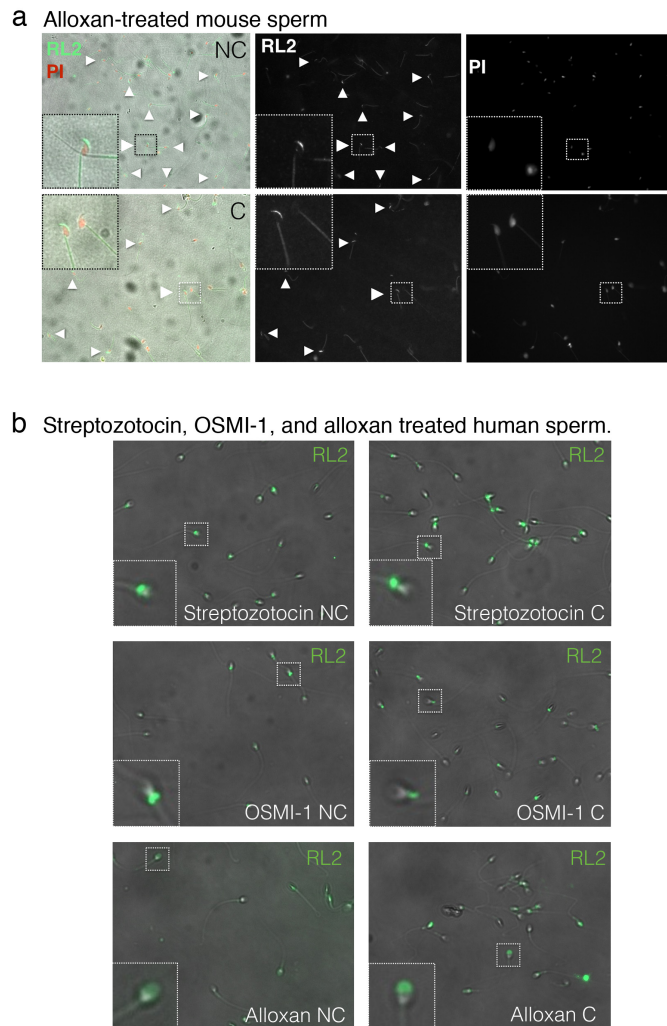


Figure 4.4: O-GlcNAc staining after OGT and/or OGA inhibition. Sperm from human and mouse were treated with OGT and OGA inhibitors before exposure to a non-capacitating or a capacitating buffer. Fixed mouse and human sperm were stained with the anti-O-GlcNAc antibody RL2 via immunofluorescence. Mouse sperm treated with alloxan showed an increase in O-GlcNAc staining in the acrosomal cap region of both NC and C sperm (4a, white arrowheads). Human sperm treated with streptozotocin or OSMI-1 showed no change in O-GlcNAc staining in the connecting piece after exposure to NC or C buffers (4b, upper and middle panels). Alloxan treatment induced acrosomal cap staining in both NC and C sperm (4b, lower panels).

