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Sugar-Coated Sperm: Unraveling the Functions of the Mammalian Sperm Glycocalyx

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SUMMARY

Mammalian spermatozoa are coated with a thick glycocalyx that is assembled during sperm development, maturation, and upon contact with seminal fluid. The sperm glycocalyx is critical for sperm survival in the female reproductive tract and is modified during capacitation. The complex interplay among the various glycoconjugates generates numerous signaling motifs that may regulate sperm function and, as a result, fertility. Nascent spermatozoa assemble their own glycans while the cells still possess a functional endoplasmic reticulum and Golgi in the seminiferous tubule, but once spermatogenesis is complete, they lose the capacity to produce glycoconjugates de novo. Sperm glycans continue to be modified, during epididymal transit by extracellular glycosidases and glycosyltransferases. Furthermore, epididymal cells secrete glycoconjugates (glycophosphatidylinositol-anchored glycoproteins and glycolipids) and glycan-rich microvesicles that can fuse with the maturing sperm membrane. The sperm glycocalyx mediates numerous functions in the female reproductive tract, including the following: inhibition of premature capacitation; passage through the cervical mucus; protection from innate and adaptive female immunity; formation of the sperm reservoir; and masking sperm proteins involved in fertilization. The immense diversity in sperm-associated glycans within and between species forms a remarkable challenge to our understanding of essential sperm glycan functions.

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``[S]perm glycans seem to be in a prime place to convey a large and varied amount of information in a small and sweet package.''

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STRUCTURAL DIVERSITY OF THE GLYCOCALYX

All living cells are enveloped in a glycocalyx, a ''sugar coat'', consisting of a many diverse glycoconjugates. The glycocalyx has been described as a molecular ''forest'' (Cohen and Varki, 2010), where the polypeptide cores of glycoproteins form ''tree trunks'' that stretch away from the membrane and extend into a canopy of glycans. Some of these glycans are branched and decorated with an assortment of monosaccharide ''leaves'' at their termini. Others form ''vines'', composed of long polysaccharide chains carrying various functional groups (Fig. 1). Extensive diversity in glycan structures exists in every layer of this

Abbreviations: ADAM, alpha disintergrin-associated metalloprotease; BSG, basigin; Cer, ceramide; DEFB126, beta defensin 126; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GDS, glycodelin S; Glc, glucose; GPI, glycophosphatidylinositol; Man, mannose; [mrt]CD52, [male reproductive tract] CD52; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PH20/SPAM1, PH20 hyaluronidase/sperm adhesion molecule 1

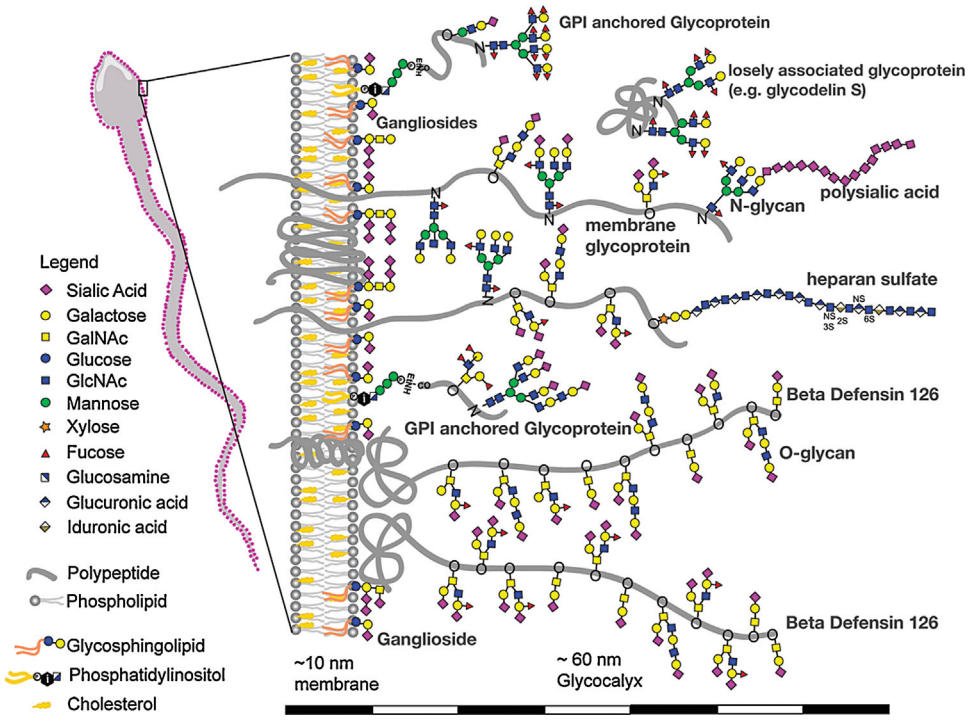

Figure 1. The major glycan and glycoconjugate classes of the sperm glycocalyx. Monosaccharides are coded by colored symbols explained in the legend. Proteins and lipids are gray, except cholesterol (in yellow), and the lipids of glycosphingolipids (in orange). Mammals synthesize most glycans with a dozen different monosaccharide-building blocks; some of these monosaccharides can be further modified by sulfation and/or acetylation.

molecular forest. O- and N-linked glycans fill the canopy with monosaccharide leaves. In mammals, the terminal monosaccharides are often sialic acids. Proteoglycans harboring long glycosaminoglycan (e.g., heparan sulfate or chondroitin sulfate) ''vines'' overlap with and extend up from the canopy. At the cell membrane, or the ''forest floor'', glycolipids carry various oligosaccharide structures. Above these are membrane-associated glycoproteins, or short ''shrubs'', that display glycans away from the membrane but not nearly as far away as the glycans associated with the canopy.

The glycocalyx of gametes is embellished with molecules that participate in fertilization- specific activities. The mammalian egg is enveloped by a glycocalyx, consisting of an interwoven meshwork of zona pellucida glycoproteins and surrounded by a giant matrix of hyaluronic acid-rich vestment containing cumulus cells and cumulus-specific glycoconjugates, such as glycodelin C (Chiu et al., 2007a). In sperm, abundant glycophosphatidylinositol (GPI)-anchored glycoproteins add thickness to the ''forest'' understory and loosely associated glycoproteins stick to the canopy like ''epiphytes'', with no direct anchor to the membrane below.

SYNTHESIS AND ASSEMBLY OF THE MAMMALIAN SPERM GLYCOCALYX

The mammalian sperm glycocalyx is 20–60-nm thick, and includes many different glycoproteins, glycolipids, and GPI-anchored glycoproteins. Sperm synthesize some of these glycoconjugates, whereas others are assembled or made by paternal somatic cells (Schröter et al., 1999). Nascent sperm cells synthesize glycans as they develop in the seminiferous tubules. De novo synthesis of glycoconjugates occurs co- and post-translationally in the endoplasmic reticulum/Golgi, where a series of glycosyltransferases sequentially modify growing glycan chains on glycoproteins or glycolipids (Fig. 2). In contrast to polypeptides, glycan structures are not directly encoded in the genome; instead, glycan structures are generated by the activity of successive transferases whose activity depends on the pool of available nucleotide-sugar donors (Varki et al., 2009). Glycans on proteins can be O-linked (to serine or threonine) or N-linked (to asparagine). N- and O-linked glycans are synthesized by different sets of enzymes, each with its own characteristic monosaccharide compositions (Varki et al., 2009). Glycolipids are similarly synthesized by the addition of monosaccharide units, forming sphingolipids (Schnaar et al., 2009).

