<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Glycodelin-A interacts with fucosyltransferase on human sperm plasma membrane to inhibit spermatozoa-zona pellucida binding</th>
</tr>
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<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Chiu, PCN; Chung, MK; Koistinen, R; Koistinen, H; Seppala, M; Ho, PC; Ng, EHY; Lee, KF; Yeung, WSB</td>
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**Introduction**

Glycodelin is a glycoprotein previously known as placental protein-14 or progesterone-associated endometrial protein. The molecule has three reported isoforms that are named according to their source where they are most abundant: glycodelin-A (amniotic fluid isoform), glycodelin-S (seminal plasma isoform) and glycodelin-F (follicular fluid isoform) (Seppala et al., 2002; Chiu et al., 2003a). The glycodelin isoforms have a molecular size of 28-30 kDa. They have the same protein core but differ in their glycosylation.

Glycodelin isoforms modulate sperm functions (Yeung et al., 2006). Glycodelin-A is abundant in the secretory endometrium (Seppala et al., 2002), and is highly upregulated in the endometrium at implantation (e.g. Kao et al., 2002). It is the first endogenous glycoprotein found to inhibit spermatozoa-zona pellucida binding (Ohnberger et al., 1995) and its absence in the endometrium in the periovulatory period may be related to the opening of a fertilization window (Seppala et al., 2002). The fallopian tube also expresses glycodelin in a hormone-dependent manner (Seppala et al., 2002). Glycodelin-S maintains the spermatozoa in an uncapacitated state before their migration into the uterine cavity (Chiu et al., 2005), and glycodelin-F prevents premature progesterone-induced acrosome reaction (Chiu et al., 2003b).

The first step in fertilization involves the interaction between molecules on the spermatozoa and the zona pellucida of an oocyte. There is ample evidence that sperm surface carbohydrate-binding proteins mediate gamete recognition and interact with high affinity and specificity to the zona pellucida glycoproteins (Chapman and Barratt, 1996; Wassarman, 1999; Ozgur et al., 1998). Despite the fact that several carbohydrate-binding proteins have been proposed to be responsible for spermatozoa-zona pellucida binding (Nixon et al., 2001; Leyton et al., 1992; Cheng et al., 1994), the exact identity of these sperm surface protein(s), especially those for humans, remains unclear.

The mechanism by which glycodelin-A inhibits spermatozoa-zona pellucida binding is unknown. We hypothesize that glycodelin-A competes with zona pellucida glycoproteins for their sperm membrane receptors. Previous studies showed that glycodelin-A binds specifically to human spermatozoa (Chiu et al., 2003a) and the binding was greatly reduced after acrosome reaction or in the presence of zona pellucida glycoproteins (Chiu et al., 2003b; Chiu et al., 2004). The carbohydrate moieties of glycodelin-A are crucial for its zona-binding inhibitory activity as well as binding on human spermatozoa as deglycosylated glycodelin loses such activities in vitro (Chiu et al., 2003a; Chiu et al., 2004). Coincidentally, the spermatozoa-zona pellucida interaction is also a carbohydrate-mediated event (Wassarman, 1999). These data...
suggest that the glycodelin receptor(s) and zona pellucida
glycoprotein receptor(s) are closely related. The objectives of
this study were to identify the receptor of glycodelin-A in
human spermatozoa and to characterize its interaction with
human zona pellucida.

Results
Fucosyltransferase 5 (FUT5) is a sperm surface
glycodelin-A binding protein

The results of identification of glycodelin-A-bound sperm
surface protein(s) are shown in Figs 1, 2. Sulfosuccinimidyl-
2-[6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]ethyl-
1,3′-dithioproionate (Sulfo-SBED)-conjugated glycodelin-A
was covalently bound to spermatozoa after photoactivation.

Treatment of the glycodelin-A–sperm surface protein
complexes with dithiothreitol (DTT; reducing conditions)
resulted in reduction of the azo linkage and transfer of the
biotin label from glycodelin-A to the sperm proteins. Thus,
biotinylated glycodelin-A (Fig. 1A,B, lane 1) showed a single
biotin band with molecular size ~30 kDa under non-reducing
conditions, which was lost after DTT treatment (Fig. 1A,B,
lane 2).

One major biotinylated complex with size ~93 kDa appeared
after incubation of glycodelin-A (Fig. 1A,B, lane 3) with
the sperm extract. Treatment with DTT revealed a major biotin-
labeled complex of size ~65 kDa (Fig. 1A,B, lane 4). Some
minor bands of smaller sizes were also seen. By contrast,
glycodelin-A did not interact with fibroblasts and non-
specifically bound glycodelin-A was removed by washing of
the cells after incubation. This is evidenced by the absence of
a band in sodium dodecyl sulphate-polyacrylamide gel
electrophoresis (SDS-PAGE; Fig. 1A,B, lane 6).

Deglycosylated glycodelin-A did not interact with human
spermatozoa (data not shown).

Mass-spectrometry (MS) analysis of the tryptic digests and
a database search identified the ~65 kDa glycodelin-A bound
protein as FUT5 (Table 1). The six peptide sequences showed
>90% match to the MS spectra and confirmed the protein
identity at high confidence level. Two of the identified
peptides, SFSWALAFCK and TELVAWAVSNWKPSAR,
were unique to FUT5 among fucosyltransferase 3 (FUT3),
FUT5 and fucosyltransferase 6 (FUT6). The identity of the
protein was confirmed by western blot analysis using the anti-
FUT5 antibody, N-18 (Fig. 1C). The immunoreactivity was
associated with bands of sizes ~93 and ~65 kDa, corresponding
to the glycodelin-A–FUT complex in non-reducing conditions
(Fig. 1C, lane 3) and FUT in reducing conditions (Fig. 1C, lane
4), respectively.

