Identification by proteomics of oviductal sperm-interacting proteins

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Abstract

The interactions between oviductal fluid (OF) proteins and spermatozoa play major roles in sperm selection, storage and capacitation before fertilization. However, only a few sperm-interacting proteins in the OF has been identified and very little is known about the regulation of sperm-oviduct interactions across the estrous cycle. Samples of bovine frozen-thawed sperm from three bulls were incubated with OF at pre-, post-ovulatory stages (Pre-/Post-ov) or luteal phase (LP) of the estrous cycle (7 mg/mL proteins, treated groups) or with a protein-free media (control). The proteomes of sperm cells were assessed by nanoLC-MS/MS and quantified by label-free methods. A total of 27 sperm-interacting proteins originating in the OF were identified. Among those, 14 were detected at all stages, eight at Post-ov and LP and five only at LP. The sperm-interacting proteins detected at all stages or at LP and Post-ov were on average more abundant at LP than at other stages ($P < 0.05$). At Pre-ov, OVGP1 was the most abundant sperm-interacting protein while at Post-ov, ACTB, HSP27, MYH9, MYH14 and OVGP1 were predominant. Different patterns of abundance of sperm-interacting proteins related to the stage were evidenced, which greatly differed from those previously reported in the bovine OF. In conclusion, this study highlights the important regulations of sperm-oviduct interactions across the estrous cycle and provides new protein candidates that may modulate sperm functions.

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Introduction

After mating or insemination in mammals, a limited number of spermatozoa enter the oviduct, where they interact with both the oviductal fluid (OF) and the oviduct epithelial cells before the time of ovulation and fertilization (Hunter & Wilmot 1984). There is evidence that interactions between oviductal secretions and spermatozoa play important roles in sperm selection, survival and capacitation before fertilization in livestock (Killian 2004, 2011, Ghersevich *et al.* 2015). *In vitro*, bovine spermatozoa incubated with OF displayed higher sperm survival (Abe *et al.* 1995a), motility (McNutt & Killian 1991, Abe *et al.* 1995a) and capacitation (Parrish *et al.* 1989, McNutt & Killian 1991, Bergqvist *et al.* 2006) than did controls incubated without OF. Furthermore, the *in vitro* exposure of bovine sperm to OF yielded higher percentages of fertilized oocytes than those of controls without OF (McNutt & Killian 1991, Grippo *et al.* 1995). The proteins in the OF may play a central role in modulating sperm functions. Incubation with oviductal protein extracts had beneficial

effects on bovine sperm viability (Boquest *et al.* 1999, Kumaresan *et al.* 2005, 2006), motility and acrosomal integrity and reduced sperm membrane damage during freezing and thawing (Kumaresan *et al.* 2005, 2006). Furthermore, the oviductal proteins from non-luteal (or follicular) stages of the estrous cycle maintained higher motility and viability and limited better the membrane damage after thawing than did proteins from the luteal phase (Kumaresan *et al.* 2006).

Using various experimental approaches, it was shown that oviductal proteins ranging in size from around 20 to 140 kDa associate with sperm membranes (McNutt *et al.* 1992, Rodriguez & Killian 1998, Killian 2004). Using immunohistochemistry and Western immunoblotting, some oviductal proteins interacting with sperm were identified. For instance, OVGP1, also known as oviductin, has been shown to bind to bull spermatozoa and a positive effect of OVGP1 on sperm capacitation, viability, motility and fertilization ability was demonstrated (King *et al.* 1994, Abe *et al.* 1995b, Killian 2004). However, up to now, only a few sperm-interacting proteins in the OF were identified and very

little is known about the regulation of sperm–oviduct interactions across the estrous cycle.

The OF is a complex and dynamic fluid originating from the secretions of oviduct epithelial secretory cells, transudate from the circulating serum and hypothetical inputs from the pre-ovulatory follicle (Leese *et al.* 2001, 2008). It is well known that the composition of the OF varies across the female cycle (Leese *et al.* 2008). We recently showed that out of 485 proteins identified in the bovine OF by high-resolution mass spectrometry, a limited number (<22) was specific to a given stage of the estrous cycle. However, the abundance of up to 20% of these proteins significantly fluctuated between cycle stages in a given side relative to ovulation (Lamy *et al.* 2016a). We hypothesized that these stage-dependent changes in the OF protein content may modulate interactions between oviductal proteins and spermatozoa.

Thus, the objectives of the present study were to: (1) develop a strategy to identify and quantify oviductal proteins that interact with spermatozoa; (2) study the regulation of these interactions in three stages of the estrous cycle, namely the post-, pre-ovulatory and luteal phases.

Materials and methods

Unless otherwise specified, all reagents used were from Sigma-Aldrich.