Most cell-surface and secreted proteins in mammals are glycosylated. Glycosylation in the endoplasmic reticulum is often required for the proper folding of nascent proteins when they interact with chaperone proteins. For example, the chaperones calnexin and calreticulin bind to special high-mannose N-glycans with a terminal glucose residue (Glc1Man9GlcNAc2) on a nascent protein (Ruddock and Molinari, 2006). Alpha disintegrin- associated metalloprotease (ADAM) family

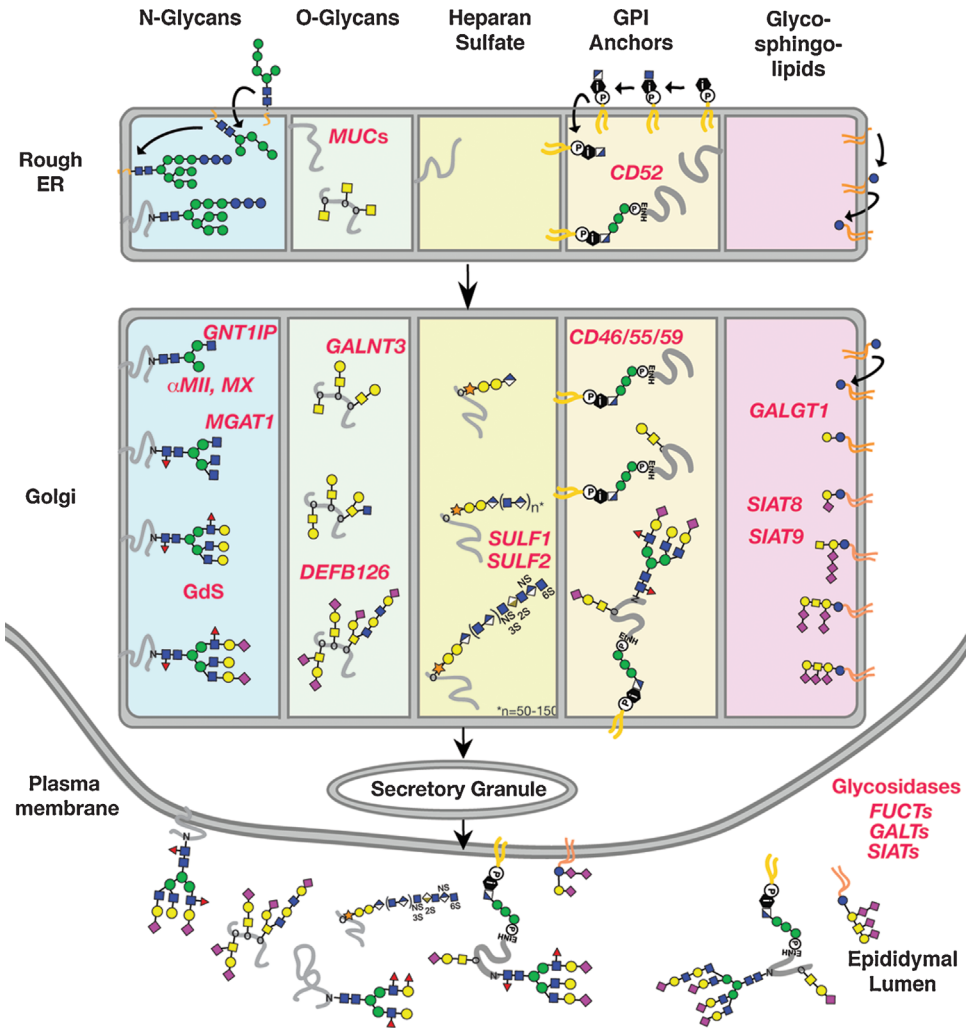

Figure 2. Synthesis of major glycan classes in the endoplasmic reticulum/Golgi of nascent sperm, and subsequent modification in the lumen of the epididymis. Genes or names for glycosylation enzymes discussed in the text are highlighted in red.

members, which play critical roles at the sperm surface, also require N-glycosylation to interact with endoplasmic-reticulum chaperones, including calreticulin and testes-specific calmegin, during meiosis (Yamaguchi et al., 2006; Ikawa et al., 2011).

Gene transcription, translation, and post-translational modification continue during meiotic divisions (Schultz et al., 2003) (see Figs. 3 and 4A). As a consequence, some glycoconjugates are synthesized by haploid sperm cells, making it possible that each sperm possesses an individual complement of glycans that results from its inherited set of enzyme alleles. True individuality, however, is limited by the persistence of inter-spermatid cytoplasmic bridges that allow for diffusion of haploid gene products (Ventela et al., 2003). As a consequence, sperm are more likely to possess a molecular composition mostly representative of the diploid parent than its inherited haploid genome (Dadoune et al., 2004).

Once spermatogenesis is complete, spermatozoa enter the epididymis and lose the ability to synthesize glycans, although existing glycans are still modified and new glycans are adsorbed to the sperm glycocalyx in the epididymis (Fig. 4). The activity of glycosidases and glycosyl transferases that are secreted into the lumen of the epididymis modifiy glycans that were made in the testis (Bernal et al., 1980; Tulsiani, 2006). The sperm glycocalyx is further modified as glycoconjugates and microvesicles (epididymosomes), secreted by epithelial cells along the male reproductive tract or by male accessory glands, are incorporated into the sperm membrane (Kirchhoff and Hale, 1996; Sullivan et al., 2007) (Fig. 4B). These post-testicular events further diversify the layers of glycoconjugates, adding so-called sperm-coating antigens (Dravland and Joshi, 1981; Flickinger et al., 1990) and other immune-modulatory glycoconjugates, such as CD59 (Rooney et al., 1993), to the sperm glycocalyx (Fig. 4C).

