REPRODUCTION
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Siglec expression on the surface of human, bull and ram sperm

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Abstract

Sialic acid (Sia) is a major constituent of both the sperm glycocalyx and female reproductive mucosal surface and is involved in regulating sperm migration, uterotubal reservoir formation and oocyte binding. Siglecs (sialic acid-binding immunoglobulin – like lectins) commonly found on immune cells, bind to Sia in a linkage- and sugar-specific manner and often mediate cell-to-cell interactions and signalling. Proteomic and transcriptomic analysis of human and bovine sperm have listed Siglecs, but to date, their presence and/or localisation on sperm has not been studied. Therefore, the aim of this study was to characterise the presence of Siglecs on the surface of bovine, human and ovine sperm using both immunostaining and Western blotting. Siglec 1, 2, 5, 6, 10 and 14 were identified and displayed both species- and regional-specific expression on sperm. Almost universal expression across Siglecs and species was evident in the sperm neck and midpiece region while variable expression among Siglecs, similar among species, was detected in the head and tail regions of the sperm. The possible role for these proteins on sperm is discussed.

Reproduction (2018) **155** 361–371

Introduction

Both the plasma membrane of sperm (Schroter *et al.* 1999, Tecle & Gagneux 2015) and the female reproductive tract epithelial cells (Chávez & Anderson 1985, Carson *et al.* 1998, Dellmann 2006) possess a dense glycocalyx, comprising a diverse array of glycolipids and glycoproteins. The glycans associated with these structures are often arranged in chains and are commonly terminated in peripheral galactose, N-acetylgalactosamine as well as sialic acid and fucose sugars (Cohen & Varki 2010). The glycocalyx protects sperm from the external environment but may also influence their physical interactions due to an overall electronegative surface charge, largely imparted by Sialic acids (Sia) (Tollner *et al.* 2008*a*, Tecle & Gagneux 2015).

Sia comprise a nine-carbon backbone (with at least 11 carbons in total) and are ubiquitous on cell surfaces and are particularly abundant on the sperm plasma membrane (Warren 1959, Schroter *et al.* 1999, Varki & Schauer 2009). They display two parent forms, N-acetyl-neuraminic acid, which is converted by CMP-Neu5Ac hydroxylase (CMAH) into N-glycolyl-neuraminic acid (Shaw & Schauer 1989). These two Sia may undergo extensive substitution resulting in approximately 50 different types having been described (Cohen & Varki 2010). In humans, N-glycolyl-neuraminic acid is absent due to a mutation within the *CMAH* gene (Varki 2009). Sia are commonly found in α 2–3, 2–6 or 2–8 linkages to other sugars, which along with their extensive substitution, result in a tremendous diversity of carbohydrate structures (Mahajan & Pillai 2016).

Sia are involved in cell protection and also present a complex array of ligands that are recognised by Sia-binding proteins and can also mask endogenous cell surface Sia receptors (Varki & Schauer 2009) that are central to cell-to-cell communication and cell signalling. Several studies, in different species, have identified roles for Sia, both on sperm and within the female reproductive tract. Sperm evoke a rapid immune response when deposited within the female reproductive tract (Brandtzaeg 1997, Robertson & Sharkey 2001) and Sia on the sperm surface may facilitate immune tolerance (Toshimori *et al.* 1991, Ma *et al.* 2016). Sperm penetration through cervical mucus is enhanced by mutual repulsion via negatively charged Sia on sperm and mucin glycans (Tollner *et al.* 2008*a*) and lower levels of sperm negatively charged glycans correlates with subfertility associated with reduced cervical mucus penetration in both the chicken and human (Steele & Wishart 1996, Tollner *et al.* 2011). Sia levels and their selective removal also appear to be critical for

ISSN 1470–1626 (paper) 1741–7899 (online)
https://doi.org/10.1530/REP-17-0475
Online version via www.reproduction-online.org

sperm–oviductal and oocyte interactions; for example, Sia on sperm are implicated in the formation of the sperm-oviductal reservoir in several species including primates and rodents (Cortés *et al.* 2004, Tollner *et al.* 2008*b*, Kadirvel *et al.* 2012, Miller 2015), while in murine sperm, reduced levels of sialylation are evident after capacitation (Focarelli *et al.* 1995, Ma *et al.* 2012). In humans, a deficiency in sialidase expression correlates with idiopathic subfertility (Ma *et al.* 2012) and the removal of sperm Sia has been shown to enhance sperm-zona pellucida binding (Lassalle & Testart 1994). Conversely, Sia on the human and bovine zona pellucida are involved in sperm binding to as yet unidentified sperm ligands (Focarelli *et al.* 1995, Velásquez *et al.* 2007, Pang *et al.* 2011, Clark 2013, Takahashi *et al.* 2013).

Sia are recognised by a diverse group of Sia-binding proteins that effect Sia-dependent cell responses (Varki *et al.* 2009, Bochner *et al.* 2015). Siglecs (Sialic acid binding immunoglobulin-like lectins) comprise the largest group of Sia-binding proteins and are widely expressed on, and regulate, a range of cells associated with adaptive and innate immune responses (Crocker *et al.* 2007, Pillai *et al.* 2012). They are a subset of type 1 membrane proteins containing a V-set immunoglobulin domain, which bind specific sia linkages. They also possess an extended region composed of a C2 immunoglobulin domain, a transmembrane region and most have a cytoplasmic tail usually involved in activating or inhibitory intracellular signalling via cytoplasmic immunoreceptor tyrosine-based inhibitory or activating motifs (Varki & Angata 2006, Pillai *et al.* 2012, Macauley *et al.* 2014).

In vertebrates, more than 20 Siglecs have been identified which, based on their common architecture, are sub-divided into 2 groups (Crocker *et al.* 2007). CD22 Siglecs include Siglec 1, 2, 4 and 15 and are conserved structurally between humans, rodents and most mammals displaying about 25–30% identity. A second group, referred to as 'CD33-related Siglecs', differ in number between species. However, within species, they show high sequence similarity to each other in their extracellular regions (50–85%) and usually contain tyrosine-based signalling motifs in their cytoplasmic regions. Most Siglecs have short extended regions and are, consequently, located close to the cell surface, preventing them from binding to target ligands on other cells. In addition, the presence of abundant Sia on the same cell surface (endogenous cell surface Sia) allows *cis* interactions in which they bind ligands on the same cell surface. This interaction may play a role in regulating Siglec function (Crocker *et al.* 2007).