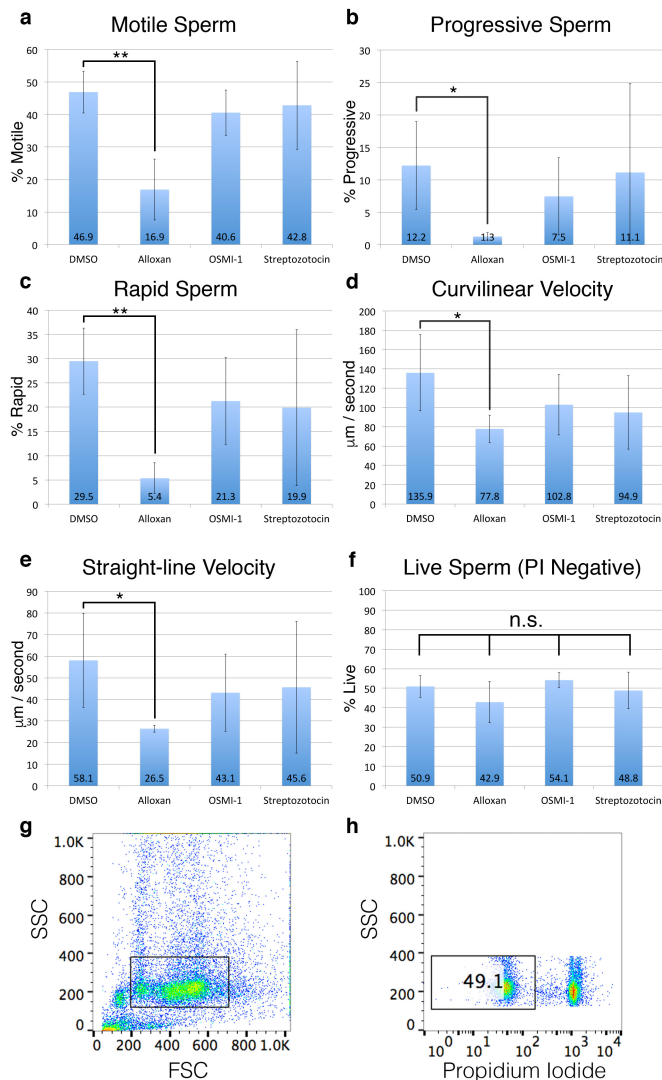


Figure 4.5: Motility characteristics of OGT and/or OGA inhibitor-treated mouse sperm. Mouse sperm were incubated with OGT and/or OGA inhibitors and measured for motility characteristics on a computer assisted semen analysis machine. The percentages of motile, progressive, and rapidly moving sperm were significantly reduced following treatment with alloxan (5a-c). The average curvilinear and straight-line velocity was also reduced after alloxan treatment (5d-e). Sperm viability was measured via flow cytometry and gated on forward and side scatter characteristics as well as the absence of the membrane-impermeable dye propidium iodide (5g-h, boxes represent gates. Shown are the gates of a representative sperm sample treated with DMSO). No treatment significantly affected sperm viability (5f). * indicates $p < .05$, ** indicates $p < .01$.

