REPRODUCTION
RESEARCH

Deleted in malignant brain tumor 1 is secreted in the oviduct and involved in the mechanism of fertilization in equine and porcine species

Barbara Ambruosi[1,2,3,4], Gianluca Accogli[5], Cécile Douet[1,2,3,4], Sylvie Canepa[1,2,3,4], Géraldine Pascal[1,2,3,4], Philippe Monget[1,2,3,4], Carla Moros[6], Uffe Holmskov[7], Jan Mollenhauer[7,8], Catherine Robbe-Masselot[9,10,11], Olivier Vidal[9,10,11], Salvatore Desantis[5] and Ghylène Goudet[1,2,3,4]

[1]INRA, UMR 85, Physiologie de la Reproduction et des Comportements, 37380 Nouzilly, France, [2]CNRS, UMR 7247, 37380 Nouzilly, France, [3]Université François Rabelais, 37000 Tours, France, [4]IFCE, 37380 Nouzilly, France, [5]Veterinary Clinic and Animal Productions Unit, Department of Emergency and Organ Transplantation, University of Bari, 70010 Valenzano, Bari, Italy, [6]Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia, 30100 Murcia, Spain, [7]Institute for Molecular Medicine, Department of Cardiovascular and Renal Research, University of Southern Denmark, DK-5000 Odense, Denmark, [8]Lundbeckfonden Center of Excellence NanoCAN, University of Southern Denmark, DK-5000 Odense, Denmark, [9]Université Lille Nord de France, 59000 Lille, France, [10]USTL, UGSF, IFR 147, 59650 Villeneuve d'Ascq, France and [11]CNRS, UMR 8576, 59650 Villeneuve d'Ascq, France

Correspondence should be addressed to G Goudet at INRA, UMR 85, Physiologie de la Reproduction et des Comportements; Email: ghylene.goudet@tours.inra.fr

Abstract

Oviductal environment affects preparation of gametes for fertilization, fertilization itself, and subsequent embryonic development. The aim of this study was to evaluate the effect of oviductal fluid and the possible involvement of deleted in malignant brain tumor 1 (DMBT1) on IVF in porcine and equine species that represent divergent IVF models. We first performed IVF after pre-incubation of oocytes with or without oviductal fluid supplemented or not with antibodies directed against DMBT1. We showed that oviductal fluid induces an increase in the monospermic fertilization rate and that this effect is canceled by the addition of antibodies, in both porcine and equine species. Moreover, pre-incubation of oocytes with recombinant DMBT1 induces an increase in the monospermic fertilization rate in the pig, confirming an involvement of DMBT1 in the fertilization process. The presence of DMBT1 in the oviduct at different stages of the estrus cycle was shown by western blot and confirmed by immunohistochemical analysis of ampulla and isthmus regions. The presence of DMBT1 in cumulus–oocyte complexes was shown by western blot analysis, and the localization of DMBT1 in the zona pellucida and cytoplasm of equine and porcine oocytes was observed using immunofluorescence analysis and confocal microscopy. Moreover, we showed an interaction between DMBT1 and porcine spermatozoa using surface plasmon resonance studies. Finally, a bioinformatic and phylogenetic analysis allowed us to identify the DMBT1 protein as well as a DMBT1-like protein in several mammals. Our results strongly suggest an important role of DMBT1 in the process of fertilization.

Introduction

Deleted in malignant brain tumor 1 (*DMBT1*) was first identified as a candidate tumor-suppressor gene for brain tumors (Mollenhauer *et al*. 1997). It was then proposed as a candidate tumor suppressor gene for lung and gastrointestinal tumors caused by homozygous deletions or by a lack of expression (Mori *et al*. 1999, Wu *et al*. 1999, Mollenhauer *et al*. 2000). DMBT1 is involved in epithelial differentiation and mucosal innate immunity by binding to a large panel of pathogens including HIV (Mollenhauer *et al*. 2000, 2004, Ligtenberg *et al*. 2010, Madsen *et al*. 2010, Al-Awqati 2011).

The *DMBT1* gene, located on human chromosome 10q26.13, encodes a secreted high-molecular-weight glycoprotein of the scavenger receptor cysteine-rich (SRCR) group B protein family (Mollenhauer *et al*. 1997). The protein is composed of 14 SRCR domains separated by SRCR-interspersed domains, which are subjected to interindividual polymorphism, two CUB (Clr/Cls Uegf Bmp1) domains, and a zona pellucida (ZP) domain toward the C-terminus (Mollenhauer *et al*. 1999, 2002).

The *DMBT1* gene encodes orthologs in rabbit (hensin (Takito *et al*. 1999)), mouse (CRP-ductin (Cheng *et al*. 1996, Madsen *et al*. 2003) and vomeroglandin (Matsushita *et al*. 2000)), rat (ebnerin (Li & Snyder 1995)), bovine (bovine gallbladder mucin (Nunes *et al*. 1995, Mollenhauer *et al*. 2001)), human (gp-340 in lung (Holmskov *et al*. 1997, 1999) and salivary agglutinin in saliva (Prakobphol *et al*. 2000, Ligtenberg *et al*. 2001)), and porcine (sperm binding glycoprotein (SBG; Teijeiro *et al*. 2012)) species. The pig SBG has been shown to be present in the oviduct, at the apical surface of isthmic and ampullar epithelial cells (Perez *et al*. 2006), to bind to the heads of pig sperm (Marini & Cabada 2003) and to be involved in sperm selection in the oviduct (Teijeiro *et al*. 2008).

The oviduct is an essential organ in reproductive biology, as it supports gamete transport, maturation, capacitation, fertilization, early embryonic growth, and embryo transport to the uterus. Its role is of particular importance during the process of fertilization. Oviduct epithelial cells co-culture promotes *in vitro* production of embryos in human (Bongso *et al*. 1991, Kervancioglu *et al*. 1997), bovine (Chian & Sirard 1995, Way *et al*. 1997, Martus *et al*. 1998), porcine (Romar *et al*. 2001, McCauley *et al*. 2003), deer (Locatelli *et al*. 2005), and dromedary (Khatir *et al*. 2004) species. Moreover, some oviductal proteins have been shown to interact with gametes and to improve efficiency of IVF in porcine (McCauley *et al*. 2003, Coy *et al*. 2008), bovine (Martus *et al*. 1998), human (O'Day-Bowman *et al*. 1996), and equine (Mugnier *et al*. 2009) species. Some of these proteins, such as osteopontin, have been identified (Hao *et al*. 2006, Goncalves *et al*. 2008, Monaco *et al*. 2009) in cattle and pigs and atrial natriuretic peptide A (Anderson *et al*. 1994, Zamir *et al*. 1995, Zhang *et al*. 2006) and oviductin (oviduct-specific glycoprotein; Martus *et al*. 1998, Buhi 2002, McCauley *et al*. 2003) in cattle, pigs, and humans.

Our aim was to evaluate a putative role of DMBT1 in the mechanisms of fertilization, particularly in the interaction between oocyte and spermatozoa. For this purpose, we used two models in which the oviductal secretions are of particular importance for fertilization: the porcine and equine species. In porcine species, IVF rates are higher than 80%, and the reported levels of polyspermy after IVF often exceed 50% (Abeydeera & Day 1997, Funahashi & Day 1997, Day 2000, Nagai *et al*. 2006). The exposure of oocytes to porcine oviductal epithelial cells or oviductal fluid before IVF improves the rate of monospermy (Romar *et al*. 2001, Coy *et al*. 2008). In equine species, IVF rates are low and no repeatable IVF technique is available yet (Palmer *et al*. 1991, Dell'aquila *et al*. 1996, Alm *et al*. 2001, Hinrichs *et al*. 2002, McPartlin *et al*. 2009). We have shown a beneficial effect of homologous and heterologous oviduct cells on equine IVF rates (Mugnier *et al*. 2009). Thus, we studied the role of DMBT1 in gamete interaction during equine and porcine fertilization.

Materials and methods

All procedures on animals were conducted in accordance with the guidelines for the care and use of laboratory animals issued by the French Ministry of Agriculture and with the approval of the ethical review committee (Comité d'Ethique en Expérimentation Animale Val de Loire) under the number 2011/6. All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

Collection and preparation of porcine oviduct fluid

Collection of oviducts

Genital tracts from gilts and sows were obtained from a commercial abattoir and transported to the laboratory at room temperature. The cyclic stage of females was assessed on the basis of ovarian morphology on both ovaries from the same female. Oviducts were classified as early follicular, mid follicular, late follicular, or luteal phase, according to the diameter of follicles and the presence of corpora lutea, as described previously (Carrasco *et al*. 2008). Porcine oviducts at the preovulatory or post-ovulatory stage were collected from slaughtered Meishan gilts from our experimental pigsty (Unité Expérimentale de Physiologie Animale de l'Orfrasière, UEPAO, Nouzilly, France). Adult cyclic gilts received a daily dose of 5 ml Regumate (20 mg/gilt per day of altrenogest, Intervet S.A., Angers, France) *per os* for 18 days. An i.v. injection of human chorionic gonadotropin (hCG, Chorulon, 500 IU/gilt, Intervet S.A.) was performed 3 days later. Gilts were killed 36 h after hCG injection (4 h before ovulation) or 46 h after hCG injection (6 h after ovulation). Equine oviducts were collected from mares slaughtered in a commercial abattoir; the cyclic stage of females was unknown. For both species, the two oviducts were immediately collected and transported to the laboratory within a few minutes at room temperature.

Collection of oviductal fluid

The oviducts were dissected free from surrounding tissues, transferred to Petri dishes on ice, and washed in Medium 199 with Earles Salts, 25 mM HEPES, and $NaHCO_3$ supplemented with 80 mg/ml gentamicin. The oviductal fluid from the ampulla was expelled by gentle squeezing using a sterile microscope slide. It was collected by aspiration with an automatic pipette by introducing the tip into the ampulla and making a manual ascendant pressure from the isthmus to the ampulla (Carrasco *et al*. 2008). After centrifugation at 10 000 *g* for 15 min, the supernatant containing secreted and intracellular components was immediately stored at K20 °C until use as 'oviductal fluid'.

Collection and in vitro *maturation of porcine and equine oocytes*

Equine and porcine immature cumulus–oocyte complexes (COCs) were collected from slaughtered mares and gilts. Ovaries were obtained from local slaughterhouses immediately after females were killed and transported to the laboratory within 2 h in 0.9% (w/v) NaCl at 32–38 °C. COCs were

collected by aspiration as described previously (Goudet *et al*. 2000) and recovered under a stereomicroscope.

For *in vitro* maturation, porcine immature COCs were washed in Dulbecco's PBS solution (DPBS, Dulbecco A, Paris, France) supplemented with 25 mg/ml gentamicin and then in maturation medium. The maturation medium was Medium 199 with Earle's salts supplemented with 10 ng/ml epidermal growth factor (EGF), 400 ng/ml FSH (PRIMUFOL, Rhône Mérieux, Lyon, France), and 570 mM cysteamine (Marchal *et al*. 2003). Porcine COCs were then cultured in a group of 30–40 for 44 h in 500 ml maturation medium in an atmosphere of 5% CO_2 in air at 39 °C in 100% humidity.

