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A Mouse Model for Dietary Xenosialitis

*ANTIBODIES TO XENOGLYCAN CAN REDUCE FERTILITY**

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Humans can incorporate the xenoglycan *N*-glycolylneuraminic acid (Neu5Gc) from the diet into reproductive tissues and secretions. Most humans also have circulating antibodies specific for this dietary xenoglycan. The potential for inflammation induced by incorporated Neu5Gc and circulating anti-Neu5Gc antibodies, termed xenosialitis, has been discussed as a factor influencing several human diseases. Potential effects of xenosialitis on human fertility remain unknown. Here, we investigate possible adverse effects of the presence of Neu5Gc on sperm or endometrium combined with anti-Neu5Gc antibodies in semen or uterine secretions in a mouse model. We use *Cmah*$^{-/-}$ mice, humanized for Neu5Gc deficiency. We find that the viability, migration, and capacitation of sperm with incorporated Neu5Gc are negatively affected when these are exposed to anti-Neu5Gc antibodies. In addition, we find that after copulation, activated uterine neutrophils and macrophages show increased phagocytosis of sperm in the presence of anti-Neu5Gc antibodies via the complement receptor 3 (C3R) and FcγI/II/III (Fc receptor). Furthermore, Neu5Gc in endometrial cells combined with the presence of anti-Neu5Gc antibodies alters the receptivity and decidualization of endometrial explants. These studies provide mechanistic insights on how Neu5Gc on sperm and/or endometrium combined with anti-Neu5Gc antibodies in semen and uterine fluid might contribute to unexplained human infertility.

Infertility is a current global health issue affecting ~8–10% of couples in industrialized countries (1). It is a multifactorial problem, and a large fraction of cases (up to 30%) are classified as unexplained infertility (2, 3). Human anti-sperm antibodies (ASA)[2] have been linked to human infertility, and have been detected in up to 12% of infertile couples as well as in a small fraction of fertile individuals (<2.5% of fertile men and <1.4% of fertile women) (4, 5). The mechanisms of the adverse effects of ASA on fertility include antibody binding to sperm, resulting in the inhibition of sperm movement, agglutination, impairment of cervical mucus penetration by sperm, suppression of recognition processes, and impairment of entry into the ovum (6). Furthermore, such adverse effects could also include complement-mediated effector function in the female reproductive tract (7–9). Additional immune factors could also be involved in idiopathic infertility. It has been proposed that circulating anti-sialic acid antibodies target incorporated non-human sialic acid and contribute to chronic inflammation in various tissues (10–12). Our objective is to determine whether xenoglycan-antibody interactions also affect human infertility (13) given tentative evidence from a clinical study (14). Sialic acids (Sias) are nine-carbon backbone, acidic, amino sugars that decorate the surfaces of all vertebrate cells in high abundance (tens to hundreds of millions of molecules per cell). Sias are typically found as terminal sugars on the branches of *N*-linked and *O*-linked glycans and glycolipids. These cell surface sugar molecules have many roles in immunity including their contribution as self-associated molecular patterns (15, 16). The two most common mammalian Sias are *N*-acetylneuraminic acid (Neu5Ac) and Neu5Gc. Neu5Gc differs from Neu5Ac by a single oxygen atom added to the donor sugar nucleotide CMP-Neu5Ac by the enzyme CMP-Neu5Ac hydroxylase (CMAH) (17). The single copy *CMAH* gene was inactivated in humans over 2 million years ago (18) by an *Alu*-mediated deletion of 487 bp including a 92-bp exon resulting in a frameshift mutation. As a consequence, humans are genetically incapable of producing Neu5Gc. Nevertheless, Neu5Gc has been detected in human tissues, both healthy and diseased. These include endothelia of blood vessels, certain epithelia (11, 19), fetal tissues (20), and carcinomas, especially of prostate, ovary, and breast (21–23). The absence of any alternative synthetic pathway for Neu5Gc was confirmed by characterizing the Sias of transgenic *Cmah*$^{-/-}$ mice, which bear the same *Cmah* gene mutation as found in humans (24). Furthermore, Neu5Gc can be metabolically incorporated into cultured human cells *in vitro*. In both normal and cancerous cells, exogenous Neu5Gc was shown

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[2] The abbreviations used are: ASA, anti-sperm antibodies; BWW, Biggers, Whitten, and Whittingham medium; CASA, computer-assisted sperm analysis; SCSA, sperm chromatin structural assay; CMAH, cytidine monophosphate *N*-acetylneuraminic acid hydroxylase; PSM, porcine submaxillary mucin; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; NTHi, nontypeable *Haemophilus influenzae*; SVF, seminal vesicle fluid; Sia, sialic acid; FcR, Fc receptor; LIF, leukemia inhibitory factor; PI, propidium iodide; D4, gestation day 4.

to reach lysosomes via pinocytic/endocytic pathways, get imported in free form into the cytosol, and subsequently become available for activation and transfer to glycoconjugates (25). Dietary incorporation of Neu5Gc explains the presence of Neu5Gc in some human tissues. An experiment with human volunteers confirmed that ingested Neu5Gc can be taken up and incorporated into human sialoglycoconjugates such as mucins (19). Human diets in most industrialized countries are typically rich in red meats (pork, beef, and lamb), which contain high levels of Neu5Gc and are thus likely to account for the accumulation of Neu5Gc in human tissues (26–29). Furthermore, the last decades have seen a strong increase in consumption of Neu5Gc-rich red meat in the United States (30). The *Cmah*$^{-/-}$ mouse (human-like model) has been used in dietary studies to prove that Neu5Gc can be metabolically incorporated following ingestion of Neu5Gc-rich glycoprotein (31). These experiments have shown that Neu5Gc is incorporated into the small intestine and subsequently appears in circulation at a steady-state level for several hours, followed by metabolic incorporation into multiple peripheral tissues (31).

The human immune system regards Neu5Gc-containing glycan structures as "foreign," resulting in a polyclonal humoral response, with variable titers of circulating anti (α)-Neu5Gc antibodies among most humans (32, 33). The incorporation and accumulation of dietary Neu5Gc in the presence of an ongoing immune response against this non-human epitope make Neu5Gc the first known example of a "xeno-autoantigen" contributing to cancer and chronic inflammation (11, 34).