Collection and preparation of oviductal fluid

Bovine OF samples were collected and prepared as previously described (Lamy *et al.* 2016b) with slight modifications. Briefly, both oviducts and ovaries from individual adult cows were collected at a local slaughterhouse (Vendôme, France), immediately placed on ice and transported to the laboratory. The oviducts were classified into three stages of the estrous cycle based on the morphology of ovaries and corpus luteum, as previously described (Ireland *et al.* 1980): pre-ovulatory (Pre-ov, days 19–21 of the estrous cycle), post-ovulatory (Post-ov, days 1–4) and the whole luteal phase (LP, days 5–18). The oviducts ipsilateral to the side of ovulation (to the pre-ovulatory follicle at Pre-ov and to the corpus luteum at Post-ov and PL) were cleaned of surrounding tissues and the infundibulum and utero-tubal junction were cut off. Then, their content (OF+cells) was collected in a Petri dish by applying once a gentle pressure on the entire oviduct with a glass slide. This content was then aspirated with a pipette and put into a conical 1.5-mL tube. The cells were then separated from the OF by centrifugation at 2000g for 10 min at 4°C. The supernatants were then centrifuged for 10 min at 12,000g at 4°C to eliminate cellular debris. The resulting supernatants were pooled to constitute only one OF sample per stage of the estrous cycle (100–160 µL): two cows were used at Pre-ov and three at Post-ov and at LP. Protein concentrations in the OF samples were determined using the Uptima BC Assay kit (Interchim, Montluçon, France) according to the manufacturer's

instructions and using bovine serum albumin as a standard. Protein concentrations were 45.1, 50.7 and 53.7 mg/mL for pre-ov, post-ov and LP, respectively. OF samples were aliquoted in small volumes and stored at –80°C until use.

Sperm incubation with oviductal fluid and preparation of protein samples

Bovine semen previously frozen in a protein-free preservation medium (OptiXcell, IMV Technologies, L'Aigle, France) from three fertile bulls (*Bos taurus*, 0.25 mL straws, approximately 20×10^6 spermatozoa/straw) was used. Straws were thawed in a water bath at 35°C for 2 min, and then washed three times by suspension in 2 mL of phosphate-buffered saline (PBS) at 37°C followed by a centrifugation at 700g for 5 min. After the last centrifugation, the concentration of spermatozoa in the pellet was measured using a spectrophotometer (Eppendorf, Montesson, France). For each bull, a total of 20×10^6 spermatozoa were incubated for 1 h at 37°C in PBS-containing OF at Pre-ov, Post-ov or LP (final protein concentration 7 mg/mL; treated groups) or the same volume of a protein-free medium (SOF, Minitube, Tiefenbach, Germany; control group). At a given stage, the same OF sample was used for the three different bulls.

After incubation, spermatozoa were washed three times by suspension in 1 mL PBS followed by centrifugation at 2000g for 4 min. Spermatozoa were then lysed in 50 µL of Trizma base at 10 mM containing 2% (w/v) of sodium dodecyl sulfate (SDS) and 0.05% (v/v) of a protease inhibitor cocktail (P2714) for 2 min at ambient temperature and homogenized by pipetting. The samples were then centrifuged for 10 min at 15,000g. The protein concentration in the supernatant was determined using the Uptima BC Assay kit (Interchim). A fraction of each sample was kept at –80°C for immunoblots, then each sample (50 µg of proteins per lane) was fractionated separately on a 10% SDS-PAGE (50V, 30 min). The gel was stained with Coomassie (G250) and each lane was split horizontally into three bands of similar size for analysis by nanoliquid chromatography coupled to tandem mass spectrometry (nanoLC–MS/MS).

NanoLC–MS/MS analysis

Each band was in-gel digested with bovine trypsin (Roche Diagnostics GmbH) as previously described (Labas *et al.* 2015). All experiments were performed on triplicate (three technical replicates for each bull) on a LTQ Orbitrap Velos mass spectrometer coupled to an Ultimate 3000 RSLC Ultra High Pressure Liquid Chromatographer (Thermo Fisher Scientific). Five microliters of peptide extract were loaded on trap column for desalting and separating using a nano-column as previously described (Labas *et al.* 2015). The gradient consisted of 4–55% B for 90 min at 300 nL/min flow rate.

Data were recorded using Xcalibur software (version 2.1; Thermo Fisher Scientific). The instrument was operated in positive data-dependent mode. Resolution in the Orbitrap was set to $R=60,000$. In the scan range of m/z 300–1800, the 20 most intense peptide ions with charge states ≥ 2 were sequentially isolated and fragmented using collision-induced dissociation (CID). The ion selection threshold was 500 counts

for MS/MS, and the maximum allowed ion accumulation times were 200ms for full scans and 50ms for CID-MS/MS in the LTQ. The resulting fragment ions were scanned at the 'normal scan rate' with $q=0.25$ activation and activation time of 10ms. Dynamic exclusion was active during 30s with a repeat count of 1. The lock mass was enabled for accurate mass measurements. Polydimethylcyclsiloxane (m/z , 445.1200025, $(\text{Si}(\text{CH}_3)_2\text{O})_6$) ions were used for internal recalibration of the mass spectra.