For *in vitro* maturation, equine immature COCs were washed once in Medium 199 with Earle's Salts, 25 mM HEPES, and $NaHCO_3$ supplemented with 20% (v/v) FCS and 25 mg/ml gentamicin and then once in maturation medium. The maturation medium was Medium 199 with Earle's salts supplemented with 20% (v/v) FCS and 50 ng/ml EGF (Goudet *et al*. 2000). Equine COCs were then cultured in a group of 20–30 for 26–30 h in 500 ml maturation medium in an atmosphere of 5% CO_2 in air at 38.5 °C in 100% humidity.

After *in vitro* maturation, porcine and equine COCs were partially denuded by flushing in maturation medium and processed for pre-incubation as described below. Some porcine and equine COCs were immediately processed for assessment of nuclear status as described below.

Preparation of equine and porcine spermatozoa

Frozen porcine spermatozoa ($800X10^6$/ml) from one ejaculate of three 'PietrainXLarge-White' boars were thawed at 37 °C for 20 s and washed by centrifugation at 100 *g* for 10 min in Beltsville-Thawing-Solution (BTS; COBIPORC, Saint-Gilles, France). Motile spermatozoa were obtained by centrifugation of the pellet at 700 *g* for 30 min on a discontinuous gradient of Percoll (Amersham Pharmacia Biotech; 2 ml 45% in BTS over 2 ml 90%). The 90% Percoll solution contained 80 mM NaCl, 10 mM HEPES, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.4 mM $MgCl_2$, 2 mM $CaCl_2$, and 21.6 mM lactic acid (Eid *et al*. 1994, Parrish *et al*. 1995). Sperm cells collected at the bottom of the 90% fraction were washed by centrifugation at 100 *g* for 10 min in 4 ml modified Tris-buffered medium (mTBM; Marchal *et al*. 2003). This medium contained 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 20 mM Tris (MP Biomedicals, Illkirch, France), 11 mM glucose, 5 mM sodium pyruvate, 1 mM caffeine, and 0.1% BSA. The concentration of the pelleted spermatozoa was determined by counting on a Thoma chamber under a microscope (Olympus, IMT-2, Paris, France) and the spermatozoa were diluted at $10X10^6$/ml in mTBM. The motility was visually evaluated under a microscope (Olympus, IMT-2).

Fresh equine semen was collected with a closed artificial vagina from two adult Welsh pony stallions of proven fertility from our experimental study (UEPAO). Semen was filtered through gauze, sperm motility was visually evaluated under light microscopy on a heated stage, and sperm concentration was assessed using a spectrophotometer (Ciba-Geigy, Basel, Switzerland). Then, 1 ml semen was diluted in 2 ml pre-warmed non-capacitating modified Whittens medium (MW; 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose (anhydrous), 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt, and 1 mM pyruvic acid, pH 7.25; McPartlin *et al*. 2009). Diluted sperm was transported to the laboratory (5 min) at 37 °C and centrifuged in 15 ml conical tubes at 100 *g* for 1 min at 37 °C to remove particulate matter and dead spermatozoa. The supernatant was then transferred to a 14 ml round-bottomed tube and centrifuged at 600 *g* for 5 min at 37 °C. The pellet was resuspended in 1.5 ml non-capacitating pre-warmed MW, and the concentration was determined by counting on a Thoma chamber under a microscope (Olympus, IMT-2). Spermatozoa were then diluted at $10X10^6$/ml in capacitating pre-warmed MW, which was a non-capacitating MW supplemented with 25 mM $NaHCO_3$ and 7 mg/ml BSA, pH 7.25 (McPartlin *et al*. 2009). Spermatozoa were incubated in 500 ml aliquots in polyvinyl alcohol-coated 5 ml round-bottomed tubes at 37 °C in a humidified atmosphere for 6 h. The motility was visually evaluated under a microscope (Olympus, IMT-2) at the beginning and at the end of the incubation period. Spermatozoa were then diluted at $1X10^6$/ml in capacitating MW supplemented with 5 mM procaine to induce hyperactivation. Droplets of 100 ml spermatozoa suspension were laid down onto culture dishes and covered with mineral oil.

Pre-incubation of porcine and equine oocytes

Pre-incubation with oviductal fluid collected at the post-ovulatory stage

Porcine oocytes were washed in mTBM. They were pre-incubated in droplets of 50 ml mTBM or post-ovulatory oviductal fluid supplemented with 0, 1, or 2 mg/ml anti-gp340 antibodies for 30 min in an atmosphere of 5% CO_2 in air at 39 °C in 100% humidity. Pre-incubation with post-ovulatory oviductal fluid supplemented with preimmune serum was used as a negative control. The anti-gp340 antibody is directed against gp340, the human ortholog of DMBT1, and was kindly provided by Prof. U Holmskov from the University of Odense, Denmark (Holmskov *et al*. 1997). Its specificity was confirmed by Holmskov *et al*. (1997). The preimmune serum was provided by Prof. U Holmskov.

Equine oocytes were washed in capacitating MW. They were pre-incubated in droplets of 50 ml capacitating MW or post-ovulatory oviductal fluid supplemented with 0 or 2 mg/ml of anti-gp340 antibodies for 30 min in an atmosphere of 5% CO_2 in air at 38.5 °C in 100% humidity. Pre-incubation with post-ovulatory oviductal fluid supplemented with preimmune serum was used as a negative control.

Pre-incubation with human recombinant DMBT1

Porcine oocytes were washed in mTBM. They were pre-incubated in droplets of 50 ml mTBM supplemented with 0, 0.01, 0.1, 1, 10, or 100 mg/ml human recombinant DMBT1 (hrDMBT1) for 30 min in an atmosphere of 5% CO_2 in air at 39 °C in 100% humidity. The hrDMBT1 was kindly provided by Prof. C Robbe-Masselot from the University of Lille, France (End *et al*. 2005, Rossez *et al*. 2011). Owing to the low availability of equine ovaries in abattoirs and therefore of

equine oocytes for experiments, the effect of pre-incubation of equine oocytes with hrDMBT1 was not tested.

IVF of porcine and equine oocytes

After pre-incubation, porcine oocytes were washed in mTBM and groups of 30–40 were transferred to droplets of 100 µl mTBM. Then, 10 µl spermatozoa diluted at $10X10^6$/ml in mTBM were added to the oocytes and co-incubated for 24 h in an atmosphere of 5% CO_2 in air at 39 °C in 100% humidity. After pre-incubation, equine oocytes were washed in capacitating MW and groups of ten were transferred to droplets of 100 µl spermatozoa suspension diluted at $1X10^6$/ml and co-incubated for 24 h in an atmosphere of 5% CO_2 in air at 38.5 °C in 100% humidity. To evaluate the activation rate, some equine oocytes were washed in capacitating MW and transferred to droplets of 100 µl capacitating MW supplemented with 5 mM procaine without spermatozoa and incubated for 24 h in an atmosphere of 5% CO_2 in air at 38.5 °C in 100% humidity.

Assessment of nuclear status

After gamete co-incubation, oocytes were washed by aspiration in and out of a pipette in DPBS and fixed in 4% paraformaldehyde in DPBS for 1 h at room temperature. After washing in DPBS, oocytes were stained with 2.5 µM bisbenzimide fluorescent dye (Hoechst 33342) in DPBS:glycerol (1:3 v/v) and mounted on microscope slides. The slides were kept at 4 °C in darkness until observation. Oocytes were observed under an inverted epifluorescence microscope with a 365 nm excitation filter to assess the nuclear status.

Fertilization was defined by the presence of at least two pronuclei in the cytoplasm. Oocytes containing two pronuclei were considered monospermic, and oocytes containing more than two pronuclei were considered polyspermic. Oocytes with one pronucleus without sperm cells were considered activated oocytes. Oocytes with metaphase II and one polar body were considered mature non-fertilized oocytes. The fertilization rate was calculated as the percentage of oocytes containing at least two pronuclei (fertilized oocytes) with respect to the number of mature, activated, and fertilized oocytes. The monospermy rate was calculated as the percentage of oocytes containing two pronuclei with respect to the number of fertilized oocytes.

Gel electrophoresis and immunoblotting

In order to analyze the proteins in the equine and porcine oviducts, the protein concentration in oviductal fluids was evaluated using the CooAssay Protein Determination Small Kit (Interchim, Montluçon, France). Oviductal fluids were diluted in electrophoresis Laemmli buffer (0.062 mol/l Tris-HCl, pH 6.8, 5% (v/v) glycerol, 1% (w/v) SDS, 0.5% (w/v) bromophenol blue and 2% (v/v) 2-b-mercaptoethanol at final concentration) and boiled at 95 °C for 3 min.

In order to analyze the proteins in the porcine COCs, 200 COCs were diluted in a lysis buffer (b-glycerophosphate 80 mM, EDTA 20 mM, $MgCl_2$ 15 mM, HEPES 20 mM, and dithiothreitol 1 mM) supplemented with 0.2% protease inhibitors cocktail. They were frozen in liquid nitrogen and thawed at 25 °C three times, diluted in electrophoresis Laemmli buffer, and boiled at 95 °C for 3 min.

Proteins were separated using 6% SDS-PAGE. Acrylamide/bis-acrylamide (37.5-1) solution was purchased from Interchim. For each lane, 50 µg total proteins from oviductal fluid, or 50 µg hrDMBT1, or a volume equivalent to 5–100 COCs were loaded on the gel. The proteins were then transferred onto a PVDF membrane (hybond-P PVDF membrane transfer; Amersham Pharmacia Biotech) for 90 min at 4 °C.

The membrane was washed with TBS (1.21 g/l Tris base and 9 g/l NaCl, pH 7.4) containing 0.1% (v/v) Tween 20 (TBS-T), incubated overnight in blocking solution (5% (w/v) non-fat dry milk, 0.2% (v/v) IGEPAL, pH 7.4, in TBS), and incubated for 4 h with the primary antibodies (anti-gp340 antibodies) diluted 1/2000 in TBS-T. The membrane was washed twice with TBS-T, incubated for 1 h in blocking solution, incubated for 1 h with peroxidase-conjugated secondary antibodies (donkey anti-rabbit IgG, GE Healthcare, VWR, Strasbourg, France) diluted 1/2000 in blocking solution, and washed three times with TBS-T. The peroxidase activity was revealed using the ECL-Plus Western blotting detection system (GE Healthcare, VWR).

Light microscopy and immunohistochemistry on oviduct sections

After collection of the pig oviducts, tissue fragments ($1X0.5$ cm) were cut from the ampulla and the isthmus regions. The tissue fragments were fixed overnight in 4% (w/v) phosphate-buffered paraformaldehyde at 4 °C. After fixation, the tissues were washed and dehydrated in ethanol series, cleared in xylene, and embedded in paraffin wax. Then, 4 µm-thick sections were cut, submitted to de-waxing with xylene and hydration in an ethanol series of descending concentration, and stained with Mayer's hematoxylin and eosin (to study the general morphology) or processed for immunohistochemistry as follows.