Presence of FUT protein in human spermatozoa

Recombinant FUT proteins were expressed in inclusion bodies
and constituted for approximately 50% of the total protein of
the transformed Escherichia coli. The yield of soluble protein
was approximately 30-66 mg/l of culture. The expected sizes
of recombinant FUT3, FUT5 and FUT6 are 51, 58 and 61 kDa,
respectively. These recombinant FUTs were used to determine
the specificities of the anti-FUT antibodies against different
regions of the enzymes.

Owing to the high sequence similarity (~90%) at the C-
terminus, the antibody D-17 raised against this region of FUT5
recognized the three recombinant FUTs (Fig. 2A, lanes 3, 4
and 5). However, antibodies raised against the variable N-
terminus of FUT5 (N-18: Fig. 2C, lane 4) and FUT6 (G-16:
Fig. 2D, lane 5) reacted only with their respective recombinant
isoform only. The antibody N-16 is raised against the N-
terminus of FUT6 but cross-reacts with that of FUT3
(manufacturer information). It reacted strongly with FUT3
(Fig. 2B, lane 3) and FUT6 (Fig. 2B, lane 5) and very weakly
with FUT5 (Fig. 2B, lane 4).

The antibodies D-17 (Fig. 2A, lane 2) and N-18 (Fig. 2C,
lane 2) recognized two major protein bands of sizes ~79 and
~65 kDa in the human sperm extract. Several smaller
components of much lower intensity were also found. They
might result from proteolysis of sperm FUT during sample
preparation. The ~79 kDa band was also recognized by N-16
Sperm-zona pellucida binding

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Sperm-zona pellucida binding (Fig. 2B, lane 2), although the signal was weaker than that of D-17 and N-18. The antibody G-16 (Fig. 2D, lane 2) did not react with any protein in the sperm protein extract.

The antibody D-17 localized FUT immunoreactivity to the acrosome region and the midpiece (Fig. 3A) in 90-95% of spermatozoa with or without permeabilization. Immunoreactivity was found in the midpiece in 88-96% of non-permeabilized spermatozoa when N-18 antibody was used (Fig. 3C). The acrosome region in 83-90% of the spermatozoa was also strongly stained after permeabilization, suggesting that the N-terminal epitope of FUT5 in the acrosome region was intracellular. The omission of FUT antibodies and the use of antibodies preabsorbed with the appropriate blocking peptide greatly reduced the signal (data not shown). The antibodies N-16 and G-16 did not produce any signal (Fig. 3B,D).

Acceptors of FUT reduced the binding of glycodelin-A to spermatozoa

Unlabeled glycodelin-A was the best competitor (LogEC50: 2.31±0.08 pmol/ml) in the binding of 125I-glycodelin-A to spermatozoa (Fig. 4A), consistent with previous observations (Chiu et al., 2003b; Chiu et al., 2004). Among the FUT-acceptors, 2'-fucosyllactose, lacto-N-fucopentaose-I and asialofetuin competed with 125I-glycodelin-A for binding sites with LogEC 50 of 2.81±0.14, 3.48±0.20 and 2.50±0.12 pmol/ml, respectively (Fig. 4B). Phenyl-β-D-galactoside melibiose, RNAse and glycodelin-S did not affect the binding of 125I-glycodelin-A to spermatozoa. The competitors at the concentrations used did not affect sperm viability, acrosomal status and motility (data not shown).

Purification of the sperm FUT

Ion-exchange chromatography separated the sperm protein extracts into ten fractions (fractions 1-10; Fig. 5A). The FUT in these fractions were further purified by guanosine 5'-diphosphate (GDP)-agarose affinity chromatography. The purified sperm FUT appeared as a single major band of size ~65 kDa in fractions 5-8 in silver-stained SDS-PAGE (Fig. 5B) and in western blot analysis using antibody N-18 (Fig. 5C). The identity of the isolated band was confirmed to be FUT5 by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; data not shown). The FUT activity in these fractions positively correlated with the appearance of the protein band (Table 2). There were 36.3-88.7-fold increases in FUT activity in fractions 5-8 relative to that in the total detergent-extractable protein (data not shown). Fractions 5-7 with a higher percentage of enzyme activity recovery (10.2-38.7%) were pooled for further experimentation.

Co-immunoprecipitation of glycodelin-A with FUT

The interaction between FUT and glycodelin-A was confirmed by co-immunoprecipitation using the anti-glycodelin antibody and the anti-FUT antibody, N-18. Glycodelin-A co-precipitated with recombinant FUT3 and FUT5, but not with FUT6 (Fig. 6A). It also interacted strongly with sperm FUT (Fig. 7). Deglycosylated glycodelin-A did not co-precipitate with recombinant and purified sperm FUTs (Fig. 6B), consistent with the previous observation that the interactions between glycodelin-A and spermatozoa were glycosylation dependent (Chiu et al., 2003b; Chiu et al., 2004). The addition of solubilized zona pellucida reduced the binding of

Table 1. Identification of glycodelin-A interacting protein by MALDI-TOF MS

<table>
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<tr>
<th>Peptides</th>
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<th>Mass-to-charge ratio matched (m/z)</th>
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<th>End</th>
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<td>1158.5532</td>
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<td>202</td>
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</table>

Protein sequence of human FUT5 (SwissProt accession no. Q11128) as retrieved from a ProteinProspector MS-Fit search shows a sequence coverage of 25% and MOWSE score of 69 (prospector.ucsf.edu). Peptides 2 and 4 (in bold) were unique to FUT5 among FUT3, FUT5 and FUT6.
glycodelin-A to sperm FUT in a dose-dependent manner (Fig. 7, Lane 1-3).