To date, much of the characterisation of Siglecs has focused on immune-related human and murine cells. Within the bovine genome, 11 Siglecs (Siglec 1–6, 8, 10, 13, 14, 15) have been identified and are similar to Siglecs from other species including human and mouse (https://www.ncbi.nlm.nih.gov/). In the context of reproduction, murine B cells have been shown to bind to murine sperm via Siglec 6 (Ma *et al.* 2012), and this may be involved in mediating Sia-dependent immune tolerance within the female reproductive tract. Several transcriptomic and proteomic studies on human and bovine sperm have listed the presence of Siglecs including Siglec 1, 2, 5, 6, 10, 12 and 14 (Lalancette *et al.* 2008, Peddinti *et al.* 2008, Gu *et al.* 2011, Baker *et al.* 2013, Amaral *et al.* 2014). In addition, Siglec-10 was found to be differentially expressed in sperm from bulls of high and low fertility (Lalancette *et al.* 2008).

To our knowledge, the expression and particularly localisation of Siglecs on sperm has not been studied in detail. Here, we characterise the expression of Siglecs on human, bovine and ovine sperm using immunohistochemical staining and Western blotting. Expression of several different Siglecs in all three species was detected, and these were confined to discrete sperm regions including the acrosomal cap, as well as the parts of the tail including the midpiece. The possible roles for Siglecs in different sperm regions and species are discussed.

Materials and methods

Human, bovine and ovine sperm

Fresh semen (2 mL) from three bulls was obtained from Progressive Genetics, (Enfield, Ireland) and was transported to the laboratory at 37°C within 1 h of collection. Sperm were counted and diluted to approximately 20×10^6/mL in phosphate buffered saline (PBS) prior to use. Extended semen from three human donors was purchased from Cryos International Aps (Aarhus, Denmark). Each straw had a volume of 500 µL and contained approximately 2×10^8/mL sperm in extender and had been screened for common genetic abnormalities as well as sexually transmitted diseases. Semen from three Vendeen rams was a gift from Prof. Mark Crowe (University College Dublin, Dublin, Ireland). Each straw contained in total approximately 85×10^6 sperm in 200 µL of extender. All frozen semen was stored in liquid nitrogen and thawed at 37°C for 30 s in a water bath and held in a 1.5 mL Eppendorf tube during processing. In addition, bovine epididymal sperm were collected from the cauda of the epididymides of three mature bulls, within 30 min of slaughter, at a local abattoir. A small incision was made through the wall of the cauda epididymis, and the sperm was gently squeezed into clean sterile collection tubes. All sperm samples were centrifuged to remove seminal plasma and diluents (370 ***g***, 5 min, RT) and the supernatant was discarded. The pellet was diluted in 400 µL of PBS (Thermo Fisher Scientific) and then re-centrifuged at 370 ***g*** for 5 min. The PBS was removed and the pellet was resuspended in 200 µL of PBS. Approximately 15 µL of this suspension was then added to Superfrost Plus slides (Thermo Fisher Scientific) to generate a thin smear, which was air-dried and stored at −20°C until use.

Antibodies for immunohistochemical staining and Western blotting

Antibodies against human Siglecs 1, 2, 5, 6, 10 and 14 that recognise extracellular epitopes in regions and domains that are conserved between human and other species were employed and are detailed in Table 1 and Supplementary Table 5A and B (see section on supplementary data given at the end of this article). Antibodies were used at a dilution between 1:35 and 1:200. Positive staining was detected by secondary staining with either FITC-conjugated or biotinylated antibodies. Secondary antibodies employed were polyclonal rabbit anti-mouse (F0232, FITC conjugated), polyclonal rabbit anti-mouse (E0464, biotinylated), polyclonal rabbit anti-goat (F0250, FITC conjugated) and polyclonal rabbit anti-goat (E0466, biotinylated), all from DakoCytomation.

Confirmation that human Siglec antibodies cross-react with other species

As no bovine or ovine Siglec antibodies are currently available, antibodies against human Siglecs were employed to screen for the presence of Siglecs on bovine and ovine sperm. To assess whether these human antibodies can cross-react with bovine Siglecs, bovine white blood cells (WBCs) and bovine and ovine spleen sections were screened. Blood films were fixed with 70% alcohol before being processed for immunostaining as described below. Spleen from mature cows and sheep ($n=3$, age range 2–5 years, Kildare Chilling, Kildare, Ireland) were collected from animals within one hour of slaughter. Tissue was dissected as appropriate and samples were collected in 10% buffered formalin. Tissue samples were fixed in 10% buffered formalin for 36 h and then processed by dehydration through a series of ascending ethanol solutions (30, 60, 90, 100%), cleared in xylene and impregnated with paraffin wax to form tissue blocks for sectioning. Suitably orientated tissue blocks were sectioned at 4 µm and tissue integrity was confirmed by microscopic examination after haematoxylin and eosin staining. Subsequently, serial sections were prepared and mounted on Superfrost plus slides for immunostaining.

Fluorescent immunostaining

Fluorescent immunostaining was employed to localise and visualise the expression patterns of each Siglec using fluorescent microscopy. A circular area was demarcated on sperm-smeared slides or tissue sections using a hydrophobic marker pen (Dako). Slides were then rinsed in PBS for 5 min and 80 µL of normal rabbit serum (NRS) (Invitrogen), diluted 1:20 in PBS, was added to each circle and incubated for 20 min at RT. Following removal of the NRS, the primary antibody (80 µL in PBS) at the appropriate dilution was added to each slide and incubated for 1 h at RT. Slides were washed three times with PBS × 5 min. The appropriate fluorescent secondary antibody was added at a suitable dilution and incubated for 2 h at RT. Slides were covered in tinfoil during this step to restrict light access to the reagent. Following incubation, slides were washed with PBS three times for 5 min. A cover slip was placed over each circle using aqueous non-fluorescent mounting medium. Slides were stored at 4°C and visualised using a fluorescence microscope (Nikon, Eclipse E400) with a FITC filter. Appropriate controls using primary antibody alone and secondary antibody alone were included.