Human α-Neu5Gc antibodies appear during late infancy, correlating with weaning, exposure to dietary Neu5Gc (in formula based on bovine milk), and exposure to common bacteria, such as non-typeable *Haemophilus influenza*e (NTHi), which incorporate Neu5Gc into their cell surface lipooligosaccharides (35). Proposed as a human-specific mechanism, xenosialitis could exacerbate vascular pathologies such as atherosclerosis, and was also found to stimulate tumor growth in a human-like mouse model (36). A recent study demonstrated that Neu5Gc from diet increased the incidence of cancer in a mouse model (12).

We have previously shown that α-Neu5Gc antibodies can enter the female reproductive tract in mice and target Neu5Gc-positive sperm (37). Female mice genetically engineered with a human-like *Cmah*$^{-/-}$ mutation and immunized to express α-Neu5Gc antibodies show reduced fertility when mated with Neu5Gc-positive wild type males (13, 24). Furthermore, human α-Neu5Gc antibodies are also capable of targeting paternally derived antigens and can mediate cytotoxicity against Neu5Gc-bearing chimpanzee sperm *in vitro* (13), (37). Here, we investigate the possible consequences of the presence of Neu5Gc and α-Neu5Gc antibodies in the reproductive system in a mouse model humanized for its sialic acid and α-xenoglycan immune response.

Results

Mouse α-Neu5Gc Antibodies Bind to Neu5Gc on Motile Mouse Sperm—Cmah$^{-/-}$ Mice immunized against Neu5Gc produce antibodies in their sera (Fig. 1*A*) as well as in their uterine secretions (Fig. 1*C*). The binding of the antibody to motile sperm represents a crucial first step for its potential effect on sperm. We co-incubated WT C57/BL6 mouse sperm that have 20% of their Sias in the form of Neu5Gc (13) with

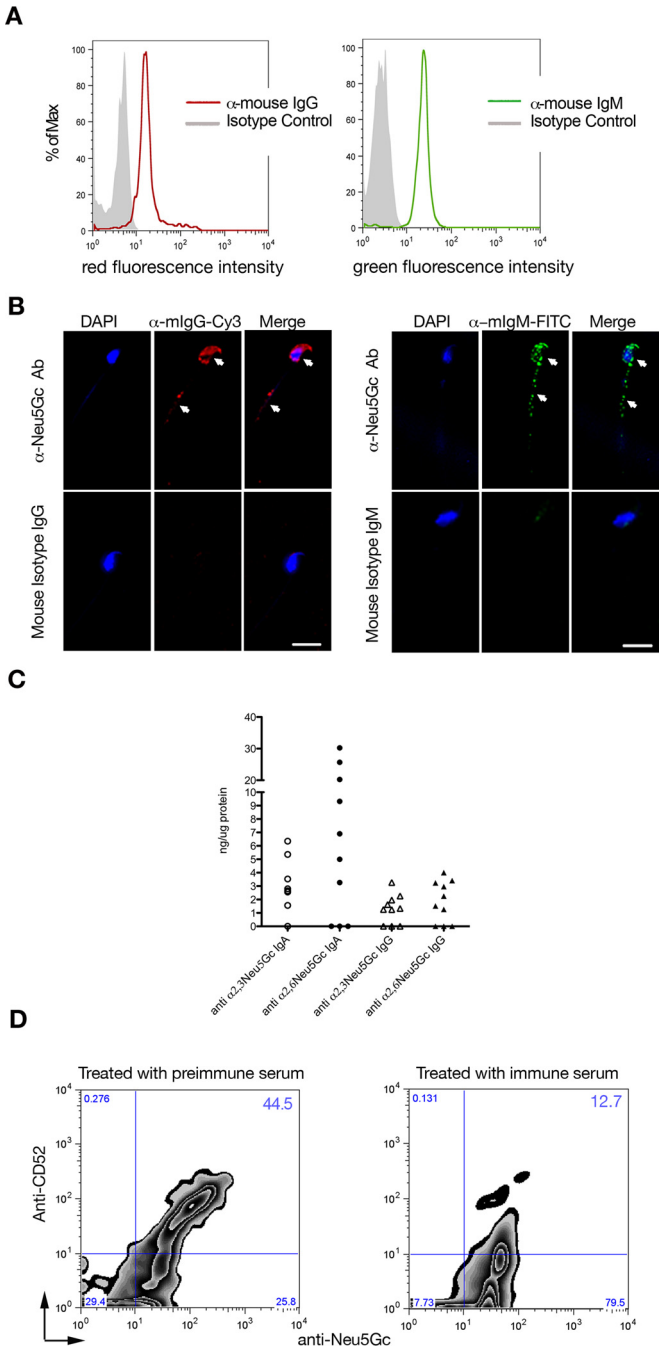

FIGURE 1. **Serum α-Neu5Gc IgG and IgM binding of Neu5Gc on WT motile sperm.** *A*, serum from immunized mice was incubated with WT motile sperm. Antibody binding to the sperm was characterized by staining with fluorescently labeled polyclonal goat-anti-mouse α-IgG and α-IgM antibodies (Santa Cruz Biotechnology) using flow cytometry, 10,000 events measured per sample. *%ofMax*, percentage of maximum. *B*, binding by antibodies from sera of immunized *Cmah*$^{-/-}$ mice visualized by fluorescent microscopy was found mainly on the sperm head; *red*, IgG; *green*, IgM. *Scale bar* = 15 μm. *C*, production of α-Neu5Gc IgA and IgG antibodies targeting 2,3/2,6-linked Neu5Gc in *Cmah*$^{-/-}$ mouse uterine fluid immunized with NTHi 6 months earlier and detected by ELISA. *D*, mouse α-Neu5Gc antibodies targeting Neu5Gc on seminal fluid CD52 sialoglycoprotein after incubation of *Cmah*$^{-/-}$ mouse sperm with Neu5Gc-containing WT seminal fluid. Neu5Gc on CD52 was targeted by mouse α-Neu5Gc antibody during preincubation as revealed by reduction in subsequent staining by commercial α-Neu5Gc antibody.