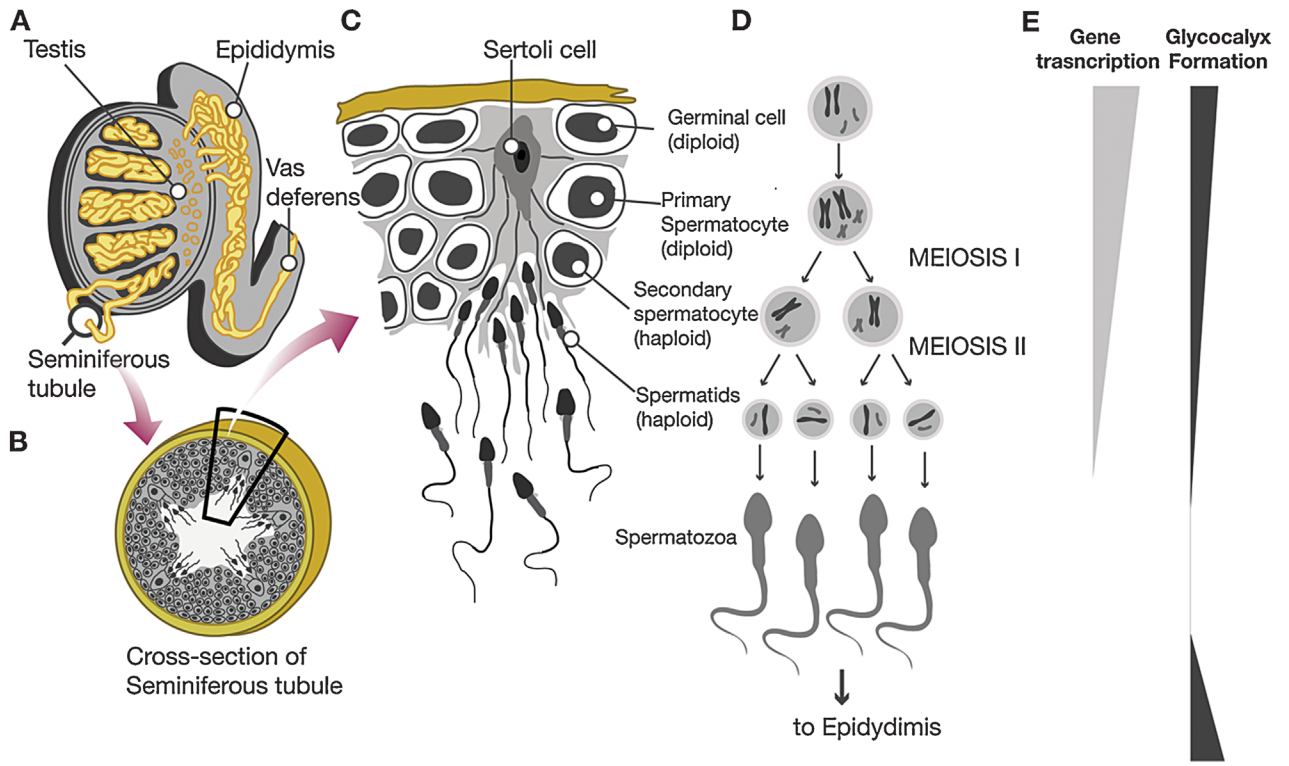

Figure 3. Mammalian spermatogenesis. **A**: Spermatogenesis takes place in the seminiferous tubules inside the testes. **B**: Primordial germ cells (spermatogonia) differentiate into primary and secondary spermatids, spermatocytes, and spermatozoa. **C**: Sertoli cells provide the environment for successful spermatogenesis. **D**: Schematic of meioses I and II. **E**: Comparison of the levels of gene transcription and glycocalyx formation during sperm maturation, according to the parallel timelines of 'C' and 'D'.

FUNCTIONS OF THE MAMMALIAN SPERM GLYCOCALYX IN THE FEMALE REPRODUCTIVE TRACT

A mature glycocalyx allows sperm to penetrate the cervical mucus in species that utilize vaginal insemination (Gilks et al., 1989; Tollner et al., 2008b), and protects sperm from humoral and cellular immunity in the uterus, where sperm encounter female antibodies, complement, and immune cells, such as macrophages and neutrophils (Pandya and Cohen, 1985; Thompson et al., 1992) (Fig. 4D). The sperm glycocalyx displays sialic acids (Toshimori et al., 1991; Varki, 2011) and bisecting, fucosylated N-glycans on its surface. Sialic acids form ''self-associated molecular patterns''—that is, these highly abundant terminal cell-surface molecules are recognized by numerous innate immune receptors such as Siglecs, which are known to modulate (mostly inhibit) the immune response (Toshimori et al., 1991; Varki, 2011). Bisecting, fucosylated N-glycans have also been shown to contribute immune-modulatory activity in the uterus (Pang et al., 2007) (Fig. 4E).

Passage from the uterus to the oviduct requires sperm to transit through the uterotubal junction (Fig. 4F), where female anatomy is complex and selection against sperm with misfolded proteins has been reported (Nakanishi et al., 2004). Glycan-mediated interactions are involved in the attachment of sperm to the oviduct epithelium (Suarez and Pacey, 2006b; Suarez, 2008; Tollner et al., 2008a) during formation of the mammalian sperm reservoir (Pollard et al., 1991) in the oviductal isthmus (Fig. 4G).

Before fertilization, sperm undergo capacitation, which involves dramatic modification of the sperm glycocalyx (Fig. 4H). Glycoconjugates (GPI-anchored proteins and beta defensins) are shed and sialic acids are cleaved by sperm sialidases during this process (Tollner et al., 2012; Ma et al., 2012). Capacitation also involves a redistribution of glycoconjugates, such as gangliosides (associated with lipid rafts) and glycan-modifying enzymes (e.g., sperm hyaluronidase PH20/sperm adhesion molecule 1 (SPAM1)) on the sperm surface, which is required for capacitated sperm to successfully fertilize an egg (Diekman, 2003) (Fig. 4I).

THE GLYCOCALYX CREATES AND CONSTRAINS SPERM INDIVIDUALITY

A panoramic view of the synthesis, assembly, and modification of the sperm glycocalyx reveals various points at which heterogeneity in a sperm population could be established. Differences due to post-meiotic, haploid gene expression of enzymes involved in sperm glycan