Anti-FUT antibody and FUT acceptors inhibited spermatozoa-zona pellucida binding

An anti-FUT antibody D-17 at concentrations of 1, 5, 10 and 20 μg/ml significantly decreased the number of spermatozoa bound to hemizona ($P<0.05$) when compared with the control. The hemizona binding index (HZI) decreased in a dose-dependent manner (Fig. 8A). Preabsorption with blocking peptide abolished the inhibitory effect of the antibody on spermatozoa-zona pellucida binding (Fig. 8A).

The results of different concentrations of FUT acceptors on spermatozoa-zona pellucida binding are shown in Fig. 8B. The zona-binding capacity of treated spermatozoa was inhibited by 2'-fucosyllactose, lacto-N-fucopentaose-I and asialofetuin in a dose-dependent manner ($P<0.05$). At 3000 pmol/ml, the HZI was 60.6±4.1%, 72.6±2.8% and 52.8±4.3%, respectively. No significant inhibition was observed with phenyl-β-D-galactoside, melibose and RNAse. Anti-FUT antibodies with or without blocking peptide preabsorption and FUT acceptors at the concentrations used did not affect sperm viability, acrosomal status and motility (data not shown).

Sperm FUT bound to human zona pellucida

Fluorescent-labeled sperm FUT specifically bound to the zona pellucida of human oocytes (Fig. 9A). The bound signal was removed in the presence of an excess of unlabeled sperm FUT (Fig. 9B). Fluorescent-labeled ovalbumin did not bind to intact zona pellucida (Fig. 9C). The binding of labeled sperm FUT to solubilized zona pellucida
Sperm-zona pellucida binding increased with the amount of FUT used and reached a plateau at concentrations >200 pmol/ml (data not shown). Scatchard plotting revealed an equilibrium dissociation constant ($K_d$) of 42.82 pmol/ml.

**Discussion**

Experimental evidence suggests that lipocalins, e.g. glycodelin-A (Rachmilewitz et al., 2003), $\beta$-lactoglobulin (Mansouri et al., 1998) and retinol-binding protein (Sundaram et al., 1998) bind to cell surface receptors. Glycodelin-A inhibits T-cell activity partly by binding to CD45, a major surface tyrosine phosphatase in T-cells (Rachmilewitz et al., 2003). Chemical cross-linking followed by mass spectrometric analysis of the cross-linked molecules is useful to elucidate ligand-receptor interactions (Williams et al., 2000; Bleil and Wassarman, 1990). Using such an approach, we identified FUT5 as a glycodelin-A binding protein on human spermatozoa. Several observations support this conclusion: (1) sulfo-SBED linked glycodelin-A to a sperm surface protein of size ~65 kDa, which was identified as FUT5 by MS; (2) an anti-FUT5 antibody recognized glycodelin-A–sperm protein complexes and a ~65 kDa protein band in sperm extract; (3) indirect immunofluorescence staining localized FUT5.

**Fig. 5.** Purification of sperm FUT. (A) Representative Mono-Q chromatogram of sperm extracts. The protein fractions (1-10) were desalted and further purified using a GDP-agarose column. They were then subjected to 12% SDS-PAGE analysis with silver staining (B) and western blot analysis using the anti-FUT5 antibody N-18 (B).

**Fig. 6.** Co-immunoprecipitation of FUT with glycodelin-A or deglycosylated glycodelin-A. (A) Glycodelin-A. Lane 1: marker. Lane 2: 5 µg FUT3 + 30 pmol/ml glycodelin-A. Lane 3: 5 µg FUT5 + 30 pmol/ml glycodelin-A. Lane 4: 5 µg FUT6 + 30 pmol/ml glycodelin-A. (B) Deglycosylated glycodelin-A. Lane 1: marker. Lane 2: 5 µg FUT3 + 30 pmol/ml deglycosylated glycodelin-A. Lane 3: 5 µg FUT5 + 30 pmol/ml deglycosylated glycodelin-A. Lane 4: 5 µg FUT6 + 30 pmol/ml deglycosylated glycodelin-A. Lane 5: 5 µg sperm FUT + 30 pmol/ml deglycosylated glycodelin-A.

**Table 2. Purification of sperm FUT**

<table>
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<tr>
<th>Fractions</th>
<th>Protein (µg)</th>
<th>FUT activities (pmol product/h)</th>
<th>Specific activities (activity/µg protein)</th>
<th>Recovery (% activity)</th>
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<td>248.2</td>
<td>0.302</td>
<td></td>
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<tr>
<td>Mono-Q + GDP-agarose</td>
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<tr>
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<td>8</td>
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<tr>
<td>10</td>
<td>2.1</td>
<td>0.4</td>
<td>0.190</td>
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The table shows the typical activity of FUT from 100×10^6 spermatozoa in different fractions of the purification protocol. A similar pattern was seen in two other experiments.
acrosome region of human spermatozoa, a region that binds glycodelin-A (Chiu et al., 2003a); (4) α1,3 and α1,4, but not α1,2 FUT acceptors, non-FUT-related sugars (melibiose) and glycodelin-S, inhibited the binding of glycodelin-A to human spermatozoa; and (5) co-immunoprecipitation of glycodelin-A with recombinant FUT5 and purified sperm FUT. Moreover, in a cross-linking experiment, the glycodelin-A–FUT5 complex could only be obtained from spermatozoa, but not from fibroblasts, consistent with our previous finding that the binding of glycodelin is cell type-specific (Tse et al., 2002). The inability of deglycosylated glycodelin-A to co-immunoprecipitate with sperm FUT and recombinant FUT5 is also in line with the documented importance of carbohydrate moieties on spermatozoa-glycodelin interaction (Chiu et al., 2003b; Chiu et al., 2004).