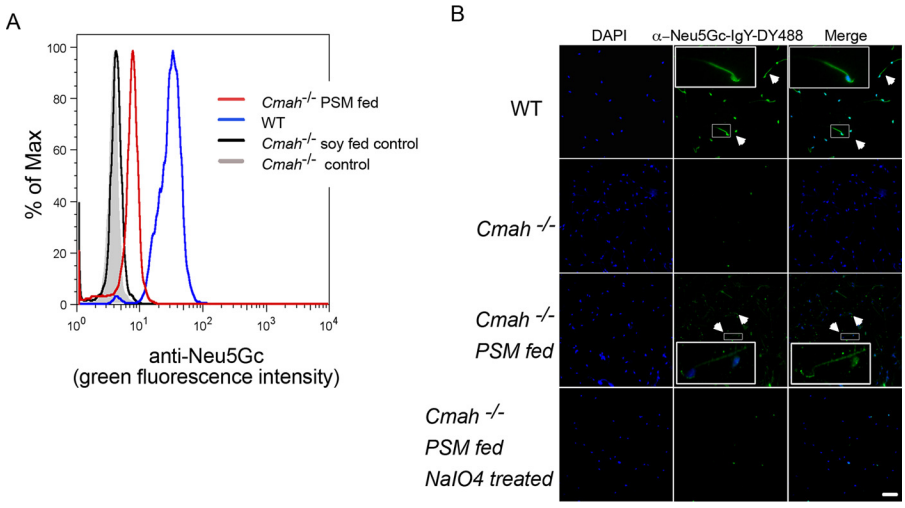

FIGURE 2. **Incorporation of Neu5Gc on sperm fed with PSM and production of uterine α-Neu5Gc Ab in *Cmah*$^{-/-}$ mice.** *A*, incorporation of Neu5Gc on epididymal sperm on *Cmah*$^{-/-}$ mice fed with PSM for 9 months as quantified by staining with anti-Neu5Gc antibody and flow cytometry analysis when compared with sperm from wild type, unfed, and control fed mice. *% of Max*, percentage of maximum. *B*, fluorescence microscopy comparing sperm from WT, *Cmah*$^{-/-}$, and PSM-fed *Cmah*$^{-/-}$ mice. $NaIO_4$ treatment truncates the side chain of Neu5Gc and confirms the specificity of the antibody (cells were examined in a DeltaVision real-time fluorescence microscope, Applied Precision). *Scale bar*: 15 μm.

α-Neu5Gc antibodies from NTHi-immunized *Cmah*$^{-/-}$ mice (35). We detected serum IgG and IgM α-Neu5Gc Ab bound to motile sperm by flow cytometry analysis (Fig. 1*A*). Immunohistology revealed that most of the binding occurred to the sperm head (Fig. 1*B*). The target of mouse uterine α-Neu5Gc antibodies was measured by ELISA using glycan probes carrying the two most common linkage types of Neu5Gc: α2,3 and α2,6 (Fig. 1*C*). To verify the Neu5Gc specificity of the mouse antibodies, we also incubated *Cmah*$^{-/-}$ mouse sperm with seminal vesical fluid (SVF) from WT mice to decorate the Neu5Gc negative sperm with seminal sialoglycoproteins such as CD52 from Neu5Gc-positive WT mice. CD52 is a highly sialylated glycophosphoinositol-anchored glycopeptide abundantly transferred from SVF to the membrane of mature sperm during ejaculation (38). These SVF-preincubated sperm were then exposed to immune or control sera and subsequently stained in parallel with α-CD52 and commercial affinity-purified α-Neu5Gc IgY antibodies. The immune serum strongly reduced the staining for CD52, indicating that the Neu5Gc on CD52 targeted by the mouse α-Neu5Gc antibodies partially blocked CD52 staining (Fig. 1*D*).

Cmah$^{-/-}$ *Mice Fed Neu5Gc-rich Chow Incorporate Neu5Gc in Their Sperm*—Mice fed porcine submaxillary mucin (PSM)-containing chow for 6 months produce sperm that carry intermediate levels of Neu5Gc when compared with regular or soy chow-based controls that lack Neu5Gc, and wild type C57BL/6 mice that have about 20% of all their sperm sialic acids as Neu5Gc (Fig. 2, *A* and *B*). The specificity of the antibodies for sialic acid was confirmed by mild periodate treatment, which cleaves the side chain of sialic acid.

Effects of α-Neu5Gc Antibodies on WT Mouse Sperm prior to Fertilization—We explored the effects of α-Neu5Gc antibody binding to motile sperm containing Neu5Gc. Wild type epididymal sperm showed reduced viability after exposure to mouse sera with high titers of α-Neu5Gc antibody. SYBR-14 (live) and propidium iodide (PI, dead) staining followed by fluorescence measurements using FACS analysis revealed a strong increase in the fraction of dead and dying epididymal sperm after exposure to immune serum (Fig. 3*A*). Motility measured as curvilinear velocity and velocity (straight line velocity) measured by computer-assisted sperm analysis (CASA) were not affected even after exposure to immune sera with variant titers (Fig. 3*B*). There was no evidence for any effects of the antibodies on levels of sperm DNA damage as assayed by sperm chromatin structure assay (SCSA) (Fig. 3*C*) nor on the ability of sperm to bind eggs using a homologous sperm-egg binding assay (Fig. 3*D*).

On the other hand, a sperm migration assay through a filter showed reduced migration by epididymal sperm treated with high titer mouse α-Neu5Gc antibody from both sera and uterine fluid (Fig. 3*E*). This might reflect the ability of sperm to penetrate cervical mucus and/or to navigate morphologically complex passages in the female reproductive tract.

Calcium influx into the sperm cell is associated with the capacitation process, a key event for fertile sperm (39). We also stained sperm exposed to immune or control sera for intracellular calcium using the calcium specific fluorescent dye fluo3 and analyzed the sperm populations by flow cytometry. Calcium influx was reduced by an order of magnitude in sperm exposed to immune serum (Fig. 3*F*).