In order to identify the proteins, MS/MS ion searches were performed using the MASCOT search engine (version 2.2; Matrix Science, London, UK) via Proteome Discover 1.4 software (Thermo Fisher Scientific) against a local database (369,225 entries). From the NCBI non-redundant database (download 15/01/2017), a sub-database was generated using Proteome Discover 1.4 software from keywords targeting mammalian taxonomy. The parameters used for database searches include trypsin as a protease with two missed cleavage allowed, carbamidomethylcysteine (+57Da), oxidation of methionine (+16) and N-terminal protein acetylation (+42) as variable modifications. The tolerance of the ions was set at 5 ppm for parent and 0.8Da for fragment ion matches. Mascot results from the target and decoy databases were incorporated to Scaffold software (version 4.4.4, Proteome Software, Portland, USA). Peptide identifications were accepted if they could be established with greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller *et al.* 2002). Peptides were considered distinct if they differed in sequence. Protein identifications were accepted if they could be established with greater than 95.0% probability as specified by the Protein Prophet algorithm (Nesvizhskii *et al.* 2003) and contained at least two identified peptides (false discovery rate (FDR) < 0.01%).

Label-free protein quantification, identification of sperm-interacting proteins and statistical analysis

Protein quantification was based on a label-free approach using spectral counting, as previously described (Liu *et al.* 2004, Old *et al.* 2005). All proteins with more than two peptides identified were considered for protein quantification. Scaffold Q+ software (version 4.4, Proteome Software; www.proteomesoftware.com) was used using the Spectral Count quantitative module. Quantifications performed with normalized spectral counts were therefore carried out on distinct proteins. Spectral count quantification was performed using protein cluster analysis and 'Weighted Spectra' option. The 'weight' of a given spectrum measures how much this spectrum is shared by other proteins. The normalization of spectra among samples was realized in Scaffold by adjusting the sum of the selected quantitative values for all proteins within each MS sample to a common value, which was the average of the sums of all MS samples present in the experiment. This was achieved by applying a scaling factor for each sample to each protein or protein group. Thus, numbers of normalized weighted spectra (NWS) were tabulated using experiment-wide protein clusters. The reproducibility linked directly to the nanoLC-MS/MS methodology was evaluated by the quantitative variance for each stage of the estrous cycle

(PL, Post-ov, Pre-ov, control) considering three biological replicates in three technical replicates and for each protein group (coefficients of variation in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article).

Proteins were defined as sperm-interacting proteins originating in the OF if they met the following conditions: (1) detection by nanoLC-MS/MS at a minimum level of five NWS in the three technical replicates of one or more bulls in at least one treated group (Pre-ov, Post-ov and/or LP) and (2) no detection in the control group without OF.

Mean levels of detection are presented as the means of NWS obtained from all replicates in the samples from three bulls. Data were analyzed using the GraphPad software (Prism, version 5). For interacting proteins detected at all stages, differences in protein levels between stages were assessed using the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. For interacting proteins detected at two stages, differences between stages were assessed using Mann-Whitney's test. Differences were considered significant when $P < 0.05$.

Immunoblotting

In order to validate the mass spectrometry data, four sperm-interacting proteins (ANXA2, GRP78, HSP90B1 and MYH9) detected at all stages, among which three (ANXA2, GRP78 and MYH9) were previously identified in the bull sperm proteome (Peddinti *et al.* 2008, Kasvandik *et al.* 2015) were selected. Primary antibodies used in immunoblotting were rabbit polyclonal anti-MYH9 (1:1000; sc-98978; Santa Cruz Biotechnology), rabbit polyclonal anti-GRP78 (1:400; sc-13968; Santa Cruz Biotechnology), rat monoclonal anti-HSP90B1 (1:1000; ADI-SPA-850; Enzo Life Science, Farmingdale, NY, USA) and rabbit polyclonal anti-ANXA2 (1:1000; CSB-PA001840HA01HU; Cusabio, College Park, MD, USA). All antibodies were diluted in Tris-buffered saline supplemented with 0.5% Tween 20 (TBST) and supplemented with lyophilized low-fat milk (5% w/v; TBST-milk). Secondary antibodies were goat anti-rat conjugated to horseradish peroxidase (HRP; 1:5000, sc-2006; Santa Cruz Biotechnology) or goat anti-rabbit HRP (1:5000, A6154, Sigma-Aldrich). Sperm extracts for the three bulls (30 μg proteins/lane) and OF samples (10 μg proteins/lane) were migrated on an 8–16% gradient SDS-PAGE. Liquid transfer was performed overnight at 4°C. The Western blots were blocked in TBST-milk. Ponceau red staining was used to check homogeneous loading among lanes in each blot and to normalize the data, as previously described (Romero-Calvo *et al.* 2010). Ponceau staining was quantified on the whole lane by densitometry using an Image Scanner (Amersham Biosciences, GE HealthCare Lifesciences) and analyzed using the TotalLab Quant software (version 11.4, TotalLab, Newcastle upon Tyne, UK). Then, membranes were incubated with primary antibodies under mild agitation at 37°C for 1.5 h or overnight at 4°C, and then washed and incubated with secondary antibodies for 1 h at 37°C. The peroxidase was revealed with chemiluminescent substrates (SuperSignal West Pico and West Femto Chemoluminescent Substrates, Thermo Scientific) and the images were digitized with a cooled CDD Camera (ImageMaster VDS-CL, Amersham Biosciences). The