De-waxed and rehydrated tissue sections were incubated for 30 min in a solution of 0.3% (v/v) H_2O_2 in methanol to inhibit endogenous peroxidase activity. They were rinsed with PBS-1% (w/v) BSA, and non-specific binding sites for immunoglobulins were blocked by 5% (v/v) normal goat serum (NGS) in PBS-BSA for 30 min. Sections were incubated for 2 h at 37 °C in a moist chamber with a 1:250 dilution of primary antibody anti-gp340 and then incubated for 30 min with diluted 1:50 biotinylated universal secondary antibody of Vectastain Elite ABC kit (Universal; Vector, Burlingame, CA, USA). After washing for 15 min in PBS-BSA, immunohistochemical visualization was obtained using the Vecta-lab 'Elite' (ABC) kit (Vector). Peroxidase activity was visualized by incubating with 0.01% H_2O_2 and 0.05% diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer, pH 7.2, for 5 min to reveal the brown immunoreactive cells. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted.

To confirm the specificity of the immunoreaction, the following control procedures were performed: i) replacement of primary antibody with NGS and ii) omission of the primary

antibody incubation step. These procedures yielded no immunostaining (Fig. 1B1 and C1).

Immunocytochemical staining and confocal laser scanning microscopy

Immature oocytes, *in vitro* matured oocytes, and *in vitro* matured oocytes pre-incubated with oviductal fluid were partially denuded by flushing in DPBS. They were fixed in 4% (w/v) paraformaldehyde in DPBS for 20 min at 37 °C, washed in DPBS, and kept at 4 °C in DPBS containing 0.05% (v/v) sodium azide (NaN_3) and 1 mM phenylmethylsulphonyl fluoride. Nonspecific reactions were blocked by pre-incubation of oocytes for 2 h at room temperature in DPBS containing 2% (w/v) BSA (A-7030). Oocytes were incubated overnight at 4 °C with anti-gp340 antibodies diluted 1:200 in DPBS containing 2% BSA, washed in DPBS containing 0.2% BSA, incubated for 2 h at 4 °C with AlexaFluor 488-conjugated-anti-rabbit antibodies diluted 1:800 in DPBS containing 2% BSA, and washed in DPBS containing 0.2% BSA. Oocytes were then stained with 1 mg/ml bisbenzimide (Hoechst 33342) in DPBS containing 0.2% BSA for 5 min, washed in DPBS three times, and mounted on microscope slides in Mowiol V4-88 (133 mg/ml; Hoechst, Frankfurt, Germany) and *n*-propyl gallate (5 mg/ml). The slides were kept at 4 °C in darkness until observation. Oocytes were observed under a confocal laser scanning microscope (LSM 700, Zeiss, Jena, Germany) with diodes of 488 and 405 nm wavelength. Controls were performed using no primary antibodies to ascertain the absence of non-specific binding or no secondary antibodies to ascertain the absence of auto-fluorescence.

Surface plasmon resonance

Preparation of spermatozoa

Frozen porcine spermatozoa (12 straws, 800×10^6/ml per straw) from one ejaculate of three 'PietrainXLarge-White'

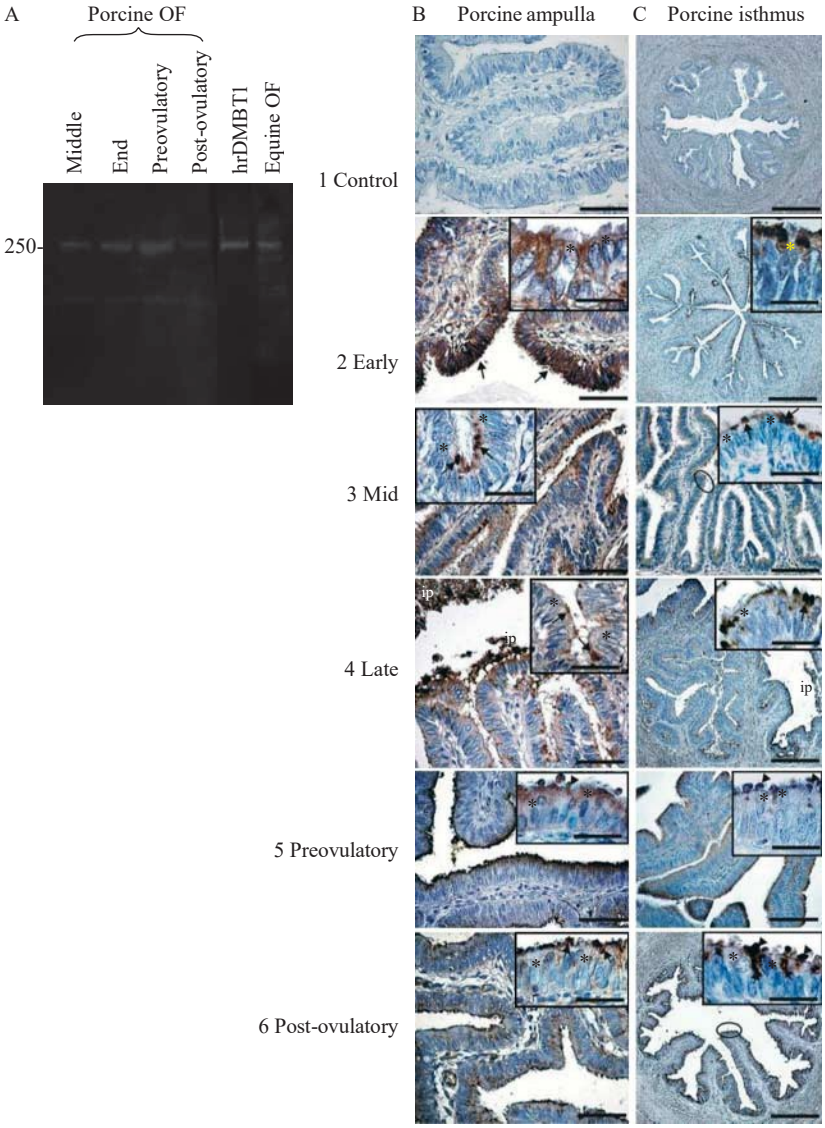

Figure 1 DMBT1 expression (A) in porcine and equine oviductal fluid (OF), hrDMBT1 was used as a positive control; DMBT1 localization in porcine ampulla (B2, B3, B4, B5 and B6) and porcine isthmus (C2, C3, C4, C5 and C6) sections collected at early follicular phase, mid follicular phase, late follicular phase, preovulatory stage (4 h before ovulation), or post-ovulatory stage (6 h after ovulation); B1 and C1 show the negative control stainings; ip, intraluminal immunoreactivity; arrow, apical region of non-ciliated cells; arrow head, apical blebs; *, ciliated cells. Circles in C3 and C6 indicate the areas showed at high magnification in the insets. Scale bars: B1–50 mm; B2–60 mm; B3–60 mm; B4–55 mm; B5–60 mm; B6–55 mm; insets in B2–30 mm; B3, B4, B5 and B6–40 mm; C1–600 mm; C2–160 mm; C3 and C4–130 mm; C5–100 mm; C6–500 mm; insets in C2–35 mm; C3, C4 and C5–40 mm; C6–30 mm.

boars were thawed at 37 °C for 20 s, diluted in HBS (10 mM HEPES and 150 mM NaCl, pH 7.4), and centrifuged at 100 *g* for 10 min at 20 °C. Resulting pellets were pulled and resuspended in 10 ml HBS and centrifuged at 100 *g* for 10 min. The pellet was resuspended in 10 ml HBS and subjected to nitrogen cavitation (10 min, 45 bar) in a cell disruption bomb (Flesch *et al*. 1998). The cavitate was slowly extruded into a tube containing 100 µl protease inhibitors (Fermentas, Saint Remy les Chevreuses, France) and 100 µl EDTA 0.05 M and centrifuged at 1000 *g* for 10 min at 20 °C. The supernatant was centrifuged at 6000 *g* for 10 min at 20 °C, and the resulting supernatant was centrifuged at 110 000 *g* for 3 h 30 min at 4 °C. The pellet, containing sperm plasma membranes, was resuspended in HBS supplemented with 1% protease inhibitors and 0.05 M EDTA and the supernatant was kept as a negative control. The protein content was evaluated with the BCA protein kit (Interchim) and samples were stored at −80 °C. The quality of the preparation of sperm plasma membranes was evaluated using transmission electron microscopy.

Surface plasmon resonance

Surface plasmon resonance experiments were performed on BIAcore T100 (GE Healthcare). The anti-gp340 antibodies were immobilized at a level of 1350 resonance units on two flow cells (FC) of a CM3 sensor chip (GE Healthcare), using standard amine coupling according to the manufacturer's instructions. One of the two FC was the test FC while the other was the control FC. Binding analyses were carried out at 25 °C. The hrDMBT1 was diluted at 6 µM in the running buffer (DPBS complemented with 0.9 mM calcium chloride and 0.5 mM magnesium chloride) and injected for 600 s at a flow rate of 5 µl/min on the test FC. Porcine sperm plasma membrane preparations were sonicated on ice, filtered on Durapore filters (0.2 µm), diluted at 20 µg/ml proteins in the running buffer, and injected for 600 s at a flow rate of 5 µl/min on the test FC and the control FC. The supernatant of the sperm plasma membrane preparations as well as a BSA solution (20 µg/ml) was injected under the same conditions as negative controls. Dissociation was studied over 600 s. Regeneration of the surfaces was performed at a flow rate of 30 µl/min with two subsequent injections for 60 s of a solution containing 100 mM HEPES, 1.5 M NaCl, and 0.5% (v/v) Tween 20 followed by an injection of a solution of 20 mM NaOH, 1 M NaCl for 60 s, an injection of a solution of 50 mM NaOH, 1 M NaCl for 60 s, and a stabilization for 120 s.

Identification and phylogeny of DMBT1 proteins in mammals

To broaden the study to other mammals, we searched for the presence of DMBT1 proteins for human, chimpanzee, mouse, rat, bovine, horse, pig, and dog species in UniProtKB databases (http://www.uniprot.org/). UniProtKB consists of two sections: Swiss-Prot, which is manually annotated and reviewed, and TrEMBL, which is automatically annotated and is not reviewed. The proteins that are manually annotated and reviewed were included directly. The proteins that are automatically annotated and not reviewed were compared with the previous ones using a Basic Local Alignment Search Tool (BLAST; Altschul *et al*. 1997) program to discard redundant data.

To identify other eventual DMBT1 proteins, we submitted for each species the amino acid sequences of DMBT1 to the tblastn Software against the genome. Tblastn takes a protein query sequence and compares it against a nucleotide database, which has been translated in all six reading frames. When we observed an alignment, the localization of the sequences on the chromosomes was visualized using MapViewer (http://www.ncbi.nlm.nih.gov/mapview/) and the amino acid sequence was extracted from NCBI (http://www.ncbi.nlm.nih.gov/protein) or Ensembl databases (http://www.ensembl.org) (Flicek *et al*. 2012).

To make sure that DMBT1 and DMBT1-like proteins are two distinctive proteins, alignment of DMBT1 and DMBT1-like proteins for each species was performed. This was done using the Needle Software that builds Needleman-Wunsch global alignments of two sequences (http://mobyle.pasteur.fr; Neron *et al*. 2009).

