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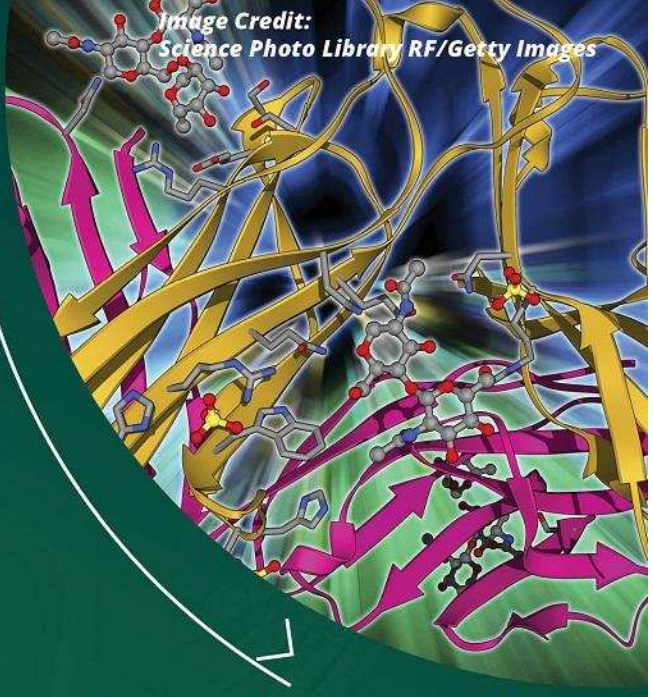

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RESEARCH ARTICLE

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Sperm binding to porcine oviductal cells is mediated by SRCR domains contained in DMBT1

María Lorena Roldán[1] | Juan Manuel Teijeiro[2,3] | Jimena Ruiz Álvarez[2] | Patricia Estela Marini[1,2,4]

[1] Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Rosario, Argentina

[2] Laboratorio de Medicina Reproductiva, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

[3] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rosario, Argentina

[4] Consejo de Investigaciones de la Universidad Nacional de Rosario (CIUNR), Rosario, Argentina

Correspondence
Dra Patricia Estela Marini, Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Rosario, Argentina, LMR, FCByF, Suipacha 590, Rosario S2002LRK, Argentina.
Email: pmarini@fbioyf.unr.edu.ar; marini@ibr-conicet.gov.ar

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Abstract

The oviduct is an organ in which a subpopulation of sperm is stored in a reservoir, preserving its fertilizing potential. In porcine, two oviductal proteins have been identified in relation to sperm binding, Annexin A2 and Deleted in Malignant Brain Tumor 1 (DMBT1). DMBT1 is a multifunctional, multidomain glycoprotein, and the characteristics of all of its domains, as well as its carbohydrates, make them candidates for sperm binding. In this work, we challenge sperm for binding to pig oviductal cells on primary culture, after treatment with antibodies specific for the different domains present in DMBT1. Only anti-SRCR antibodies produced inhibition of sperm binding to cells. Thus, SRCR is the main domain in DMBT1 promoted sperm binding to form the reservoir in the oviduct, and this function is probably elicited through the polypeptide itself.

KEYWORDS

DMBT1, oviduct, sperm reservoir, SRCR

1 | INTRODUCTION

The oviduct is a dynamic organ in which events essential for reproduction, such as gamete transport and encounter, and sperm final maturation and selection take place. Sperm selection through the formation of a reservoir occurs in many mammalian species such as human, sheep, cow, horse, pig, hamster, and mice (for review see Ref.[1]). The reservoir is formed by binding of a subpopulation of sperm with favorable characteristics and in non-capacitated state to the oviductal epithelium. While being in contact with the epithelium, sperm show extended viability and preserved fertilizing ability[2]; and capacitation and hyperactivation are delayed.[3] Sperm release occurs in limited number, providing a suitable amount of viable, potentially fertile spermatozoa at the time of ovulation.

The mechanism of sperm binding to the epithelial cells is still under study, however, many of its components have been identified. Carbohydrates contained in oviductal glycoproteins are considered main components of sperm-oviduct interaction, with different saccharides providing species specificity. In pig, oligomannose, terminal mannose and galactose residues,[4] and Lewis X related moieties[5] have been identified as important for sperm binding. Also in pig,

spermadhesins, a family of proteins with carbohydrate binding capability predominantly found in seminal plasma and/or peripherally associated to sperm,[6] have been involved in sperm binding to the oviduct: DQH, through mannose,[7] and AQN1, through galactose,[8] and the sperm protein lactadherin through Lewis X trisaccharide.[9]

Oviductal proteins have also been proposed to interact with sperm for reservoir formation. In pig, ANXA2[10] and Deleted in Malignant Brain Tumors 1, DMBT1, formerly called sperm binding glycoprotein, SBG,[11,12] have been related to this function. The interaction between DMBT1 and sperm was first inferred by purification of the glycoprotein based on affinity to sperm periacrosomal membranes[11] and further confirmed by plasmon resonance studies.[13] DMBT1 purified from pig oviductal epithelial cells exposes O-linked Galβ1-3GalNAc, which might be recognized by the spermadhesin AQN-1, adsorbed to spermatozoa.[8,11] Also, DMBT1 has been shown to interact in vitro with another sperm protein present in porcine as well as in human sperm, S100A7 (Psoriasin).[12]