intensity of the signals was quantified using the TotalLab Quant software (TotalLab). Data were analyzed using the GraphPad software (Prism, version 5). Differences in normalized signals between stages were assessed using the Kruskal–Wallis test followed by Dunn's test for multiple comparisons.

Analysis of molecular functions, biological process, networks and regulation of sperm-interacting proteins

The gene names of interacting proteins were determined from the protein NCBI accession numbers using UniProt Knowledgebase (UniProtKB) 'Retrieve/ID mapping' tool (<http://www.uniprot.org/uploadlists/>). The Gene Ontology (GO) analysis and pie graphs were obtained using the Protein Analysis Through Evolutionary Relationships (PANTHER) database (<http://pantherdb.org/>). Functional networks between sperm-interacting proteins were built using the STRING database, version 10.5 (<https://string-db.org/>) (Szklarczyk *et al.* 2017). STRING networks with a score of 0.4 or greater were generated using the 'Multiple proteins' module, selecting *Bos taurus* as organism and based on experimentally determined interactions, database annotation, co-expression and text mining. The initial input was the list of the 27 gene names, and then the proteins with no interaction were suppressed from the list.

In order to compare the abundance of interacting proteins on sperm with their initial abundance in the OF, interacting proteins were searched among proteins previously quantified by nanoLC–MS/MS at different stages of the estrous cycle in the bovine OF (Lamy *et al.* 2016a). The abundance of proteins in the OF at LP was calculated as the mean number of NWS in mid and late luteal phases.

Results

Identification and functional analysis of oviductal sperm-interacting proteins

Among 319 protein clusters, a total of 558 proteins were detected in sperm samples incubated in the control group or in OF at Pre-ov, Post-ov or PL (Supplementary Table 1). Among those, 27 (4.8%) were identified as oviductal sperm-interacting proteins: their names, molecular weight and known biological functions are listed in Table 1.

According to the GO analysis, 50% of the sperm-interacting proteins were classified as structural, 25% as catalytic and 22% as binding proteins. A wide range of biological process was evidenced, among which the major ones were cellular processes (31%), metabolism (22%) and cellular organization or biogenesis (21%). All molecular functions, biological processes and pathways of oviductal sperm-interacting proteins are presented in Supplementary Fig. 1. The interactions between sperm-interacting proteins were predicted from the STRING database (Fig. 1; see the list and scores of all interactions in Supplementary Table 2).

Regulation of oviductal sperm-interacting proteins according to the stage of the estrous cycle

The sperm-interacting proteins were differentially detected across the estrous cycle: 14 proteins (52%) were detected at all stages (Pre-ov, Post-ov and LP), eight (30%) at Post-ov and LP and five (18%) only at LP. The mean level of detection of the 14 proteins detected at all stages was four times higher at PL than at Pre-ov (32.3 vs 8.6 NWS, $P < 0.001$) and twice higher at PL than at Post-ov (32.3 vs 16.3 NWS; Fig. 2 and Table 1). Similarly, for the eight proteins detected only at LP and Post-ov, the mean level of detection was three times higher at PL than at Post-ov (5.5 and 1.8 NWS; $P < 0.01$; Fig. 2). For the proteins detected at all stages (Fig. 3A) or at Post-ov and LP (Fig. 4A), different patterns of variation according to the stage were identified. However, those greatly differed from the reported regulation of the same proteins in the OF (Figs 3B and 4B). By immunoblotting, ANXA2, GRP78, MYH9 and HSP90B1 were highly detected in OF samples at all stages while no signal could be seen for GRP78, MYH9 and HSP90B1 in control sperm samples (Fig. 5). In sperm samples incubated with OF, the mean ratios of Western signal intensities between stages were globally in accordance with proteomic data (Fig. 5). The effect of the stage of the estrous cycle on signal intensities was significant for HSP90B1 ($P < 0.001$) and tended to be significant for MYH9 and ANXA2 ($P = 0.08$ and 0.1 , respectively), without significant differences in pairwise comparisons.