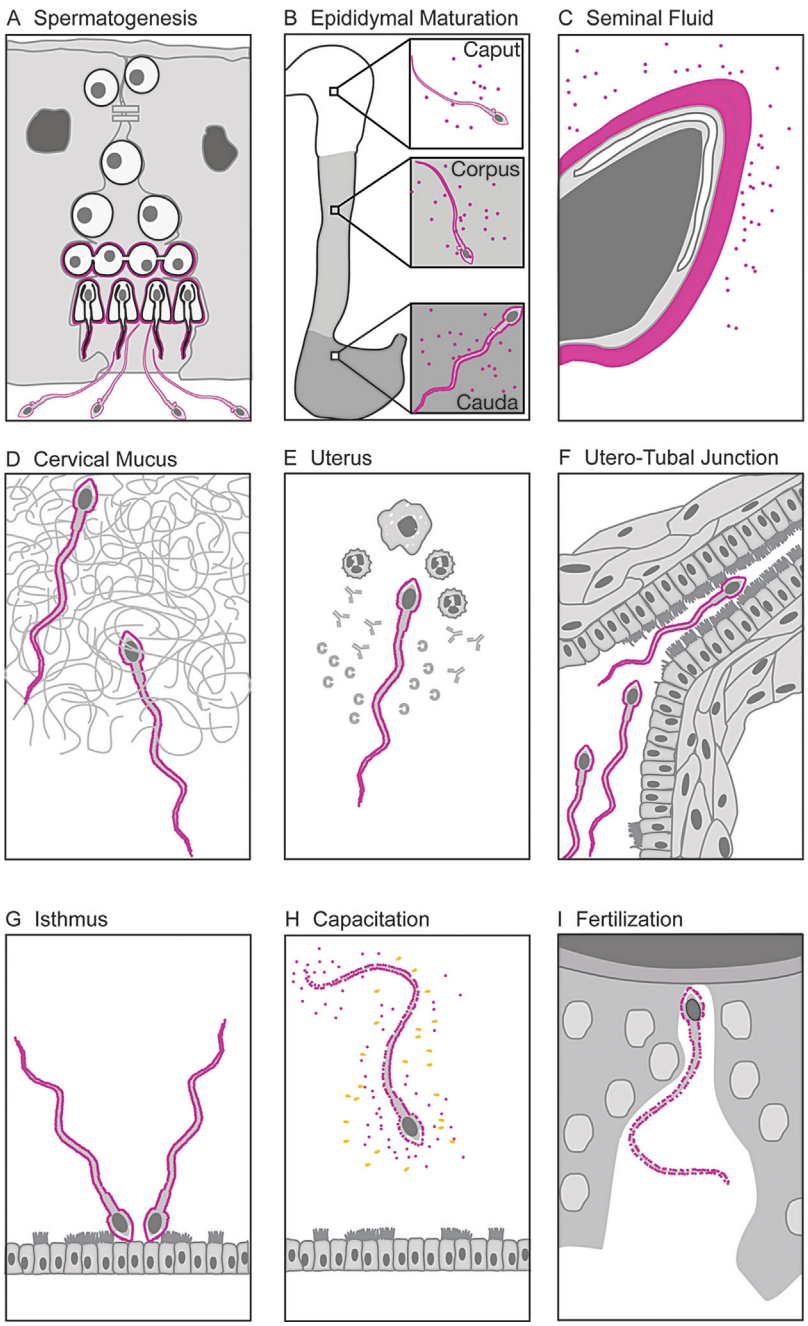

Figure 4. Functions of the sperm glycocalyx. **A**: N-glycosylation-dependent protein folding during spermatogenesis and spermatid-Sertoli cell adhesion. **B**: Spatially defined sperm glycan modification in the epididymis. **C**: Seminal components added to the glycocalyx maintain sperm in a non-capacitated, acrosome-intact state. **D**: Cervical mucus transit requires sialylated beta- defensin glycoproteins in primates. **E**: Immune modulatory glycans, including ''self-associated molecular patterns'' and immuno-suppressive signals, aid sperm survival in the uterus. **F**: Sperm passage through the uterotubal junction facilitated by glycosylation-dependent folding of ADAM proteins. **G**: Formation of the sperm reservoir by glycan-dependent adherence of sperm to the oviductal epithelium. **H**: Loss of glycans, glycoproteins, and cholesterol during capacitation. **I**: Exposure of masked functional molecules on the sperm cell surface, due to loss of glycans and glycoconjugates, allow for sperm–egg interactions.

synthesis in the testes; differential modifications during epididymal transit; and variability in the addition of glycoconjugates via accessory glands all contribute to a sperm's individuality. These differences could provide the female with a means to select certain sperm, considering that sperm glycans may reflect its quality. Conversely, epididymis-derived sperm-associated glycoproteins may mask such variability, perhaps initially protecting the sperm from female immunity but later shed to reveal the individuality of each spermatozoon's underlying functional groups at fertilization. Masking could also help mediate the evolutionary conflict between diploid males and their haploid gene products, whereby selfish meiotic- drive elements increase their propagation by biasing the likelihood of their transmission to another generation at the cost of the male's fitness (Springer and Gagneux, 2013).

In the following review, we detail the function of different glycoconjugates during the life of a spermatozoon. First, the importance of glycan synthesis in spermatogenesis will be discussed. Next, the assembly of somatically supplied glycoconjugates from the epididymis will be reviewed. Finally, we will focus on the function and modifications of male- specific glycans in the female reproductive tract.

GLYCANS ARE SYNTHESIZED BY SPERM IN THE TESTIS, AND ARE REQUIRED DURING SPERMATOGENESIS

A Specific Class of Glycosphingolipids Is Required During Spermatogenesis

Glycosphingolipids are common components of the outer leaflet of cell membranes, and are characterized by a ceramide lipid (a fatty acid linked to a long chain amino alcohol) attached to a disaccharide of glucose and galactose that can be variably extended. The enzyme GALGT1 (*N*-acetylneuraminyl-galactosylglucosylceramide *N*- acetylgalactosaminyltransferase) is responsible for adding *N*-acetylgalactosamine to the trisaccharide on nascent gangliosides, which are sialylated glycosphingolipids (Fig. 2). GALGT1 is expressed in the seminiferous epithelium, from 2 to 8 weeks of age in mice. *Galgt1*-null mutant males are infertile (Takamiya et al., 1998): At 4–6 weeks of age, *Galgt1*- mutants have severely smaller testis weight as compared to wild-type mice, and *Galgt1*-null adult males are aspermic due to a failure to complete spermatogenesis within the testes. Histologically, giant multi-nucleated cells, which are degenerating spermatids that fail to remain in contact with Sertoli cells, accumulate in the testis when *Galgt1* is missing. In addition, abnormal vacuolization was observed in mutant Sertoli cells; such vacuolization has been shown to occur when spermatogenesis is impaired (Takamiya et al., 1998).

Both a and b type gangliosides (Schnaar et al., 2009) are present in whole mouse testis. Gangliosides present in the seminiferous tubules of wild-type mice include **GM1** (Galβ1,3GalNAcβ1,4(Neu5Acα2-3)Galβ1,4Glcβ1,1Cer); **GD3** (Neu5Acα2,8Neu5Acα2-3Galβ1,4Glcβ1,1Cer); **GD1a** (Neu5Acα2,3Galβ1,3GalNAcβ1,4(Neu5Acα2,6)Galβ1,4Glcβ1,1Cer); **GT1b** (Neu5Acα2,3Galβ1,3GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,3)Galβ1,4Glcβ1,1Cer); **GD1b** (Galβ1,3GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,3)Galβ1,4Glcβ1,1Cer)— each of which can bind to testosterone in vitro. Although normal testosterone levels were present in *Galgt1*-null mouse testes, circulating levels in the serum were only 5% that of the wild-type mice; indeed, testosterone was highly enriched in the Leydig cells of *Galgt1*-mutant compared to wild-type mice. On the other hand, radiolabeled testosterone injected into the testis of mutant mice could not be found in seminal fluid (Takamiya et al., 1998).

While the requirement for *Galgt1* in spermatogenesis has been established, the precise types of gangliosides required were called into question after the development of knockout mice lacking two sialyltransferases, *Siat9* (encoding lactosylceramide α2,3-sialyltransferase) and *Siat8* (encoding α N-acetyl neuraminide α2,8-sialyltransferase 1). These enzymes cap the glycan chains on gangliosides with terminal sialic acid, and function downstream of *Galgt1* during ganglioside synthesis. Both *Siat9*- and *Siat8*-knockout mice lack a and b series of complex gangliosides, just like *Galgt1*-null males. Yet *Siat8*- and *Siat9*-mutant males are fertile. Profiling of the neutral glycosphingolipids and acidic glycosphingolipids (gangliosides) from testes of the *Siat8*-, *Siat9*-, *Galgt1*-mutant, and wild-type mice revealed a class of novel fucosylated neutral glycosphingolipids containing polyenoic, very-long-chain polyunsaturated fatty acid residues in all males except *Galgt1* mutants. Mass spectrometry analysis revealed the four fucosylated neutral glycosphingolipids to be: A) Fuc α 1,2 Gal β 1,3 GalNac β 1,4 Gal β 1,4 Glc β 1,1, Cer; B) Gal α 1,3(Fuc α 1,2) Gal β 1,3 GalNac β 1,4 Gal β 1,4 Glc β 1,1, Cer; C) GalNac α 1,3 (Fuc α 1,2) Gal β 1,3 GalNac β 1,4 Gal β 1,4 Glc β 1,1, Cer; D) GalNac β 1,3 Gal α 1,3 (Fuc α 1,2) Gal β 1,3 GalNac β 1,4 Gal β 1,4 Glc β 1,1, Cer, which were all sialylated in wild-type testis homogenates but not the *Siat* mutants. Antibodies specific to two of the four fucosylated neutral glycosphingolipids localized these glycosphingolipids to cells undergoing spermatogenesis (not spermatogonia) and Sertoli cells (Sandhoff et al., 2005).