DMBT1 is a glycoprotein present in multiple organisms and several cell types, where it exerts functions in the innate immune defence against pathogens,[14] epithelial homeostasis,[15] inflammation, and tumor suppression.[16] In reproductive organs, it has been reported in the porcine and women's oviduct in a membrane bound form[11,17,18] with ability to bind sperm and, also, probably involved in the homeostasis of the epithelia covering the female tract.[18] In the oviduct of equine and porcine species, DMBT1 has been found in a secreted form related to reproduction.[13] Likewise, DMBT1 has been detected in the lumen of ewe cervix in a proteomic study, being more abundant in luteal phase,[19] and in women's cervix.[20] Also, DMBT1 mRNA has been detected in the uterus of monkeys and rats.[21]

Concerning the structure of DMBT1, it is a member of the scavenger receptor cysteine-rich (SRCR) family[22] and is formed by a combination of domains that include SRCR, CUB (for complement C1r/C1s, Uegf, Bmp1), and ZP (zona pellucida).[14] All of the domains that compose DMBT1 have been shown to mediate specific binding to cells and proteins (for review see Ref.[23]). The aim of this work is to analyze the importance of each type of domain in sperm reservoir formation. To this end we challenge sperm binding ability to primary cultures of porcine oviductal epithelial cells treated with antibodies specific for the different domains.

2 | MATERIALS AND METHODS

2.1 | Reagents

Unless stated, reagents were from Sigma-Aldrich, Buenos Aires, Argentina.

2.2 | Antibodies

Anti-CUB antibodies, RRID: AB_2714150, were developed in rabbit in a previous work.[18] The antigen was a recombinant polypeptide containing amino acids 578-803 of pig DMBT1 including domains CUB1, scavenger interspersed domain 4 (SID4), and 50 amino acids from SRCR4 (Figure 1A). These

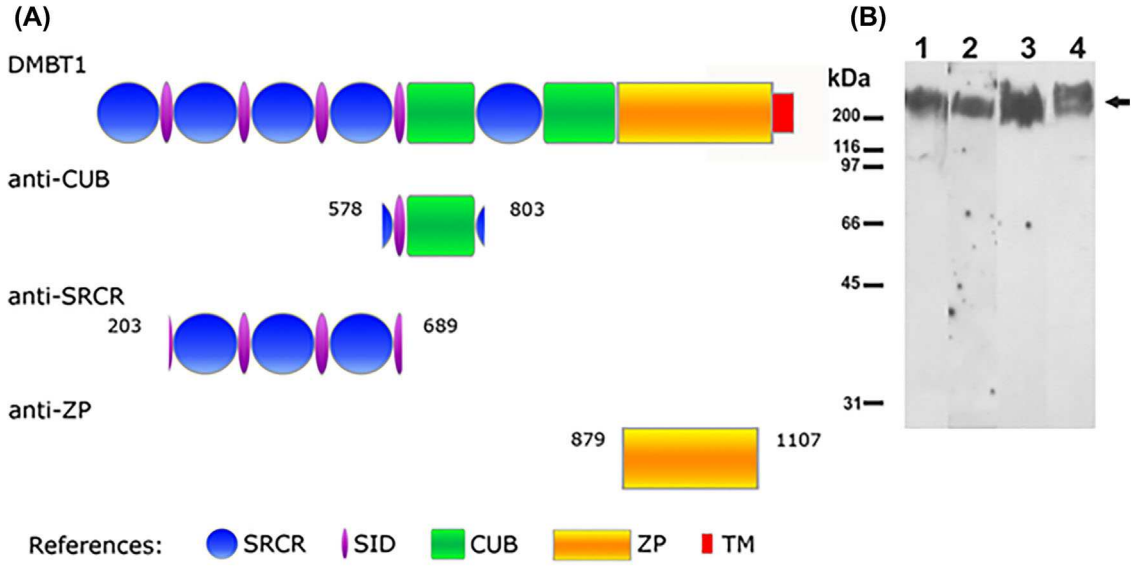

FIGURE 1 Antibodies specificity. A, Diagram of the domain composition of porcine oviductal DMBT1. Protein fragments used for antibody development are shown for anti-CUB and anti-SRCR, and the domain potentially recognized by anti-ZP is indicated. B, Western blot of 100 μg of protein extracts from primary cultures of oviductal cells with (1) anti-DMBT1p84, (2) anti-SRCR, (3) anti-CUB, (4) anti-ZP

antibodies have been used in Western-blot and immunohistochemistry showing specificity for porcine and human DMBT1, and in immunoprecipitation of the porcine oviductal glycoprotein.[18] Anti-DMBT1p84 polyclonal rabbit antibodies against human DMBT1, gently provided by Dr Jan Mollenhauer, University of Southern Denmark, Denmark,[24] were used as control.

