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Modulation of The Oviductal Environment by Gametes

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The notion of a gamete recognition system that alerts females to the presence of gametes in their reproductive tract profoundly influences our understanding of the physiology of events leading to conception and the bearing of offspring. Here, we show that the female responds to gametes within her tract by modulating the environment in which pregnancy is initially established. We found distinct alterations in oviductal gene expression as a result of sperm and oocyte arrival in the oviduct, which led directly to distinct alterations to the composition of oviductal fluid *in vivo*. This suggests that either gamete activates a cell-type-specific signal transduction pathway within the oviduct. This gamete recognition system presents a mechanism for immediate and local control of the oviductal microenvironment in which sperm transport, sperm binding and release, capacitation, transport of oocytes, fertilization, and early cleavage-stage embryonic development occur. This may explain the mechanisms involved in postcopulatory sexual selection, where there is evidence suggesting that the female reproductive tract can bias spermatozoa from different males in the favour of the more biologically attractive male. In addition, the presence of a gamete recognition system explains the oviduct's ability to tolerate spermatozoa while remaining intolerant to pathogens.

Keywords: Oviduct • Spermatozoa • Oocyte • Maternal Communication • Quantitative Proteomics

Introduction

The mammalian oviduct is the venue of important events leading to the establishment of pregnancy. The oviductal environment is currently considered to be solely regulated by ovarian hormones which influence the composition of oviductal secretions in order to support events such as sperm and oocyte transport, sperm binding and release, sperm capacitation, fertilization, and early-embryonic development within the oviduct.[1,2]

However, current understanding of oviductal physiology and regulation of the oviductal environment cannot explain a number of biological phenomena. For example, the mechanisms by which allogenic spermatozoa are tolerated by the oviduct are unknown. Despite highly active innate and adaptive immune oviductal defences,[3] allogenic spermatozoa are selectively sequestered by the oviduct, and their viability is actively maintained prior to fertilization.[4] Immune cells such as neutrophils and antibodies are present in greater concentrations during oestrus compared with any other stage of the reproductive cycle,[5] and it is generally accepted that the susceptibility of the genital tract to infection is reduced during oestrous.[6] Yet, this is exactly the time that mating occurs and allogenic spermatozoa traverse the female reproductive tract. Components of seminal plasma are thought to confer protection against immune attack in the lower regions of the female reproductive tract.[7] However, there is little likelihood that seminal plasma reaches the oviduct after mating due to the filtering nature of the utero−tubual junction.[8] The masking of sperm antigens as a mechanism for evasion of the immune response has also been the subject of speculation.[9]

Furthermore, current understanding of oviduct function cannot provide an explanation for the mechanisms involved in postcopulatory sexual selection, where the ability of the female reproductive tract to preferentially select spermatozoa from males with so-called 'good genes' or reject genetically incompatible spermatozoa has been demonstrated.[10−12] The inability of spermatozoa carrying genetic disorders to move through the utero−tubal junction into the oviduct provides an example.[13,14]

The molecular basis of spermatozoa tolerance within the female reproductive tract, and postcopulatory sexual selection

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by the female reproductive tract, can be explained if it could be demonstrated that a gamete recognition system exists in the female reproductive tract. To prove the existence of such a system in the female reproductive tract, we hypothesized that the presence of gametes in the oviduct *in vivo* leads to an alteration of the oviductal secretory proteomic profile. Furthermore, we anticipated that alterations in the oviductal microenvironment in response to gametes *in vivo* are specific. Therefore, we additionally hypothesized that the oviduct's reaction to spermatozoa would be different from its reaction to oocyte(s).