Phylogenetic analyses were carried out using http://www.phylogeny.fr. Multiple sequence alignments were performed using the MUSCLE algorithm. Curation was performed using Gblocks with non-stringent selection parameters. Phylogenetic trees were reconstructed using BioNJ (neighbor joining) and PhyML (maximum of likelihood) methods. Bootstrap values were estimated with 100 replications and the tree was rooted on the opossum DMBT1 protein (ENSMODP00000010153).

Statistical analysis

The fertilization and monospermy rates were compared among groups using c^2 analysis. Differences were considered statistically significant at $P \leq 0.05$.

Results

Pre-incubation of equine and porcine oocytes with oviductal fluid collected at the post-ovulatory stage

In this experiment, the *in vitro* maturation rate of porcine oocytes was 74% (of the 498 oocytes cultured *in vitro*, 368 were mature). The fertilization rate of porcine oocytes pre-incubated with oviductal fluid (70%, 64/92) was similar to that of the control group (65%, 57/87) (Fig. 2A). In contrast, the monospermic fertilization rate was significantly higher in the oviductal fluid group (77%, 49/64) compared with the control group (53%, 30/57; $P \leq 0.01$) (Fig. 2B). The addition of anti-gp340 antibodies during porcine oocyte pre-incubation with oviductal fluid showed a significant decrease in the monospermic fertilization rate (50%, 33/66 with 1 mg/ml anti-gp340; 41%, 23/56 with 2 mg/ml anti-gp340) compared with oviductal fluid (77%, 49/64; $P \leq 0.01$), canceling the positive effect of oviductal fluid (Fig. 2B). To make sure that the effect observed with anti-gp340 antibodies was specifically due to the antibodies, oocytes were pre-incubated with oviductal fluid

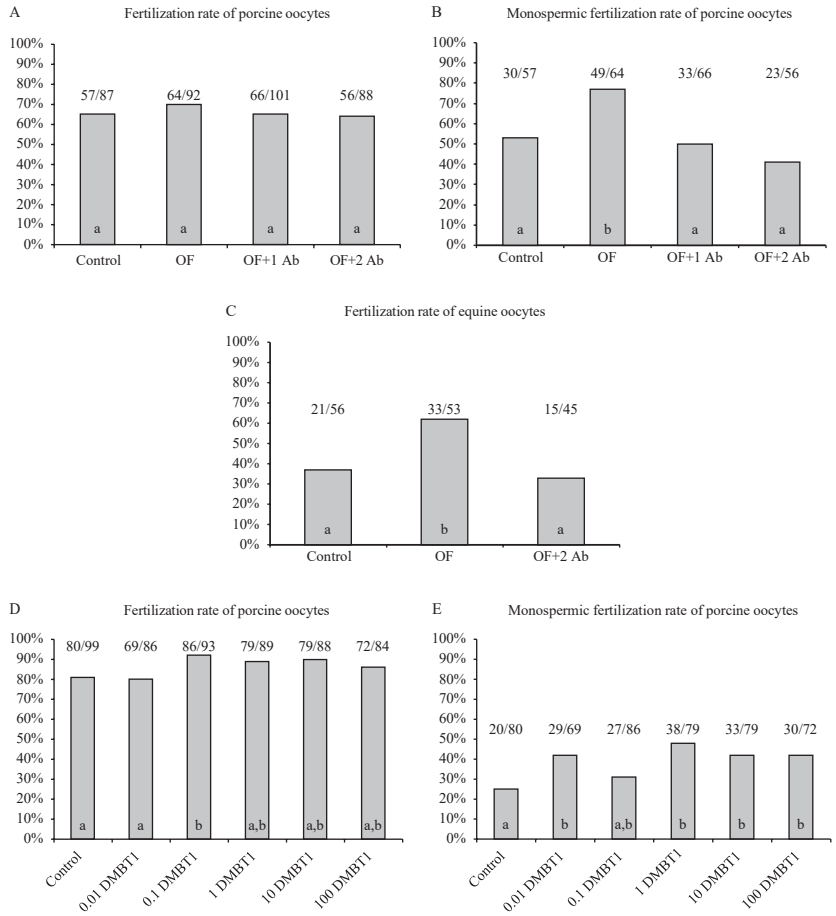

Figure 2 Fertilization rate (A and C) and monospermic fertilization rate (B) of porcine (A and B) and equine (C) oocytes pre-incubated in the control medium or in post-ovulatory oviductal fluid supplemented with 0 mg/ml (OF), 1 mg/ml (OFC1 Ab), or 2 mg/ml (OFC2 Ab) anti-gp340 antibodies. The porcine monospermic fertilization rate was significantly higher in the oviductal fluid group compared with the control group, the addition of anti-gp340 antibodies showed a significant decrease in the monospermic fertilization rate compared with oviductal fluid. The equine fertilization rate was significantly higher in the oviductal fluid group compared with the control group, and the addition of anti-gp340 antibodies showed a significant decrease in the fertilization rate compared with the oviductal fluid. The porcine and equine fertilization rate and the porcine monospermic fertilization rate were not different between oocytes pre-incubated in oviductal fluid and oocytes pre-incubated in oviductal fluid supplemented with preimmune serum (data not shown). Fertilization rate (D) and monospermic fertilization rate (E) of porcine oocytes pre-incubated in the control medium supplemented with 0, 0.01, 0.1, 1, 10, or 100 mg/ml human recombinant DMBT1. The addition of hrDMBT1 significantly increased the fertilization rate (0.1 mg/ml hrDMBT1) and the monospermic fertilization rate (0.01, 1, 10, or 100 mg/ml). The fractions represent the number of fertilized oocytes out of the number of mature, activated, and fertilized oocytes in (A, C and D), and the number of monospermic oocytes out of the number of fertilized oocytes in (B) and (E). Statistical differences are indicated by different letters ($P < 0.05$).

supplemented with preimmune serum. Their fertilization rate and their monospermic fertilization rate were similar to those for oocytes pre-incubated with oviductal fluid.

The *in vitro* maturation rate of equine oocytes was 61% (of the 254 oocytes cultured *in vitro*, 154 were mature). Equine oocytes pre-incubated with oviductal fluid showed a significant increase in the fertilization rate compared with that of the control group (62%, 33/53 vs 37%, 21/56 for oviductal fluid and control groups respectively; $P < 0.01$; Fig. 2C). The addition of 2 mg/ml anti-gp340 antibodies during equine oocyte pre-incubation with oviductal fluid showed a significant decrease in fertilization rates (33%, 15/45 for oviductal fluid and 2 mg/ml anti-gp340 vs 62%, 33/53 for oviductal fluid; $P < 0.01$), canceling the positive effect of oviductal fluid (Fig. 2C). To make sure that the effect observed with anti-gp340 antibodies was specifically due to the antibodies, oocytes were pre-incubated with oviductal fluid supplemented with preimmune serum. Their fertilization rate was not different from those for oocytes pre-incubated with oviductal fluid. All equine fertilized oocytes contained two pronuclei and no polyspermy was observed. When equine oocytes were incubated for 24 h in droplets of 100 ml capacitating MW supplemented with 5 mM procaine without spermatozoa, no activation was observed.

Pre-incubation of porcine oocytes with hrDMBT1

To make sure that DMBT1 was really involved in the effect observed earlier, oocytes were pre-incubated with the recombinant molecule. In this experiment, the *in vitro* maturation rate of porcine oocytes was 81% (of the 664 oocytes cultured *in vitro*, 539 were mature). The fertilization rate of porcine oocytes pre-incubated with 0.1 mg/ml of hrDMBT1 (92%, 86/93) was significantly higher than the control group (81%, 80/99; $P \leq 0.02$) (Fig. 2D). The monospermic fertilization rate was significantly higher for porcine oocytes pre-incubated with 0.01 mg/ml (42%, 29/69), 1 mg/ml (48%, 38/79), 10 mg/ml (42%, 33/79), or 100 mg/ml (42%, 30/72) DMBT1 compared with the control group (25%, 20/80; $P \leq 0.003$) (Fig. 2E).

Presence of DMBT1 in the equine and porcine oviduct and localization in the porcine oviduct

We analyzed the presence of DMBT1 using gel electrophoresis and immunoblotting in equine oviductal fluids collected at an unknown stage of the estrus cycle and in porcine oviductal fluids collected in the ampulla from females killed in the middle of follicular phase, at the end of follicular phase, at the preovulatory stage (4 h before ovulation), or at the post-ovulatory stage (6 h after ovulation). The antibody raised against DMBT1 revealed a band in equine and porcine samples, and in the four stages of the estrus cycle (Fig. 1A). The bands were close to 340 kDa, at the level of the band obtained for hrDMBT1 (Fig. 1A).

We analyzed the localization of DMBT1 using immunohistochemistry and light microscopy on porcine oviduct sections. The data obtained on the ampulla sections are presented in Fig. 1B. The DMBT1 antiserum immunoreacted with cells of the epithelium lining the ampulla mucosal folds. Staining was observed mainly in non-ciliated cells, but also in other cell types. The immunostaining pattern was different in the different estrous cycle stages. At the beginning of the follicular phase, epithelial cells of the upper zone of some mucosal folds expressed immunoreactivity in the entire cytoplasm (Fig. 1B2). In the middle phase, DMBT1 was expressed throughout the lining epithelium in the apical region of the epithelial cells (Fig. 1B3). These cells displayed immunoreactivity from the supra-nuclear region to luminal surface during the final follicular phase. Immunopositive material was also present in the luminal microenvironment (Fig. 1B4). In the preovulatory phase, the epithelial cells throughout the lining epithelium showed immunostaining from supra-nuclear region to the apical region from where immunopositive apical blebs detached and fell into the lumen (Fig. 1B5). Finally, 6 h post-ovulation, the immunostaining was still present in the entire supra-nuclear region of epithelial cells but no immunoreactive apical blebs were found (Fig. 1B6).

The data obtained on the isthmus sections are presented in Fig. 1C. In the epithelium lining the mucosal folds of the isthmus, the antiserum immunoreacted mainly with the non-ciliated cells, as well as other cell types. At the beginning of follicular phase, DMBT1 was not expressed in the epithelial cells of the base of mucosal trabeculae (Fig. 1C2). The immunopositive epithelial cells showed staining mainly at the apical region (inset of Fig. 1C2). In the middle and final stages, DMBT1 was expressed in the apical region of epithelial cells, constituting also the base of mucosal trabeculae (Fig. 1C3 and C4). Particularly, in the final follicular phase, the epithelial cells secreted immunopositive substance, which accumulated in the lumen (Fig. 1C4). In the preovulatory phase, immunopositive blebs detached from the apical region of immunoreactive epithelial cells along the entire isthmus (Fig. 1C5), whereas after 6 h post-ovulation, a decrease in the number of immunopositive epithelial cells occurred (Fig. 1C6). The negative control stainings yielded no immunostaining (Fig. 1B1 and C1).