Anti-ZP and anti-SRCR (RRID: AB_2714151) antibodies were developed using the same protocol as for anti-CUB, which has been approved by Facultad de Ciencias Bioquímicas y Farmacéuticas Committee for Use and Care of Laboratory Animals, UNR, RES. N° 935/2015. For anti-SRCR antibodies development, the 1460 bp cDNA fragment corresponding to amino acids 203-689 (SRCR 2-4, SID 2-4, and part of SID 1; Figure 1A) was cloned in pRSETB vector (Invitrogen, Buenos Aires, Argentina). To this aim epithelial cells were obtained by scrapping the isthmic region of porcine oviducts with a scalpel blade, RNA was prepared using TRIzol-reagent (Invitrogen) and reverse transcription was performed with SuperScript-II reverse transcriptase (Invitrogen). The cDNA was used as template in PCR with the primers: 5′-TC**GGATCC**CACAGCAACTTCTTCGTT-3′ and 5′-TT**GAATTC**GACACAGTTGGCATTGTT-3′. The fragment was cloned and sequenced, and expression was induced in *Escherichia coli* DH5α by 1 mM IPTG. The recombinant protein was purified based on its His-tag and used to generate polyclonal antibodies. Anti-SRCR antibodies were checked by Western blot of extracts of oviductal cells showing specificity and affinity similar to anti-DMBT1 p84 and anti-CUB (Figure 1B). Control experiments were performed using pre-immunization serum and secondary antibodies alone (not shown).

Anti-ZP, gently gifted by Dr Silvia Arranz, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR; were obtained using purified porcine ZP[25] as immunogen. When tested on immunoblots of porcine oviductal cell extracts, anti-ZP showed specificity and affinity for DMBT1 comparable to the described for anti-DMBT1 p84, anti-CUB, and anti-SRCR (Figure 1B).

2.3 | Western blots

Western blots were performed as described previously.[26] Briefly, protein extracts of cells from primary cultures were prepared using buffer 50 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 M phenylmethanesulfonyl fluoride (PMSF), 2 μg/mL aprotinin, 0.1% v/v 2-mercaptoethanol. Total protein concentration was assessed by the Bradford protein assay.[27] Extracts (100 μg of proteins) were subjected to SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, Buenos Aires, Argentina). Membranes were blocked with 5% dry milk in TBS-T (TBS: 50 mM Tris-HCl pH 7.5, 0.150 M NaCl; plus 0.05% Tween-20) for 2 h and incubated with rabbit anti-CUB, anti-SRCR, or anti-ZP (1:1000) serum, or anti-DMBT1 p84 (1:400) for 2 h at room temperature. After TBS wash, membranes were incubated with anti-rabbit IgG-HRP (GE Healthcare) (1:10 000 v/v) in TBS for 1 h at room temperature. After washing with TBS, peroxidase activity was revealed using an ECL kit (GE Healthcare).

2.4 | Oviductal cell primary cultures

Ovaries were collected at a local abattoir and transported to the laboratory in ice. After trimming from other reproductive tissues and washing with sterile PBS, the oviducts were cut in halves and used to prepare epithelial cell monolayers.[28] Oviducts were subjected with pins and flushed with 1 mL PBS while gently extruding with tweezers. The recovered cells were washed by centrifugation and suspended in M199 medium supplemented with 10% bovine fetal serum (Internegocios, Mercedes, Argentina), and 100 UI/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamycin, 0.25 μg/mL amphotericin B. Cells were counted on Neubauer chamber and vitality was determined by Trypan blue staining (vitality was greater than 85%) and ciliary beating. Live cells (2×10^6) were added to 3.5 cm Petri dishes containing coverslips and the mentioned culture medium, and grown at 38.5°C on 5% CO_2 and 100% humidity until confluence greater than 80% (5-7 days).

2.5 | Sperm preparation

Semen was collected by the gloved-hand method from adult fertile boars routinely used for artificial insemination. The sperm-rich fraction was diluted in Cronos (Laboratorio Medi Chimica, Reggio Emilia, Italy) and conserved at 16°C until use (no more than 24 h from collection). Samples were analyzed for motility, viability, concentration, acrosomal, and morphological parameters[29] to determine their quality. Sperm were washed in Tyrode's Salts (1.8 mM Cl_2Ca, 2.7 mM KCl, 1 mM Cl_2Mg, 137 mM NaCl, 0.4 mM NaH_2PO4, 21.8 mM HEPES, 12 mM NaHCO3) by centrifugation and suspended in TALP medium (Tyrode's Salts supplemented with 19.6 mM sodium lactate, 1 mM sodium pyruvate and 6 mg/mL Bovine Serum Albumin, pH 7.2), which simulates the oviductal fluid.[30]

2.6 | Binding assay

Cellular monolayers were washed with temperate PBS (3×) and incubated with increasing amount of antibodies or preimmune serum (4, 8, and 12 μL for every serum and additionally 2 and 6 μL for anti-SRCR and 16 μL for anti-ZP), for 20 min at 38.5°C on 5% CO_2 and 100% humidity. After washing three times with PBS, sperm (2×10^6 sperm in 1 mL TALP) were added and incubation was continued under the same conditions for 1 h. After incubation, non-

bound sperm were removed by sorrow washing, and remaining sperm-cells were fixed with 2.5% V/V glutaraldehyde. Cover slips were removed from dishes and mounted in 50% glycerol, 10 mM Tris-HCl pH 8. Bound sperm were counted, fifteen 2 mm^2 fields/coverslip (400×), with Olympus BFX40 microscope (Japan). As control, cell monolayers without antibody treatment were used. In order to minimize errors due to particular cultures and animals or samples, the amount of bound sperm for every condition was expressed as percentage of the spermatozoa bound in the control without any serum treatment. Sperm/oviductal cells co-incubations were done by quintuplicate.