Here, we report altered oviductal protein secretions in response to the presence of spermatozoa and oocytes in the oviduct *in vivo* at the time of ovulation. Using minimally invasive surgical technologies, oviductal fluid from a gamete-stimulated oviduct was directly compared to a non-gamete-stimulated oviduct that originated from the same animal. Limiting our experimental approach to individual animals avoided analytical complications arising from the high degree of biological variation in the precise composition of oviductal fluid between different individuals.[15] Isotope coded affinity tag (ICAT) technology[16,17] was used to relatively quantify oviductal secretory protein changes specific to each gamete. Results were verified in several candidate proteins using Western blot analysis, and analysis of gene expression identified a correlation between altered gene and protein expression profiles. All of the altered proteins identified are well-known to influence gamete maturation, viability, and function; yet, this is the first report to demonstrate their regulation by both of the gametes *in vivo*. This report points to the existence of a gamete recognition mechanism *in vivo* that alerts females to the presence of gametes in their reproductive tract.

Materials and Methods

Animal Preparation. Experiments were conducted on an experimental pig farm under environmentally controlled conditions at the University of Murcia (Murcia, Spain). All experiments were performed after obtaining local ethical committee approval. Sows were observed for signs of *oestrus*. A total of 15 sows *in oestrus* were selected and randomly assigned to three different experimental groups in equal numbers.

Experimental Design. Experiments were designed to examine the effect of (i) sperm presence in the oviduct, (ii) oocyte presence in the oviduct, and (iii) surgical interventions on the oviduct (control). Sows were anaesthetized with sodium thiopental (7 mg/kg body weight), and maintained under anesthesia with halothane (3.5–5%). They were individually prepared for laparoscopic surgery. Figure 1 provides a schematic overview of the surgical model employed. In the experiments examining the effect of sperm presence in the oviduct, both oviducts were clipped at the infudibulum using titanium clips (Endo Clip II 10 mm; Tyco Healthcare, Mansfield, MA) to prevent oocytes from entering the oviducts, and one uterine horn was cut using titanium staples (EndoGIA Universal 60/4.8; Tyco Healthcare, Mansfield, MA) to prevent sperm from entering that oviduct (Figure 1a). Following surgery, sows were returned to their accustomed environment. Twenty-four hours following surgery, sows were artificially inseminated with Beltsville Thawing solution (BTS)-diluted semen (3000×10^6 spermatozoa in a dose of 100 mL), or with BTS-diluent only (no sperm; surgical control). In the experiments examining the effect of oocyte presence in the oviduct, one oviduct was clipped to prevent oocytes from entering that oviduct (Figure 1b). All sows were then examined for signs of physiological ovulation by transrectal ultrasonography (Pie Medical SC100 Scanner, Maastricht, The Netherlands). Twenty-four hours after ovulation and artificial insemination (AI), sows were anesthetized as above, and the oviducts were exposed via a midventral laparotomy under aseptic conditions. Ovaries were visually inspected for signs of ovulation.

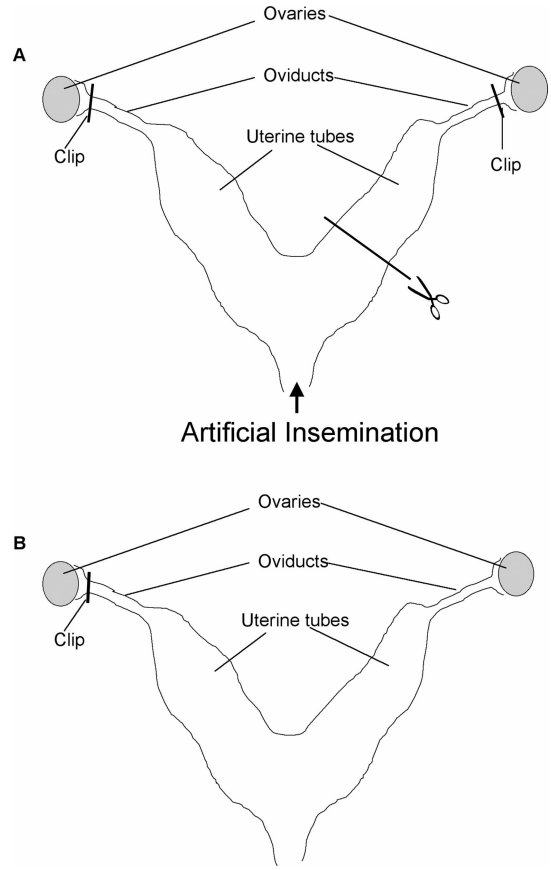

Figure 1. Sows were subjected to laparoscopic surgery. (A) In the experiments examining the effect of sperm, oviducts were clipped to prevent oocytes from entering the oviduct, and one uterine horn was cut to prevent sperm from entering that oviduct. Sows were then artificially inseminated with diluted semen (sperm) or diluent only (surgical control). (B) In the experiments examining the effect of oocytes, one oviduct was clipped to prevent oocytes from entering that oviduct after ovulation.