Presence and localization of DMBT1 in the COCs

In order to check for an interaction of DMBT1 on the COCs, porcine and equine oocytes were pre-incubated with oviductal fluid and processed for immunocytochemical staining using anti-gp340 antibodies and confocal laser scanning microscopy. In the pig, staining of both the ZP and the cytoplasm was observed for immature oocytes (with a germinal vesicle), *in vitro* matured oocytes (with a metaphase II and a polar body), and *in vitro* matured oocytes pre-incubated with oviductal fluid (Fig. 3A). Staining was also observed for cumulus cells whatever the stage of the oocyte (Fig. 3A). No labeling was observed in the absence of primary antibodies regardless of the group (Fig. 3A). No auto-fluorescence was observed in the absence of secondary antibodies. In the horse, as in the pig, staining of both the ZP and the cytoplasm was observed for immature and *in vitro* matured oocytes pre-incubated or not with oviductal fluid, as well as cumulus cells (data not shown). No labeling was observed in the absence of primary or secondary antibodies.

To confirm the presence of DMBT1 in the COCs, gel electrophoresis and immunoblotting were performed on porcine COCs collected from the ovaries of females killed in a commercial abattoir. In the lanes where 50 and 100 COCs have been loaded, the anti-gp340 antibodies revealed a band close to 340 kDa (Fig. 3B). The low availability of equine ovaries in the abattoirs did not allow us to collect enough equine COCs for gel electrophoresis and immunoblotting.

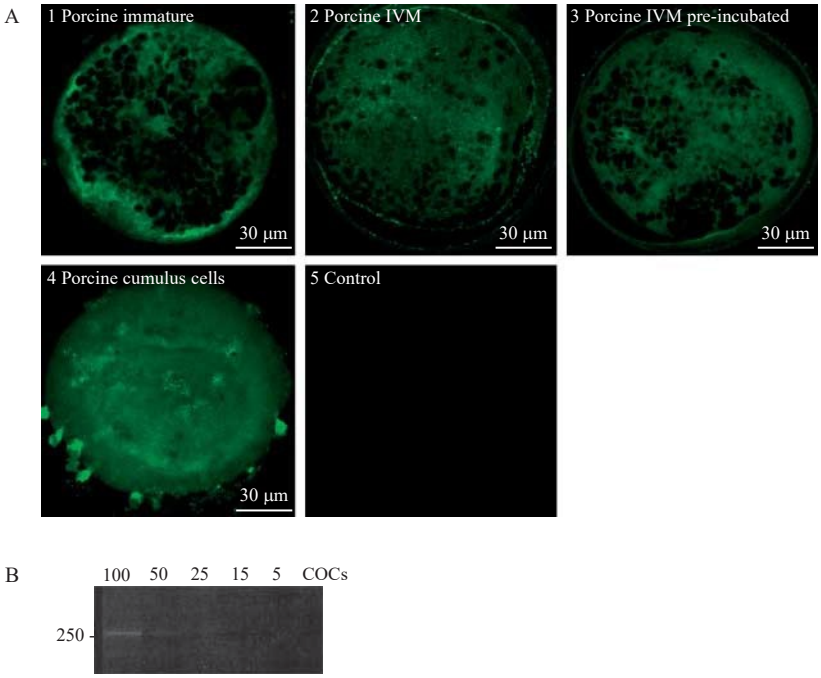

Figure 3 (A) Optical sections in the equatorial plane of porcine immature oocyte (1), *in vitro* matured oocyte (2) *in vitro* matured oocyte pre-incubated with oviductal fluid (3 and 5) and in the upper-surface plane of *in vitro* matured oocyte with cumulus cells (4) incubated with anti-gp340 antibodies (1, 2, 3 and 4) or no primary antibodies (5) and observed with a confocal laser scanning microscope. (B) Gel electrophoresis and immuno-blotting on porcine COCs collected from the ovaries of slaughtered females. Five to 100 COCs have been loaded onto each lane and revealed with the anti-gp340 antibody.

Interaction of DMBT1 with porcine spermatozoa using surface plasmon resonance

In order to check for an interaction between DMBT1 and spermatozoa, we performed surface plasmon resonance experiments. We showed that the injection of porcine sperm plasma membrane preparations on the test FC resulted in a binding to hrDMBT1 captured by the immobilized anti-gp340 antibodies (Fig. 4A). The same membrane preparation injected simultaneously into the control FC (immobilized anti-gp340 antibodies without pre-injection of hrDMBT1) resulted in a low non-specific binding signal (Fig. 4B). A low non-specific binding signal was also observed after the injection into the two FC of the supernatant of the sperm plasma membrane preparations (Fig. 4C and D) or the BSA solution (data not shown). These results show an interaction of DMBT1 with porcine sperm plasma membrane preparations.

Identification and phylogeny of DMBT1 proteins in mammals

To broaden the study to other mammals, we searched for the presence of DMBT1 proteins in databases. Table 1 shows an updated list of the DMBT1 proteins in human, chimpanzee, mouse, rat, bovine, horse, pig, and dog species. In all species, except the chimpanzee, two proteins were identified, generally called DMBT1 and DMBT1-like protein in the databases. In the chimpanzee, the tblastn of human DMBT1 vs chimpanzee genome showed two matches located on chromosome 10. The first one was called DMBT1 protein-like. For the second one (LOC745251), no amino acid sequence was found.

In all species, except the pig, the two *DMBT1* genes are located in a syntenic region on the same chromosome. In the pig, the two genes are located on chromosomes 6 and 14 respectively. The pig chromosome 14 region that contains *DMBT1* also contains FAM24A-like (XP_003133260), which was also found in the syntenic region that contains *DMBT1* in other species.

To make sure that DMBT1 and DMBT1-like proteins are two distinct proteins, global alignments of DMBT1 and DMBT1-like proteins for each species were done. They showed low percentages of identity and low percentages of similarity (data not shown).

A first phylogenetic tree was reconstructed using all the sequences presented in Table 1 (data not shown). However, the phylogenetic tree and the multiple alignment data showed that the amino acid sequence of dog-DMBT1-like (XP_548815) was highly divergent from the other sequences. Thus, this sequence was removed from the dataset of sequences used for new phylogenetic reconstructions. The phylogenetic trees obtained using BioNJ (Neighbor Joining) and PhyML (Maximum of Likelihood) methods are presented in Fig. 5. Both trees contain two groups of proteins, the DMBT1 protein and the DMBT1-like protein, each having a common ancestral gene. The branches of the DMBT1-like proteins are generally longer than the branches of the DMBT1 proteins. The so-called chimpanzee DMBT1-like protein is in the group of DMBT1 proteins, suggesting that this chimpanzee protein could be DMBT1 and should be

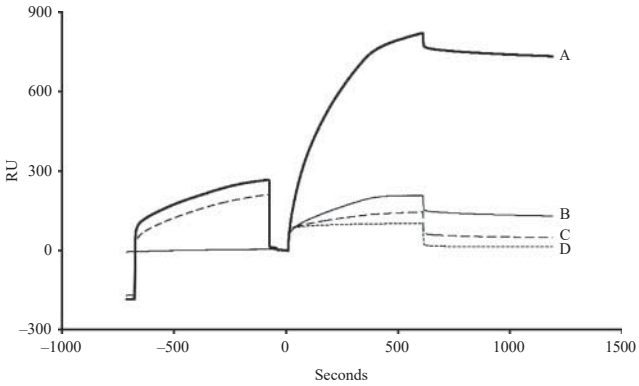

Figure 4 Surface plasmon resonance binding data. On the test flow cell (FC), 6 mM hrDMBT1 were injected onto immobilized anti-gp340 antibodies, and then porcine sperm plasma membrane preparations (A) or the supernatant of the sperm plasma membrane preparations (C) were injected. In the control FC (immobilized anti-gp340 antibodies without pre-injection of hrDMBT1), porcine sperm plasma membrane preparations (B) or the supernatant of the sperm plasma membrane preparations (D) were injected. The test FC sensorgrams show a first association phase (increasing response) during the 600-s injection of hrDMBT1, followed by a second association phase during the 600-s injection of sperm membranes (A) or a low signal during the injection of the supernatant (C), followed by a dissociation phase (decreasing response). The control FC sensorgrams show no signal in the first part, followed by a low signal during the injection of sperm membranes (B) or the supernatant (D).

reclassified as chimpanzee DMBT1. The pig *DMBT1* gene located on chromosome 14 (ENSSSCP00000011425) is in the group of DMBT1-like proteins, suggesting that this gene encodes a DMBT1-like protein and should be reclassified as pig DMBT1-like.

Discussion

To our knowledge, this is the first study analyzing the role of DMBT1 in the mechanisms of fertilization. For this purpose, we decided to study two models, equine and porcine, that have different efficiencies in IVF rates. Our results suggest an involvement of DMBT1 in the fertilization process in both species. Our first objective was to evaluate the role of oviductal secretions in fertilization and the involvement of DMBT1 in this process.

In our conditions, pre-incubation of porcine and equine oocytes with oviductal fluid significantly increased monospermic IVF rates. These positive effects were canceled once anti-gp340 antibody was added to oviductal fluid, suggesting the involvement of DMBT1 (or a factor attached to DMBT1) in the fertilization process. This hypothesis has been confirmed after further experiments in which hrDMBT1 was used. In fact, pre-incubation of porcine oocytes with hrDMBT1 at different concentrations significantly increased both fertilization and monospermic fertilization rates. Owing to the low availability of equine ovaries in abattoirs and therefore of equine oocytes for experiments, we did not analyze the effect of pre-incubation of equine oocytes with hrDMBT1 on fertilization yet.

Our results confirm the beneficial effect of oviductal fluid showed in previous studies (Coy *et al*. 2008, Lloyd *et al*. 2009). These studies reported that exposure of oocytes to periovulatory oviductal fluid increased ZP resistance to proteolytic digestion and monospermic rates after IVF in porcine and bovine species. Moreover, pre-incubation of porcine oocytes increased cleavage and blastocyst developmental rates. These findings showed that oviductal fluid is capable of providing oocytes with components over a very short period of time between IVM and IVF, which benefit fertilization, embryo development, and embryo quality, and from our results, DMBT1 seems to be one of these components.

In previous studies, DMBT1 (previously called SBG in the pig) has been proposed to be implicated in sperm selection through acrosome alteration and suppression of motility of a subpopulation of sperm that have begun capacitation prematurely (Teijeiro *et al*. 2008, Teijeiro & Marini 2012*a*). Using surface plasmon resonance experiments, we showed an interaction between DMBT1 and sperm plasma membrane preparations. Thus, from our results, we can provide new evidence on the relevance of this molecule during the fertilization process.