2.7 | Glycosylation prediction

The potential for N- and O-glycosylation sites found on porcine DMBT1 were determined using the free GlycoEP Prediction Service (http://www.imtech.res.in/raghava/glycoep/index.html).[31] This site's predictions are based in the analysis of a large dataset of glycosylation sites with redundancy reduction. Prediction based on Composition Profile of Patterns (CPP, 20 amino acids) was used. Accuracy is higher than 95.67% and 91.89% for N- and O-glycosylation, respectively.

Amino acids were considered potential glycosylation sites when thresholds were higher than 1.

2.8 | Statistics

The assumption of normality of the data was determined by Q-Q plot and by Barlett test. For statistical analysis, the Linear mixed Models with Heterogeneous Variance or ANOVA and the multiple comparison test of LSD Fisher (software infoSTAT) were used. Differences were considered significant for $P < 0.05$.

3 | RESULTS

3.1 | Anti-SRCR antibodies inhibit sperm binding to oviductal cells

Anti-SRCR polyclonal antibodies were developed against a DMBT1 fragment expressed in *E coli*, corresponding to SRCR 2, 3, and 4 domains, and including SID 2 and 3 and a portion of SID1 and 4 (Figure 1A). Treatment of primary oviductal cell cultures with 4, 8, and 12 µL of anti-SRCR serum prior to sperm addition produced strong, statistically significant ($P < 0.01$) inhibition of sperm binding (Figure 2A, Table 1). This indicates that SRCR domains are involved in sperm binding to oviductal epithelial cells. A possible incidence of the SID regions present in the polypeptide used for anti-SRCR development must be considered. Other dilutions of antibodies (2, 6 µL) were assayed in order to evaluate concentration dependent effects. However, similar results were obtained for every amount of anti-SRCR (Figure 1A, Table 1), indicating that the lowest concentration provides almost saturating masking of sperm recognition sites. Cells conserved the ability to bind approximately 60% of sperm disregard the dilution of anti-SRCR used, in agreement with the conception of sperm/cell binding as a complex multicomponent event.

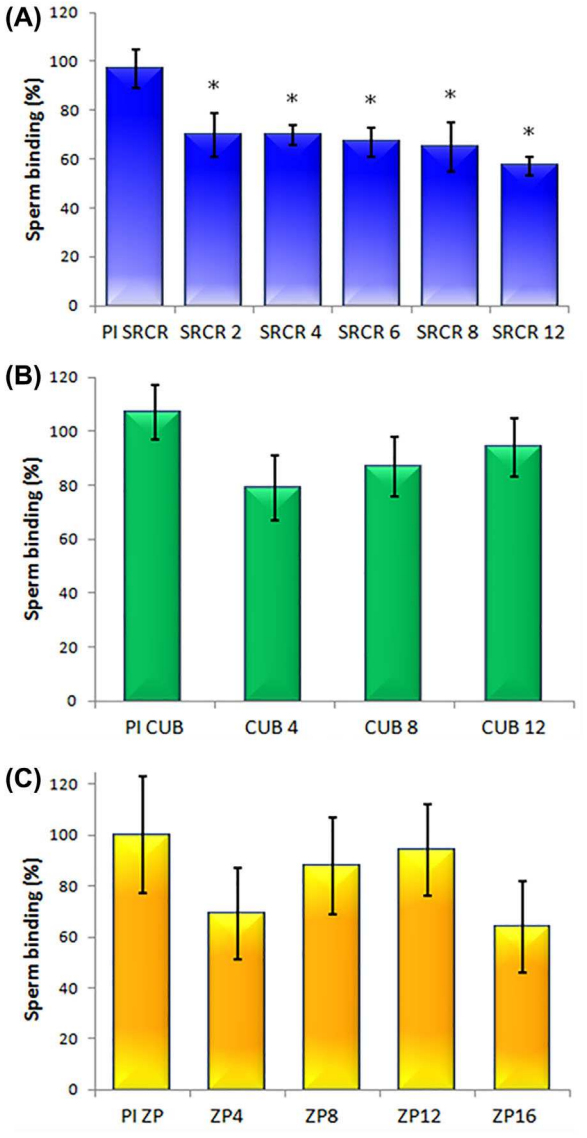

FIGURE 2 Inhibition of sperm binding to oviductal cells in primary culture by specific antibodies. Diagram of the percentage of sperm bound to oviductal cells in vitro, after treatment with specific antibodies. A, anti-SRCR ($P < 0.01$), (B) anti-CUB ($P < 0.05$), (C) anti-ZP ($P < 0.05$). $n = 5$. *Indicates significant differences. PI, preimmune serum for every antibody (mean of 4, 8, and 12 µL); motif names followed by numbers indicate the specificity of the antibodies and the volume of serum used in each condition (eg, SRCR 4, 4 µL of anti-SRCR; PI SRCR, preimmune serum of anti-SRCR)