Oviductal Fluid Collection. Oviducts were trimmed free of surrounding tissue and ligated at the infundibulum and at the isthmus−uterus junction. Oviducts were flushed within 30−90 s after excision with 20 mL of PBS using a needleless syringe, and all fluid was collected. A protease inhibitor solution (phenylmethylsulfonyl fluride; PMSF; Sigma, Dorset, U.K.) was added to the collected media to a final concentration of 1 mM. All samples were immediately centrifuged at 12 000*g* for 5 min to remove spermatozoa, oocytes, detached oviductal cells, or any other debris. The supernatant was collected, immediately snap-frozen in liquid nitrogen, and stored at −70 °C until further processing. In all cases, the pellet was examined under a light microscope for the presence of oocytes to confirm whether oocytes had successfully entered the oviducts or, in the experiments where oocyte entry was unwanted, to confirm that clipping the oviduct prevented oocyte entry into the

oviduct after ovulation. In cases where clips had failed, or it was unsure whether the animal had ovulated, samples were discarded.

Isolation of Oviductal Epithelial Cell RNA. After oviductal fluid collection, epithelial cells were isolated by opening the oviduct longitudinally and scraping the mucosal epithelial layer with a sterile glass slide. This resulted in obtaining highly enriched samples of oviductal epithelial cells. Cells were transferred immediately to Trizol (Invitrogen, Paisley, U.K.), and total RNA was isolated according to the manufacturer's instructions. The purified total RNA pellet was resuspended in ethanol, immediately snap-frozen in liquid nitrogen, and stored at −70 °C until further processing.

Sample Preparation for ICAT Labelling. Oviductal fluid samples were thawed and ultracentrifuged at 100 000*g* for 30 min. The supernatant was collected, and samples from animals in the same experimental group were pooled. Samples were then albumin- and IgG-depleted using a ProteoPrep Blue Albumin depletion kit (Sigma) according to the manufacturer's instructions. A Plus-one 2D clean up kit (Amersham Biosciences, Buckinghamshire, U.K.) was used according to the manufacturer's instructions to purify, desalt, and remove all impurities from the protein samples. The resulting protein pellets were dissolved in denaturing buffer (50 mM Tris and 0.1% SDS), for ICAT labelling.

Labelling Proteins with ICAT Reagents. A Bicinchoninic acid (BCA) assay was performed as previously described by Smith et al.[17] to determine the protein concentration of each oviductal fluid sample. Briefly, 10 μL of each protein sample was added to 200 μL of 2% (v/v) copper sulphate solution in BCA and incubated at 37 °C in the dark for 30 min. Absorbance was read at 570 nm using a Benchmark 96 well plate reader (Bio-Rad). One-hundred micrograms of each oviductal fluid protein sample was dissolved in 80 μL of denaturing buffer (50 mM Tris and 0.1% SDS). Protein samples were reduced by adding 2 μL of 50 mM Tris(2-carboxy-ethyl)phosphine hydrochloride (TCEP; Sigma). The samples were boiled at 100 °C for 10 min. A reduced protein sample from the sperm-oviduct or control oviduct was transferred into a vial containing ICAT heavy or light reagent (Applied Biosystems, Framingham, MA), respectively. Samples were vortexed thoroughly. After a brief centrifugation, samples were incubated for 2 h at 37 °C in the dark. The isotope-labelled samples were then combined into one tube and dried using a vacuum centrifuge.