Chromogenic immunostaining

Chromogenic immunostaining was employed to determine Siglec expression on spleen and distribution on sperm and to support and complement results obtained from fluorescent immunostaining. A circular area was demarcated using a hydrophobic marker on sperm-smeared slides, which were then rinsed in PBS for 5 min and 80 µL of 3% H_2O_2 in PBS added for 10 min to block endogenous peroxidase. Slides were washed under running tap water for 5 min and then immersed in PBS. A 1:20 dilution of NRS was added to each slide and incubated for 20 min at RT. NRS was removed and primary antibody (80 µL in PBS) at the appropriate dilution was added to each slide and incubated for 1 h at RT. Slides were washed three times with PBS for 5 min each. The appropriate biotinylated secondary antibody was added (1:400) and incubated for 30 min at RT. Slides were then washed three times with PBS for 5 min each time. Avidin–biotin complexed with horseradish peroxidase (ABC) (Vectastain Elite ABC kit, PK-6100, Vector Labs, Peterborough, UK) was added to each slide for 20 min. Slides were washed 3 times with PBS for 5 min. Colour was developed by adding 3,3′-diaminobenzidine tetrahydrochloride chromogen substrate and incubating for 5 min. Slides were then washed under running tap water for 5 min, dehydrated using 2 changes of 70% ethanol followed by two 2 changes of 100% ethanol and cleared in 2 changes of xylene for 3 min each. Cover slips were then applied using a synthetic mountant (DPX) and visualised using a light microscope

Table 1 Siglec 1, 2, 5, 6, 10 and 14 antibody source and antigen.

Siglec	Source	Antigen
Siglec 1	CD169 (N-20), goat polyclonal Sc-23594, (Santa Cruz Biotechnology)	Peptide mapping near the N terminus of human Siglec 1
Siglec 2	CD22 (N-20), goat polyclonal Sc-7031, (Santa Cruz Biotechnology)	Peptide mapping at N terminus of Human CD22
Siglec 5	MAB1072, mouse mono-clonal, (R&D Systems)	Mouse myeloma cell line NS0-derived recombinant human Siglec-5/CD170 Lys18-Thr434
Siglec 6	N-15 Sc-51427, goat polyclonal, (Santa Cruz Biotechnology)	Peptide mapping within an N terminal extracellular domain of human Siglec 6
Siglec 10	N-13 Sc-240882, goat polyclonal, (Santa Cruz Biotechnology)	Peptide mapping within an N terminal extracellular domain of human Siglec 10
Siglec 14	Siglec 5/14 AF1072, goat polyclonal, (R&D Systems)	Human Siglec 5 Glu17→Thr434