TABLE 1 Inhibition of sperm binding by anti-domain antibodies

Serum	Sperm binding (%)
PI SRCR	97.00 ± 8.00
SRCR 2	70.00 ± 9.00
SRCR 4	70.00 ± 4.00
SRCR 6	67.00 ± 6.00
SRCR 8	65.00 ± 10.00
SRCR 12	57.00 ± 4.00
PI CUB	107.00 ± 10.00
CUB 4	79.00 ± 12.00
CUB 8	87.00 ± 11.00
CUB 12	94.00 ± 11.00
PI ZP	100.00 ± 23.00
ZP4	69.00 ± 18.00
ZP8	88.00 ± 19.00
ZP12	94.00 ± 18.00
ZP16	64.00 ± 18.00

Means and standard deviations of sperm bound upon treatment with different dilutions of anti-SRCR, anti-CUB and anti-ZP antibodies are indicated. Motif names followed by numbers indicate de specificity of the antibodies and the volume of serum used in each condition (eg, SRCR 4, 4 μL of anti-SRCR; PI SRCR, preimmune serum of anti-SRCR).

3.2 | Anti-CUB antibodies have no effect on sperm binding to oviductal epithelial cells

The use of anti-CUB antibodies developed against a fragment of DMBT1 that includes CUB1 and SID4 domains, and a portion of SRCR 4 and 5 (Figure 1A), in experiments of inhibition of sperm binding to cultured oviductal epithelial cells had no effect ($P < 0.05$) (Figure 2B, Table 1). The homology between CUB 1 and CUB2 domains, and the use of polyclonal antibodies, allows inferring that these motifs are not involved in DMBT1 mediated sperm-oviduct binding. Also, as SID4 is included in the polypeptide used for anti-CUB development, the possible involvement of SID domains in sperm binding, considered in the previous item, where anti-SRCR was used, is not supported by this result.

3.3 | Anti-ZP antibodies do not significantly inhibit sperm binding to porcine oviductal cells in primary culture

The binding of sperm to oviductal epithelial cells was challenge with anti-ZP antibodies at different dilutions. Although these polyclonal antibodies recognize DMBT1 with an affinity similar to that of anti-SRCR, they did not significantly ($P < 0.05$) modify the number of sperm bound to oviductal cells in primary culture (Figure 2C, Table 1), indicating that this DMBT1 moiety is probably not involved in sperm binding for reservoir formation.

3.4 | Glycosylation prediction for different DMBT1 domains

As DMBT1 is a highly glycosylated protein with O-linked saccharides that expose Galβ1-3GalNAc groups, which probably interact with sperm for reservoir formation, the potential of the different domains of DMBT1 for glycosylation was analyzed. As shown in Table 2, using a threshold of 1, potential N-glycosylation sites are scarce, and predominant in ZP followed by CUB domains. Potential O-glycosylation sites instead are predominant in SIDs, with stretches of consecutive or close amino acids with high O-glycosylation possibilities in SID1 and SID4. Of the five SRCR domains, only SRCR1 shows a potential O-glycosylation site. Despite the high threshold used, potential N- and O-glycosylation sites are detected at the unlikely intracellular region.

4 | DISCUSSION

In many animals and diverse organs, DMBT1 is localized in the lumen and in epithelial cells.[32] This is also the case for porcine oviductal DMBT1.[13,17,19] When purified from oviductal cells and solubilized, DMBT1 shows deleterious effects over the acrosomes of sperm that have begun

TABLE 2 Potential O- and N-glycosylation sites determined by GlycoEP Predicton Service

DOMAIN	O-glycosylation	N-glycosylation
NH_2 terminal	S62, S63, T66	D49
SRCR1	S136	
SID1	T202, T203, T204, T206, S207, S208, T211	
SRCR2		
SID2		
SRCR3		
SID3	T512	
SRCR4		
SID4	T651, T652, T653, S659, S660	
CUB1		D738, D749
SRCR5		
aa 883-896		D894
CUB2		
ZP		D1019, D1077, D1191
TM		
Intracellular region	S1411, S1412, T1415	D1398

Threshold > 1.

capacitation, at least in vitro.[26] As sperm with acrosome alterations have been detected on the oviductal lumen in vivo,[33] the soluble form of DMBT1 is proposed to be part of a mechanism of negative selection of sperm.[26] DMBT1 exposed at the apical surface of epithelial cells, instead, would be part of the sperm reservoir formation mechanism. This form has been shown to interact with galectin 3 and other proteins, and is considered to take part in cellular homeostasis.[18] The luminal and cellular forms of DMBT1 might represent distinct isoforms with different functions; alternatively, a single isoform might show different function according to its environment and local interaction with different partners. In favor of the first hypothesis, DMBT1 present in the lumen and in the cells of the oviduct have been reported to have different apparent molecular masses.[11,13] Also, isoforms formed by differential glycosylation have been reported in human tears[34] and splicing variants have been found in mice intestine, one of which lacks the transmembrane domain.[35] So far, splicing variants or RNA lacking the transmembrane region has not been detected for pig DMBT1 and a signal peptide has not been identified,[36,37] indicating DMBT1 excretion probably occurs through a non-canonical mechanism. In favor of DMBT1 acquiring different properties according to its environment, the same glycoprotein shows different bacteria binding capacities when it is solubilized or in a surface bound form,[38] and a dual role has been proposed for bound and soluble DMBT1 in complement activation.[32]