Fucosyltransferases catalyze fucose transfer from GDP-fucose to different oligosaccharide acceptors in H9251, H9251, H9251, and H9251-linkages. They are type II transmembrane proteins consisting of a small N-terminal cytoplasmic tail, a transmembrane region and a C-terminal catalytic domain usually oriented towards the lumen of the Golgi complex. Six human FUTs have been cloned (de Vries et al., 2001). Three FUTs, namely FUT3, FUT5 and FUT6, cluster on human chromosome 19p13.3 (Reguigne-Arnould et al., 1995; McCurley et al., 1995). They share ~90% sequence homology; the main differences between them are located in the N-
terminal region, whereas their C-terminal regions are almost identical (de Vries et al., 2001).

Fucosyltransferase 3, FUT5 and FUT6 are found only in humans and chimpanzees (Costache et al., 1997). The expression of FUT3 and FUT6 are abundant in gastrointestinal tissues and minimal in spleen, lung and cervix uteri. Compared with FUT6, FUT3 is not expressed in liver and is less abundant in kidney. The expression of FUT5 is weak and restricted to liver, colon, spleen, testis and hematopoietic cells (Cameron et al., 1995). Human FUTs differ in their acceptor substrate specificity (de Vries et al., 2001); FUT3 and FUT5 are α1,3/1,4-FUTs and interact with both type 1 (Galβ1-3GlcNAc) and type 2 (Galβ1-4GlcNAc) acceptors, whereas FUT6 is a α1,3-FUT and uses only type 2 acceptors. Among this group of enzymes, FUT5 has the broadest acceptor specificity. In addition to type 1 and 2 acceptors, FUT5 also works well with 3-sulfo-, 6-sulfo-, α2,3-sialylated and α1,2-fucosylated forms of these acceptors as well as glycans having the GalNAcβ1-4GlcNAc structure (Pykar et al., 2000; de Vries et al., 2001).

Among the human FUTs, only FUT3, FUT5 and FUT6 are inhibited by N-ethylmaleimide, a method for discriminating FUTs (de Vries et al., 1995). In a separate experiment, we found that treatment with 10 mM N-ethylmaleimide for 1 hour at 4°C abolished the enzymatic activity of sperm FUT. This sensitivity towards N-ethylmaleimide treatment together with their high similarity in protein sequence and exclusive presence in higher primates meant only FUT5 and FUT6 were considered as possible alternatives to FUT5 in this study.

Glycodelin-A contains approximately 17.5% of carbohydrate moieties (Bohn et al., 1982) with two glycosylation sites, Asn-28 and Asn-63, containing mainly complex-type glycans (Dell et al., 1995). The major non-reducing epitopes in the complex-type glycans include Galβ1-4GlcNAc and antennae containing GalNAcβ1-4GlcNAc (Dell et al., 1995). The latter is rare in glycoproteins of higher animals. Both are substrates of FUT5, which may serve as the site of interaction between glycodelin-A and sperm FUT5. By contrast, glycodelin-S has little Galβ1-4GlcNAc and no GalNAcβ1-4GlcNAc structure (Morris et al., 1996). Its glycans are unusually rich in fucose. The differences in carbohydrate moieties may explain the inability of glycodelin-S to bind to sperm FUT; and is consistent with the observation that the binding sites of glycodelin-S are different from other glycosidase isoforms (Chiu et al., 2005). However, glycodelin-F competes with glycodelin-A for binding to spermatozoa. This is in line with our previous binding kinetic experiments demonstrating that human spermatozoa possess two receptors for glycodelin-F, one of which is common with glycodelin-A (Chiu et al., 2003; Chiu et al., 2004). Our unpublished observation also shows that sperm FUT binds glycodelin-F.

This report provides the first demonstration of the isolation of FUT5 from human spermatozoa. Soluble FUTs are present in semen (Tulsiani et al., 1990) and adsorbed onto the sperm surface (Tulsiani et al., 1993). These exogenous FUTs are unlikely to be involved in spermatozoa-zona pellucida binding as our preliminary data showed that they are easily removed by density gradient centrifugation, consistent with the previous observation that Percoll processing shed loosely attached seminal plasma components including glycosyltransferase from spermatozoa (Flesch et al., 2001; Tulsiani et al., 1990). In this study, these exogenous FUTs were further removed from percoll-processed and capacitated spermatozoa with high-salt solutions known to release peripheral but not integral membrane proteins (Khosoo et al., 2003). Triton X-100 was then used to solubilize the integral membrane proteins (Shur and Neely, 1988). Our results showed that human sperm FUT activity was detergent but not high-salt extractable. The purified protein with FUT activity had a size (~65 kDa) similar to that of the FUT5 band identified in the cross-linking experiment. Its identity was confirmed to be FUT5 based on western blot analysis using an anti-FUT5 antibody and MS analysis. These observations indicated that the purified sperm FUT5 was an integral membrane component on the sperm surface. Immunostaining using antibodies against different regions of FUT5 with and without permeabilization further suggested that FUT5 had its C-terminal oriented externally whereas its N-terminal was intracellular.