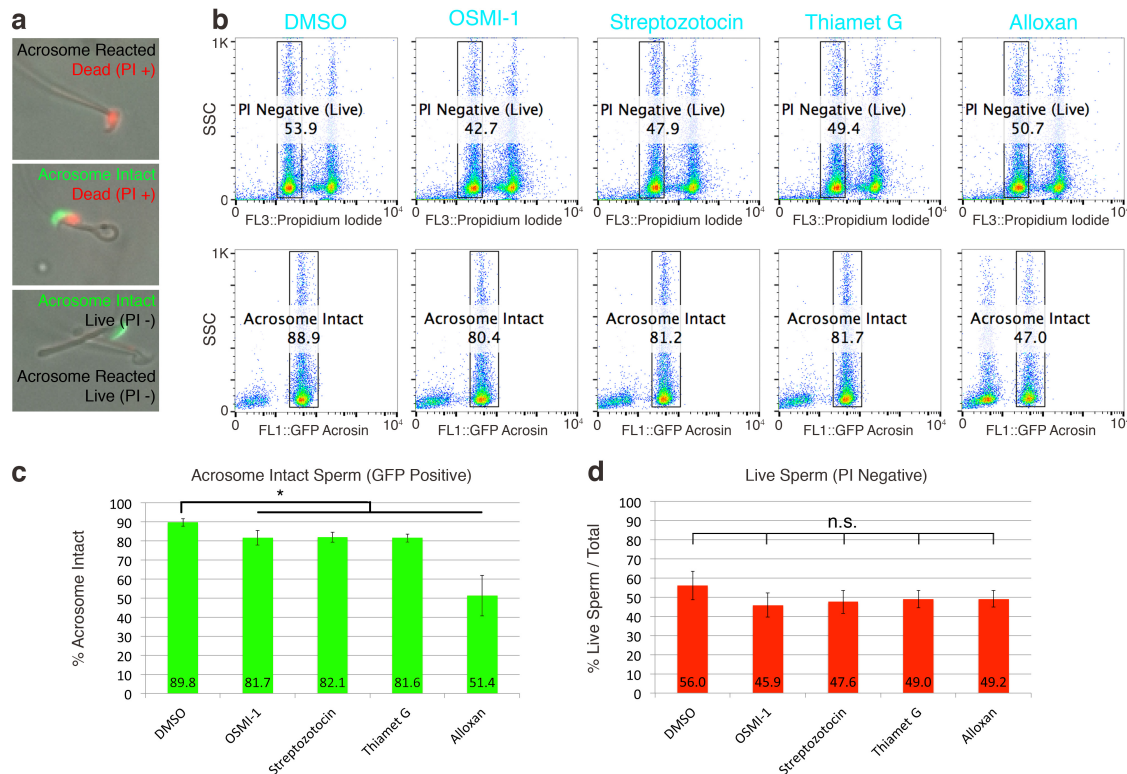


Figure 4.6: Flow cytometry analysis of acrosome intact sperm following OGT and/or OGA inhibition. Sperm from transgenic mice with fluorescently tagged GFP:Acrosin in their acrosome were incubated with OGT and/or OGA inhibitors and measured for acrosome intactness via flow cytometry. Sperm were considered acrosome intact if they retained GFP signal and excluded the membrane-impermeable dye propidium iodide (PI) (a). Sperm were gated first by their exclusion of PI, and then by the retention of GFP:Acrosin (b). The percentage of intact sperm was significantly reduced after treatment with the OGT/OGA inhibitors OSMI-1 (81.7%), streptozotocin (82.1%), thiamet G (81.6%), or alloxan (51.4%) compared to control (DMSO; 89.8%) (c). Inhibitor treatment did not significantly reduce sperm viability (d). * indicates $p < .05$.

Testicular size is altered in OGA $-/-$

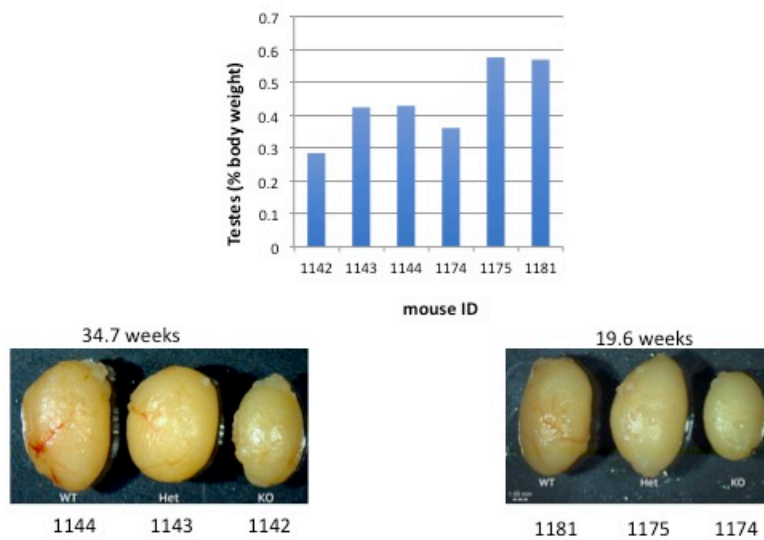


Figure 4.7: O-GlcNAcase Knockout (KO) mice show reduced testis size. Testis from WT, Heterzygous and Knockout mice were measured and weighed and showed a dramatic reduction in size. A summary of these measurements are quantified in the panel at the top of the figure.