Carbohydrate motifs have been shown to participate in sperm binding to form the oviductal reservoir and DMBT1 exposed glycans may be responsible for its interaction with sperm. However, many of the peptidic domains that compose DMBT1 also show cell and protein specific binding capabilities.[23]

The sequences of the SRCR domains contained in DMBT1 allow classifying this protein as of group B of the SRCR family. This group is composed mainly of transmembrane proteins, frequently found on immune cells.[39] These SRCR domains show specific binding capacities, for example they participate in viral infection to produce porcine reproductive and respiratory syndrome.[40] Moreover, SRCR domains are considered to be able to discriminate between different species and strains of bacteria,[41] and also between proteins, as in binding of human DMBT1 to IgA, surfactant proteins and lactoferrin.[42]

When binding of porcine sperm to oviductal cells in primary culture was challenged by prior incubation of the cells with antibodies directed against diverse domains, only anti-SRCR antibodies produced a decrease on the number of bound sperm (Figure 2A). This inhibition strongly supports that SRCR motifs are main components of the sperm attachment mechanism for reservoir formation. The anti-SRCR antibodies used in this work were developed by rabbit immunization with a recombinant protein expressed in a bacterial system, a non-glycosylated polypeptide. The antibodies are able to specifically recognize both the non-glycosylated, and the oviductal glycosylated forms of DMBT1 by Western blot (Figure 1B). When the potential of SRCR domains for glycosylation was analyzed, only SRCR1 showed a possible O-glycosylation site (Table 2). Also, SRCR polypeptides may specifically bind to bacteria,[43] arguing in favor of a possible polypeptide rather than glycoside recognition of DMBT1 by sperm. However, it is commonly accepted that reservoir formation proceeds through carbohydrate recognition and the polypeptide used for anti-SRCR development includes the potentially glycosylated amino acids of SID domains (Figure 1B, Table 2). The present assay does not allow distinguishing if the peptide itself or the carbohydrates bound to SID domains are responsible for sperm attachment to oviductal cells. However, SID4 is present in the peptide used for anti-CUB development, and these antibodies do not inhibit sperm binding (Figure 2B). Also, a structure has been proposed for human DMBT1 in which the high density of glycans present in SIDs would produce an extended conformation similar to that of mucins that would alternate stretched SIDs with globular SRCR domains. If a similar structure is present in pig oviductal DMBT1, anti-SRCR antibodies would probably recognize the globular SRCR motifs, but not the SID stretches in which the polypeptide would be surrounded by extensive O-glycosylation, supporting sperm binding to polypeptidic SRCR domains. In addition the polypeptide GRVEVLYR**G**SW has been identified as binding motif in human DMBT1's SRCR,[23] and porcine DMBT1 SRCR3 domain contains a similar polypeptide, with only one mismatch: GRVEVLY**Q**GSW.

CUB domain crystal structures were first resolved using boar spermadhesins porcine seminal plasma protein I and II (PSP I/PSPII), and bovine acidic seminal fluid protein, all of which bind to sperm. CUB domains also exhibit bacteria binding capability in mice and human DMBT1. These characteristics support the possible involvement of DMBT1 CUB domains in sperm binding for reservoir formation. However, anti-CUB antibodies did not produce significant inhibition of sperm binding to primary cultures of oviductal cells (Figure 2B). The CUB domains contained in DMBT1 belong to the cbCUB type of Ca^{2+} dependent binding domains, different in properties from the CUB domains contained in spermadhesins (for review see Ref.[44]). The binding abilities of both types of CUB domains are different, and disparity would be expected in the binding properties of the proteins that contain them. The inability of DMBT1's CUB domains to bind sperm exposed by the present results (Figure 2B), are in accordance with these differences. Anti-CUB antibodies were developed against CUB1, however, the homology between CUB1 and CUB2 and the polyclonal nature of the antibodies, together with the necessity of two cbCUB domains for binding to this type of motifs, support the idea that CUB domains present in DMBT1 are not involved in sperm binding for reservoir formation.

ZP domains are predominant in zona pellucida glycoproteins which form the matrix that covers the egg and, under physiological conditions, are responsible for spermatozoa

binding, induction of acrosome reaction, polispermy prevention, and early embryo protection.[45] In mammals, the zona pellucida is composed by three or four ZP proteins that present variations between species, but all of which contain characteristic ZP domains. The variable capacities of ZP proteins, their domains and saccharides, to bind to diverse regions of sperm under different physiological conditions,[45] allows questioning if the ZP domain contained in DMBT1, or its potentially bound saccharides may participate in non-capacitated sperm binding to the ewe's oviduct. Anti-ZP antibodies show no effect on sperm binding to oviductal cell cultures (Figure 2C). It is to note that most experiments performed to study sperm binding to the oocyte use capacitated or acrosome reacted sperm,[45] while sperm involved in reservoir formation are considered to be non-capacitated.[46] The region of sperm involved in ZP binding is strongly dependent on the acrosomal state, and shows strong variations among species.[45] The results (Figure 2C) support that spermatozoa expose ZP binding elements on their surface upon capacitation and acrosome reaction, which would not be available in the surface of non-capacitated sperm.