Our second aim was to clarify the role of DMBT1. We analyzed the expression of DMBT1 in the oviductal fluids, and we found that DMBT1 is present in both equine and porcine oviducts. Moreover, DMBT1 is present in the pig oviduct in all the examined stages of the estrus cycle. The presence of DMBT1 in the oviduct was then confirmed by immunohistochemical analysis. We analyzed the localization of DMBT1 in different sections of porcine oviduct (ampulla and isthmus) during the different estrous cycle stages. In both regions, the immunostaining was present mainly in non-ciliated cells, which are secretory cells mainly involved in the synthesis and release of glycoproteins that dissolve in the oviductal fluid (Buhi 2002, Abe & Hoshi 2007, Desantis *et al*. 2011), but also in other cell types. The secretion of DMBT1 seems to follow the stages of the estrus cycle, as the presence of the protein is observed from the end of follicular phase in both isthmus and ampulla. This result is also confirmed by the observation that the immunoreactivity at the beginning of the follicular phase is in the cytoplasm and in the apical region of epithelial cells. From the middle of the follicular phase, then, it is mainly concentrated in the apical surface, indicating that cells are preparing for DMBT1 secretion. Moreover, we found that at the end of follicular stage and in the preovulatory phase, DMBT1 is present in the apical region of secretory cells as well as in the lumen of both ampulla and isthmus segments. The evidence that in the post-ovulatory stage, the expression of DMBT1 was found in the apical region of epithelial cells seems to show a continuous epithelium

Table 1 Updated list of the DMBT1 proteins in mammals with their UniProt, GenBank, and Ensembl accession numbers and localization. For humans and rats, the accession numbers of the two longest transcripts have been mentioned.

Species	Name of the protein	UniProtKB	GenBank	Ensembl	Localization
Homo sapiens (human)	Deleted in malignant brain tumors 1 protein (the gene has 15 transcripts)	Q9UGM3	XP_003403680 BAA78577	ENSP00000357911 ENSP00000342210	10q26.13
H. sapiens (human)	Putative DMBT1-like protein	A6NE64			10q26.13
Pan troglodytes (chimpanzee)	Deleted in malignant brain tumors 1 protein-like		XP_003339241 XP_003312842 XP_003312844	ENSPTRP00000042778	Chromosome 10
Mus musculus (mouse)	Deleted in malignant brain tumors 1 protein (the gene has two transcripts)	Q60997	AAH49835 BC049835	ENSMUSP00000006366 ENSMUSP00000081556	Chromosome 7
M. musculus (mouse)	Putative DMBT1-like protein	Q8BZE1			Chromosome 7
Rattus norvegicus (rat)	Deleted in malignant brain tumors 1 protein (the gene has six transcripts)	Q8CIZ5	NP_074040 XP_003749045	ENSRNOP00000051794 ENSRNOP00000027919	Chromosome 1
R. norvegicus (rat)	Deleted in malignant brain tumors 1 protein-like		XP_003749046		Chromosome 1
Bos taurus (bovine)	DMBT1	F1MD73	DAA14688	ENSBTAP00000015890	Chromosome 26
B. taurus (bovine)	Deleted in malignant brain tumors 1 protein-like		XP_003584177		Chromosome 26
Equus caballus (horse)	Deleted in malignant brain tumors 1 protein	F6TJS5	XP_001495444	ENSECAP00000006236	Chromosome 1
E. caballus (horse)	Deleted in malignant brain tumors 1 protein-like			ENSECAP00000016995	Chromosome 1
Sus scrofa (pig)	Deleted in malignant brain tumors 1 protein	Q4A3R3	CAJ27171 CAJ14977 NP_001041653	ENSSSCP00000003229	Chromosome 6
S. scrofa (pig)	Deleted in malignant brain tumors 1 protein	F1SEE7	XP_003133265	ENSSSCP00000011425	Chromosome 14
Canis lupus familiaris (dog)	Deleted in malignant brain tumors 1 protein (the gene has three transcripts)	F1PRV8 F1PSK2 F1PSL5	XP_544052	ENSCAFP00000018537 ENSCAFP00000018515 ENSCAFP00000018502	Chromosome 28
C. lupus familiaris (dog)	Deleted in malignant brain tumors 1 protein-like		XP_548815		Chromosome 28

secretion in order to guarantee an optimal oviductal environment to maintain the capacity of the isthmus for sperm selection and the ampulla for receiving oocytes.

In a previous study (Perez *et al*. 2006), immunohistochemical analysis of DMBT1 in oviducts obtained from gilts of approximately 120 days of age. Advances in *in vitro* production of pig embryos was performed. The results showed that DMBT1 localizes preferentially to the apical surface of epithelial cells, it is concentrated at the lumen rather than at the bottom of the folds and crypts of the fallopian tube, and there is a bigger amount of DMBT1 in the isthmus than in the ampulla. Our results are in agreement with these findings even if we did not find any difference between isthmus and ampulla. It is important to notice that the previous study was performed in the prepubertal stage and that they did not compare the different estrus cycle stages as we did in this study.

Immunofluorescence studies and confocal microscopy showed localization of DMBT1 in the ZP and cytoplasm of both porcine and equine oocytes, as well as cumulus cells. The presence of DMBT1 in porcine COCs was also confirmed by immunoblotting analysis. This is the first study, to our knowledge, in which the presence of DMBT1 in the oocytes has been analyzed. These findings indicate that DMBT1 could act as a bridge between oocytes and sperm cells or oocytes and oviductal cells. DMBT1 could mediate their interaction by linking other associated proteins that can interact with DMBT1 and mediate its effect. DMBT1 has been shown to react with integrins, molecules that are involved in gamete interaction (Vijayakumar *et al*. 2008), and a recent study (Teijeiro & Marini 2012*b*) showed that S100A7 (psoriasin), a protein localized at the head of sperm cells, interacts with DMBT1. Moreover, our results, obtained by surface plasmon resonance, showed an interaction between DMBT1 and porcine spermatozoa, giving more emphasis on this hypothesis.

Previous studies reported the hypothesis that DMBT1 is involved in sperm-negative selection, by damaging those spermatozoa that have begun capacitation when they arrive at the oviduct (Teijeiro *et al*. 2008, Teijeiro & Marini 2012*a*). Our results add more information on the role of DMBT1 showing that it can be involved not only in this negative selection but also in pathways, such as fertilization, in which oocytes, sperm cells, and the oviduct are involved.

Our bioinformatics analyses allowed us to identify two distinctive proteins, generally called DMBT1 and

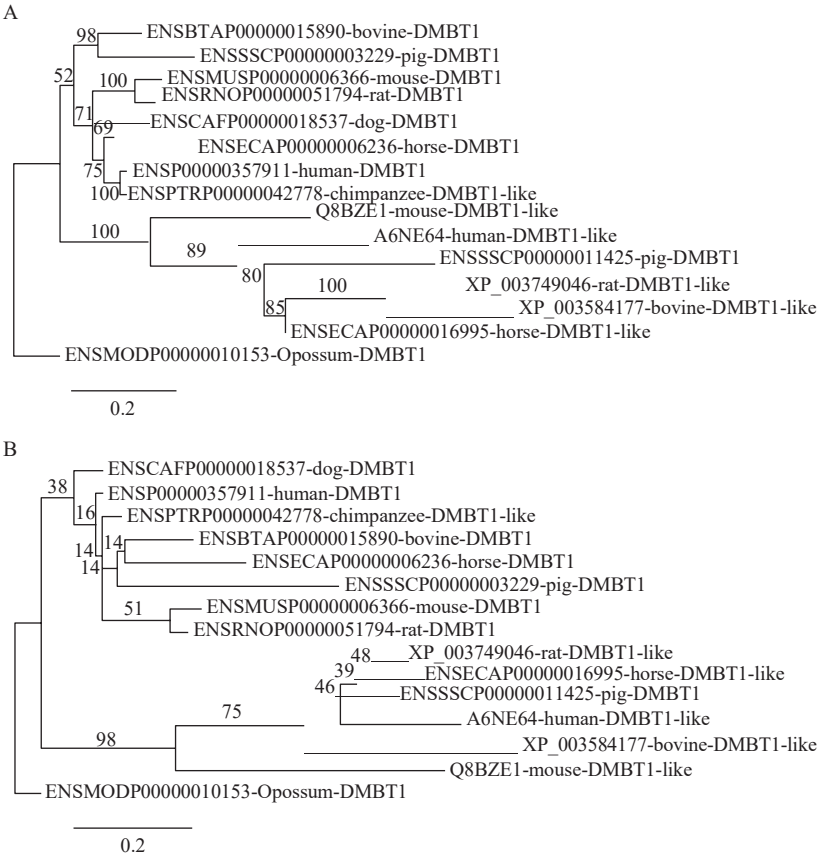

Figure 5 Phylogenetic trees of the DMBT1 proteins reconstructed using BioNJ (A) and PhyML (B) methods. Branch lengths indicate rates of evolution. The values at the tree nodes represent bootstrap values.

DMBT1-like in the databases, in human, mouse, rat, bovine, horse, and dog species. In the pig, the two proteins are called DMBT1. The pig *DMBT1* genes are located on two different chromosomes, 6 and 14. The localization of the sequences on the chromosomes using MapViewer showed that the region of chromosome 14 containing the pig *DMBT1* gene that encodes the protein ENSSSCP00000011425 also contains the gene *FAM24A*-like, as in the dog, cow, chimpanzee, mouse, and rat species. Moreover, the region of chromosome 6 containing the pig *DMBT1* gene that encodes the protein ENSSSCP00000003229 also contains the spermadhesins *PSP-I*, *PSP-II*, *AQN1*, and *AWN1*, as in the bovine species (*DMBT1* and *DMBT1*-like genes are located close to the spermadhesins *SPADH1* and *SPADH2*). This synteny analysis would suggest a breaking of synteny in the pig. According to the phylogenetic trees, we suggest that chromosome 6 contains the pig orthologous gene of the human *DMBT1* gene as the pig *DMBT1* located on chromosome 6 (corresponding to ENSSSCP00000003229) shares a common ancestral gene with the other *DMBT1* genes. The pig *DMBT1* located on chromosome 14 (corresponding to ENSSSCP00000011425) shares a common ancestral gene with the other *DMBT1*-like genes. In all species except the pig, the two proteins are located on the same chromosome. Reconstruction of phylogenetic trees could suggest that *DMBT1* genes share a common ancestral gene and that *DMBT1*-like genes share another common ancestral gene. The low percentages of identity and similarity between the DMBT1 and DMBT1-like proteins as well as the longer branches of the phylogenetic trees for the DMBT1-like proteins suggest that the *DMBT1*-like genes have accumulated a high number of mutations during evolution. The evolution of this gene results probably from chromosomal rearrangements. Evolution of *DMBT1*-like genes could confer a specific role of the DMBT1-like protein, i.e. an alternative and/or complementary function to DMBT1 protein.

Finally, under our experimental conditions, we observed that during equine IVF, we obtained IVF rates of 62%, after pre-incubation of oocytes with oviductal fluid. This result is interesting, considering that conventional IVF has only limited success in equine species (Palmer *et al*. 1991, Dell'aquila *et al*. 1996, Hinrichs *et al*. 2002, McPartlin *et al*. 2009, Mugnier *et al*. 2009). We chose the technique developed by McPartlin *et al*. (2009) in which spermatozoa were hyperactivated using procaine. In this study, they obtained IVF rates of 61% but their results do not seem to be repeatable, and we obtained 37% under the same conditions (our control group). A recent study (Ortgies *et al*. 2012) analyzed the effect of procaine, pentoxifylline, and trolox on capacitation and hyperactivation of stallion

spermatozoa. Their findings showed that the combination of capacitating MW and procaine gave the best results for the induction of capacitation and hyperactivation in stallion spermatozoa. Our result is therefore encouraging for future successful IVF in equine species. Moreover, the improvement of monospermic fertilization rates that we observed in the pig is also encouraging in this species as polyspermy is still a problem.