Separation of Labelled Proteins by SDS-PAGE and In-Gel Trypsin Digestion. The dried ICAT-labelled proteins (100 μg from sperm-oviducts and 100 μg from control oviducts) were reconstituted in 40 μL of SDS electrophoresis sample buffer prior to separation on a 12% SDS-polyacrylamide gel. The gel was briefly stained with Coomassie blue. The gel lane containing the ICAT-labelled samples was cut horizontally into 13 bands of similar size. Each gel band was diced into approximately 1 mm^3 pieces and then destained with 200 mM ammonium bicarbonate with 40% (v/v) acetonitrile. The gel pieces were incubated overnight in a trypsin solution of 0.4 μg of trypsin (Promega, Southampton, U.K.) and 50 μL of 50 mM ammonium bicarbonate. The next day, peptides were extracted in four sequential extraction steps using 30 μL of 25mM NH_4CO_3 (10 min, room temperature), 30 μL of acetonitrile (15 min, 37 °C), 50 μL of 5% (v/v) formic acid (15 min, 37 °C), and finally, with 30 μL of acetonitrile (15 min, 37 °C). All extracts were pooled and dried in a vacuum centrifuge.

Affinity Purification and Cleavage of ICAT-Labelled Peptides. To affinity-purify the ICAT-labelled peptides, dried peptides were resuspended in 500 μL of PBS (20 mM NaH_2PO_4 and 300 mM NaCl, pH 7.2) prior to loading the ICAT-labelled peptides onto an avidin cartridge (Applied Biosystems). The column-bound peptides were washed sequentially with 1 mL of PBS (10 mM NaH_2PO_4 and 150 mM NaCl, pH 7.2), 1 mL of 50 mM ammonium bicarbonate with 20% methanol (pH 8.3), and 1 mL of distilled and deionized water before peptides were eluted with 30% (v/v) acetonitrile and 0.4% (v/v) formic acid (pH 2). The eluate from the avidin cartridge was dried in a vacuum centrifuge. ICAT-labelled peptides were resuspended and incubated with a 95:5 ratio of ICAT cleavage reagent A and cleavage reagent B (Applied Biosystems) at 37 °C for 2 h to cleave the affinity tag (biotin) from isotope-coded peptides. Peptides were dried in a vacuum centrifuge.

Identification and Quantification of Proteins by Mass Spectrometry. The lyophilized ICAT-labelled peptide mixture was resuspended in 0.1% (v/v) formic acid in 3% acetonitrile. This mixture was separated on a PepMap C18 RP capillary column (LC-Dionex, Leeds, U.K.), and eluted in a 60-min gradient via a LC packings Ultimate nanoLC directly into the mass spectrometer. The compositions of the hydrophilic and hydrophobic solvents were 5% (v/v) acetonitrile and 0.1% (v/v) formic acid, and 95% (v/v) acetonitrile and 0.1% (v/v) formic acid. An Applied Biosystems/MDS-Sciex QStarXL electrospray ionisation quadrapole time-of-flight tandem mass spectrometer (ESI-qQ-TOF) was used for mass spectrometric analysis. Analyst Qs software (service pack 8; Applied Biosystems) was used for data acquisition and data analysis. The data acquisition was performed in the positive ion mode using Information Dependent Acquisition (IDA). After each TOF-MS scan, three peaks with charge states two or three were selected for tandem mass spectrometry.

The ProICAT software package (version 1.0, service pack 2; Applied Biosystems) was used for the identification and quantitation of proteins based on the peptides analysed during the LC−MS/MS experiment. Manual examination of all MS spectra was additionally performed to confirm software-based quantitation. Quantification was only considered valid for spectra that appeared dramatically above background ions and contained no contaminating ion peaks. The peptide-based protein matches provided by ProICAT were additionally confirmed using MASCOT 2.0 software (www.matrixscience.com; MASCOT script for Analyst Qs version 1.6b10). A sequence query search was performed using the NCBInr data base (November, 2004; 4684543 entries). The taxonomy was limited to filter for only mammalian matches, and trypsin was used as enzyme, with one missed cleavage site allowed. The peptide tolerance was set to 1.0 Da, and the MS/MS tolerance was set to 0.25 Da. ICAT modifications of cysteine residues were set as variable modifications. Only individual ion scores indicating extensive homology ($p < 0.05$) were considered as valid.