Multiple glycosylation sites are predicted in the different domains of DMBT1 (Table 2) and strong O-glycosylation has been shown for this protein.[11] However, the absence of sperm binding inhibition by anti-CUB and anti-ZP, together with the scarce potential glycosylation sites on SRCRs, allows favoring the hypothesis of sperm interaction with polypeptidic SRCR. As sperm binding to oviductal cells surfaces should be promoted by the concerted action of several molecules, the carbohydrates shown to mediate this interaction would probably be exposed by other oviductal proteins. In whole, SRCR domains contained in oviductal cellular DMBT1 seem to be crucial for sperm binding.

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ORCID

Patricia Estela Marini http://orcid.org/0000-0002-3064-1808

REFERENCES

1. Kölle S. Transport, distribution and elimination of mammalian sperm following natural mating and insemination. *Reprod Domest Anim*. 2015;50:2–6.
2. Hunter RH, Léglise PC. Polyspermic fertilization following tubal surgery in pigs, with particular reference to the role of the isthmus. *J Reprod Fertil*. 1971;24:233–246.
3. Rodríguez-Martínez H, Saravia F, Wallgren M, et al. Boar spermatozoa in the oviduct. *Theriogenology*. 2005;63:514–535.
4. Wagner A, Ekhlasi-Hundrieser M, Hettel C, et al. Carbohydrate-based interactions of oviductal sperm reservoir formation-studies in the pig. *Mol Reprod Dev*. 2002;61:249–257.
5. Machado SA, Kadirvel G, Daigneault BW, et al. LewisX-containing glycans on the porcine oviductal epithelium contribute to formation of the sperm reservoir1. *Biol Reprod*. 2014;91:140.
6. Sanz L, Calvete JJ, Mann K, Schäfer W, Schmid ER, Töpfer-Petersen E. The complete primary structure of the boar spermadhesin AQN-1, a carbohydrate-binding protein involved in fertilization. *Eur J Biochem*. 1992;205:645–652.
7. Maňásková P, Pěknicová J, Elzeinová F, Tichá M, Jonáková V. Origin, localization and binding abilities of boar DQH sperm surface protein tested by specific monoclonal antibodies. *J Reprod Immunol*. 2007;74:103–113.
8. Ekhlasi-Hundrieser M, Gohr K, Wagner A, Tsolova M, Petrunkina A, Töpfer-Petersen E. Spermadhesin AQN1 is a candidate receptor molecule involved in the formation of the oviductal sperm reservoir in the pig1. *Biol Reprod*. 2005;73:536–545.
9. Silva E, Frost D, Li L, Bovin N, Miller DJ. Lactadherin is a candidate oviduct Lewis X trisaccharide receptor on porcine spermatozoa. *Andrology*. 2017;5:589–597.
10. Teijeiro JM, Ignotz GG, Marini PE. Annexin A2 is involved in pig (*Sus scrofa*) sperm-oviduct interaction. *Mol Reprod Dev*. 2009;76:334–341.
11. Marini PE, Cabada MO. One step purification and biochemical characterization of a spermatozoa-binding protein from porcine oviductal epithelial cells. *Mol Reprod Dev*. 2003;66:383–390.
12. Teijeiro JM, Marini PE. S100A7 is present in human sperm and a homologous pig sperm protein interacts with sperm-binding glycoprotein (SBG). *Andrologia*. 2012;44:772–779.
13. Ambruosi B, Accogli G, Douet C, et al. Deleted in malignant brain tumor 1 is secreted in the oviduct and involved in the mechanism of fertilization in equine and porcine species. *Reproduction*. 2013;146:119–133.
14. Madsen J, Mollenhauer J, Holmskov U. Review: Gp-340/DMBT1 in mucosal innate immunity. *Innate Immun*. 2010;16:160–167.
15. Kang W, Reid KBM. DMBT1, a regulator of mucosal homeostasis through the linking of mucosal defense and regeneration? *FEBS Lett*. 2003;540:21–25.
16. Ligtenberg AJM, Veerman ECI, Nieuw Amerongen AV, Mollenhauer J. Salivary agglutinin/glycoprotein-340/DMBT1: a single molecule with variable composition and with different functions in infection, inflammation and cancer. *Biol Chem*. 2007;388:1275–1289.
17. Pérez FA, Roma SM, Cabada MO, Marini PE. Sperm binding glycoprotein is differentially present surrounding the lumen of isthmus and ampulla of the pig's oviduct. *Anat Embryol (Berl)*. 2006;211:619–624.
18. Roldán ML, Marini PE. First evidence of the interaction between deleted in malignant brain tumor 1 and galectin-3 in the mammalian oviduct. *Histochem Cell Biol*. 2014;141:181–190.
19. Soleilhavoup C, Riou C, Tsikis G, et al. Proteomes of the female genital tract during the oestrous cycle. *Mol Cell Proteomics*. 2016;15:93–108.
20. Patyka M, Malamud D, Weissman D, Abrams WR, Kurago Z. Periluminal distribution of HIV-binding target cells and Gp340 in