The local immune system is stimulated as sperm enter the female genital tract, and by 12–14 h after copulation, female cells outnumber sperm, especially in the uterus, where many sperm are phagocytosed and killed in neutrophil extracellular traps (40–42). To explore how the phagocytosis of sperm might be affected by α-Neu5Gc antibodies, we used WT-GFP mouse sperm (expressing green fluorescent protein driven by CAG promotor in their midpieces) as targets, exposing these to immune and control sera before *in vitro* co-incubation with activated uterine leukocytes from *Cmah*$^{-/-}$ females mated with a wild type male 14 h after mating. We then measured phagocytosis of GFP sperm using fluorescent cytometry to measure green fluorescence and staining by several fluorescent antibodies. We stained for neutrophils with anti-Ly6G antibody (marker: 6y-Gr1) and for macrophages using anti-F4/80

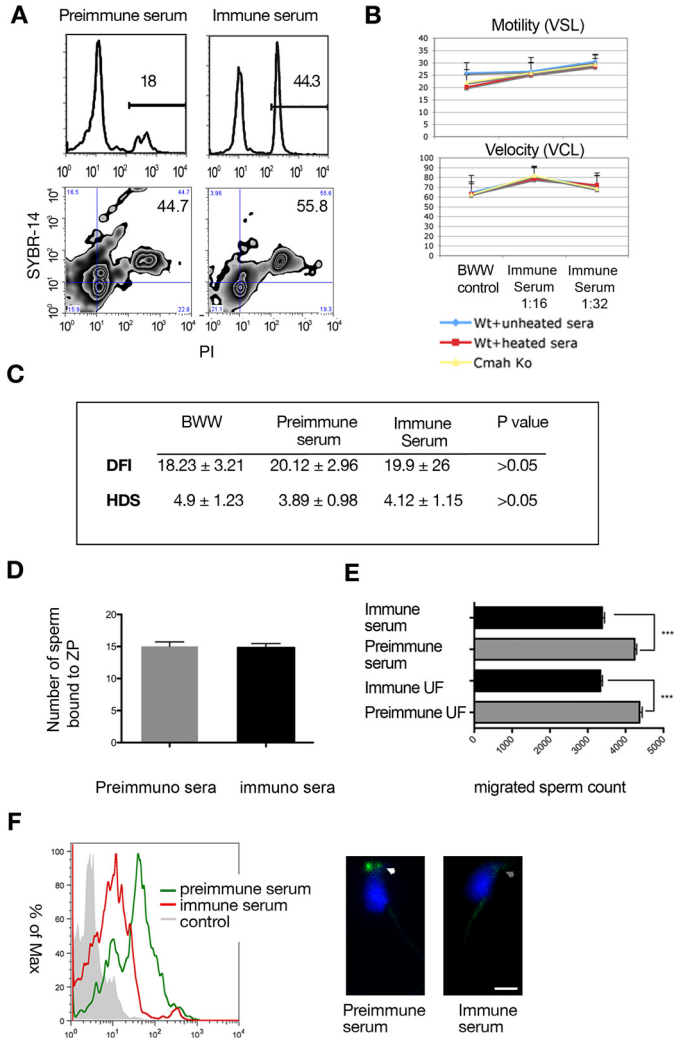

FIGURE 3. **Effect of α-Neu5Gc Ab on Neu5Gc-containing sperm.** *A*, the viability of WT sperm treated with α-Neu5Gc antibody from mouse sera analyzed with by flow cytometry. Values indicate dying and dead sperm, stained by PI above and doubly stained by SYBR-14/PI below (10,000 event measured for each sample). *B*, motility in sperm treated with α-Neu5Gc ab from mouse sera by CASA. *VSL*, straight line velocity; *VCL*, curvilinear velocity. *C*, DNA analysis in sperm treated with α-Neu5Gc ab from mouse sera by SCSA. *DFI*, DNA fragmentation index; *HDS*, high DNA stainability. *D*, sperm-egg binding assay in sperm treated with α-Neu5Gc antibodies from mouse sera. *E*, migration of WT sperm treated with α-Neu5Gc antibody from sera and uterine fluid using filter system. ***, $p < 0.0001$. *F*, calcium influx (*green*) in sperm exposed to *in vitro* capacitation medium, analyzed by flow cytometry (*left panel*, 10,000 events measured for each sample) and microscopy (*right panel*, *arrow*). Cells were examined in a DeltaVision real-time fluorescence microscope (Applied Precision). *% of Max*, percentage of maximum. *Scale bar* = 15 μm. *Error bars* represent S.D.

antibodies and found that phagocytosis was enhanced by 10.10 ± 2.3 and 5.6 ± 1.8%, respectively ($n = 4$, representative experiment shown in Fig. 4*A*). This indicates that α-Neu5Gc antibodies binding to sperm can recruit and activate leukocytes. To test for involvement of complement, we stained for C3R (complement receptor 3, marker CD11b) and found that FcR II/III (CD16/32) and FcR I (CD64) on neutrophils and macrophages were significantly occupied in the group treated with immune serum as quantified by FACS analysis. This suggests that these three complement receptors might play roles during phagocytosis mediated by α-Neu5Gc antibodies and indicates that the process is in part mediated by complement (Fig. 4*B*). Our experiments indicate that CD11b (C3R) might play a bigger role than FcR II/III,I (CD16/32,CD64) on neutrophils and macrophages (Fig. 4*C*).

Effects of α-Neu55Gc Antibodies on Receptivity and Decidualization of Endometrium—Results from our clinical study with *in vitro* fertilization patients pointed to failure of implantation as the main reason for low clinical pregnancy rates in women with xenoglycan and α-Neu5Gc antibodies in their uterus (14). No Neu5Gc was found in $Cmah^{-/-}$ mouse endometrium even after long time feeding with PSM in our present study, and the failure of this model might be due to differences in mouse and human endometrial metabolism and regeneration. Thus, we exposed mouse estrus endometrium to free Neu5Gc *in vitro* to load the tissues with the xenoglycan and test the possibility of Neu5Gc accumulating in these tissues. Exogenous Neu5Gc accumulated in the luminal and glandular epithelia after exposure of endometrial explants to 5 mM Neu5Gc for 2 days (Fig. 5*A*). We then tested the effect of co-occurrence of Neu5Gc and α-Neu5Gc antibodies on the receptivity and decidualization of endometrium. Wild type endometrium explants of D4 (gestation day 4) were co-cultured with high serum levels of α-Neu5Gc antibodies for 2 days. We detected a strong reduction in both leukemia inhibitory factor (LIF), used as a marker of receptivity, and metalloproteinase inhibitor 3 (TIMP3), used as a marker of decidualization, indicating a possible effect of the xenoglycan and α-Neu5Gc antibodies on the endometrium's receptivity and decidualization (Fig. 5, *B* and *C*).