In conclusion, we showed the involvement of DMBT1 in the fertilization process in the pig and the horse, its presence in the COCs, and confirmed and further analyzed its presence in the oviduct as well as an interaction between DMBT1 and spermatozoa. The presence of DMBT1 in other species such as human, mouse, rat, cattle, and dog allows us to hypothesize a role in the fertilization process in these species.

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 study was supported by the 'Institut Français du Cheval et de l'Equitation' (IFCE). B Ambruosi was financially supported by a post-doctoral grant from the 'Université François Rabelais de Tours'. C M Nicolás is supported by the Grant AGL2009-12512-C02-02.

Acknowledgements

The authors would like to thank Guy Duchamp, Eric Venturi, and the staff of the experimental stud and pigsty at the 'Unité Expérimentale de Physiologie Animale de l'Orfrasière (UEPAO)'. They also thank Maryse Meurisse, Fabrice Vincent, Sophie Picard, Gaël Ramé, Thierry Delpuech, Pascal Papillier, Jean-Noël Couet, and the staff of the experimental slaughterhouse at the 'Unité Mixte de Recherche de Physiologie de la Reproduction et des Comportements (UMR PRC)' for technical help. They thank Thomas Loyau for his contribution to this work Sylvia Bedford-Guaus and Lori McPartlin at Cornell University (Ithaca, USA) for constructive discussion.

References

Abe H & Hoshi H 2007 Regional and cyclic variations in the ultrastructural features of secretory cells in the oviductal epithelium of the Chinese Meishan pig. *Reproduction in Domestic Animals* 42 292-298. (doi:10.1111/j.1439-0531.2006.00781.x)

Abeydeera LR & Day BN 1997 Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified Tris-buffered medium with frozen-thawed ejaculated spermatozoa. *Biology of Reproduction* 57 729-734. (doi:10.1095/biolreprod57.4.729)

Al-Awqati Q 2011 Terminal differentiation in epithelia: the role of integrins in hensin polymerization. *Annual Review of Physiology* 73 401-412. (doi:10.1146/annurev-physiol-012110-142253)

Alm H, Torner H, Blottner S, Nurnberg G & Kanitz W 2001 Effect of sperm cryopreservation and treatment with calcium ionophore or heparin on *in vitro* fertilization of horse oocytes. *Theriogenology* 56 817-829. (doi:10.1016/S0093-691X(01)00610-0)

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25 3389-3402. (doi:10.1093/nar/25.17.3389)

Anderson RA, Feathergill KA, Drisdel RC, Rawlins RG, Mack SR & Zaneveld LJ 1994 Atrial natriuretic peptide (ANP) as a stimulus of the human acrosome reaction and a component of ovarian follicular fluid: correlation of follicular ANP content with *in vitro* fertilization outcome. *Journal of Andrology* 15 61-70. (doi:10.1002/i.1939-4640.1994.tb01685.x)

Bongso A, Ng SC, Fong CY & Ratnam S 1991 Improved fertilization rates of human oocytes in coculture. *Journal of In Vitro Fertilization and Embryo Transfer* 8 216-221. (doi:10.1007/BF01130808)

Buhi WC 2002 Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction* 123 355-362. (doi:10.1530/rep.0.1230355)

Carrasco LC, Romar R, Aviles M, Gadea J & Coy P 2008 Determination of glycosidase activity in porcine oviductal fluid at the different phases of the estrous cycle. *Reproduction* 136 833-842. (doi:10.1530/REP-08-0221)

Cheng H, Bjerknes M & Chen H 1996 CRP-ductin: a gene expressed in intestinal crypts and in pancreatic and hepatic ducts. *Anatomical Record* 244 327-343. (doi:10.1002/(SICI)1097-0185(199603)244:3⁋327::AID-AR5O3.0.CO;2-V)

Chian RC & Sirard MA 1995 Fertilizing ability of bovine spermatozoa cocultured with oviduct epithelial cells. *Biology of Reproduction* 52 156-162. (doi:10.1095/biolreprod52.1.156)

Coy P, Canovas S, Mondejar I, Saavedra MD, Romar R, Grullon L, Matas C & Aviles M 2008 Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *PNAS* 105 15809-15814. (doi:10.1073/pnas.0804422105)

Day BN 2000 Reproductive biotechnologies: current status in porcine reproduction. *Animal Reproduction Science* 60–61 161-172. (doi:10.1016/S0378-4320(00)00079-8)

Dell'aquila ME, Fusco S, Lacalandra GM & Maritato F 1996 *In vitro* maturation and fertilization of equine oocytes recovered during the breeding season. *Theriogenology* 45 547-560. (doi:10.1016/0093-691X(95)00402-T)

Desantis S, Zizza S, Accogli G, Acone F, Rossi R & Resta L 2011 Morphometric and ultrastructural features of the mare oviduct epithelium during oestrus. *Theriogenology* 75 671-678. (doi:10.1016/j.theriogenology.2010.10.007)

Eid LN, Lorton SP & Parrish JJ 1994 Paternal influence on S-phase in the first cell cycle of the bovine embryo. *Biology of Reproduction* 51 1232-1237. (doi:10.1095/biolreprod51.6.1232)

End C, Lyer S, Renner M, Stahl C, Ditzer J, Holloschi A, Kuhn HM, Flammann HT, Poustka A, Hafner M *et al.* 2005 Generation of a vector system facilitating cloning of DMBT1 variants and recombinant expression of functional full-length DMBT1. *Protein Expression and Purification* 41 275-286. (doi:10.1016/j.pep.2005.02.010)

Flesch FM, Voorhout WF, Colenbrander B, van Golde LM & Gadella BM 1998 Use of lectins to characterize plasma membrane preparations from boar spermatozoa: a novel technique for monitoring membrane purity and quantity. *Biology of Reproduction* 59 1530-1539. (doi:10.1095/biolreprod59.6.1530)

Flicek P, Amode MR, Barrell D, Beal K, Brent S, Carvalho-Silva D, Clapham P, Coates G, Fairley S, Fitzgerald S *et al.* 2012 Ensembl 2012. *Nucleic Acids Research* 40 D84-D90. (doi:10.1093/nar/gkr991)

Funahashi H & Day BN 1997 Advances in *in vitro* production of pig embryos. *Journal of Reproduction and Fertility. Supplement* 52 271-283.

Goncalves RF, Chapman DA, Bertolla RP, Eder I & Killian GJ 2008 Pre-treatment of cattle semen or oocytes with purified milk osteopontin affects *in vitro* fertilization and embryo development. *Animal Reproduction Science* 108 375-383. (doi:10.1016/j.anireprosci.2007.09.006)

Goudet G, Belin F, Mlodawska W & Bezard J 2000 Influence of epidermal growth factor on *in vitro* maturation of equine oocytes. *Journal of Reproduction and Fertility. Supplement* 56 483-492.

Hao Y, Mathialagan N, Walters E, Mao J, Lai L, Becker D, Li W, Critser J & Prather RS 2006 Osteopontin reduces polyspermy during *in vitro* fertilization of porcine oocytes. *Biology of Reproduction* 75 726-733. (doi:10.1095/biolreprod.106.052589)

Hinrichs K, Love CC, Brinsko SP, Choi YH & Varner DD 2002 *In vitro* fertilization of *in vitro*-matured equine oocytes: effect of maturation medium, duration of maturation, and sperm calcium ionophore treatment, and comparison with rates of fertilization *in vivo* after oviductal transfer. *Biology of Reproduction* 67 256-262. (doi:10.1095/biolreprod67.1.256)

Holmskov U, Lawson P, Teisner B, Tornoe I, Willis AC, Morgan C, Koch C & Reid KB 1997 Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein-D binding molecule. *Journal of Biological Chemistry* 272 13743-13749. (doi:10.1074/jbc.272.21.13743)

Holmskov U, Mollenhauer J, Madsen J, Vitved L, Gronlund J, Tornoe I, Kliem A, Reid KB, Poustka A & Skjodt K 1999 Cloning of gp-340, a putative opsonin receptor for lung surfactant protein D. *PNAS* 96 10794-10799. (doi:10.1073/pnas.96.19.10794)

Kervancioglu ME, Saridogan E, Atasu T, Camlibel T, Demircan A, Sarikamis B & Djahanbakhch O 1997 Human fallopian tube epithelial cell co-culture increases fertilization rates in male factor infertility but not in tubal or unexplained infertility. *Human Reproduction* 12 1253-1258. (doi:10.1093/humrep/12.6.1253)

Khatir H, Anouassi A & Tibary A 2004 Production of dromedary (*Camelus dromedarius*) embryos by IVM and IVF and co-culture with oviductal or granulosa cells. *Theriogenology* 62 1175-1185. (doi:10.1016/j.theriogenology.2004.01.016)

Li XJ & Snyder SH 1995 Molecular cloning of Ebnerin, a von Ebner's gland protein associated with taste buds. *Journal of Biological Chemistry* 270 17674-17679. (doi:10.1074/jbc.270.30.17674)

Ligtenberg TJ, Bikker FJ, Groenink J, Tornoe I, Leth-Larsen R, Veerman EC, Nieuw Amerongen AV & Holmskov U 2001 Human salivary agglutinin binds to lung surfactant protein-D and is identical with scavenger receptor protein gp-340. *Biochemical Journal* 359 243-248. (doi:10.1042/0264-6021:3590243)

Ligtenberg AJ, Karlsson NG & Veerman EC 2010 Deleted in malignant brain tumors-1 protein (DMBT1): a pattern recognition receptor with multiple binding sites. *International Journal of Molecular Sciences* 11 5212-5233. (doi:10.3390/ijms1112521)

Lloyd RE, Romar R, Matas C, Gutierrez-Adan A, Holt WV & Coy P 2009 Effects of oviductal fluid on the development, quality, and gene expression of porcine blastocysts produced *in vitro*. *Reproduction* 137 679-687. (doi:10.1530/REP-08-0405)

Locatelli Y, Cognie Y, Vallet JC, Baril G, Verdier M, Poulin N, Legendre X & Mermillod P 2005 Successful use of oviduct epithelial cell coculture for *in vitro* production of viable red deer (*Cervus elaphus*) embryos. *Theriogenology* 64 1729-1739. (doi:10.1016/j.theriogenology.2005.04.002)

Madsen J, Tornoe I, Nielsen O, Lausen M, Krebs I, Mollenhauer J, Kollender G, Poustka A, Skjodt K & Holmskov U 2003 CRP-ductin, the mouse homologue of gp-340/deleted in malignant brain tumors 1 (DMBT1), binds gram-positive and gram-negative bacteria and interacts with lung surfactant protein D. *European Journal of Immunology* 33 2327-2336. (doi:10.1002/eji.200323972)

Madsen J, Mollenhauer J & Holmskov U 2010 Review: Gp-340/DMBT1 in mucosal innate immunity. *Innate Immunity* 16 160-167. (doi:10.1177/1753425910368447)