OGA KO testis display altered spermatogenesis

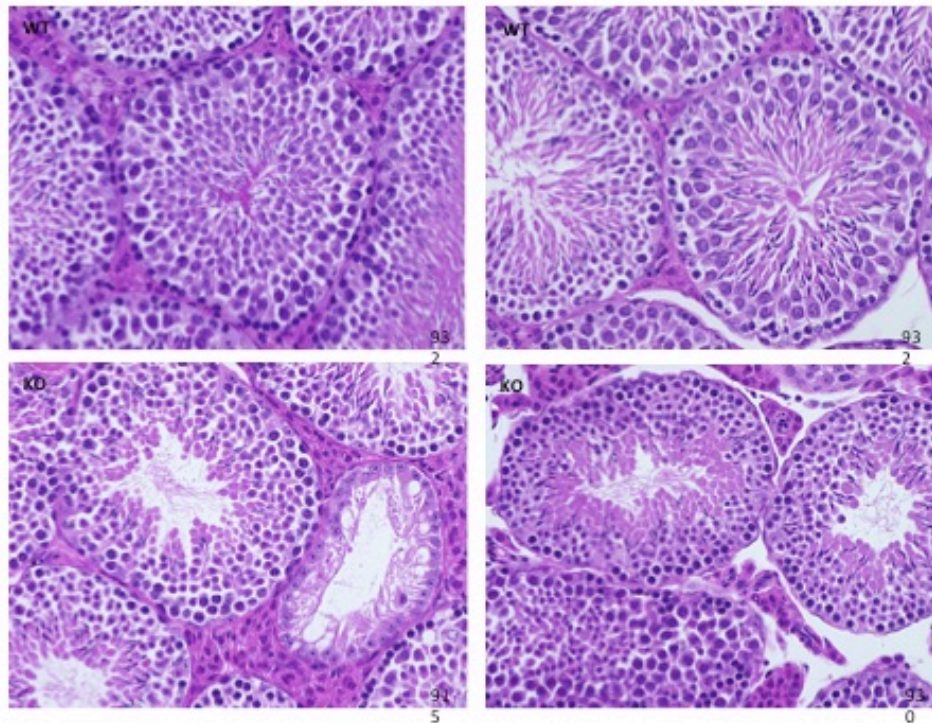


Figure 4.8: O-GlcNAcase Knockout (KO) mice exhibit disrupted spermatogenesis compared to wildtype controls (WT). Sections of the testes reveal disorganized structure and paucity of sperm in the O-GlcNAcase knockout animals (bottom panels, labeled KO) compared to control (top panels, labeled WT). The seminiferous tubules show major disorganization and the number of sperm is significantly reduced.

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CHAPTER 5:

CONCLUSIONS

All the studies described in this dissertation investigated changes within the sperm glycome. The goal was to determine whether the presence of specific glycans, either on in the intracellular compartment, affected critical sperm functions. Specifically, my studies sought to determine the presence of specific glycans, their dynamics during capacitation, and their effects on fertility and/or immune activation. Several hypotheses were supported, while others were falsified. Specifically, the hypothesis that Neu5Gc presence reduces fertility was supported. The presence of a potentially fertility-reducing, non-human sialic acid was not found on the sperm of fertile fathers. The hypothesis that capacitation-dependent reductions in sialylation increase immune cell activation was not supported. Neutrophil activation was not consistently increased in capacitation. Finally, O-GlcNAc, an intracellular form of glycosylation, was observed in the sperm cells of human and mouse. Disruption of the cycling of O-GlcNAc appears to interfere with acrosomal integrity.

Chapter 2 presented the investigation of a non-human sialic acid, Neu5Gc, on the sperm of human fathers. This study used immunoblot analysis to determine whether the sperm of confirmed fertile men contained Neu5Gc. We concluded that no fertile men had detectable levels of Neu5Gc, whereas a subset of subfertile men had sperm which did contain Neu5Gc. These results, together with the presence of circulating anti-Neu5Gc antibodies in the serum and uterine lavages of subfertile

women, suggest that Neu5Gc on sperm may reduce fertility in human males. Furthermore, our results indicate a need to change the formula of the extender media used to incubate frozen semen samples, as the extender media contained the antigenic nonhuman glycan Neu5Gc.

The presence of Neu5Gc on the sperm of fertility patients, and corresponding absence in fertile men, is consistent with the hypothesis that Neu5Gc presence on sperm is a debilitating factor in human reproduction. If so, this has potential ramifications for couples trying to conceive, and may be treatable with diet modification. Though the diet questionnaire did not show a significant correlation between the amount of Neu5Gc in a person's diet and the presentation of Neu5Gc antigen on their cells, the diet is still the only plausible route of Neu5Gc entry into the body. Furthermore, the rapid rate of sperm production in human males means that a diet change to Neu5Gc-free foods may quickly reflect in the glycan composition of newly generated sperm cells.