Although the antibodies D-17 and N-18 recognized two major protein bands with masses of ~79 and ~65 kDa in human sperm extracts, we were successful in purifying the ~65 kDa protein only. The reason for this is unclear. One possibility is that the ~79 kDa protein may be a new isoform of FUT highly similar to FUT5 but with different surface-charge properties and/or GDP-binding affinities making it unable to be purified by the present purification protocol. It has been shown that the specificity and affinity of FUTs to substrate and donor can be greatly altered by mutating a few or even one amino acid residues (Jost et al., 2005; Vo et al., 1998). This hypothesis is being investigated in our laboratory.

The molecular mass of the purified sperm FUT5 (~65 kDa) differs from that of recombinant FUT5 (~58 kDa). The difference could be because of glycosylation of the native enzyme, whereas the recombinant FUT5 derived from E. coli did not possess glycosylation. The human FUT5 contains four potential N-glycosylation sites with two of them in the N-terminal region and the other two closer to the C-terminal region (Christensen et al., 2000a; Christensen et al., 2000b). The N-linked glycans of glycosyltransferases are involved in protein folding, prevention of proteolytic degradation and intracellular transport and localization. In addition, glycosylation in FUTs affects the biochemical activity of the enzyme (Christensen et al., 2000a; Christensen et al., 2000b).

Glycodelin-A inhibits spermatozoa-zona pellucida binding (Chiu et al., 2003a; Oehninger et al., 1995). Evidence suggests that there is a close relationship between glycodelin-A receptors and zona pellucida glycoprotein receptors (Chiu et al., 2003b; Chiu et al., 2004). The present study showed that FUT5 was involved in spermatozoa-zona pellucida binding and that glycodelin-A inhibited such binding by interacting with FUT5. This conclusion comes from three observations: (1) sperm FUT5 bound to intact or solubilized human zona pellucida; (2) co-incubation with solubilized zona pellucida dose-dependently reduced the binding of glycodelin-A to sperm FUT5; (3) an anti-FUT5 antibody and FUT acceptors dose-dependently decreased the number of bound spermatozoa in a hemizona binding assay. It was noted that an anti-FUT5 antibody could not completely block spermatozoa-zona pellucida binding. This may be because of the presence of multiple receptors for zona pellucida proteins (see below).

It is generally accepted that the specific interaction between spermatozoa and zona pellucida is a carbohydrate-mediated event (Wassarman, 1999; Ozgur et al., 1998; Chapman and
In the past two decades, several carbohydrate-binding proteins on the sperm plasma membrane such as β1,4-galactosyltransferase (Nixon et al., 2001), α-mannosidase (Tulsiani et al., 1989), β-N-acetylgalcosaminidase (Godknecht and Honegger, 1991) and selectin-like receptors (Oehninger, 2001) have been proposed to be involved in such interaction. However, none has received wide acceptance (Wassarman, 1999) and no carbohydrate-binding protein has so far been purified from human spermatozoa and confirmed to bind to human zona pellucida. The exact identity of the sperm protein(s) responsible for spermatozoa-zona pellucida binding in human remains unknown.

The evidence presented in this study shows that human sperm FUT5 has all the characteristics required to play a role in spermatozoa-zona pellucida binding. It is an intrinsic component of the sperm plasma membrane located in the acrosomal region of human spermatozoa and has its catalytic domain (C-terminal) oriented towards the sperm surface. Its ability to bind to intact zona pellucida raises the possibility that it may be a zona pellucida glycoprotein receptor. This is in line with our previous conclusion that the glycodelin-A receptor is closely related to a zona pellucida glycoprotein receptor (Chiu et al., 2003b; Chiu et al., 2004).

Apart from their usual intracellular location, FUT activities have been demonstrated on the cell surface of diverse cell types, such as prostatic epithelial cells (Marker et al., 2001) and rat Sertoli cells (Raychoudhury and Millette, 1997). It is generally believed that glycosyltransferases on the cell surface and rat Sertoli cells (Raychoudhury and Millette, 1997). It is generally believed that glycosyltransferases on the cell surface do not perform the glycosyltransferase function because of a lack of sugar nucleotide donors. Consequently, their function is confined to their carbohydrate-binding ability (Colley, 1997). These cell surface FUTs mediate adhesion of spermatogenic cells to Sertoli cells (Raychoudhury and Millette, 1997) and among neurons during synaptogenesis in culture (Matsui et al., 1986).

The spermatozoa of rat (Raychoudhury and Millette, 1995), mouse (Ram et al., 1989; Cardullo et al., 1989) and human (Tulsiani et al., 1990) express FUT activities. In human and mouse, FUT activity resides in the plasma membrane and is retained after capacitation (Ram et al., 1989; Cardullo et al., 1989; Tulsiani et al., 1990). Carbohydrate moieties, sialyl and non-sialyl N-acetyllactosamines recognized by FUT are present on the zona pellucida of boar (Mori et al., 2000), mouse (Dell et al., 2003; Chalabi et al., 2006) and pig (Yonezawa et al., 1999). Oligosaccharides or artificial carbohydrate chains with N-acetyllactosamine terminals inhibit the binding of spermatozoa to the mouse oocyte (Litscher et al., 1995). Our unpublished observation also shows that glycodelin-A interacts with sperm FUT to regulate spermatozoa-endosalpingeal epithelial interaction in humans. Experiments are being performed to confirm this hypothesis.