Considering the Pre-ov stage (at which spermatozoa enter the oviduct), OVGP1 was by far the most abundant sperm-interacting protein with a mean level of detection of 69.1 NWS vs 4.0 NWS on average for the 13 other proteins detected at this stage (Fig. 3A). At Post-ov (when fertilization occurs), five proteins were predominant: ACTB, HSP27, MYH9, MYH14 and OVGP1, with means of 35.9 NWS vs 3.7 NWS for the 17 other proteins detected at this stage.

Discussion

In this study, we identified 27 sperm-interacting proteins in the bovine OF. To our knowledge, this is the first time a mass spectrometry-based approach was used to identify and quantify proteins that interact with spermatozoa. All the proteins identified as interacting with sperm cells except three (KRT1, MYL12A and TUBA1A) were previously reported in oviductal secretions in the bovine (Lamy *et al.* 2016a). Furthermore, KRT1, MYL12A and TUBA1A were reported in the OF in the equine (Smits *et al.* 2017) and/or ovine (Soleilhavoup *et al.* 2016) species. Proteins that are regulated in abundance between non-luteal (or peri-ovulatory) and luteal stages are potential candidates for a role in gamete maturation and fertilization. It is of note that nine of the sperm-interacting proteins identified (ANXA1, ANXA2,

Table 1 Bovine oviductal fluid proteins identified as interacting with sperm cells and their mean levels of detection (in normalized weighted spectra) at the pre-ovulatory (Pre-ov), post-ovulatory (Post-ov) and luteal (LP) phases of the estrous cycle (a dash means no detection).

Protein name	Accession number	Molecular weight	Gene name	Pre-ov	Post-ov	LP	Biological functions
16S ribosomal protein	AAA03646.1	16 kDa	<i>RPS16</i>	–	1.8	7.4	Biosynthetic process. Cellular component biogenesis. Cellular process
40S ribosomal protein S5	XP_014335836.1	27 kDa	<i>RPS5</i>	–	–	6.2	Biosynthetic process. Cellular component biogenesis. Cellular process
40S ribosomal protein SA	XP_014968691.1	19 kDa	<i>RPSA</i>	–	2.7	5.1	RNA localization. Biosynthetic process. Cellular component biogenesis
60S ribosomal protein L12	XP_004636380.1	18 kDa	<i>RPL12</i>	1.3	6.8	12.3	Biosynthetic process. Cellular component biogenesis. Cellular process
60S ribosomal protein L7	ELW70554.1	26 kDa	<i>RPL7</i>	–	2.5	8.3	Biosynthetic process. Cellular component biogenesis. Cellular process
60S ribosomal protein L8	ELR49951.1	24 kDa	<i>RPL8</i>	–	2.5	6.4	Biosynthetic process. Cellular process, translation
78 kDa glucose-regulated protein	NP_001068616.1	72 kDa	<i>GRP78</i> or <i>HSPA5</i>	4.1	5.8	41.4	Protein folding, response to a stress
Actin, cytoplasmic 1	OBS83020.1	42 kDa	<i>ACTB</i>	7.4	20.9	23.2	Cellular component organization. Cytokinesis. Endocytosis
Annexin A2	XP_008581381.1	39 kDa	<i>ANXA2</i>	0.6	3.5	11.5	Fatty acid metabolic process
Annexin A1	CAX39971.1	39 kDa	<i>ANXA1</i>	–	0.4	4.2	Fatty acid metabolic process
Elongation factor 1-alpha 1	XP_008572262.1	49 kDa	<i>EEF1A1</i>	8.1	13.5	16.7	Biosynthetic process. Catabolic process. Cellular process
Endoplasmic	NP_777125.1	92 kDa	<i>HSP90B1</i>	2.3	4.1	36.0	Protein folding, Response to a stress
Ezrin	NP_776642.1	69 kDa	<i>EZR</i>	–	–	3.0	Cellular component morphogenesis. Cellular process
Heat shock 27 kDa protein 1	ANO81440.1	22 kDa	<i>HSP27</i>	0.4	31.4	72.8	Immune system process. Protein folding, Response to a stress
Keratin, type II cytoskeletal 1	XP_009246044.1	39 kDa	<i>KRT1</i>	–	–	1.9	Cellular component morphogenesis. Cellular process
Myosin regulatory light chain 12A	BAD96289.1	20 kDa	<i>MYL12A</i>	–	1.5	3.2	Mesoderm development. Muscle contraction
Myosin-14	XP_010822105.1	229 kDa	<i>MYH14</i>	0.1	64.4	81.5	Cellular component morphogenesis. Cellular component movement. Cytokinesis
Myosin-9	NP_001179691.1	227 kDa	<i>MYH9</i>	15.9	42.4	36.3	Cellular component morphogenesis. Cellular component movement. Cytokinesis
Myosin-VI	NP_001193001.1	149 kDa	<i>MYO6</i>	1.3	2.1	14.4	Cellular component morphogenesis. Cellular component movement. Cytokinesis
Oviduct-specific glycoprotein 1	XP_010801455.1	61 kDa	<i>OVGP1</i>	69.1	20.3	72.6	Catabolic process. Nitrogen compound metabolic process
Protein disulfide isomerase A6	AAI48887.1	50 kDa	<i>PDI A6</i>	–	–	14.6	Cellular process. Response to a stress
Protein disulfide isomerase A4	DAA30269.1	72 kDa	<i>PDI A4</i>	–	–	4.1	Cellular process. Response to a stress
Protein disulfide isomerase A3	NP_776758.2	57 kDa	<i>PDI A3</i>	1.0	1.9	23.2	Cellular process. Response to a stress
Ribosomal protein L6	ABY75291.1	20 kDa	<i>RPL6</i>	–	2.5	3.1	Biosynthetic process. Cellular component biogenesis. Cellular process
Ribosomal protein S8	BAC40485.1	24 kDa	<i>RPS8</i>	–	0.4	6.1	Biosynthetic process. Cellular component biogenesis. Cellular process
Tubulin alpha-3 chain	XP_003801738.2	46 kDa	<i>TUBA1</i>	2.5	7.0	5.3	Cellular component morphogenesis. Cellular component movement. Chromosome segregation
Uncharacterized protein C1orf194	OBS65308.1	16 kDa	<i>C1orf194</i>	6.4	3.5	5.1	Transmembrane transporter activity