Western Blot Analysis. Oviductal fluid proteins (10 μg of protein) that had not been albumin-depleted were separated on self cast homogeneous 12% polyacrylamide SDS-PAGE gels. Resolved proteins were transferred to a Polyvinylidene fluoride (PVDF) Immobilon P^{SQ} transfer membrane (0.2 μm pore size) (Millipore) using a BioRad Mini Trans-blot electrophoretic transfer cell (Bio-Rad Laboratories Ltd., Herts, U.K.). Following transfer, membranes were blocked with 5% (w/v) non-fat milk

powder in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) overnight at 4 °C. Blocked membranes were then incubated with either of the following antibodies: anti-fibrinogen (Autogen Bioclear), goat anti-complement C3 monoclonal antibody (Calbiochem), rabbit anti-retinol binding protein polyclonal antibody (Dakocytomation), or rabbit anti-oviduct specific glycoprotein. Antibodies were diluted 1:1000, 1:1000, 1:200, and 1:2000, respectively, in 5% (w/v) milk powder in TBST and incubated for 1 h at room temperature. After three washes with TBST, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h. After three more washes with TBST, immunoreactive proteins on the membranes were detected using SuperSignal West Dura chemiluminescent reagents (Perbio Science UK Ltd., Northumberland, U.K.) and exposure to Hyperfilm ECL high-performance chemiluminescent film (Amersham).

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction. Oviductal total RNA was treated with DNase I (DNA-free Kit; Ambion, Huntingdon, U.K.) to remove genomic DNA contamination from the samples. First-strand cDNA synthesis was performed using the Superscript II reverse transcription system (Invitrogen), following the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using an iCycler iQ Real-Time PCR detection system (Bio-Rad Laboratories, Ltd.) and iQ SYBR Green supermix (Bio-Rad Laboratories, Ltd.). The PCR primers used were as follows: complement C3 forward, CGGTGTTGCCAAACTGAGC; complement C3 reverse, TCGTGATGGTCAAGGGCAG; retinol binding protein (RBP) forward, TTCCGAGTCAAAGAGAACTTCG; RBP reverse, TCATAGTCCGTGTCGATGATCC; oviductin forward, TGACCGCTGGAACTTTCTCT; oviductin reverse, GTTGTGTCCCGTGACCTTCT; beta-actin forward, ACCACTGGCATTGTCATGGACTCT; beta-actin reverse, ATCTTGATCTTCATGGTGCTGGGC.

All real-time PCR reactions were carried out for 40 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. The quantification data were analyzed with the iCycler iQ software package (Bio-Rad Laboratories, Ltd.). Quantification of PCR products was performed by comparing the threshold cycles (Ct) of all samples run in triplicate. All values were normalized against beta-actin housekeeping gene Ct values, using the '2(-delta delta Ct) method'[16] to give an expression value relative to the internal control of β-actin expression. A student's t test was used to test the significance of difference between expression values of sperm versus no sperm, oocyte versus no oocyte, and diluent versus no diluent oviducts.

Results

Oviductal fluid was collected from oviducts following 24 h exposure to sperm, oocytes, or diluent, and their respective controls (Figure 1). Oviductal fluid proteins were then processed for ICAT labelling.

ICAT. After ICAT labelling, oviductal fluid proteins were fractionated by SDS-PAGE. Each SDS-PAGE lane was cut into 13 bands of approximately equal size, these subsequently were digested and analysed by LC-ESI−MS/MS. Figure 2 provides an overview of the experimental workflow followed.

Sequence database searching with the collision induced dissociation (CID) spectra obtained by this study resulted in approximately 1200 identified, cysteine-containing peptides that were matched to 271 proteins by ProICAT software. Peptide and protein matches were accepted only when confirmed by significant database matching scores after the data was re-analysed using Mascot. The quantification data calculated for each peptide pair by ProICAT was accepted only when visual examination of the Tof-MS spectra revealed that peptides appeared dramatically above background ions, and contained no contaminating peaks. Detailed information of peptides identified is provided in Supporting Information.