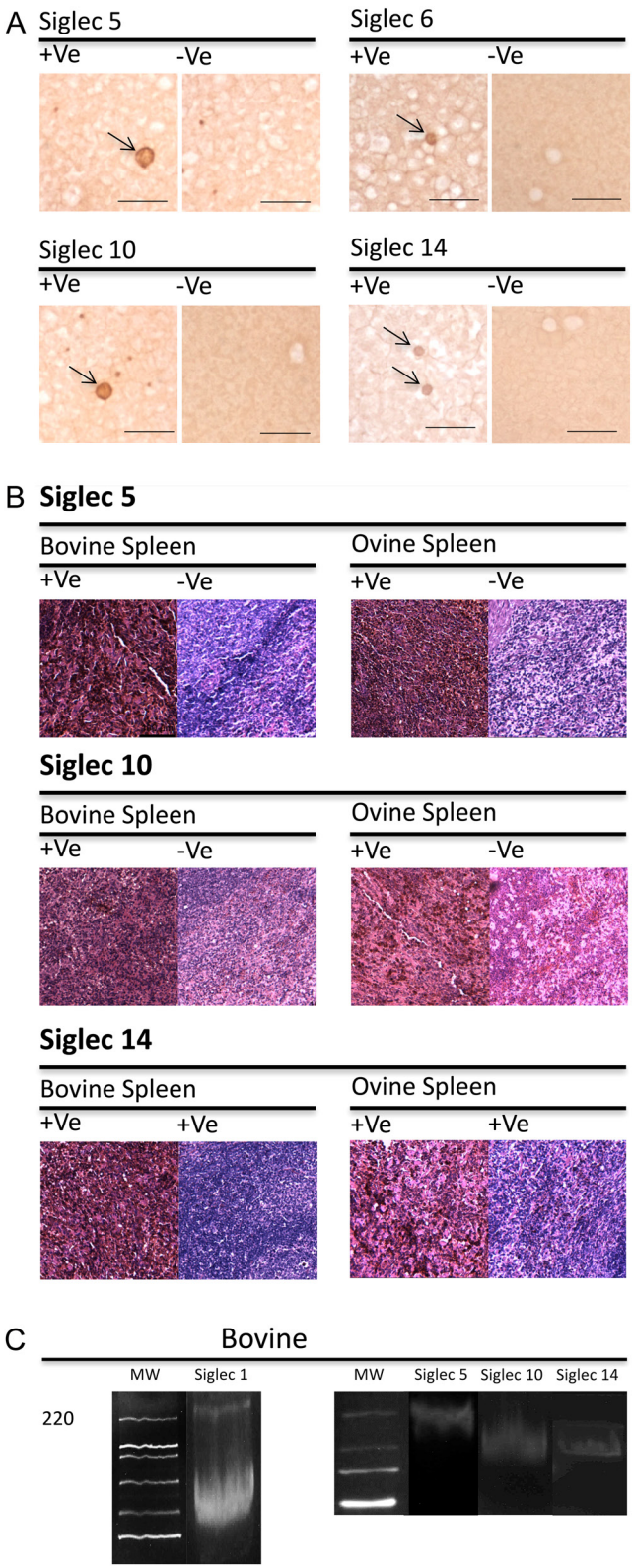

Figure 1 (A) Immunostaining of bovine blood with human anti-Siglec antibodies. Bovine blood was smeared on glass slides and probed with Siglec 1, 2, 5, 6, 10 and 14 antibodies. Positive staining was detected by secondary staining with biotinylated antibodies and visualised by chromogenic substrate (DAB). Size Bar 10 µm, –ve staining with secondary antibody only, +ve staining with primary antibody detected with secondary antibody. (B) Immunostaining of bovine and ovine spleen tissue with human anti-Siglec antibodies. Bovine and ovine spleen tissue sections proteins were immunostained with human anti-Siglec antibodies for Siglec 1, 2, 5, 6, 10 and 14. Positive staining was detected by secondary staining with biotinylated antibodies and visualised by chromogenic substrate (DAB). Magnification ×10, –ve staining with secondary antibody only, +ve staining with primary antibody detected with secondary antibody. (C) Western blot of bovine spleen lysates with human Siglec antibodies. (C) Bovine spleen lysate proteins were separated by electrophoresis on a 10% SDS PAGE gel and probed with Human anti-Siglec antibodies for Siglec 1, 2, 5, 6, 10 and 14 and detected by HRP-conjugated secondary antibody and enhanced chemiluminescence (ECL).

(model Nikon, Labophot-2A). Appropriate primary only antibody and secondary only antibody controls were included for all samples.

Human and bovine sperm lysates

To extract protein, for each individual ($n=3$), 20×10^6 human sperm (1 straw) were thawed and washed in 200 µL of buffer (10 mM HEPES, pH 7.4, 150 mM NaCl), centrifuged (5 min, RT, 10,000 rpm) and the pellet was re-suspended in 100 µL of buffer. The sample (buffer + pellet) was ground using a pestle and mortar with liquid nitrogen for 5 min. A 100 µL volume of HEPES buffer was added, and the lysate was decanted into a microcentrifuge tube. Fifty microliters of 5× Laemmli sample buffer containing 5% 2-mercaptoethanol (v/v) was added, and the sample was boiled for 5 min prior to loading on SDS-PAGE. For bovine sperm, the raw ejaculate ($500–2000\times10^6$ sperm/mL) was centrifuged (5 min, RT, 10,000 rpm) and the seminal plasma was discarded. The pellet was re-suspended in HEPES buffer at approximately 20×10^6 sperm/mL and sperm lysate was prepared as described earlier.

Bovine spleen lysate

250 mg of bovine spleen tissue was obtained fresh from an abattoir and placed on ice. The tissue was ground in liquid nitrogen using a mortar and pestle in 500 µL of HEPES buffer. The sample was centrifuged (20 min, 4°C, 14,000 rpm) and the pellet was re-suspended in 50 µL of 5× sample buffer containing 5% 2-mercaptoethanol (v/v) and boiled for 5 min prior to loading.

SDS-page and Western blotting

Protein concentration for sperm lysates was determined using a BCA kit (Pierce, Thermo Fischer Scientific) using bovine serum albumin as the protein standard according to the manufacturer's instructions. Twenty µg of sperm or spleen lysate were added to 5× buffer (5:1), boiled at 95°C for 5 min and separated on a 10% SDS-PAGE under reducing conditions (Laemmli 1970). Precision protein standards (BioRad) with a suitable molecular weight range were used as molecular

weight markers. Proteins were then transferred for 18 h at 15V and 4°C to an Immobilon-P Polyvinylidene Difluoride (PVDF) membrane (Millipore) using a Bio-Rad Transblot apparatus (BioRad) according to the manufacturer's instructions. After the transfer, the PVDF membrane was re-permeabilised with methanol, rinsed in dH_2O and blocked with blocking solution (PBS with 0.05% Tween20 (Sigma) and 3% (w/v) skimmed milk powder) for 60 min. Siglec antibodies at appropriate dilution (1:500 to 1:1000) were added in blocking solution and incubated with shaking overnight. Membranes were washed in blocking solution and probed with a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP, Santa Cruz Biotechnology), diluted 1:5000 in blocking solution. Blots were developed using Enhanced Chemiluminescence (ECL, Thermo Fischer Scientific) and visualised using Molecular Imager ChemiDoc XRS System (BioRad).

Results

Confirmation of detection of Siglecs in bovine and ovine tissue using human antibodies

The ability of the human Siglec antibodies to detect Siglecs in other species was confirmed for all Siglecs by immunostaining of human, bovine and ovine spleen and leucocytes. All Siglec antibodies tested gave a positive result on human, bovine and ovine WBCs in the presence of the primary antibody, and a signal was not detected using the secondary antibody alone (Fig. 1A, Siglec 5, 6, 10 and 14 bovine leucocyte immunostaining shown). In addition, all Siglecs tested showed positive immunostaining staining of bovine and ovine spleen sections (Fig. 1B, Siglec 5, 10 and 14) and bovine spleen lysates Western blotted with Siglec 1, 5, 10 and 14 antibodies displayed bands corresponding to bovine Siglecs (Fig. 1C). In addition, human, bovine and ovine sequence alignments were conducted, and in general, they show a high degree of conservation across species and within the epitope regions for their respective antibodies (Table 1 and Supplementary Table 5A and B).

Immunohistochemistry to detect Siglecs on sperm

Siglec expression patterns on sperm from different species are summarised in Table 2 and detailed in Fig. 2. Siglec expression was detected in all species examined and in sperm from the bovine cauda epididymis tail, and no differences in the patterns of expression were evident between different individuals from the same species. Siglec expression was specific to both species and sperm region. Immunoreactivity was detected only in the presence of the primary antibodies, and non-specific staining was not detected using the secondary antibodies alone. Siglec 1 expression was strongly detected on the acrosomal region with less intense staining of the equatorial band, neck and midpiece in bovine sperm while on human sperm, Siglec 1 expression was evident on the neck and midpiece. Siglec 2 expression was detected most strongly on the apical region of the acrosomal cap, and on the neck and principal piece in bovine sperm, and the same pattern was evident in epididymal bovine sperm where the

Table 2 Summary of Siglec immunostaining patterns on human, ovine and bovine ejaculated sperm and bovine epididymal sperm.