Discussion

This study demonstrates in a mouse model that exogenous Neu5Gc and α-Neu5Gc antibodies can contribute to immune-related reduction of fertility in males and females. Their co-occurrence in a mouse model was associated with several adverse effects. In humans, α-Neu5Gc antibodies appear after weaning, possibly promoted by the combination of dietary Neu5Gc and its presentation on antigens of NTHi (35). Together with the finding of reduced clinical pregnancy rates in the presence of Neu5Gc antigen and directed antibodies in human fertility patients (14), our results suggest that the uterine mucosal immune system can produce α-Neu5Gc antibodies independently in $Cmah^{-/-}$ mouse model immunized by NTHi (intraperitoneal injection). We detected dietary Neu5Gc on epididymal sperm from $Cmah^{-/-}$ mice fed with PSM without blood-testis barrier damage, suggesting that there might be another underlying mechanism for Neu5Gc accumulation on human sperm where the majority of men do not show incorporated Neu5Gc despite life-long red meat diets. In our previous study, we documented *in vivo* fertility reduction in female $Cmah^{-/-}$ mice with high titer α-Neu5Gc antibody and mated with WT C57B/L (13), suggesting that in humans, the presence of Neu5Gc on sperm and high titer α-Neu5Gc antibodies in female genital tract or in male semen could contribute to unexplained infertility.

Based on our analyses of the distribution of Neu5Gc and α-Neu5Gc antibodies in the human genital system and the physiology of the fertilization process, we propose that dietary xenosialitis could negatively affect human fertility in a variety of ways (Fig. 5). Binding of α-Neu5Gc antibodies to motile sperm

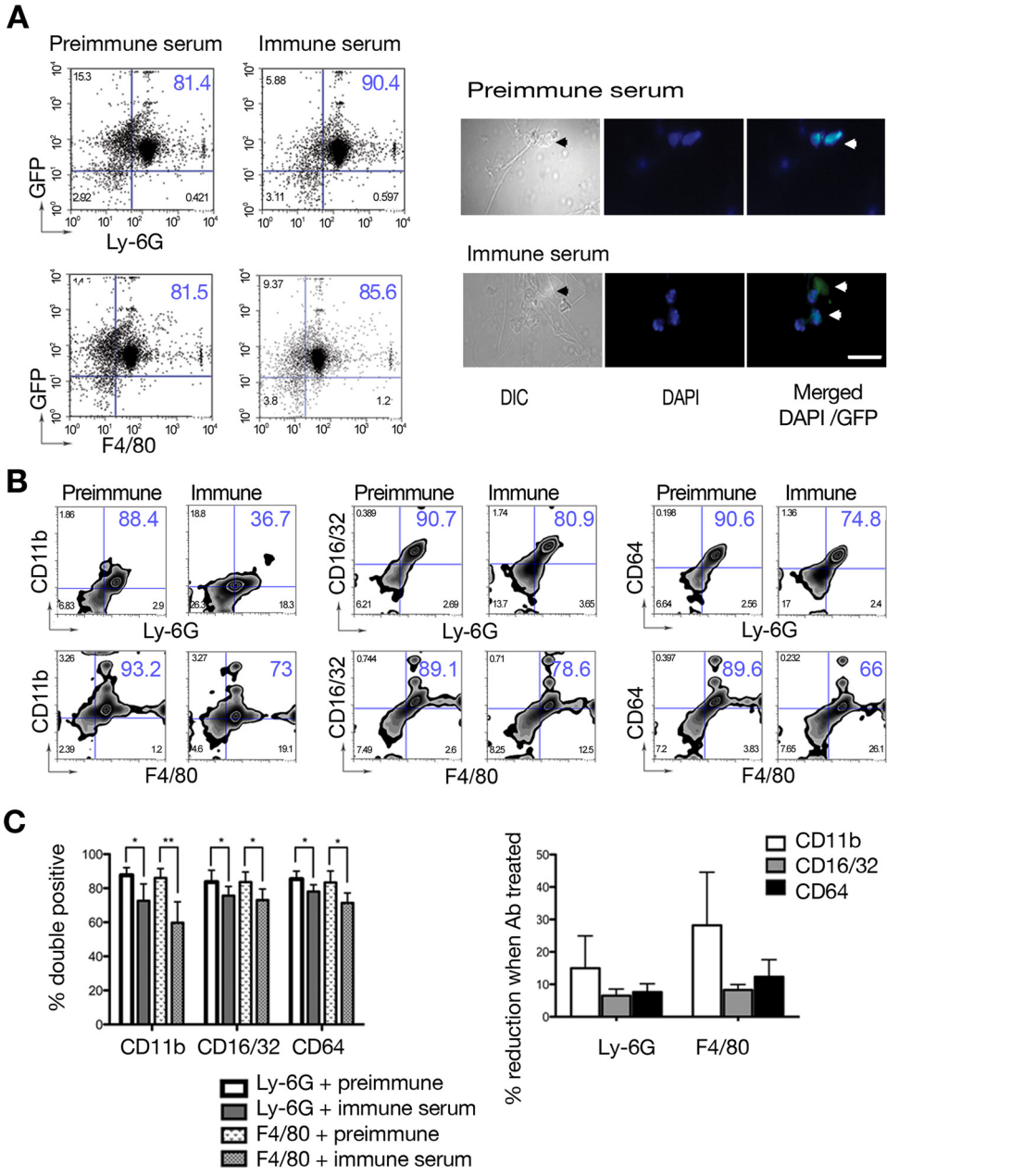

FIGURE 4. **Effect of α-Neu5Gc antibody on interactions between Neu5Gc-containing sperm and uterine immune cells.** *A*, phagocytosis of GFP sperm by activated uterine neutrophils and macrophages analyzed by flow cytometry (*left panel*) and microscopy (*right panel*). *Arrows* indicate phagocytosed sperm (cells were examined in a DeltaVision real-time fluorescence microscope, Applied Precision). *Scale bar* = 15 um. *B*, representative results from flow cytometry analysis of Fc receptors on neutrophils and macrophages. *C*, quantification of Fc receptors on neutrophils and macrophages in three independent experiments. * indicates >0.5, and ** indicates >0.05. *Error bars* represent S.D.

in vivo might critically contribute to the failure of sperm prior to fertilization, in line with reported mechanisms suggested from research on effects of human ASA (43, 44). Our results seem concordant with reports that ASA affect sperm fertilization capability after antibodies bind to antigen (Neu5Gc)-containing sperm through multiple key mechanisms. These include decreased viability, migration, calcium influx, and the increase of phagocytosis via the receptors of complement 3R (CD11b) and Fc IgG I/II/III (CD16/32/64) on mouse activated (15 h) neutrophils and macrophages entering the mated uterus as part of the leukocytic reaction. In contrast, we found no apparent effects of α-Neu5Gc antibody exposure on sperm motility, DNA damage, and the ability of sperm binding to the egg *in vitro*. With regard to the female reproductive tract, we found that free Neu5Gc was incorporated into the glandular and lumenal epithelia of mouse endometrium *in vitro* by means of three-dimensional culture system (45) and resulted in detrimental effects on decidualization and receptivity. This was apparent from staining with two markers for receptivity and decidualization, respectively, leukemia inhibitory factor (LIF) and tissue inhibitors of metalloprotease (TIMP3) (46, 47).