Biological functions were retrieved from PANTHER database.w

Figure 1 STRING network showing the interactions between the sperm-interacting proteins. The line thickness is proportional to the strength of data support (Supplementary Table 2 for the scores obtained for each interaction).

EEF1A1, EZR, GRP78, HSP90B1, MYH9, OVGP1, PDIA4) were differentially abundant in the bovine OF between Pre- or Post-ov and LP, all of them being more abundant around the time of ovulation than during the luteal phase (Lamy *et al.* 2016a).

Proteins were defined as interacting with sperm cells if they were detected at significant levels (>5 NWS) in at least one group supplemented with OF and not detected in the control group, which contained only sperm proteins. However, 10 out of the 27 sperm-interacting proteins were also previously reported as endogenous bull sperm proteins using mass spectrometry: ACTB, ANXA1, ANXA2, EEF1A1, GRP78, MYH9, MYH14, PDIA6, RPL8 and TUBA3 (Peddinti *et al.* 2008). Peddinti *et al.* (2008) used differential detergent fractionation of sperm-protein extracts, allowing the detection of 3799 proteins in bull sperm. Thus, the 10 endogenous sperm proteins were probably under the detection limit of the method used, leading to their absence in the control group. Nevertheless, using the same quantification method, most of these proteins (ANXA1, ANXA2, MYH14, MYH9, GRP78 and PDIA6) were among the most abundant proteins quantified in the bovine OF (>47 NWS on average across the estrous cycle) (Lamy *et al.* 2016a). Furthermore, by Western blot analysis, MYH9 and GRP78 could not be detected in sperm-protein extracts from the control samples while the signal for ANXA2 was very weak. Thus, it is likely that these proteins present at high level in the OF interacted with sperm cells and were identified only as interacting proteins despite their presence at probably much lower levels among endogenous sperm proteins.

The GO analysis of those 27 proteins showed their main involvement in structural function and intracellular process, which may be surprising for proteins originating from the OF. Although the cells and cell debris were eliminated by centrifugations from the OF before sperm

incubation, the presence of proteins released from cells in the OF at the time of collection could not be excluded. Nevertheless, similar proportions of proteins classified as non-secreted intracellular were previously reported in the OF in several mammals including the bovine (Lamy *et al.* 2016a) ovine (Soleilhavoup *et al.* 2016), porcine (Georgiou *et al.* 2005) and equine (Smits *et al.* 2017). Furthermore, the oviductal epithelium is known to release extracellular vesicles (EVs) in the OF: these oviductosomes have been identified in the murine and bovine species (Al-Dossary & Martin-Deleon 2016). Based on the recently reported protein content of bovine *in vivo*-derived oviductosomes (Alminana *et al.* 2017), 21 out of the 27 identified sperm-interacting proteins could originate from EVs, including OVGP1, GRP78, ANXA1, ANXA2, MYH 9 and 14. Moreover, there is evidence that EVs regulate sperm maturation via the direct transfer of essential proteins to sperm in both male and female genital tracts (Barkalina *et al.* 2015). In particular, the plasma membrane Ca²⁺ ATPase 4 (PMCA4), a sperm-protein essential for fertilizing ability, has been shown to be delivered by fusion with oviductal EVs in murine sperm (Al-Dossary *et al.* 2015). These

Figure 2 Mean abundance of sperm-interacting proteins across the estrous cycle for proteins detected at all stages (A; *n* = 14) or at LP and Post-ov (B; *n* = 8). LP, luteal phase; NSC, normalized spectral counts; Post-ov, post-ovulatory phase; Pre-ov, pre-ovulatory phase of the estrous cycle.