In summary, the present investigation provides evidence for the presence of FUT5 on the plasma membrane of human spermatozoa. Its unusual plasma membrane location and its specific interaction with glycodelin-A and zona pellucida protein suggest that it is a candidate receptor molecule for the zona pellucida proteins and glycodelin-A on human spermatozoa. Further characterization of this protein is necessary to reveal the details of interaction of spermatozoa with the zona pellucida. Data showed that glycodelin-A interacts with sperm FUT to inhibit spermatozoa-zona pellucida binding.

Materials and Methods
Semen samples
The Ethics Committee of the University of Hong Kong approved the research protocol. Spermatozoa from normal semen (World Health Organization, 1999) were processed by Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation (Chiu et al., 2003a). After capacitation in Earle’s balanced salt solution (EBSS; Flow Laboratories, Irvine, UK) supplemented with sodium pyruvate, penicillin-G, streptomycin sulfate and 3% bovine serum albumin (BSA) for 3 hours, Percoll-processed spermatozoa were resuspended in EBSS containing 0.3% BSA (EBSS/BSA).
Determination of acrosomal status and motility of sperm
Fluorescent isothiocyanate-labeled peanut (Pisum sativum) agglutinin (FITC-PSA; Sigma, St Louis, MO, USA) and Hoechst staining techniques were used to evaluate the acrosomal status of spermatozoa (Chiu et al., 2002). The fluorescence patterns of 300 spermatozoa in randomly selected fields were determined under a fluorescence microscope (Zeiss, Oberkochen, Germany) with 600× magnification. Spermatozoa without Hoechst and FITC-PSA staining or with FITC-PSA staining confined to the equatorial segment only were considered as acrosome reacted. A Hobson Sperm Tracker System (Hobson Tracking Systems, Sheffield, UK) was used to study sperm motility. The set-up parameters of the system and the procedures were described elsewhere (Chiu et al., 2003b).

Hemizona binding assay
The hemizona binding assay was performed as described (Yao et al., 1996). Unfertilized oocytes from our assisted reproduction program were micro-bisected into two identical hemizonae. Each hemizona was incubated with 2 × 10^6 spermatozoa/ml in a 100 μl droplet of EBSS-BSA under mineral oil for 3 hours at 37°C in an atmosphere of 5% CO₂ in air. The numbers of tightly bound spermatozoa on the outer surface of the hemizonae were counted. The HZI was defined as the ratio of the number of bound spermatozoa in the test droplet to that in the control droplet multiplied by 100.

Purification of glycodelin isoforms
A monoclonal anti-glycodelin antibody (clone F43-7F9) sepharose column was used to purify glycodelin-A and glycodelin-S from amniotic fluid and seminal plasma, respectively (Riittinen et al., 1991; Chiu et al., 2003b). The bound glycodelin was eluted with 0.1% trifluoroacetic acid and dialysed against 100 mM sodium phosphate buffer (pH 7.2). Glycodelin-S was further purified by anion-exchange chromatography (Chiu et al., 2005). The concentrations of the purified glycodelin isoforms were determined with a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The N-Glycosidas re De Glycosylation Kit (Bio-Rad) was used to deglycosylate glycodelin-A (Chiu et al., 2003b). The deglycosylated protein obtained after three successive protein precipitations was redissolved in 20 μl phosphate buffered saline (PBS) and purified by gel filtration chromatography in an AKTA system (Pharmacia). The purity of the deglycosylated protein was analyzed by SDS-PAGE.

Expression and purification of FUT3, FUT5 and FUT6
The full-length cDNAs coding for FUT3, FUT5 and FUT6 were PCR amplified using gene-specific primers (Table 3) and human testis Marathon cDNA (Clontech, Palo Alto CA, USA). The amplified fragments were column purified and cloned into the pET108-D/TOP vector (Invitrogen, Carlsbad, CA, USA) for protein expression. Expression of the FUT fusion proteins were performed in E. coli BL21 (DE3) Star by 0.5 mM IPTG induction for 3 hours. The FUT proteins were expressed as inclusion bodies in the bacteria and purified with the Protein Refolding kit (Novagen; EMD Biosciences, Madison, WI, USA) according to the manufacturer protocol. In brief, bacterial cell pellet was resuspended in 0.1 culture volume of 1× IB wash buffer (20 mM Tris-HCl, 10 mM EDTA, pH 7.5, 1% Triton X-100) and lysozyme at a final concentration of 100 μg/ml was added. The mixture was incubated at 30°C for 15 minutes and sonicated for 5×20 seconds on ice. Inclusion bodies were collected by centrifugation at 10,000 g for 10 minutes, washed twice with 1× IB wash buffer and dissolved in 1× IB solubilization buffer containing 1 mM DTT and 0.3% N-lauroylsarcosine in 50 mM CAPS (pH 11.0). The soluble enzyme fraction in the supernatant was collected after centrifugation and dialyzed with 500 ml of 20 mM Tris-HCl (pH 8.5) containing 0.1 mM DTT, and then with 500 ml of the same buffer but without DTT. Quantification of the recombinant proteins was performed as described above.