Fig. 6 depicts scenarios for potential interference in males or females. Anti-Neu5Gc antibodies of the male and/or female can bind to motile sperm and negatively affect a number of events prior to fertilization, including sperm viability, motility, migration, chromatin structure, ability to capacitate, phagocytosis by

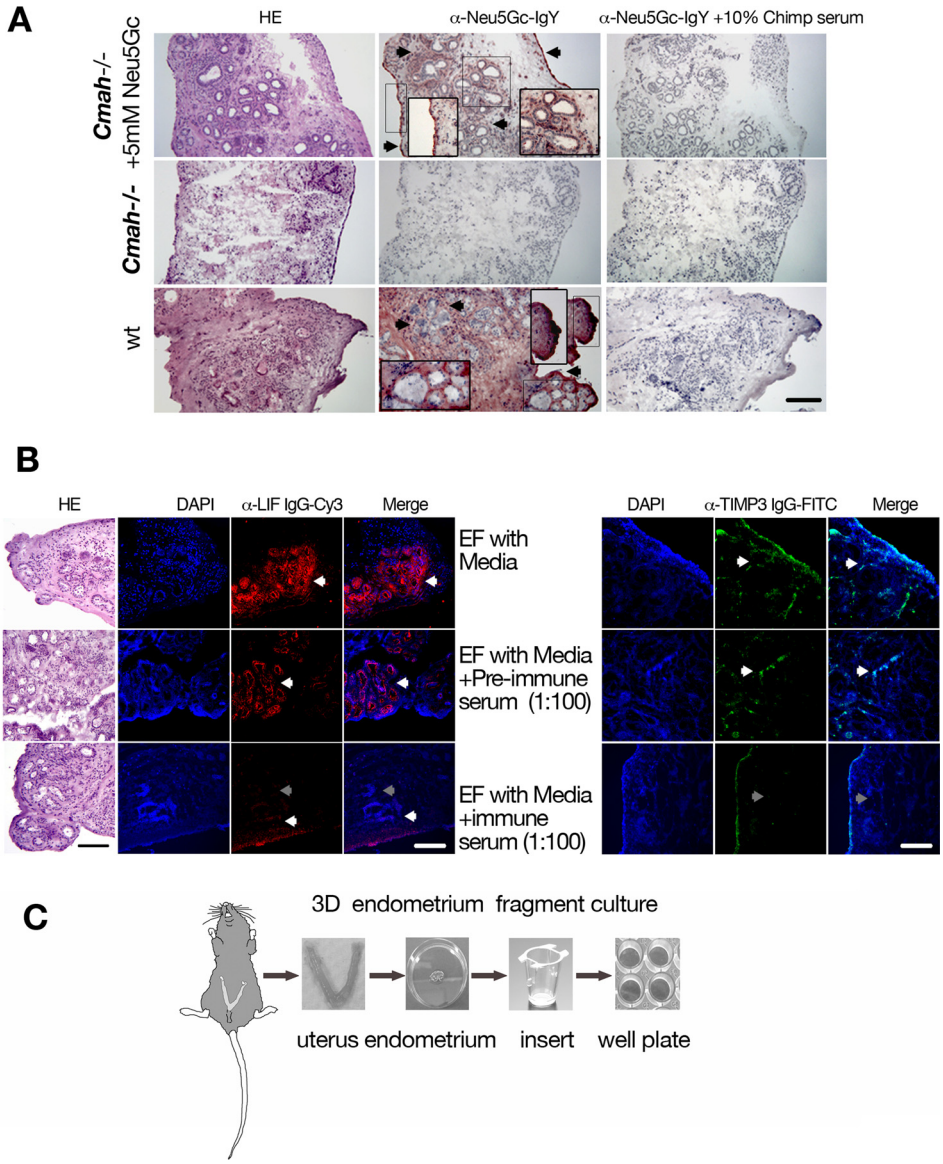

FIGURE 5. **Incorporation of Neu5Gc, effect of α-Neu5Gc antibody on the receptivity and decidualization of the endometrium.** *A*, incorporation of Neu5Gc in *Cmah*$^{-/-}$ estrus endometrium fragment (three-dimensional culture) *in vitro* for 48 h. Exogenous Neu5Gc was incorporated on epithelia of lumen and gland *in vitro* (*black triangles*). *Scale bar*: 200 μm. *B* and *C*, the effect of α-Neu5Gc antibody on the receptivity and decidualization of WT D4 endometrium (three-dimensional culture) *in vitro* for 48 h. *Red*, LIF; *green*, TIMP3; *blue*, DAPI; *gray arrow*, no expression; *white arrow*, positive expression. Tissues were examined in a DeltaVision real-time fluorescence microscope, Applied Precision. *Scale bar* = 200 μm. *HE*, hematoxylin/Eosin; *EF*, endometrium fragment.

leukocytes, adhesion of sperm to the oviductal epithelium, penetration of the cumulus layer, and sperm-egg binding. In addition, co-occurrence of Neu5Gc and α-Neu5Gc antibodies in the female tract could affect the receptivity and decidualization of endometrium and thus compromise implantation.

A clear answer to the age-old question of why hundreds of millions of sperm are required for the fertilization of a single ovum by a single sperm remains elusive. The factors favoring the precarious survival of a small minority of sperm that reach the fallopian tube remain poorly understood but may well involve differences in the sperm glycocalyx of individual sperm (48), differences in protein folding including haploid gene products on the sperm membrane, sperm chromatin structure and/or epigenetic modifications, and hormonal effects mediated by the female (49, 50). Unexplained infertility remains a troubling problem, and new approaches for understanding this multifactorial condition are needed. Exogenous, dietary Neu5Gc and α-Neu5Gc antibodies (xenosialitis) in humans could be novel factors contributing to unexplained human infertility. This phenomenon shares mechanisms with ASA, a well accepted cause for human infertility as an immunologic factor targeting certain substances on sperm that have been known to be antigenic for more than a century (51), but also includes autoimmunity in the female. Evidence from our findings in a humanized mouse model together with our published clinical findings (14) provide justification to study xenosialitis as a factor underlying human infertility by means of large-scale, comprehensive, and multicenter research. In light of the many profound differences between human and mouse reproductive biology, ranging from sperm count and mating behavior to female reproductive tract anatomy, diet, and immunology, our findings can only hint at potential mechanisms in humans.