		Sperm location						
Siglec	**Sperm**	Acrosomal cap	Equatorial band	Post-acrsomal cap	Neck	Midpiece	Cytoplasmic droplet	Principal piece
Siglec 1	Human	–	–	–	+	+	–	–
	Bovine	++	+	–	–	–	–	–
Siglec 2	Human	–	++	–	+	+	–	+
	Ovine	++	–	–	–	–	–	+
	Bovine	++	–	–	+	–	–	+
	Bovine epididymis	++	–	–	+	–	+	+
Siglec 5	Human	–	++	++	+	+	–	–
	Ovine	–	–	+	+	+	–	–
	Bovine	–	–	–	+	+	–	–
	Bovine epididymis	–	–	–	+	+	+	–
Siglec 6	Human	+	++	–	+	+	–	–
	Ovine	++	–	–	+	+	–	–
	Bovine	++	–	–	+	+	–	–
	Bovine epididymis	++	–	–	+	+	–	–
Siglec 10	Human	–	–	++	+	+	–	–
	Ovine	++	–	–	+	+	–	–
	Bovine	++	–	–	+	–	–	–
	Bovine epididymis	++	–	–	+	+	+	–
Siglec 14	Human	–	–	++	–	+	–	+
	Ovine	–	–	++	+	++	–	+
	Bovine	+	–	–	++	+	–	+
	Bovine epididymis	+	–	+	+	+	+	+

Sperm were stained with Siglec 1, 2, 5, 6, 10 and 14 antibodies and their relative intensity of staining qualitatively scored (++ strong staining, + weak staining, – no staining).

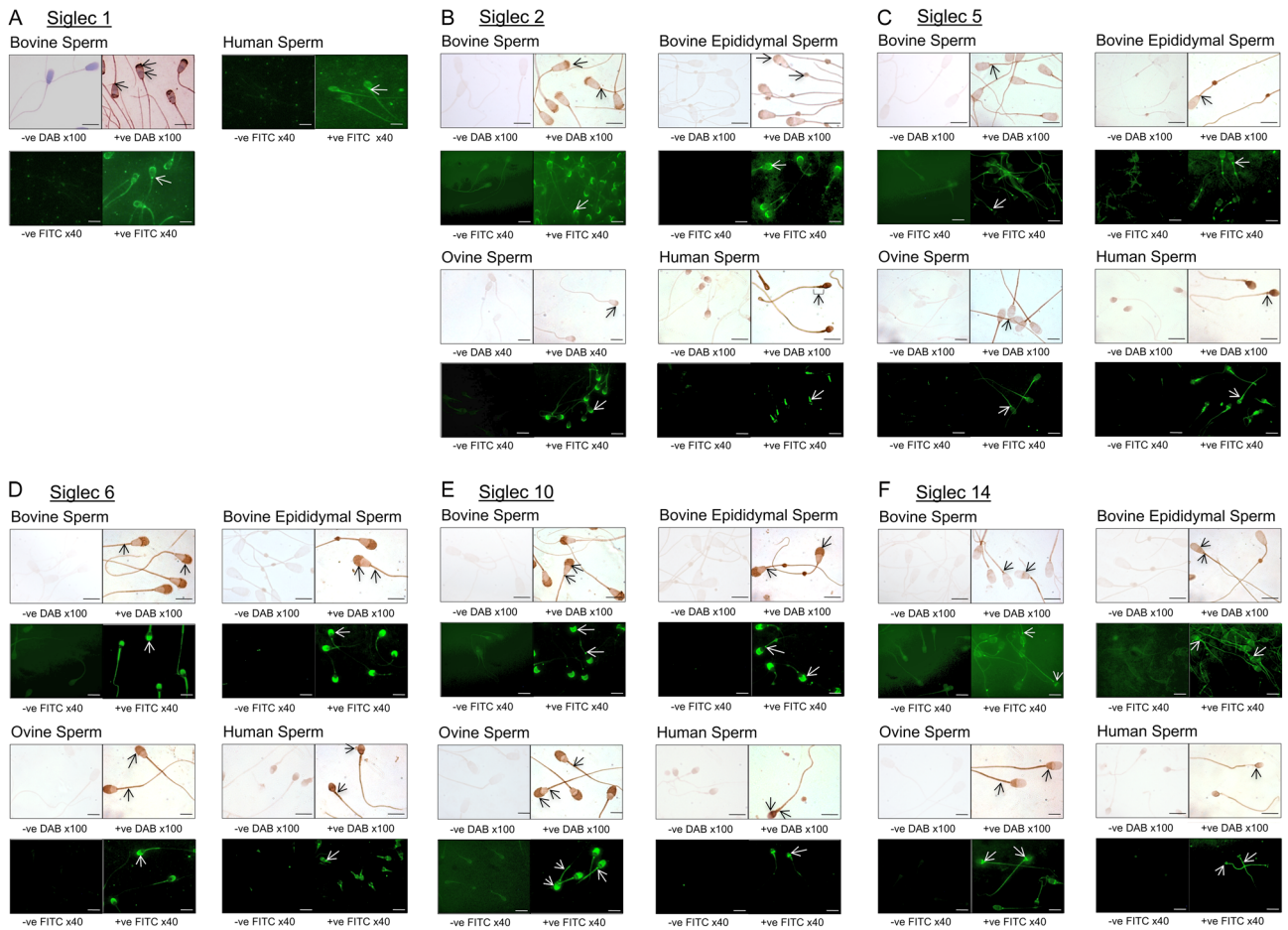

Figure 2 Immunostaining of human, bovine and ovine sperm with human Siglec antibodies. Human, bovine and ovine sperm were smeared on glass slides and probed with Siglec 1, 2, 5, 6, 10 and 14 antibodies. Positive staining was detected by secondary staining with either FITC-conjugated or biotinylated antibodies and visualised by fluorescence or chromogenic substrate (DAB). Size Bar 10 µm, −ve staining with secondary antibody only, +ve staining with primary antibody detected with secondary antibody. Panel A: Siglec 1, Panel B: Siglec 2, Panel C: Siglec 5, Panel D: Siglec 6, Panel E: Siglec 10 and Panel F: Siglec 14.