Solubilization of human spermatozoa
Capacitated spermatozoa (100×10⁶) were washed three times in PBS. Non-integral, peripheral membrane-associated proteins on spermatozoa were removed by incubating the washed spermatozoa in 1 M NaCl in PBS with gentle stirring for 30 minutes at 25°C as described (Klausen et al., 2003). The spermatozoa were then collected by centrifugation at 600 g for 10 minutes before the sperm membrane proteins were extracted by 1% Triton X-100 (Sigma) in the presence of a cocktail of protease inhibitors (Calbiochem, San Diego, CA, USA) (Chiu et al., 2003b). The insoluble fraction was discarded after centrifugation at 15,000 g for 40 minutes. The concentration of the soluble proteins was determined by Coomassie Plus-200 protein assay reagent (Pierce, Rockford, IL, USA). The supernatant was diluted in a solution of MOPS-NaOH buffer (pH 7.3) containing 0.2% Triton X-100 and 6 mM MnCl₂ (buffer A).

Identification of glycodelin-A binding protein
Glycodelin-A and glycodelin-S were conjugated to Sulfo-SBEd by a ProFound™ Sulfo-SBEd Biotin Label Transfer Kit (Pierce) according to the manufacturer's instructions. Sulfo-SBEd contains a sulfonated N-hydroxysuccinimide active ester, a photocleavable acryl azide and a transferable biotin handle. It was conjugated to glycodelin at a molar ratio of 4:1 for 2 hours at 4°C in the dark. Non-reacted cross-linker was removed by dialyzing the reaction mixture in PBS overnight at 4°C.

Capacitated spermatozoa (100×10⁶/ml) that had their non-integral, membrane-associated proteins removed by incubation in 1 M NaCl in PBS as described above were incubated with 300 pmol/ml Sulfo-SBEd-linked glycodelin-A. After 90 minutes, the spermatozoa were washed three times in PBS and cross-linked glycodelin-A with 200 μl of proteins by UV irradiation at 302 nm (High Performance Transilluminator TFM-26; UVP, Upland, CA, USA) for 15 minutes at room temperature. The integral membrane proteins of spermatozoa were isolated by 1% Triton X-100 with a cocktail of protease inhibitors (Calbiochem). The soluble proteins were diluted in buffer A, and the protein concentration was determined by Coomassie Plus-200 protein assay reagent (Pierce). The glycodelin covalently linked sperm surface proteins were then purified by immunofinity chromatography using a monoclonal anti-glycodelin antibody sepharose column as described above. One-half of the purified protein complex was treated with reducing sample buffer provided by the ProFound Sulfo-SBEd Biotin Label Transfer Kit containing 100 mM DTT to cleave the crosslinks between glycodelin and the sperm proteins at the azo linkage and to transfer the biotin label from the glycodelin to the sperm proteins. The remainder was treated with non-reducing sample buffer. Both samples were heated at 90°C for 5 minutes and analyzed by SDS-PAGE. The reduced and silver-stained glycodelin-interacting protein bands in SDS-PAGE were excised and digested in situ with trypsin (0.1 mg/ml in 25 mM NH₄HCO₃, pH 8.0). The peptides formed were recovered with Millipore C18 ZipTips and 60% acetonitrile-0.1% trifluoroacetic acid (TFA) containing the α-cyano-4-hydroxycinnamic acid matrix. The peptide-matrix samples were then analyzed with MALDI-TOF MS to obtain the peptide mass spectra (MDS Sciex, South San Francisco, CA, USA) and compared with the protein sequences in the public protein databases at the Swiss-Prot Knowledgebase (http://www.ebi.ac.uk/swissprot) as described (Lee et al., 2004).

Localization of sperm FUT
Triton X-100-extracted sperm proteins (see above) or recombinant FUTs were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane for western blot analysis as described (Lee et al., 2004). Polyclonal rabbit antibodies to FUT3 and FUT5 were generated against the N-terminus and C-terminus, respectively, of the proteins. Immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL; Amersham, Little Chalfont, UK). The identity of the antibody was confirmed by Western blot analysis of recombinant protein expressed in E. coli.

Table 3. Primer sequences used for PCR amplification of the full-length FUT cDNA for protein expression in E. coli

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer*</th>
<th>Sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUT3</td>
<td>FUT3-5′-TOPO</td>
<td>5′-CACCATGGATGATTCCTGAGTTGTCAGC-3′</td>
<td>1086 bp</td>
</tr>
<tr>
<td></td>
<td>FUT3-3′-TOPO</td>
<td>5′-TCAGTTGACCAAGCAGCGCTA-3′</td>
<td></td>
</tr>
<tr>
<td>FUT5</td>
<td>FUT5-5′-TOPO</td>
<td>5′-CAGCTGTGGATCATGAGTTGTCAGC-3′</td>
<td>1060 bp</td>
</tr>
<tr>
<td></td>
<td>FUT5-3′-TOPO</td>
<td>5′-TCAGTTGACCAAGCAGCGCTA-3′</td>
<td></td>
</tr>
<tr>
<td>FUT6</td>
<td>FUT6-5′-TOPO</td>
<td>5′-CACCATGGATGATTCCTGAGTTGTCAGC-3′</td>
<td>1080 bp</td>
</tr>
<tr>
<td></td>
<td>FUT6-3′-TOPO</td>
<td>5′-TCAGTTGACCAAGCAGCGCTA-3′</td>
<td></td>
</tr>
</tbody>
</table>