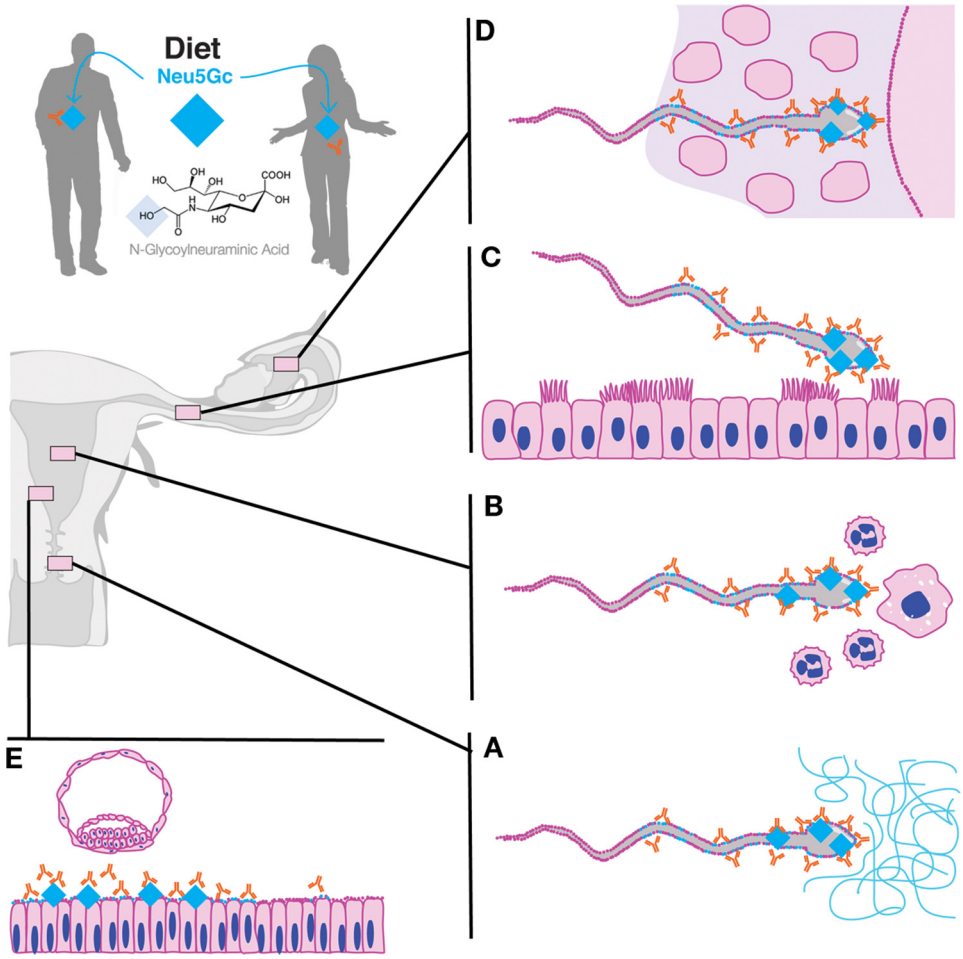

FIGURE 6. **Hypothesis of the role of exogenous (dietary) Neu5Gc and α-Neu5Gc antibody as contributing factors to unexplained human infertility.** The combination of incorporated dietary xenoglycan Neu5Gc and α-Neu5Gc antibodies in males or females could have deleterious consequences on human fertility via a number of potentially additive mechanisms, including: coating of sperm by male and/or α-Neu5Gc antibodies and/or interference with sperm movement through cervical mucins and/or other restrictive parts of the female reproductive tract (*A*), increased sperm death via female cellular and humoral immunity (*B*), interference with sperm capacitation (*C*), interference with sperm penetration of vestment, interference with sperm-egg interactions (*D*), interference with endometrial decidualization, and receptivity resulting in reduced success of implantation (*E*).

Future investigation of xenosialitis in human infertility promises to lead to important insights into causes of human idiopathic infertility and open avenues for intervention.

Experimental Procedures

Mouse Sperm Collection—WT C57BL/6 mice were purchased from Harlan Laboratories, and *Cmah*$^{-/-}$ ('24), C57BL/6 and CAG GFP mice (52) were provided by the Laboratory of Dr. Ajit Varki at University of California, San Diego. All mice were kept and utilized under University of California, San Diego Institutional Animal Care and Use Committee (IACUC) Protocol S01227. Sperm were harvested from the cauda epididymis of sacrificed 12–20-week-old males. The cauda epididymis was squashed in a drop of 50 μl of Biggers, Whitten, and Whittingham medium (BWW) or human tubal fluid (Irvine Scientific), and sperm were subjected to a swim-up procedure in 100 μl of BWW at 37 °C under 5% CO_2 for 20 min following published procedures (53).

In Vitro SVF Incubation with Sperm—Seminal vesicles were isolated from blood vessels and accessory glands by careful dissection. The gland fluid contents were manually expressed into microcentrifuge tubes. 20 μl of SVF were then mixed with 100 μl of physiological BWW and placed in a humidity chamber at 37 °C for 10 min, and then centrifuged at 1000 × *g* for 5 min, before using the supernatant for sperm incubation. Epididymal sperm were added to the diluted SVF. 10^6 sperm in 50 μl of diluted SVF were incubated at 37 °C under 5% CO_2 for 15 min (54, 55).

Mouse Model for Human Exogenous Neu5Gc Incorporation—*Cmah*$^{-/-}$ mice were fed with specially formulated chow containing Neu5Gc (added in the form of PSM (1.28 g of mucin/kg)) (56), and soy chow was fed to a control group for 6 months. Epididymal sperm and male and female genital organs were collected for measurement of incorporated Neu5Gc.