A total of 32 oviductal fluid proteins were conclusively identified and quantified on the basis of the relative abundance of heavy to light peptide pairs that originated from a particular protein (Table 1). Twenty-five (~80%) of these proteins were quantified on the basis of two or more heavy and light peptide pairs, thereby increasing confidence in quantification. The heavy-to-light ratios for all the peptide pairs identified in each complete ICAT experiment averaged a fold-change of approximately one, confirming that there was no protein loading bias in any of the experiments.

In the case of the diluent versus no diluent oviduct experiment, designed to examine the effects of surgical interventions, nearly all of the proteins showed no signs of alteration. The exception was haemoglobin beta chain, which showed less than 2-fold increased abundance in the oviductal fluid of the oviduct attached to the uterine horn that had been cut during surgery. In comparison, proteins witnessed as regulated in response to either sperm or oocyte presence in the oviduct showed regulatory changes that were far more dramatic. Of these, 19 and 3 proteins showed greater than 2-fold alteration by sperm and oocyte presence in the oviduct, respectively.

Validation of Observed Protein Expression Profiles. To confirm some of the changes in protein expression as a result of gamete presence in the oviduct, we performed Western blot analysis using antibodies against fibrinogen, complement C3, retinol binding protein (RBP), and oviduct-specific glycoprotein (OGP) (Figure 3). These were the only readily available antibodies that we found to be reactive against pig proteins. The Western blot analysis confirmed ICAT findings for all the proteins examined.

We additionally performed quantitative real-time PCR analyses to examine the gene expression profiles of a number of differentially expressed proteins. Real-time PCR was performed on pooled cDNA samples obtained from oviductal cells, and all expression data was normalized against the β-actin housekeeping gene (Figure 4). Sperm presence in the oviduct resulted in significant increases in oviductal gene expression values of complement C3, OGP, and RBP genes. Oocyte presence in the oviduct resulted in a significant decrease in oviductal C3 expression and a significant increase in oviductal RBP expression. Presence or absence of diluent resulted in no significant differences in expression of any of these genes, indicating that our surgical interventions did not affect the expression of these genes.

Discussion

Our present investigation conclusively demonstrates that the oviduct alters its microenvoinment in response to the presence of spermatozoa and oocytes in the oviduct *in vivo* at the time of ovulation by alteration of its secretory protein profile.

We had previously identified a high degree of biological variation in the precise composition of oviductal fluid between different animals.[15] This variation was presumably the result of genetic differences that exist between individual pigs; even pigs of the same breed are not genetically the same. In the present study, animal preparation by laparoscopic surgery allowed oviductal fluid from a gamete-stimulated oviduct to