Figure 3 Abundance of oviductal sperm-interacting proteins according to the stage of the estrous cycle for proteins detected at all stages (A) and variations of the same proteins in the bovine oviductal fluid proteome, adapted from [Lamy et al. \(2016a\)](#) (B). *Significant differences between Pre-ov and Post-ov; \$Significant differences between Pre-ov and LP; #Significant differences between Post-ov and LP (P -value < 0.05). LP, luteal phase; NSC, normalized spectral counts; Post-ov, post-ovulatory phase; Pre-ov, pre-ovulatory phase of the estrous cycle.

interactions between EVs of uterine or oviductal origin and spermatozoa may occur in a short time interval: even a 15-min incubation of spermatozoa with fluorescent labeled uterine EVs was sufficient to retrieve sperm with fluorescent staining, indicating the rapid fusion of EVs with sperm membrane ([Franchi et al. 2016](#)). Thus, it may be that some of the sperm-interacting proteins identified in the present study were delivered to sperm membrane after fusion with oviductal EVs. Further research is needed to elucidate which oviductal proteins bind to the sperm membrane and which may be incorporated within the sperm membrane by fusion with EVs.

A few sperm-interacting proteins identified in the present study were previously shown by immunolabeling approaches to interact with mammalian spermatozoa. This is the case for OVGP1, which was the most abundant interacting protein identified at the Pre-ov stage. OVGP1 was previously shown to interact with sperm in the bovine ([King & Killian 1994](#), [Abe et al. 1995b](#)), human ([Lippes & Wagh 1989](#)) and hamster

Figure 4 Abundance of oviductal sperm-interacting proteins detected at Post-ov and LP (left) or only at LP (A) and variations of the same proteins in the bovine oviductal fluid proteome across the estrous cycle, adapted from [Lamy et al. \(2016a\)](#) (B). *Significant differences between Post-ov and LP; #Significant differences between Pre-ov and Post-ov (P -value < 0.05). LP, luteal phase; Post-ov, post-ovulatory phase; Pre-ov, pre-ovulatory phase of the estrous cycle.

([Yang et al. 2015](#)) species. Furthermore, bovine OVGP1 has been shown to promote sperm capacitation, motility and viability *in vitro* ([King et al. 1994](#), [Abe et al. 1995b](#)). OVGP1, together with the myosins 9 and 14, HSP27 and ACTB, were among the most abundant sperm-interacting proteins at the Post-ov stage. Of interest, MYH9 has been identified on human sperm as a binding partner of OVGP1 ([Kadam et al. 2006](#)). Based on the STRING predicted networks between sperm-interacting proteins, MYH9 may also interact with MYH14 and ACTB. Thus, OVGP1, MYH9/14 and ACTB may form protein complexes in the OF before interacting with the sperm surface in order to modulate sperm capacitation around the time of fertilization.

Another network detected by the STRING database included HSP90B1 (also known as endoplasmic or GRP94), GRP78 (or HSPA5) and three protein disulfide isomerases (A3, A4 and A6). An association in the porcine OF between HSP90B1 and PDIA4 was previously proposed as playing a role in oocyte zona pellucida hardening and monospermia ([Mondejar et al. 2013](#)). Furthermore, the protein disulfide isomerases at the sperm head surface seem to play an important

Figure 5 Western blotting of myosin 9 (MYH9), endoplasmic (HSP90B1), annexin A2 (ANXA2) and 78-kDa glucose-regulated protein (GRP78, also known as HSPA5) in sperm samples incubated with or without (Control) oviductal fluid (OF) at pre- (Spz-Pre-ov), post-ovulatory (Spz-Post-ov) and luteal phase (Spz-LP) of the estrous cycle and in OF samples used for incubation (OF-Pre-ov, OF-Post-ov, OF-LP). Mean ($\pm$ S.E.M.) ratios of normalized signal intensities obtained for sperm samples between stages are indicated on the right ($n=3$ bulls).

role in gamete fusion in mammals (Ellerman *et al.* 2006, Wong *et al.* 2017). In human, the blocking of PDIA3 function by specific antibodies reduced the binding capacity of spermatozoa to bind to the zona pellucida (Wong *et al.* 2017). Moreover, the heat shock protein GRP78 has been identified on the luminal surface of oviductal epithelial cells and shown to associate with bull spermatozoa (Boilard *et al.* 2004). GRP78 was also identified in the human OF and recombinant GRP78 was able to bind to human sperm acrosomal region and to modulate sperm-zona pellucida binding (Marin-Briggiler *et al.* 2010). Taken together, these data suggest that GRP78, HSP90B1 and PDIA3/A4, acting individually or after interaction, are involved in the regulation of sperm-oocyte interaction. However, the biological functions and interactions associated with the identified proteins are mainly based on the current knowledge in the intracellular/membrane compartments and on *in vitro* data. Such hypotheses remain to be studied.