Marchal R, Caillaud M, Martoriati A, Gerard N, Mermillod P & Goudet G 2003 Effect of growth hormone (GH) on *in vitro* nuclear and cytoplasmic oocyte maturation, cumulus expansion, hyaluronan synthases, and connexins 32 and 43 expression, and GH receptor messenger RNA expression in equine and porcine species. *Biology of Reproduction* 69 1013-1022. (doi:10.1095/biolreprod.103.015602)

Marini PE & Cabada MO 2003 One step purification and biochemical characterization of a spermatozoa-binding protein from porcine oviductal epithelial cells. *Molecular Reproduction and Development* 66 383-390. (doi:10.1002/mrd.10361)

Martus NS, Verhage HG, Mavrogianis PA & Thibodeaux JK 1998 Enhancement of bovine oocyte fertilization *in vitro* with a bovine oviductal specific glycoprotein. *Journal of Reproduction and Fertility* 113 323-329. (doi:10.1530/jrf.0.1130323)

Matsushita F, Miyawaki A & Mikoshiba K 2000 Vomeroglandin/CRP-ductin is strongly expressed in the glands associated with the mouse vomeronasal organ: identification and characterization of mouse vomeroglandin. *Biochemical and Biophysical Research Communications* 268 275-281. (doi:10.1006/bbrc.2000.2104)

McCauley TC, Buhi WC, Wu GM, Mao J, Caamano JN, Didion BA & Day BN 2003 Oviduct-specific glycoprotein modulates sperm-zona binding and improves efficiency of porcine fertilization *in vitro*. *Biology of Reproduction* 69 828-834. (doi:10.1095/biolreprod.103.016444)

McPartlin LA, Suarez SS, Czaya CA, Hinrichs K & Bedford-Guaus SJ 2009 Hyperactivation of stallion sperm is required for successful *in vitro* fertilization of equine oocytes. *Biology of Reproduction* 81 199-206. (doi:10.1095/biolreprod.108.074880)

Mollenhauer J, Wiemann S, Scheurlen W, Korn B, Hayashi Y, Wilgenbus KK, von Deimling A & Poustka A 1997 DMBT1, a new member of the SRCR superfamily, on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. *Nature Genetics* 17 32-39. (doi:10.1038/ng0997-32)

Mollenhauer J, Holmskov U, Wiemann S, Krebs I, Herbertz S, Madsen J, Kioschis P, Coy JF & Poustka A 1999 The genomic structure of the DMBT1 gene: evidence for a region with susceptibility to genomic instability. *Oncogene* 18 6233-6240. (doi:10.1038/sj.onc.1203071)

Mollenhauer J, Herbertz S, Holmskov U, Tolnay M, Krebs I, Merlo A, Schroder HD, Maier D, Breitling F, Wiemann S *et al.* 2000 DMBT1 encodes a protein involved in the immune defense and in epithelial differentiation and is highly unstable in cancer. *Cancer Research* 60 1704-1710.

Mollenhauer J, Herbertz S, Helmke B, Kollender G, Krebs I, Madsen J, Holmskov U, Sorger K, Schmitt L, Wiemann S *et al.* 2001 Deleted in malignant brain tumors 1 is a versatile mucin-like molecule likely to play a differential role in digestive tract cancer. *Cancer Research* 61 8880-8886.

Mollenhauer J, Muller H, Kollender G, Lyer S, Diedrichs L, Helmke B, Holmskov U, Ligtenberg T, Herbertz S, Krebs I *et al.* 2002 The SRCR/SID region of DMBT1 defines a complex multi-allele system representing the major basis for its variability in cancer. *Genes, Chromosomes & Cancer* 35 242-255. (doi:10.1002/gcc.10115)

Mollenhauer J, Helmke B, Medina D, Bergmann G, Gassler N, Muller H, Lyer S, Diedrichs L, Renner M, Wittig R *et al.* 2004 Carcinogen inducibility *in vivo* and down-regulation of DMBT1 during breast carcinogenesis. *Genes, Chromosomes & Cancer* 39 185-194. (doi:10.1002/gcc.10309)

Monaco E, Gasparrini B, Boccia L, De Rosa A, Attanasio L, Zicarelli L & Killian G 2009 Effect of osteopontin (OPN) on *in vitro* embryo development in cattle. *Theriogenology* 71 450-457. (doi:10.1016/j.theriogenology.2008.08.012)

Mori M, Shiraishi T, Tanaka S, Yamagata M, Mafune K, Tanaka Y, Ueo H, Barnard GF & Sugimachi K 1999 Lack of DMBT1 expression in oesophageal, gastric and colon cancers. *British Journal of Cancer* 79 211-213. (doi:10.1038/sj.bjc.6690035)

Mugnier S, Kervella M, Douet C, Canepa S, Pascal G, Deleuze S, Duchamp G, Monget P & Goudet G 2009 The secretions of oviduct epithelial cells increase the equine *in vitro* fertilization rate: are osteopontin, atrial natriuretic peptide A and oviductin involved? *Reproductive Biology and Endocrinology* 7 129. (doi:10.1186/1477-7827-7-129)

Nagai T, Funahashi H, Yoshioka K & Kikuchi K 2006 Up date of *in vitro* production of porcine embryos. *Frontiers in Bioscience* 11 2565-2573. (doi:10.2741/1991)

Neron B, Menager H, Maufrais C, Joly N, Maupetit J, Letort S, Carrere S, Tuffery P & Letondal C 2009 Mobyle: a new full web bioinformatics framework. *Bioinformatics* 25 3005-3011. (doi:10.1093/bioinformatics/btp493)

Nunes DP, Keates AC, Afdhal NH & Offner GD 1995 Bovine gall-bladder mucin contains two distinct tandem repeating sequences: evidence for scavenger receptor cysteine-rich repeats. *Biochemical Journal* 310 41-48.

O'Day-Bowman MB, Mavrogianis PA, Reuter LM, Johnson DE, Fazleabas AT & Verhage HG 1996 Association of oviduct-specific glycoproteins with human and baboon (*Papio anubis*) ovarian oocytes and enhancement of human sperm binding to human hemizonae following *in vitro* incubation. *Biology of Reproduction* 54 60-69. (doi:10.1095/biolreprod54.1.60)

Ortgies F, Klewitz J, Gorgens A, Martinsson G & Sieme H 2012 Effect of procaine, pentoxifylline and trolox on capacitation and hyperactivation of stallion spermatozoa. *Andrologia* 44 (Suppl 1) 130-138. (doi:10.1111/j.1439-0272.2010.01150.x)

Palmer E, Bezard J, Magistrini M & Duchamp G 1991 *In vitro* fertilization in the horse. A retrospective study. *Journal of Reproduction and Fertility. Supplement* 44 375-384.

Parrish JJ, Krogenaes A & Susko-Parrish JL 1995 Effect of bovine sperm separation by either swim-up or Percoll method on success of *in vitro* fertilization and early embryonic development. *Theriogenology* 44 859-869. (doi:10.1016/0093-691X(95)00271-9)

Perez FA, Roma SM, Cabada MO & Marini PE 2006 Sperm binding glycoprotein is differentially present surrounding the lumen of isthmus and ampulla of the pig's oviduct. *Anatomy and Embryology* 211 619-624. (doi:10.1007/s00429-006-0114-0)

Prakobphol A, Xu F, Hoang VM, Larsson T, Bergstrom J, Johansson I, Frangsmyr L, Holmskov U, Leffler H, Nilsson C *et al.* 2000 Salivary agglutinin, which binds *Streptococcus* mutans and *Helicobacter pylori*, is the lung scavenger receptor cysteine-rich protein gp-340. *Journal of Biological Chemistry* 275 39860-39866. (doi:10.1074/jbc.M006928200)

Romar R, Coy P, Campos I, Gadea J, Matas C & Ruiz S 2001 Effect of co-culture of porcine sperm and oocytes with porcine oviductal epithelial cells on *in vitro* fertilization. *Animal Reproduction Science* 68 85-98. (doi:10.1016/S0378-4320(01)00133-6)

Rossez Y, Coddeville B, Elass E, Quinchon JF, Vidal O, Corfield AP, Gosset P, Lacroix JM, Michalski JC & Robbe-Masselot C 2011 Interaction between DMBT1 and galectin 3 is modulated by the structure of the oligosaccharides carried by DMBT1. *Biochimie* 93 593-603. (doi:10.1016/j.biochi.2010.12.002)

Takito J, Yan L, Ma J, Hikita C, Vijayakumar S, Warburton D & Al-Awqati Q 1999 Hensin, the polarity reversal protein, is encoded by DMBT1, a gene frequently deleted in malignant gliomas. *American Journal of Physiology* 277 F277-F289.

Teijeiro JM & Marini PE 2012*a* 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* 143 773-785. (doi:10.1530/REP-11-0314)

Teijeiro JM & Marini PE 2012*b* S100A7 is present in human sperm and a homologous pig sperm protein interacts with sperm-binding glycoprotein (SBG). *Andrologia* 44 (Suppl 1) 772-779. (doi:10.1111/j.1439-0272.2011.01264.x)

Teijeiro JM, Cabada MO & Marini PE 2008 Sperm binding glycoprotein (SBG) produces calcium and bicarbonate dependent alteration of acrosome morphology and protein tyrosine phosphorylation on boar sperm. *Journal of Cellular Biochemistry* 103 1413-1423. (doi:10.1002/jcb.21524)

Teijeiro JM, Roldan ML & Marini PE 2012 Molecular identification of the sperm selection involved porcine sperm binding glycoprotein (SBG) as deleted in malignant brain tumors 1 (DMBT1). *Biochimie* 94 263-267. (doi:10.1016/j.biochi.2011.10.008)

Vijayakumar S, Erdjument-Bromage H, Tempst P & Al-Awqati Q 2008 Role of integrins in the assembly and function of hensin in intercalated cells. *Journal of the American Society of Nephrology* 19 1079-1091. (doi:10.1681/ASN.2007070737)

Way AL, Schuler AM & Killian GJ 1997 Influence of bovine ampullary and isthmic oviductal fluid on sperm-egg binding and fertilization *in vitro*. *Journal of Reproduction and Fertility* 109 95-101. (doi:10.1530/jrf.0.1090095)

Wu W, Kemp BL, Proctor ML, Gazdar AF, Minna JD, Hong WK & Mao L 1999 Expression of DMBT1, a candidate tumor suppressor gene, is frequently lost in lung cancer. *Cancer Research* 59 1846-1851.

Zamir N, Barkan D, Keynan N, Naor Z & Breitbart H 1995 Atrial natriuretic peptide induces acrosomal exocytosis in bovine spermatozoa. *American Journal of Physiology* 269 E216-E221.

Zhang M, Hong H, Zhou B, Jin S, Wang C, Fu M, Wang S & Xia G 2006 The expression of atrial natriuretic peptide in the oviduct and its functions in pig spermatozoa. *Journal of Endocrinology* 189 493-507. (doi:10.1677/joe.1.06483)

Received 11 January 2013
First decision 18 February 2013
Revised manuscript received 21 May 2013
Accepted 28 May 2013