cytoplasmic droplet was also positive. In ovine sperm, Siglec 2 expression was detected on the apical part of the acrosomal cap and more weakly on the tail while on human sperm, the most intense expression was present on the equatorial band with some staining of the neck and tail also evident. Siglec 5 was detected on the neck, and midpiece in bovine sperm and in cauda epididymal sperm, a similar pattern along with staining of the cytoplasmic droplet was evident. In ovine sperm, Siglec 5 showed a similar pattern of staining to bovine sperm, but also with faint staining of the post-acrosomal cap. On human sperm, using DAB, expression was evident on the post-acrosomal cap with weaker staining on the neck and first half of the midpiece while the equatorial band showed strong expression using fluorescence detection. Expression of Siglec 6 in bovine ejaculated and cauda epididymal sperm, and ovine sperm was detected on the midpiece. In human sperm, Siglec 6 expression showed a different pattern with staining of the sperm equatorial band and neck/midpiece was detected. Siglec 10 expression was restricted to the acrosomal cap and neck on bovine sperm, both with similar intensity while in cauda epididymal sperm, staining on the acrosomal cap, neck, midpiece and cytoplasmic droplet was evident. On ovine sperm, expression was present on the acrosomal cap, neck and the midpiece. Siglec 10 expression on human sperm was the most extensive with signal present on the post-acrosomal cap, neck and midpiece. Siglec 14 shares extensive homology with Siglec 5 and the only commercially available antibody for Siglec 14 (Siglec-5/14 (N-13) R&D Systems) also recognises Siglec 5. The staining pattern of sperm with this Siglec 5/14 antibody was compared with Siglec 5 immunostaining to determine Siglec 14-specific patterns. On bovine sperm, staining was detected on the equatorial band, the post-acrosomal cap, the neck and the midpiece. No staining of the equatorial band and faint staining of the principal piece was evident for Siglec 5 suggesting that these regions may be specific for Siglec 14. In the other sperm regions, co-staining with Siglec 5 and Siglec 5/14

was evident, and thus, we cannot determine if this is also due to Siglec 14. On bovine epididymal sperm, a similar pattern was detected to bovine ejaculated sperm with the exception of the equatorial band and tail, again suggesting that Siglec 14 may be expressed in this region. On both ovine and human sperm, part of the midpiece and most of the principal piece showed 5/14 antibody-specific antibody staining suggesting that Siglec 14 may be expressed in this region.

Western blotting to analyse Siglec proteins on sperm

The expression and MW of Siglec proteins in human and bovine sperm lysates using Western blotting are summarised in Fig. 3 and Table 3. Bands corresponding to Siglec 1, 5, 10 and 14 were detected in human sperm lysates while Siglec 5, 10 and 14 were present in bovine lysates. These results concur with the immunostaining data. Expression of Siglec 2 or 6 was not detected by Western blotting possibly due to differences in sample epitope conformation or presentation with this technique. In general, the MW of the Siglec bands was in agreement with those expected for human and bovine Siglecs.

A band of ~220 kDa was detected with the Siglec 1 antibody in both human (data not shown) and bovine spleen (Fig. 1). In sperm, a band of >220 kDa was evident in total human sperm lysate while a signal was not detected in bovine sperm lysate for Siglec 1. For Siglec 5, a band of ~70–80 kDa was detected in both human (data not shown) and bovine spleen generally running as a smear under reducing conditions (Fig. 1). A band of ~100 kDa corresponding with Siglec 5 was present in human sperm lysates while a single band of ~80 kDa was detected in bovine sperm lysate. For Siglec 10, a band of ~70 kDa was detected in both human (not shown) and bovine spleen (Fig. 1). A band for Siglec 10 of >220 kDa was evident in both human and bovine sperm lysates. For Siglec 14, similar to immunostaining, the detection of this Siglec depends on differentiation of its staining pattern from Siglec 5 using the 5/14 antibody. A band of ~70 kDa was detected in bovine spleen, which is lower than the MW detected with Siglec 5, indicating that it is unique to Siglec 14. Bands of ~100 kDa and ~80 kDa were evident in human sperm in lysates suggesting that the lower band may be unique for Siglec 14. A smaller distinct band of ~45 kDa was detected in bovine sperm lysate.

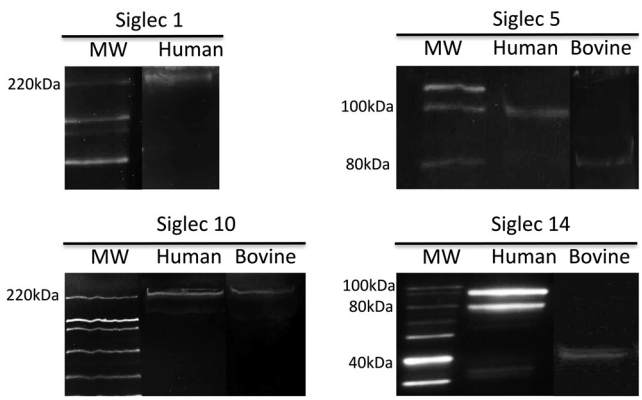

Figure 3 Western blot of human and bovine sperm lysates with human Siglec antibodies. Human and bovine sperm lysate proteins were separated by electrophoresis on a 10% SDS PAGE gel and probed with Human anti Siglec antibodies for Siglec 1, 2, 5, 6, 10 and 14 and detected by HRP conjugated secondary antibody and enhanced chemiluminescence.

Discussion

Proteomic and transcriptomic studies have previously listed the presence of Siglecs 1, 2, 5, 6, 10 and 14 in human and bovine sperm samples (Lalancette *et al.* 2008, Peddinti *et al.* 2008, Gu *et al.* 2011, Baker *et al.* 2013, Amaral *et al.* 2014), but none have mapped their topographical location on sperm. Hence, we compared Siglec expression on human, bovine and ovine sperm using immunohistochemical and Western blot analysis. In addition, Siglec expression on bovine cauda epididymal sperm, which have not been exposed to male accessory gland secretions, was analysed to determine their effect on Siglec expression.