*The primer sequences were complementary to FUT3 (accession no. NM_0000149), FUT5 (accession no. NM_0020343) and FUT6 (accession no. NM_000150). A TOPO-directional cloning sequence (CACCC) was added at the 5′-end of the forward primers, the start codon (ATG) is underlined and the stop codons (TGA, in reverse direction) are double underlined.
glycodelin-A was used instead. Purification of sperm FUT was performed as described (Muramatsu et al., 1986; Martin et al., 1988) with modifications. In brief, sperm proteins were fractionated on a Mono Q column in a SMART System (Pharmacia) pre-equilibrated in buffer A as described (Yao et al., 1998). Sperm proteins werestepwise eluted with buffer A containing 0.5 M NaCl and desalted through a Sephadex G-25 column (Pharmacia). They were further purified by a GDP-agarose column (Sigma) and eluted with buffer A containing 10 mM guanosine-monophosphate and 0.5 M NaCl. The eluates from five runs were combined and desalted. The entire procedure of purification was repeated at 4°C and for 24 hours to minimize enzyme denaturation. The purity of the purified proteins was checked by SDS-PAGE, MS and western blotting using the N-18 antibody (1:200).

The FUT activity in the fractions was measured as described (Rahina et al., 1997), briefly, sialyl-α2,3-N-acetyllactosamine-BSA (sL-N-BSA; Calbiochem)-coated wells were blocked with Tris buffered saline (TBS) and washed with TBST and water immediately before use. Purified FUTs were added and incubated at 37°C in assay buffer containing 0.1 M MOPS-NaOH (pH 7.3), 50 μM GDP-fucose, 6 mM MnCl₂, 5 mM ATP, 10 mM fucose and 0.2% Triton X-100. The product, Sialyl Lewis x (sLeX) was detected at OD₄₉₀ after successive incubation with monoclonal anti-sLex immunoglobulin M (IgM) (Calbiochem), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM (Sigma) and 3,3′,5,5′-tetramethylbenzidine (TMB) as substrate. The absorbance was determined spectrophotometrically at 492 nm. The ability of different concentrations of FUT acceptors described above. The antibody preabsorbed with 1:100 blocking peptide (Santa Cruz) was used at 37°C. Purification of sperm FUT

Complementarity of glycodelin with FUT in the presence or absence of solubilized zona pellucida.

To confirm the binding relationship between glycodelin, FUT and zona pellucida, co-immunoprecipitation was performed. Human zona pellucida was prepared from the oocytes using glass micropipettes under a microscope (Franken et al., 1994) and heat-solubilized at 70°C for 90 minutes in distilled water with the pH adjusted to 9 using Na₂CO₃ (Dunbar et al., 1980). A Seize X Mammalian Immunoprecipitation Kit (Pierce) was used to detect the interaction between glycodelin and purified sperm FUT by recombinant FUTs according to the manufacturer’s instructions. Briefly, recombinant or purified FUT was incubated with glycodelin-A in the presence of different concentrations of solubilized zona pellucida glycodelin-A for 2 hours at 37°C. After 2 hours of incubation with gentle shaking, monoclonal anti-glycodelin antibody immobilized on Protein G beads was used to precipitate the glycodelin-A-FUT complex, which was analyzed by western blot using the N-18 antibody. The procedure for the control was similar to that described for glycodelin-A except that deglycosylated glycodelin-A was used instead.

Effects of FUT acceptors and anti-FUT antibody

The binding of saturation concentration of ¹²⁵I-labeled glycodelin-A to human spermatozoon was determined (Chiu et al., 2003b) in the presence of different concentrations of low molecular mass FUT acceptors, including phenyl-β-D-galactoside, 2'-fucosyllactose and lacto-N-fucopentaose-I (Sigma), which had been used to demonstrate the presence of α(1-2) (Scudder and Chantler, 1981), α(1-3) (Priels et al., 1981) and α(1-4)-FUT activity (Priels et al., 1981), respectively, or the high molecular mass acceptor, asialofetuin (Sigma) (de Vries et al., 1995). Non-FUT acceptors were introduced to the binding assay as described above. The antibody preabsorbed with 1:100 blocking peptide (Santa Cruz) was used as control. The ability of different concentrations of FUT acceptors to inhibit spermatozoon-pellucida binding was also studied. The effect of an anti-FUT antibody and FUT acceptors on sperm motility, viability and acrosomal status was determined as described above.

Binding of sperm FUT to zona pellucida

Intact human oocytes were incubated with Alexa Fluor 488 (Alexa Fluor® 488 Protein Labeling Kit; Molecular Probes, Carlsbad, CA, USA)-conjugated sperm FUT in buffer A for 30 minutes at 37°C, and observed under a fluorescence microscope. Oocytes incubated with labeled sperm FUT in the presence of 50-fold excess of unlabeled sperm FUT were labeled as controls. The binding kinetics of sperm FUT to zona pellucida were studied by incubation of Alexa Fluor 488-labeled sperm FUT (0-500 pmol/ml) in solubilized zona pellucida (10 zona pellucida/well)-coated well microtiter plates for 2 hours at 37°C. The fluorescence intensity of bound FUT was determined using a fluoremeter with excitation and emission wavelengths of 425 and 520 nm, respectively. Specific binding of sperm FUT was determined by subtracting the optical density (OD) in the presence of a 50-fold excess of unlabeled FUT from the OD in the absence of unlabeled FUT. The experiments were performed in triplicate.

Data analysis

All the data were expressed as the mean and standard error of the mean (s.e.m.). The data were analyzed by statistical software (SigmaStat 2.03. Jandel Scientific, San Rafael, CA, USA). For all experiments, the non-parametric analysis of variance on rank test for multiple comparisons followed by the Mann-Whitney U-test were used. A probability value ≤0.05 was considered to be statistically significant.

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