Since this initial discovery, more than 13 complex neutral and acidic glycosphingolipids, eight of which were shown to be fucosylated, have been identified in the testis (Rabionet et al., 2008). Two of the identified glycosphingolipids localize to germ cells. In addition, an increase in testicular fucosylated neutral glycosphingolipids corresponded to progression through spermatogenesis. Interestingly, male mice mutant for *Slc35cl* (encoding the UDP-fucose transporter), which is expressed in developing sperm, are fertile despite not expressing any of the fucosylated neutral glycosphingolipids implicated in previous studies (Rabionet et al., 2008).

N-Linked Glycans Are Critically Required and Tightly Regulated During Spermatogenesis

All three classes of N-glycans (high mannose, hybrid, and complex N-glycans) are present on sperm. There is

also evidence that sperm have high concentrations of highly fucosylated N-glycans that exhibit immune-modulatory functions in invasive tumors (Pang et al., 2007). N-glycans are an important component of the glycocalyx, and are required for proper interaction with chaperone proteins during protein folding in the endoplasmic reticulum/Golgi. The glycans themselves also mature within the secretory pathway as they are transfered to the glycoprotein from a glycolipid donor (dolichol phosphate) and their high-mannose core is subsequently pruned to yield hybrid and complex N-glycans (Stanley et al., 2009).

Considering the high mannose content of N-glycans, mannosidases were likely to influence male fertility. One of the N-glycan-pruning enzymes is αMII (alpha-mannosidase II). The genetic absence of αMII results in decreased kidney function and an increase in autoimmune phenotypes, although male fertility is unaffected (Chui et al., 2001). Deletion of αMII did not completely abolish complex N-glycan formation, as determined by mass spectrometry, indicating that there must be an N-glycan synthesis pathway that is independent of αMII (Chui et al., 1997). Genetic ablation of a different mannosidase gene, *Mx* (encoding alpha-mannodisase IIx), however, does result in male infertility (Fukuda and Akama, 2003). *Mx* is highly expressed in spermatocytes and round spermatids, in addition to Sertoli and Leydig cells. *Mx*-mutant spermatocytes fail to adhere to Sertoli cells, resulting in the release of immature germ cells from the testicular epithelium into the epididymis. Mass spectrometry analysis revealed that wild-type testes are enriched with GlcNAc-terminating N-glycan structures, specifically a novel GlcNAc-terminated tri-antennary fucosylated N-glycan whose abundance is severely reduced in the testes of *Mx* mutants. When presented in isolation, this novel N-glycan was able to outcompete wild-type sperm for binding to Sertoli cells *in vitro*. Thus, spermatogenesis requires the formation of GlcNAc-terminating N-glycans whose synthesis is under the control of MX but not αMII (Akama et al., 2002; Fukuda and Akama, 2002, 2003).

The GlcNAc transferases MGAT1 (α1,3-mannosyl-glycoprotein 2-β-*N*- acetylglucosaminyltransferase) and MGAT2 (UDP-N-acetylglucosamine:α-6-d-mannoside β1,2-*N*-acetyl- glucosaminyltransferase II), which contribute to the formation of complex N-glycans, have both been deleted in mice. *Mgat1* knockouts are embryonic-lethal (Ioffe and Stanley, 1994), while *Mgat2* mutants do not survive past 3 weeks post birth (Wang et al., 2001) —phenotypes that make it difficult to evaluate their respective contributions to spermatogenesis. Recent work using a conditional, floxed *Mgat1* allele (Batista et al., 2012) has provided insight to the role of N-glycans in different steps of spermatogenesis by using Cre recombinase under the control of spermatogonia-, spermatocyte-, spermatid-, or Sertoli cell-specific promoters. The same experimental set up was used to perform tissue-specific ablation of genes regulating O-glycan synthesis, O-fucosylation, and Notch signaling. Surprisingly, these latter pathways were found to be dispensable for spermatogenesis, despite previously described roles of mucins (Lee and Damjanov, 1984; Lemaire and Heinlein, 1992; Seo et al., 2005) and Notch signaling (Mori et al., 2003) during spermatogenesis. Using spermatogonia-specific Cre, *Mgat1* was removed in the earliest stages of germ-cell development, resulting in infertile adult males. The abundance of complex N-glycans was substantially diminished in the *Mgat1*-null compared to wild-type testis, based on lectin staining. In addition, giant multi-nucleated cells were found in *Mgat1*-mutant seminiferous vesicles, a phenotype shared with other mutants whose germ cells fail to adhere to Sertoli cells. The testis of the mutant mice also showed a significantly elevated number of apoptotic germ cells in the seminiferous tubules compared to wild-type mice (Batista et al., 2012).

The importance of N-glycan synthesis in sperm development is so crucial that a testis-specific regulator of N-glycan synthesis, GnT1IP (GlcNAcT-I inhibitory protein), is expressed in both mouse and humans (Huang and Stanley, 2010). Murine GnT1IP has two splice variants: a long form that is membrane associated and a short form that may be secreted. The long form (GnT1IP-L) inhibits MGAT1 activity and prevents the synthesis of complex N-glycans in culture. GnT1IP-L is not expressed in spermatogonia, but is highly enriched in spermatocytes and is gradually lost as spermatocytes mature to spermatids and spermatozoa. This GnT1IP-L expression profile suggests that spermatogonia display complex N-glycans, whereas spermatocytes display mostly high-mannose N-glycans; spermatids would have a mixture of both. When expressed in Chinese hamster ovary (CHO) cells, GnT1IP-L causes the formation of high-mannose N-glycan structures that then allow for the transfected CHO cells to strongly adhere to Sertoli cells. Such binding was comparable to that of mutant CHO cells that generate high-mannose structures due to a *Mgat1* mutation (Huang and Stanley, 2010).

Although the requirement for N-glycans during spermatogenesis has been firmly established, less is known about the glycoproteins that carry these critical N-glycans. Basigin (BSG, also known as CD147) is a key component of the N-glycan-dependent pathways during spermatogenesis, as well as in the female reproductive tract during peri-implantation (Igakura et al., 1998). BSG is a transmembrane glycoprotein containing two extracellular immunoglobulin domains and a short cytoplasmic tail (Biswas et al., 1995; Toole, 2003). In the male, *Bsg* is expressed in spermatogonia, spermatocytes, spermatids, Sertoli cells, and Leydig cells (Bi et al., 2013), as well as throughout the epididymis—albeit at levels lower than in the testis (Chen et al., 2010). *Bsg*-knockout male mice are infertile due to the absence of mature sperm in the testis or epididymis. A closer investigation showed the presence of multi-nucleated giant cells and a dissociation of Sertoli cells from the basement membrane in the testis of knockout mice (Bi et al., 2013). An immortalized mouse spermatocyte cell line lacking *Bsg* fails to adhere to Sertoli cells in culture (Bi et al., 2013). Whereas BSG purified from various cell lines carry N-glycans containing polylactosamine (Galβ1−4GlcNAcβ1−3) repeats (Tang et al., 2004), BSG isolated from mouse testis carries N-glycans with terminal GlcNAc moieties (Bi et al., 2013). Moreover, lectin staining revealed substantially less GlcNAc

in the testis of *Bsg* mutants compared to wild-type mice. Together, these observations indicate that BSG is a major player in N-glycan-dependent pathways regulating spermatogenesis.