Expression of Siglecs 1, 2, 5, 6, 10 and 14, was detected on human, bovine and ovine ejaculated sperm as well as bovine epididymal sperm. While the overall patterns of Siglec expression across species were similar, some varied in both location and overall regional intensity, indicating some species-specific expression. In general, Siglecs were expressed on the sperm acrosomal cap, midpiece and to a lesser extent, principal piece. Siglec staining within individual sperm regions was specific in terms of location for each Siglec and each species studied. For example, both Siglec 1 and 2 were expressed on the human sperm head but had different patterns of expression. This variation in antibody staining may suggest distinct roles for different Siglecs in the same sperm region.

Human, bovine and ovine sperm as well as sperm from other animals share similar structural characteristics and a large number of proteins are conserved across sperm in these species human and bovine sperm (Skerget *et al.* 2013, Holland & Ohlendieck 2015, Defaus *et al.* 2016, Pini *et al.* 2016). Similar Siglecs were often expressed in the same sperm regions in the different species and these patterns of expression suggest the presence of Siglec paralogues with potentially similar roles across species. However, individual species-specific expression was also evident for some Siglecs. For example, Siglec 5 was present on the acrosomal cap, neck and midpiece of human sperm but was only found in the neck and midpiece in bovine sperm. Glycoanalysis of female reproductive tract mucus indicates that there are interspecies differences in the type and quantity of

Table 3 Summary of observed Siglec MWs in human and bull sperm Western blots.

Siglec	Species	NCBI Refseq	Peptide MW (kDa)	Reported MW (kDa)	Sperm lysate MW (kDa)
Siglec 1	Human		175	220	>220
	Bovine	XP_015313763.1	183		ND
Siglec 2	Human		95		ND
	Bovine	XP_015322920.1	95		ND
Siglec 5	Human		60	70	100
	Bovine	XP_005219620.1	61	–	77
Siglec 6	Human		49		ND
	Bovine	XP_015323116.1	59		ND
Siglec 10	Human		77	112	>220
	Bovine	NP_001193206.1	60		>220
Siglec 14	Human		44	–	100, 80
	Bovine	XP_015323126.1	43	–	43

Expected, previously reported and observed MWs of human and bovine Siglecs. ND, not detected. References for reported MWs. Siglec 1: Hartnell *et al.* 2001, Siglec 5: Cornish *et al.* 1998, Siglec 10: Li *et al.* 2001.

carbohydrates present in several species (Desouza *et al.* 1988, Lagow *et al.* 1999, Andersch-Björkman *et al.* 2007, Pluta *et al.* 2011). This may suggest unique roles for some Siglecs in certain species, perhaps reflecting different species sialomes within their respective male and female reproductive tracts.

The expression of Siglecs in both human and bovine sperm lysates was also analysed by Western blotting. Siglec 1, 5, 10 and 14 were detected in bovine spleen indicating that the human anti-Siglec antibodies detect bovine Siglecs in this format. Siglecs 5, 10 and 14 were detected in human and bovine sperm cell lysates while Siglec 1 was present in human lysates only. The fact that Siglec 1 was not detected in bovine sperm lysate may be due to a limitation of the anti-human Siglec 1 antibody to detect bovine Siglec 1 epitopes in this tissue using Western blotting. For both human and bovine Siglecs on sperm, the apparent MW was higher than that predicted for the peptide alone while Siglecs in spleen lysates also showed differences from their predicted peptide MWs. Most human Siglecs undergo glycosylation (Varki & Angata 2006) while human Siglec 2 and 5 have been shown to multimerise and the properties of other Siglecs, which may contribute to Siglec functional structure is unknown (Siddiqui *et al.* 2017). It is therefore possible that the increase in molecular weight in this study could be attributable to N-glycosylation (detailed in Table 4), differences in folding and subunit structure. Siglec 1 has a glycosylated MW of ~220 kDa (Hartnell *et al.* 2001), which is in close agreement with the MW of the human sperm-expressed protein, while Siglec 5 has a predicted MW in human of ~60 kDa (Cornish *et al.* 1998) and a respective apparent MW of ~100 kDa in human and ~80 kDa in bovine sperm, consistent again with N-glycosylation. Siglec 10 had a MW of ~220 kDa, which is larger than the predicted glycosylated MW of ~100 ± 12 kDa (Li *et al.* 2001) suggesting that it may exist as a glycosylated homodimer in common with other Siglecs such as Siglec 1 (Hartnell *et al.* 2001). The MW for human and bovine sperm Siglec 14 are consistent with the glycosylation and dimerisation of the protein subunit probably in a similar manner to Siglec 5. The relatively small size of the Siglec 14 bovine sperm lysate protein may be due to tissue-specific glycosylation resulting in altered protein conformation compared to other tissues. In addition, it suggests that it may exist as monomer rather than the expected dimer observed in human sperm. Alternatively, this band could be a splice variant of either Siglec 5 or 14 (Connolly *et al.* 2002) or possibly the result of a proteolytic cleavage event. Protein expression was not evident for Siglec 2 or 6 in either species using Western blotting. It is possible that, while the antibodies used were suitable for immunostaining studies, they were not able to detect epitopes under Western blotting conditions due to changes in epitope accessibility or that there is a relatively low level of sequence conservation between human and bovine/ovine species (Supplementary Table 5B).

A similar repertoire of Siglec expression was evident on bovine cauda epididymal sperm when compared to

Table 4 Potential human Siglec 1, 2, 5, 6, 10 and 14 glycosylation sites.