the oral, cervical and sigmoid/rectal mucosae: a mapping study. *PLoS ONE*. 2015;10:e0132942.
21. Tynan S, Pacia E, Haynes-Johnson D, et al. The putative tumor suppressor deleted in malignant brain tumors 1 is an estrogen-regulated gene in rodent and primate endometrial epithelium. *Endocrinology*. 2005;146:1066–1073.
22. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature*. 2000;406:782–787.
23. Ligtenberg AJM, Karlsson NG, Veerman ECI. Deleted in malignant brain tumors-1 protein (DMBT1): A pattern recognition receptor with multiple binding sites. *Int J Mol Sci*. 2010;11:5212–5233.
24. Renner M, Bergmann G, Krebs I, et al. DMBT1 confers mucosal protection in vivo and a deletion variant is associated with Crohn's disease. *Gastroenterology*. 2007;133:1499–1509.
25. Hedrick JL, Wardrip NJ. Isolation of the zona pellucida and purification of its glycoprotein families from pig oocytes. *Anal Biochem*. 1986;157:63–70.
26. Teijeiro JM, Marini PE. The effect of oviductal deleted in malignant brain tumor 1 over porcine sperm is mediated by a signal transduction pathway that involves pro-AKAP4 phosphorylation. *Reproduction*. 2012;143:773–785.
27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–254.
28. Caballero JN, Gervasi MG, Veiga MF, et al. Epithelial cadherin is present in bovine oviduct epithelial cells and gametes, and is involved in fertilization-related events. *Theriogenology*. 2014;81:1189–1206.
29. Althouse GC. Evaluating porcine semen for artificial insemination: standard tests. *Compendium on continuing education for the practicing veterinarian*. 1997;19:777–782.
30. Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. *Zygote*. 1994;2:371–372.
31. Chauhan JS, Rao A, Raghava GPS, Brunak S, Hansen J. In silico platform for prediction of N-, O- and C-glycosites in eukaryotic protein sequences. *PLoS ONE*. 2013;8:e67008.
32. Reichhardt MP, Holmskov U, Meri S. SALSA-A dance on a slippery floor with changing partners. *Mol Immunol*. 2017;89:100–110.
33. Tienthai P, Johannisson A, Rodriguez-Martinez H. Sperm capacitation in the porcine oviduct. *Anim Reprod Sci*. 2004;80:131–146.
34. Schulz BL, Oxley D, Packer NH, Karlsson NG. Identification of two highly sialylated human tear-fluid DMBT1 isoforms: the major high-molecular-mass glycoproteins in human tears. *Biochem J*. 2002;366:511–520.
35. Cheng H, Bjerknes M, Chen H. CRP-ductin: a gene expressed in intestinal crypts and in pancreatic and hepatic ducts. *Anat Rec*. 1996;244:327–343.
36. Haase B, Humphray SJ, Lyer S, et al. Molecular characterization of the porcine deleted in malignant brain tumors 1 gene (DMBT1). *Gene*. 2006;376:184–191.
37. Teijeiro JM, Roldán ML, Marini PE. Molecular identification of the sperm selection involved porcine sperm binding glycoprotein (SBG) as deleted in malignant brain tumors 1 (DMBT1). *Biochimie*. 2012;94:263–267.
38. Loimaranta V, Jakubovics NS, Hytonen J, Finne J, Jenkinson HF, Stromberg N. Fluid- or surface-phase human salivary scavenger protein gp340 exposes different bacterial recognition properties. *Infect Immun*. 2005;73:2245–2252.
39. Resnick D, Pearson A, Krieger M. The SRCR superfamily: a family reminiscent of the Ig superfamily. *Trends Biochem Sci*. 1994;19:5–8.
40. Ma H, Jiang L, Qiao S, et al. The crystal structure of the fifth scavenger receptor cysteine-rich domain of porcine CD163 reveals an important residue involved in porcine reproductive and respiratory syndrome virus infection. *J Virol*. 2017;91:e01897–e01816.
41. Bessa Pereira C, Bocková M, Santos RF, et al. The scavenger receptor SSc5D physically interacts with bacteria through the SRCR-containing N-terminal domain. *Front Immunol*. 2016;7:416.
42. Ligtenberg AJM, Bikker FJ, De Blieck-Hogervorst JMA, Veerman ECI, Nieuw Amerongen AV. Binding of salivary agglutinin to IgA. *Biochem J*. 2004;383:159–164.
43. Bikker FJ, Ligtenberg AJM, Nazmi K, et al. Identification of the bacteria-binding peptide domain on salivary agglutinin (gp-340/DMBT1), a member of the scavenger receptor cysteine-rich superfamily. *J Biol Chem*. 2002;277:32109–32115.
44. Gaboriaud C, Gregory-Pauron L, Teillet F, Thielens NM, Bally I, Arlaud GJ. Structure and properties of the Ca $^{2+}$-binding CUB domain, a widespread ligand-recognition unit involved in major biological functions. *Biochem J*. 2011;439:185–193.
45. Gupta SK. Role of zona pellucida glycoproteins during fertilization in humans. *J Reprod Immunol*. 2015;108:90–97.
46. Suarez SS. Mammalian sperm interactions with the female reproductive tract. *Cell Tissue Res*. 2016;363:185–194.

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