Chapter 3 probed whether less sialylated capacitated sperm increased activation of human neutrophils. Sperm incubated in a capacitating buffer showed immediate sialidase activity, indicating the removal of the "home-team jersey". However, the less-sialylated capacitated sperm did not increase neutrophil activation in all samples. In fact, capacitated sperm even decreased neutrophil inflammatory markers in one sample compared to when neutrophils were incubated alone. This result is inconsistent with my hypothesis, and necessitates further study to assess the impact of partial sialic acid loss in sperm. This is true especially in an *in vivo* setting, which includes Siglec-expressing cells of the female

reproductive tract. Besides the possibilities discussed in chapter 3, it is also likely that different classes of immune cells besides neutrophils target sperm, such as macrophages. Additionally, future experiments could further explore neutrophil activation by accounting for the increased number of DAMPs released from apoptosing sperm. This is particularly relevant due to the finding that non-capacitated sperm show a higher rate of apoptosis. I expect that with chemical or enzymatic removal of sperm sialic acids, which removes sialic acids more rapidly than endogenous sperm sialidases, I would find a difference in neutrophil activation. Finally, if capacitated sperm truly do decrease neutrophil activation, this suggests the presence of a ligand that targets inhibitory receptors on neutrophils.

Chapter 4 addressed whether or not O-GlcNAc is present in sperm. My results show that O-GlcNAc is indeed present in the sperm of humans and mice, and that capacitation induces an increase in O-GlcNAcylation. Sperm from both species contain the OGT and OGA enzymes necessary for O-GlcNAc cycling. Disruption of endogenous O-GlcNAc cycling via several chemical inhibitors, including alloxan, streptozotocin, OSMI-1, and thiamet G impacted several aspects of sperm function, including motility, tyrosine phosphorylation, and acrosomal integrity. The effect on acrosomal integrity was shared among all inhibitors, suggesting that O-GlcNAc cycling is particularly important in the maintenance of the acrosome. Alloxan induced several effects, including a capacitation-like phosphotyrosine pattern. The existence of off-target effects in chemical inhibitors called for a genetic approach. Fortunately, the Hanover lab had already produced *Oga*-null mice. Few *Oga*-null males survived to adulthood, the testes from these animals show abnormalities, and

their sperm exhibited reductions in motility. These results are consistent with the importance of O-GlcNAc cycling in sperm function.

My results generate several ideas for future research. Non-specific binding of several secondary antibodies to the sperm midpiece prevented me from determining whether the OGT enzyme is present in midpiece. As western blot indicated an OGT isoform consistent with the isoform found in the mitochondria of somatic cells, the sperm midpiece is a likely habitat for OGT. This hypothesis is consistent with the theory that the O-GlcNAc modification is reflective of the metabolic state of the cell. Additionally, given my results, it may be of interest to inspect genes related to O-GlcNAc cycling in genomic studies of infertile men. In a different vein, further classification of the unidentified cleaved fragments of the OGT enzyme found in whole sperm protein extracts may reveal a sperm-specific isoform.

The relocalization of the OGT enzyme to the acrosomal cap, as well as the increase in O-GlcNAcylation in the same area, merits further exploration. It would be interesting to observe whether the OGT enzyme is lost following acrosome reaction. The method of OGT relocalization is another exciting aspect of capacitation to consider. The OGT relocalization correlates with the mobilization of lipid rafts, suggesting OGT as a plausible member of the protein complexes previously shown to migrate towards the acrosome.

These studies expand the knowledge regarding the sperm glycome. This is a fruitful area of research, as glycosylation is relatively less understood and continues to be overlooked by many molecular biologists, in comparison to proteins and nucleic acids. Furthermore, there is strong evidence that sperm counts and fertility

is declining, at least in the rich, industrialized world (Nargund, 2009). Alarming, as many as 17% of couples in these countries rely on assisted reproductive technologies (Bhasin, 2007), and male factors are responsible for ~40% of these cases (Muratori et al., 2009). As future generations increasingly rely on artificial reproductive technologies, basic understanding of sperm biology and fertilization will be increasingly important to the continued reproductive success of aspiring parents.

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