Multiple Functional Sperm Glycoproteins Are Made During Spermatogenesis

Essential sperm glycoproteins are also made during spermatogenesis. The sperm membrane protein equatorin, for example, is an O-glycosylated protein involved in acrosome function (Hao et al., 2014). GPI-anchored glycoproteins made during spermatogenesis include prion proteins (PrP or CD230), hyaluronidase (PH20/SPAM1), basigin (BSG/CD147), and the ADAM3-interactin TEX101 (Gmachl et al., 1993; Fujisawa et al., 2004; Fujihara et al., 2014). Immune-active integral membrane glycoproteins, such as CD9 (a tetraspanin involved in sperm egg fusion), CD46 (a complement regulatory protein), and CD47 (an inhibitor of endocytosis by macrophages), are also synthesized during spermatogenesis.

Several mucin genes are expressed during spermatogenesis in humans (Seo et al., 2005). The hallmark of mucin glycoproteins is their extremely high content of O-linked glycan chains. Various mucins are expressed by the epididymis, vas deferens, and prostate, indicating that these proteins may add to or alter the sperm glycocalyx during transit through the male urogenital tract (Russo et al., 2006). Indeed, the contribution of mucin to male fertility is evident in the impaired spermatogenesis correlated to the absence of a functional *MUC1* gene (Franke et al., 2001).

SOMATIC MODIFICATIONS OF THE SPERM GLYCOCALYX

Glycan-Modifying Enzymes Secreted From the Epididymis Act on the Sperm Glycocalyx

After spermatogenesis is complete, spermatozoa exit the testis and enter the epididymal lumen. At this point, spermatozoa are incapable of producing their own glycoconjugates due to the absence of a Golgi. Yet the glycans on the sperm surface can still be modified as sperm transit through the ''Golgi-like'' epididymis.

Spatial regulation of glycosylation seems to occur throughout the epididymis, based on different lectin staining profiles in the same cell types or structures in different areas of this organ. For example, in all parts of the mouse epididymis (caput SI, caput SII-V, corpus, and cauda; see Fig. 3B), microvilli of the principle cells show PNA (peanut agglutinin), DBA (*Dolichos biflorus* agglutinin), and SNA (*Sambucus nigra* agglutinin) staining, reflecting the presence of terminal β1,3-galactose, α-*N*-acetylgalactosamine, and α2,6-linked sialic acid, respectively. Conversely, VAA (*Viscum album* agglutinin) staining, specific for β- D-galactose, is observed in the microvilli of caput S1, corpus, and cauda, but is absent from caput SII-V. MAA1 (*Maackia amurens*is agglutinin) staining is observed only in the microvilli of caput S1, whereas JAC (Jacalin, from *Artocarpus integrifolia*) staining occurs in all sections of the caput (Lohr et al., 2010). Such region-specific lectin patterns match the profile of the glycoproteins secreted into the lumen of the epididymis. While many lectins bind the lumen throughout the epididymis, the caudal lumen has the greatest number of lectin interactions, implying this region secretes the most glycoproteins (Arenas et al., 1996).

In addition to N-glycans, many glycosyltransferases (Tulsiani, 2006; Cornwall, 2014) as well as some of their nucleotide sugar donor substrates—e.g., GDP-fucose, (Tulsiani et al., 1993) and CMP-sialic acid (our laboratory, unpublished)—have been identified in the epididymis lumen. For example, galactosyltransferase, glucosaminyltrasferase, fucosyltransferase, and sialyltransferase activities were isolated from rat luminal fluid devoid of spermatozoa (Tulsiani et al., 1993; Tulsiani et al., 1998; Tulsiani, 2003), and the different activities were enriched in different sections of the epididymis (Bernal et al., 1980; Tulsiani et al., 1993). Fucosyl- and sialyltransferase activities, both enriched in the caput as compared to other regions, are crucial considering that leukocytospermia in infertile men is associated with an increase in the amount of sialic acid and fucose attached to seminal fibronectin and α1-acid glycoprotein (Kratz et al., 2003). Glycosidase activities were also isolated from the epididymal lumen. Glycosidases typically function in the acidic environment of the lysosome in other cells; however, the epididymal lumen is near neutral pH. Interestingly, the activity of these epididymis-derived variants function better in vitro at neutral than acidic pHs – e.g. β-D-galactosidase (Skudlarek et al., 1993; Tulsiani, 2003).

Few of these glycosyltransferases or glycosidases associate with the sperm membrane during epididymal transit (Tulsiani et al., 1993), yet sperm-associated proteins clearly undergo glycan changes during their residence in the epididymis. As mentioned above, basigin (BSG) is expressed in the testis and contains N-glycans that are critical for spermatogenesis (Bi et al., 2013), but loses these N-glycans during epididymal transit (Saxena et al., 2002). Maturing sperm, therefore, continues to undergo Golgi-like glycan modifications in the epididymis, making the epididymal lumen an ''extracellular'' Golgi where secreted glycan-modifying enzymes and their substrates can alter the glycocalyx of maturing sperm.

Epididymal-Derived Glycoproteins Associate With the Sperm Glycocalyx

Sperm gain an overall negative charge during their transit through the epididymis due to modifications to the sperm glycocalyx. Glycoproteins made by the epithelial epididymal cells can be secreted into the lumen, where they then associate with the sperm surface, embedded in the sperm plasma membrane by direct insertion from the fusion of epididymosomes (Schröter et al., 1999; Sullivan et al., 2007). Several GPI-anchored glycoproteins are added to the maturing sperm glycocalyx during epididymal

transit (Kirchhoff and Hale, 1996; Sullivan et al., 2007; Netzel-Arnett et al., 2009), including complement-blocking glycoproteins, such as CD52, CD55, and CD59 (Rooney et al., 1993; Kirchhoff and Hale, 1996). Interestingly, many of these epididymal-derived glycoproteins—including glycodelin S, CD52, and beta-defensin 126—are required for successful sperm navigation in the female reproductive tract. In contrast, the glycoproteins of testicular origin are required for sperm–egg fusion—consistent with a ''first-in, last-out'' functional hierarchy.

SPERM GLYCOPROTEINS FUNCTION IN THE FEMALE REPRODUCTIVE TRACT

Glycodelin S Prevents Premature Capacitation

Glycodelin S (GdS) is a male-specific glycodelin isoform (Koistinen et al., 1996) that is highly enriched in seminal plasma, but binds weakly to sperm; the amount bound to sperm is positively correlated to sperm morphology. GdS is heavily glycoslyated and has been shown to contain Lewis X and Lewis Y oligosaccharides. Lewis X- and Lewis Y-terminated N-glycans are known to inhibit both innate and adaptive immunity responses. Indeed, major-histocompatibility-class-I-negative tumors also express these glycans to evade natural killer cells and to manipulate dendritic cells (Pang et al., 2007). On the other hand, GdS is unusually devoid of sialic acids (Yeung et al., 2007), whereas the female tract produces different glycoforms of sialylated glycodelin, such as glycodelin A (Yeung et al., 2006; Chiu et al., 2007b; Yeung et al., 2007).