The annexins A1 and A2 (ANX A1-2), but also A4 and A5, have been stated as main receptors for bovine sperm on oviduct epithelial cells for the formation of the oviductal sperm reservoir (Hung & Suarez 2010, Talevi & Gualtieri 2010). To support this, ANX A1 and A2 were immunolocalized at the luminal surface of the bovine oviduct epithelium (Ignatz *et al.* 2007). Furthermore, sperm binding to explants of oviductal epithelium was inhibited *in vitro* in the presence of anti-ANXAs antibodies (Ignatz *et al.* 2007). There is also evidence that ANXA2 binds to boar sperm membranes (Teijeiro *et al.* 2009). The presence of several isoforms of annexins has been reported in the OF in several mammalian species including the ovine (Soleilhavoup *et al.* 2016), equine (Smits *et al.* 2017), porcine (Mondejar *et al.* 2012) and bovine (Lamy *et al.* 2016a). However, the roles of secreted annexins in the OF were poorly investigated. It has been hypothesized that annexins secreted at the post-ovulatory stage might promote the release of

spermatozoa from the sperm reservoir by competing for their binding sites on sperm (Coy *et al.* 2012). Consistent with this hypothesis, in the present study, ANXA2 was found to be more abundant on sperm at Post-ov than at Pre-ov. However, further research is needed to identify the exact roles of secreted annexins in sperm function.

Important variations in the abundance of sperm-interacting proteins according to the stage of the estrous cycle were evidenced. However, these variations greatly differed from those previously reported for the same proteins and using the same method of quantification in the bovine OF (Lamy *et al.* 2016a). For instance, the abundance of OVGP1 on sperm cells was 3.5 times higher at Pre-ov and LP than at Post-ov, whereas in the OF, OVGP1 was more abundant at Post-ov than at the two other stages. Another example is ANXA1, which was quantified as the most abundant protein in the bovine OF proteome at Pre-ov (Lamy *et al.* 2016a); however, sperm did interact with ANXA1 only at Post-ov and LP. Last, proteins that interact with sperm cells only at LP were detected in the OF at equivalent levels (PDIA6, RPS5) or even at higher levels (PDIA4, EZR) at other stages in the cycle. Thus, not all proteins in the OF were taken up by spermatozoa and interactions did not depend on the initial abundance of oviductal proteins. This suggests that the process of sperm-protein interaction was highly selective, in accordance with previous studies that used other experimental approaches like radiolabeled or biotin-tagged proteins (McNutt *et al.* 1992, Rodriguez & Killian 1998, Killian 2004).

Noteworthy, more sperm-interacting proteins were detected at the luteal phase than at Pre-ov and Post-ov. Furthermore, the sperm-interacting proteins detected at all stages or at LP and Post-ov were on average more abundant at LP than at peri-ovulatory stages. Although mating and insemination do not happen out of the peri-ovulatory period in domestic mammals, various effects of luteal OFs or proteins on bovine sperm physiology were reported (McNutt & Killian 1991, Abe *et al.* 1995a, Grippo *et al.* 1995, Kumaresan *et al.* 2005, 2006). In particular, oviductal proteins collected from cows in both the luteal and non-luteal phases had beneficial effects on sperm motility, viability and acrosome integrity compared with the control group (Boquest *et al.* 1999, Kumaresan *et al.* 2005, 2006). However, some of these effects were modulated according to the stage of the estrous cycle, in that non-luteal proteins were more beneficial than luteal ones (Kumaresan *et al.* 2005, 2006). Thus, it seems that some common proteins in the OF interacted with spermatozoa whatever the stage of the cycle but with different intensities, leading to different effects on sperm function. Furthermore, it has been shown that the porcine oviduct not only at peri-ovulatory but also at luteal phases of the cycle was able to store spermatozoa after intrauterine insemination, showing that *in vivo* the function of the sperm reservoir was not restricted to the ovulatory period (Brussow *et al.*

2014). Noteworthy, spermatozoa were stored in higher numbers in the oviduct after insemination at the luteal phase than at the peri-ovulatory stage but the proportion of damaged and abnormal spermatozoa in the sperm reservoir was also higher (Brussow *et al.* 2014). Taken together, these data suggest that there is a link between the ability of oviductal proteins to interact with sperm cells and the ability of the oviduct to store spermatozoa. A high abundance of interacting proteins on sperm cells and/or oviductal proteins that specifically interact at LP might have detrimental effects on spermatozoa.

In conclusion, a proteomic approach made it possible to identify and quantify for the first time potential sperm-interacting proteins originating in the OF. These include already known sperm-interacting proteins and a number of new candidates that may modulate sperm survival and function related to fertilization. Further studies will be required to determine the exact roles of these proteins and their mechanism of interaction, by binding or fusion with the sperm membrane. These interacting proteins were differentially abundant according to the stage of the cycle, highlighting important regulations of selective sperm–oviduct interactions *in vivo*.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-17-0712>.

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.

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