Mouse Model for Human Exogenous α-Neu5Gc Antibody by NTHi-immunized Mice—As described by Taylor *et al.* (35), NTHi strain 2019 or 2019siaT was grown to mid-log (A_{600}, 0.3–0.4) in Neu5Gc medium (Inalco Pharmaceuticals, San Luis Obispo, CA), washed, heat-killed, and injected (200 μl of A_{600}, 0.4) intraperitoneally into *Cmah*$^{-/-}$ mice (age 5–8 weeks, female). All mice were injected a total of three times for boosting at 2-week intervals. No adjuvant was used with any of the

bacterial injections. Serum for antibody analysis was collected after the second boost, and uterine flush was collected 6 months after initial immunization.

Determination of Neu5Gc Antigen Targeted by α-Neu5Gc Antibodies—Motile epididymal sperm from WT and *Cmah*$^{-/-}$ mice were preincubated with wild type mouse SVF (method as described above) and subsequently co-incubated with mouse sera and uterine fluid containing high levels of α-Neu5Gc Ab (detected as above) at 37 °C under 5% CO_2 for 1 h in BWW. Sperm were subsequently washed and stained with fluorescently labeled anti-mouse IgA/G/M (1:200 BioLegend) in 1% human serum albumin and PBS for 30 min at 4 °C. Changes in CD52 and Neu5Gc content on and *Cmah*$^{-/-}$ epididymal sperm after preincubation with wild type SVF were evaluated using commercial polyclonal α-Neu5Gc IgY (1:1000, Sialix) and polyclonal α-CD52 (1:500, Santa Cruz Biotechnology). $NaIO_4$ treatment truncates the side chain of Neu5Gc and confirms the specificity of the antibody (57).

Viability, Migration, and Calcium Assay of Sperm—For sperm viability analysis, sperm density was adjusted in BWW, and staining was performed according to the manufacturer's instructions using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/PI, Invitrogen) (58), prior to evaluation of the sample by flow cytometry (BD Biosciences FACSCalibur). For the sperm migration assay, 300 μl of BWW were added to each well of a 24-well plate (BD Biosciences), and then inserts (BD Biosciences, pore size 3 um) were set, and 10^5 sperm suspended in 50 μl of BWW were added to each insert and incubated at 37 °C for 30 min. Sperm that migrated into in the lower chamber were counted (this simple system was designed with the help of Noritaka Hirohashi (Ochanomizu University, Tokyo, Japan). We determined sperm capacitation status according to the instructions in Marquez and Suarez (59). 10^5 sperm were co-incubated with the loading concentration of the fluorescent calcium dye FLO3 (1 mM in DMSO, Invitrogen) and then analyzed by flow cytometry and imaged by microscopy.

Phagocytosis of Sperm by Uterine Activated Leukocytes and Determination of Potential Receptors on Neutrophils—A single male (C57BL/6) was caged with two females in estrus stage, and then females were examined the next morning for the presence of vaginal plugs as an indication of successful mating. Mated females were sacrificed 14 h after successful mating, the whole uterus was excised, the two horns were separated and clamped shut with forceps, and the uterine horns were flushed using a 26-gauge syringe with 200 μl of PBS. The flush contained uterine leukocytes (naturally activated by the mating event), which were then co-incubated with the male CAG-GFP mouse epididymal sperm in HEPES buffer with human tubal fluid (Irvine Scientific) at 37 °C under 5% CO_2 for 4 h, prior to analysis by flow cytometry. We quantified the expression of CR3, FcR RI, and FcR RII/II on neutrophils and on macrophages by staining with anti-Ly-6G (Gr-1) and anti-F4/80 for cell type, and with anti-CD11b, anti-CD64, and anti-CD16/32 for the three complement receptors (all five antibodies were fluorescently labeled and acquired from BioLegend).

Mouse Endometrial Fragment Culture in Vitro—Endometrium from *Cmah*$^{-/-}$ estrus mice mated at day 4 (D4) to WT mice (as above) was squashed in PBS (Gibco) at 4 °C following the methods described in Colette *et al.* (45). Tissues were gently dissected into small fragments (1 mm^3) using a scalpel in fresh PBS at 4 °C. Ten pieces per dish were placed on 12-mm-diameter tissue culture inserts (Teflon membrane, pore size: 0.4 μm, BD Biosciences) in 24-well culture plates. Organ cultures were maintained in serum-free conditions in Dulbecco's modified Eagle's medium/Ham F12 1:1 (DMEM/F-12, Gibco) supplemented with 1% insulin-transferrin-selenium (ITS+, Sigma, Steinheim, Germany), 0.1% EX-CYTE (Millipore). Endometrial explants were cultured for 24–48 h at 37 °C in a humidified atmosphere under 5% CO_2 in air. For the endometrium of D4 mice, 63.5 nmol/liter progesterone, 7.14 nmol/liter estradiol were added in the no-sera medium (60). Endometrial explants were cultured with and without exposure to 5 mM Neu5Gc (Inalco Pharmaceuticals) for 2 days.

Analysis of Sperm Motility and DNA Fragment—Sperm motility was measured by CASA, and both straight line velocity and curvilinear velocity data were collected and then analyzed. Sperm DNA fragmentation was determined by SCSA, measuring DNA fragmentation index and high DNA stainability, and sperm were stained by adding 1.2 ml of acridine orange (chromatographically purified, Invitrogen) for 5 min prior to analysis by flow cytometry (61).

In Vitro Capacitation of Mouse Sperm—Mouse epididymal sperm were incubated in 5 mg/ml BSA (Sigma), 20 mM $NaHCO_3$ BWW, at 37 °C under 5% CO_2 for 2 h following published methods (62).

Sperm-Egg Binding—Three-week-old female mice were superovulated by sequential administration of pregnant mare's serum gonadotropin (Sigma) and human chorionic gonadotropin (Sigma), and then the oocytes were collected by flushing the oviduct and were stripped of the cumulus cell using hyaluronidase (Invitrogen). Fresh epididymal cauda sperm were incubated in capacitation medium and then added to the oocytes for co-incubation for 30 min at 37 °C. The eggs were individually aspirated into a new drop of medium and placed on glass, kept on ice for 5 min, stained with DAPI (Sigma) at a concentration of 1:50,000 at room temperature for 2 min, and coverslipped, and sperm attached to the zona pellucida were counted (63).

Statistics—Data were graphed and analyzed using GraphPad Prism 5. Two-tailed Student's *t* tests were used to evaluate significance and calculate *p* values, with threshold values as described under "Results" or in the figure legends. Error bars represent standard deviation of mean values. A *p* value of less than 0.05 was considered significant., * indicates >0.5, ** indicates >0.05, and *** indicates >0.005.

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