In humans, spermatozoa are inseminated in the vagina and must traverse the cervical mucus and the uterus before entering the oviduct, where capacitation and fertilization occurs; the glycans of GdS are required for the protein's ability to inhibit capacitation during this period. Capacitation is the process by which spermatozoa become competent to fertilize the egg (Bedford, 1983), and is characterized, in part, by an initial albumin-dependent cholesterol efflux from sperm (Osheroff et al., 1999) (Fig. 2H). Recent findings indicate that capacitation-associated phosphorylation can be by-passed by triggering calcium influx (Tateno et al., 2013). GdS has been shown to inhibit this initial step of capacitation (Yeung et al., 2006), even though it is removed from sperm as they pass through the cervical mucus *in vivo* (Yeung et al., 2007). Taken together, the presence of GdS on ejaculated sperm is required to inhibit premature capacitation in an area of the female reproductive tract that is not the site of fertilization, e.g. from the vagina through the uterus. Moreover, the inhibitory effect of GdS has been shown to be dependent on its glycan structure as its deglycoslyation renders the protein incapable of binding sperm, thereby abolishing its ability to inhibit capacitation (Yeung et al., 2007).

CD52 May Be Immunoprotective for Sperm

CD52 is a GPI-anchored protein found in the male reproductive tract and on lymphocytes. Male reproductive tract CD52 (mrtCD52 or SAGA1) is secreted by the epididymis, inserted into the sperm plasma membrane, and bears N- and O-glycans (Diekman et al., 1999; Parry et al., 2007)—the former of which are different from those of the lymphocyte CD52. A CD52 antibody isolated from an infertile woman (termed MAb H6-3C4) was shown to recognize the N-glycan structure of mrtCD52, but not that of the lymphocyte CD52 (Hasegawa et al., 2003). Further investigation of this unique N-glycan indicated that mrtCD52 carries a version that is heavily sialylated and contains *N*-acetlylactosamine repeats (Galβ1,3GlcNAcβ1,3); this could possibly account for its ability to inhibit the classical complement pathways but not the lectin and alternative pathways (Hasegawa and Koyama, 2005; Koyama et al., 2009). Subsequent work showed that the N-glycan of mrtCD52 directly binds Cq1 (Hardiyanto et al., 2012), suggesting that mrtCD52 is an immunoprotective glycoprotein on sperm and may play a role in sperm survival during a leukocytic-mediated immune reaction.

Beta-Defensin 126 Has Multiple Functions in the Female Reproductive Tract

Beta-defensin 126 (DEFB126) associates with sperm as they transit through the distal corpus of the primate epididymis (Belleannee et al., 2012). DEFB126 is tightly associated with the sperm membrane; indeed, work with macaque DEFB126 and its mouse homolog, Defb22, has shown that this protein is inserted into the plasma membrane as a homodimer via hydrophobic amino acid residues (Tollner et al., 2012). The predicted amino acid mass of DEFB126 is 10 kDa, yet purified DEFB126 shows a size of 34–36 kDa. This significant shift in size is likely due to extensive O-glycosylation at the 20 sites of its carboxy-terminal tail, which contain glycans that are rich in α2-3 and α2-6 linked sialic acid (sialylated Tn antigen).

DEFB126 is present on ejaculated sperm, and is required for sperm to pass through the cervical mucus *in vitro*. Removal of sialic acid from DEFB126, by sialidase treatment or poly-L-lysine coating to obscure the sialic acid, decreases the number of sperm successfully traversing the cervical mucus, indicating that the terminal sialic residues are required for this sperm to penetrate the mucus (Gilks et al., 1989; Tollner et al., 2008b). Interestingly, a cohort of men with reduced fertility carry a small deletion in the *DEFB126* gene that is correlated to a significant decrease in the amount of O- glycans on the mutant sperm surface (Tollner et al., 2011). The sperm of the affected men showed normal morphology and movement, but failed to penetrate a hyaluronan gel. Hyaluronan consists of long disaccharide repeats (glucoronic acid (β1-3)-linked to N-GlcNAc (β1-4)-linked to the next disaccharide), and is routinely used as a simple mimic of cervical mucus. While hyaluronan is secreted by cervical epithelial cells and has important barrier functions (Akgul et al., 2014), cervical mucus consists mostly of highly O-glycosylated mucin glycoproteins (Andersch-Bjorkman et al., 2007).

After mating, sperm and/or seminal fluid trigger a leukocyte reaction, an immunological response wherein

circulating leukocytes—primarily neutrophils and macrophages—invade the uterus and destroy sperm (Pandya and Cohen, 1985; Katila, 2012; Morrow and Innocenti, 2012; Sharkey et al., 2012). Sperm are highly immunogenic as they harbor many male-specific proteins that are completely foreign to the female. Sperm isolated from the uterus still have DEFB126, which seems to shield male antigens from female antibodies. For example, antibodies against the sperm- specific proteins, PH20/SPAM1 and ADAM30, only bind to their antigens after DEFB126 has been removed. Moreover, rabbits immunized against whole sperm make antibodies primarily against DEFB126, whereas removal of DEFB126 or its sialic acid prior to immunization elicits a more robust immune response and results in an array of antibodies recognizing many proteins instead of just DEFB126 (Yudin et al., 2005). Taken together, these data indicate that DEFB126 and, specifically, its sialic acids, are immunoprotective for sperm in the uterus.

Of the many spermatozoa that enter the uterus, only a few manage to migrate into the oviduct. Oviduct epithelium-bound sperm seem to be maintained in a fertilization-competent state since they exhibit increased fertility and are less likely to undergo spontaneous capacitation compared to unbound sperm (Pollard et al., 1991; Dobrinski et al., 1996; Dobrinski et al., 1997). As the time of ovulation approaches, however, these reserved sperm undergo capacitation, detach from the oviductal epithelium, and swim toward the egg in the ampulla of the oviduct (Suarez and Pacey, 2006a). DEFB126 is required in the isthmus of the oviduct to form the sperm reservoir, which is a glycan-dependent process (Tollner et al., 2008a). Removal of DEFB126 or the addition of DEFB126 antibodies inhibits the ability of sperm to bind to oviductal epithelial cells *in vitro*. Similarly, sialidase treatment of DEFB126 inhibits the binding of sperm to oviductal epithelial cells. Capacitation-induced detachment from the oviductal epithelium in vivo and loss of DEFB126 under capacitating conditions in vitro suggest that DEFB126 is lost from epithelium-bound sperm during capacitation, which may facilitate their release from the oviduct.

Sialic Acids Delay Capacitation

Previous reports indicate that capacitation corresponds with an overall decrease in sialic acid content (Familiari and Motta, 1980; Focarelli et al., 1990). The mechanism by which sperm lose these terminal sugars during capacitation was previously unknown. Two sialidases, NEU1 and NEU3, are present at the sperm cell surface, and their activity is involved in capacitation in vitro (Ma et al., 2012). Inhibition of NEU1 or NEU3 leads to a decrease in tyrosine and ERK phosphorylation—both markers of capacitation—and diminished sperm binding to the zona pellucida. Moreover, some human sperm samples from infertile patients showed an absence or a decrease in NEU1 or NEU3 abundance by antibody staining (Ma et al., 2012). It would, therefore, be of great interest to determine whether NEU1 and NEU3 act on the sialic acids carried by sperm glycoproteins, such as DEFB126 or CD52, to promote capacitation in vivo.