Siglec	Nextprot reference	Number of N glycoylation sites	Potential glycan MW (kDa)
Siglec 1	https://www.nextprot.org/entry/NX_Q9BZZ2/proteomics	14	42
Siglec 2	https://www.nextprot.org/entry/NX_P20273/proteomics	11	33
Siglec 5	https://www.nextprot.org/entry/NX_O15389/proteomics	8	24
Siglec 6	https://www.nextprot.org/entry/NX_O43699/proteomics	4	12
Siglec 10	https://www.nextprot.org/entry/NX_Q96LC7/proteomics	5	15
Siglec 14	https://www.nextprot.org/entry/NX_Q08ET2/proteomics	1	3

N glycosylation sites for Siglec 1, 2, 5, 6, 10 and 14 analysed with Nextprot (https://www.nextprot.org/), listing Nextprot reference, potential number of glycosylation sites and potential glycan MW (assuming a typical N-glycan is ~3 kDa).

ejaculated sperm. However, some differences in patterns and staining intensity were detected. For example, Siglecs 2, 5, 10 and 14 were present on the sperm cytoplasmic droplet in epididymal sperm, but were not detected on ejaculated sperm. Overall, this indicates that these Siglecs are acquired or revealed during either (1) sperm development in the seminiferous tubules (as mature sperm are transcriptionally inactive (2) absorption during their passage to the epididymis or within the caput/corpus of the epididymis and (3) are lost or masked prior to ejaculation. Compared with sperm from ejaculated samples, the storage of sperm in the vas deferens, subsequent to passage through the epididymal cauda, may influence Siglec regional distribution. It is possible that the addition of male accessory gland secretions may modulate Siglec expression or function. However, it is also feasible that components of post-caudal epididymal secretions, which include Sia (Rajalakshmi & Prasad 1968, Rodríguez-Martínez *et al.* 2011), may mask Siglec epitopes in certain regions of sperm, preventing their detection using immunostaining. Interestingly, sialidase treatment of sperm to remove surface sialic acid tended to show an increase in Siglec antibody staining signal (not shown).

Siglecs show specific expression patterns in several sperm regions but predominantly on the head and parts of the tail. Depending on the Sia present within the female reproductive tract, as well as the Siglecs expressed on sperm, interactions may be promoted or impeded, which may influence sperm progression and mucus penetration. For example, cervical mucus undergoes dramatic changes during the peri-ovulatory period (Eriksen *et al.* 1998, Pluta *et al.* 2011), which is also reflected in changes in the level and type of Sia. These changes in cervical mucin Sia content may influence sperm Siglec-mediated interactions and transcervical migration. Siglecs, similar to those on B cells (Varki & Angata 2006), may also monitor the Sia population present on the sperm itself, which is known to change at various stages during sperm progression through the female reproductive tract (Ma *et al.* 2016). It is also feasible that Siglecs detecting unique Sia patterns within the female reproductive tract, or on the sperm itself, might exert a regulatory role signalling through their ITAM and ITIM signalling domains as part of their cytoplasmic tail (reviewed in McCauley *et al.* 2014). It is noticeable that Siglec expression is localised to sperm regions with specific functions associated with the regulation of sperm energy metabolism and motility.

Siglecs 1, 2, 6 and 10 were expressed on the plasma membrane in the acrosomal cap region. The acrosomal cap is central to several sperm functions including capacitation, binding to the zona pellucida, acrosomal exocytosis, and it is also a calcium store within the sperm (Wassarman 1987, Tulsiani *et al.* 1998, Herrick *et al.* 2005, Berruti & Paiardi 2011, Tulsiani & Abou-Haila 2012, Buffone *et al.* 2014). Sialylated ligands in several species are central to sperm binding to the oviductal epithelium during the formation of the sperm–oviductal reservoir as well as their subsequent release and capacitation (Hung & Suarez 2010, Talevi & Gualtiere 2010, Miller 2015), and sperm surface Siglecs may play a role in these processes. In the human and bovine species, Sia on the zona pellucida is implicated in sperm binding and oocyte penetration (Jiménez-Movilla *et al.* 2004, Velásquez *et al.* 2007, Pang *et al.* 2011, Clark 2013, Takahashi *et al.* 2013). The specific sperm ligands are yet to be identified in these processes, and it is possible that Siglecs may be involved. All the Siglecs studied were expressed in the neck and most were present in the midpiece region. These regions generate energy to promote tail movement and sperm motility and also act as a calcium store involved in regulating sperm motility (Publicover *et al.* 2008, Piomboni *et al.* 2012). It is plausible that the Siglecs in this region may play a role in regulating energy metabolism and calcium flux within the sperm in response to external environmental cues and influence sperm motility. The sperm tail is involved in multiple functions including motility, calcium influx, extracellular sensing and chemotactic response (Lishko *et al.* 2012, Alasmari *et al.* 2013). Siglec 2 and 14 expression was evident in the sperm principal piece, again suggesting that Siglecs may contribute to tail-specific functions perhaps by responding to sia patterns within the sperm environment again via their intracellular signalling domains (MaCauley *et al.* 2014).

In conclusion, several Siglecs have been identified on sperm using two independent methods (immunostaining and Western blotting), which along with previous proteomic studies (Lalancette *et al.* 2008, Peddinti *et al.* 2008, Gu *et al.* 2011, Baker *et al.* 2013, Amaral *et al.* 2014) provides strong evidence that Siglecs are part of the surface protein repertoire of both ejaculated and epididymal spermatozoa. Possible roles for these proteins include passive binding interactions with Sia present within both the male and female reproductive tract, which could impede or facilitate sperm motility. In addition, in common with Siglecs in the immune system, they may exert a regulatory role, perhaps providing a means of responding to unique Sia signatures that change within the female reproductive tract during the oestrous cycle. Finally, the presence of sperm surface Siglecs may play an important role in binding to sialylated ligands on the oviductal epithelium involved in the formation of the oviductal sperm reservoir as well as on the zona pellucida during sperm oocyte binding.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-17-0475.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Science Foundation Ireland; an Irish Research Council EMBARK Scholarship; King Faisal University Scholarship, Saudi Arabia; Saudi Arabia Government Research scholarship and Ministry of Higher Education and Scientific Research (Iraq).

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Received 30 July 2017
First decision 25 August 2017
Revised manuscript received 1 February 2018
Accepted 6 February 2018