DISCUSSION

Sperm Glycans and Glycoproteins Are Essential for Sperm Development and Function

Many glycan classes (N-glycans, glycosphingolipids, and O-glycans) and the glycoconjugates that carry them participate in sperm development and promote sperm migration and survival in the female reproductive tract. The requirement of the glycan classes reviewed herein is firmly established by experimental manipulation and/or removal of sperm glycan structures that lead to perturbations in sperm development and/or function. Yet, the contributions of two additional major glycans—polysialic acids and glycosaminoglycans—to fertilization are currently under investigation.

Polysialic acids are homopolymers of α2,8-linked sialic acids attached to N-glycans that exhibit highly restricted expression during neuronal development, stem cell-to-germ layer differentiation, and skeletal muscle development (Drake et al., 2008). A recent study reported on the presence of polysialic acid on human sperm, and speculated that it counteracts neutrophil extracellular nets (Alghamdi and Foster, 2005; Simon et al., 2013), thereby increasing sperm survival in the uterus.

Glycosaminoglycans are long, unbranched heteropolysaccharides containing disaccharide repeats of an uronic acid and an amino sugar (Bulow and Hobert, 2006). Heparan sulfate (one type of glycosaminoglycan) and syndecan-1 (a heparan sulfate proteoglycan) have been identified on ejaculated human sperm (Fig. 2), and are thought to mediate how human immunodeficiency virus 1 (HIV-1) (Ceballos et al., 2009) and human papilloma virus 16 (HPV-16) (Foresta et al., 2011) associate with sperm. Heparan sulfate modifications include 6-O, 2-O, 3-0, and NS sulfation. The importance of these modifications has been clearly established in multiple pathways, including neuronal development (Holt and Dickson, 2005), embryonic-stem-cell fate determination (Kraushaar et al., 2013), and pathological states, such as cancer (Hammond et al., 2014) and Alzheimer's disease (Ariga et al., 2010); there is also evidence that sperm heparan sulfate possess these modifications (Gagneux, unpublished data). Furthermore, the loss of SULF1 and SULF2 [heparin sulfate 6-O endosulfatases that remove 6-O-sulfate groups from heparin sulfate (Vives et al., 2014)] depletes the spermatogonial stem-cell niche (Langsdorf et al., 2011), implying that heparan sulfate helps maintain the male germ line.

Sperm Glycan Variability and Cryptic Female Choice

Given the important role of trans-glycosylation –i.e. glycosylation at the cell surface by enzyme and substrates secreted by other cells– for successful epididymal transit as well as the contribution of glycoconjugates secreted by

paternal somatic tissue, one could expect the sperm glycocalyx to be rather uniform. Surprisingly, recent reports revealing the surface heterogeneity among sperm (Cartwright et al., 1991) and variation in glycocojugate composition, such as the decoration with polysialic acid (Simon et al., 2013), pose interesting questions.

Nothing is known about the correlation of glycoclayx variation and the condition of individual spermatozoa, although it is plausible that variations in the sperm glycocalyx provide information about the content or quality of individual spermatozoa. Indeed, as the sperm glycocalyx is in direct contact with female cells, might the female filter sperm based on this glycocalyx variability as a metric of sperm quality? Cryptic female choice, whereby females actively select more advantageous sperm, remains a very appealing albeit very poorly documented phenomenon (Eberhard, 1996). Evidence exists for selective processes by cervical mucus and the uterotubal junction (Bianchi et al., 2004; Watanabe et al., 2014); indeed, retention of sperm with compromised chromatin in the cervical mucus is probably the best example of cryptic female choice (Bianchi et al., 2004).

The role of the glycocalyx in cryptic female choice remains to be formally investigated. How individual sperm characteristics, such as chromatin integrity and protein folding, might by detected by female selective factors remains an important mystery. Masking of sperm antigens by secreted epididymal glycoconjugates strongly reduces the possibility of female cryptic choice operating on membrane antigens during early stages of the sperm's journey in the female reproductive tract. Indeed, the ''parliament of glycans''—i.e., a coating of glycans provided by the paternal somatic tissue—might help counteract biased inheritance that may be initiated by a female responding to preferential marking of certain sperm that carry ''selfish genes'', which benefit the haploid sperm at the cost of the resulting diploid organism (meiotic drive) (Sandler and Novitski, 1957).

Consequences of Sperm Variability Between Closely Related Species

In the event of an inter-species mating, detection of mismatched sperm is likely to be strongly adaptive for females, given that inter-species hybridization in mammals usually incurs a loss of fitness. The sperm glycocalyx is in intimate contact with the female, so it may encode information related to its species of origin. Indeed, existing species-specific differences in glycosylation (Galili, 1993; Chou et al., 1998) allow females to discriminate against sperm from other species (Bishop and Gagneux, 2007; Ghaderi et al., 2011). Such glycan differences are recognized by anti-glycan antibodies and can induce glycan-based reproductive incompatibilities (Gagneux and Varki, 1999).

One example of a species-specific difference in glycan composition involves sialic acid. In mammals, sialic acid is found in two major forms: Neu5Gc (*N*-glycolylneuraminic acid) and Neu5Ac (*N*- acetylneuraminic acid). In contrast to chimpanzees and mice, humans lack the ability to synthesize Neu5Gc due to a mutation in the *CMAH* gene (encoding cytidine monophosphate *N*-acetylneuraminic acid hydroxylase) (Chou et al., 1998; Chou et al., 2002). As a consequence, humans make antibodies against Neu5Gc when exposed to this molecule in red meat and cow dairy products (Padler-Karavani et al., 2008; Taylor et al., 2010). Work in our laboratory has implicated sialic acid as a component of reproductive compatibility in vivo. Mice with incompatible sialic acid profiles have reduced fertility as compared to mice with compatible profiles (Ghaderi et al., 2011). In order to mimic the human condition, *Cmah*-null female mice were immunized to produce antibodies against Neu5Gc (Ghaderi et al., 2011). When these females are mated to wild-type males, whose sperm contains Neu5Gc, litter size significantly decreased as compared to non-immunized controls. This example of reproductive incompatibility mimics the natural situation that our human ancestors encountered 3.2 million year ago, when the *CMAH* gene was first inactivated. Interestingly, the loss-of-function allele rapidly swept into the species; mathematical models suggest this fixation event can be explained by reproductive incompatibility caused by female immunity (Ghaderi et al., 2011).

PERSPECTIVES AND CONCLUSION

The many functions of the sperm glycocalyx have only begun to be appreciated. While efforts are being made to define and characterize the vast array of glycans associated with sperm, less is being done to identify the functions of sperm glycans within the female. Not only does this question need to be addressed for the sake of basic science, but such an investigation could reveal novel areas of inquiry that may prove useful for understanding various reproductive health and infertility issues. In addition, by understanding the functional role of sperm glycans in reproduction, we may reveal how glycans have shaped human evolution.

We propose that the mammalian sperm glycocalyx, serves as a ''molecular beacon'' that identifies the species origin of the spermatozoa, as well as the quality of a given sperm cell to female, thus allowing her to select sperm during any part of the post-mating/pre-zygotic period. While, the role of glycans can only be completely understood in the context of the other three major classes of biomolecules (nucleic acids, lipids, and proteins) (Marth, 2008), sperm glycans seem to be in a prime place to convey a large and varied amount of information in a small and sweet package.

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