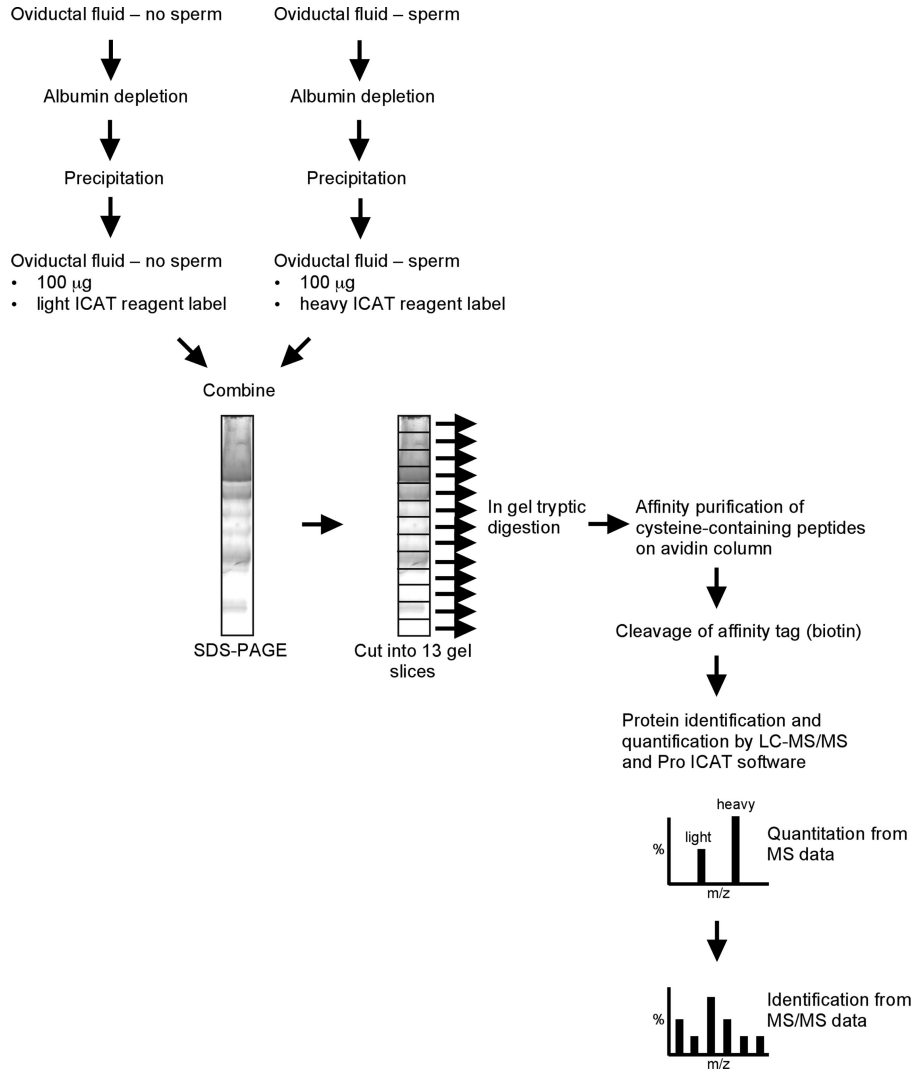

Figure 2. Workflow for the differential protein expression analysis of oviductal fluid. Following sample preparation and ICAT labelling, oviductal proteins were fractionated by SDS-PAGE. Each lane was cut into 13 bands, digested, and analysed by LC-ESI−MS/MS. Protein identification and quantification was made using the Pro ICAT software package.

be directly compared to a non-gamete-stimulated oviduct that originated from the same animal. The minimal invasiveness of laparoscopic surgery enabled us to perform *in vivo* experiments without interfering with the animal's behavior, accustomed environment, or introducing excessive stress into the animal. Because the oviducts compared were under the same hormonal influence, we can conclude that alterations in oviductal fluid concentrations occurred independently of hormonal influence. In addition, the present investigation took place around the time of natural mating in these animals. Therefore, the oviduct's reaction to gametes occurred under the influence of sex hormones that normally circulate during this stage of the reproductive cycle *in vivo*.

When ICAT technology was utilized, a total of 32 oviductal fluid proteins were conclusively identified and quantified on the basis of the relative abundance of heavy to light peptide pairs that originated from a particular protein (Table 1). In the case of the diluent versus no diluent oviduct experiment, designed to examine the effects of surgical interventions, nearly all of the proteins showed no signs of alteration. The exception was haemoglobin beta chain, which showed less than 2-fold increased abundance in the oviductal fluid of the oviduct attached to the uterine horn that had been cut during surgery. In comparison, proteins witnessed as regulated in response to either sperm or oocyte presence in the oviduct showed regulatory changes that were far more dramatic. Of these, 19 and 3 proteins showed greater than 2-fold alteration by sperm and oocyte presence in the oviduct, respectively.

We further confirmed alterations in fibrinogen, complement C3, RBP, and OGP expression at transcriptional and translational levels using real-time PCR and Western blot analysis (Figures 3 and 4). The apparent correlation between gene and protein expression provided further evidence for gamete modulation of the oviductal environment. It additionally provided evidence that the observed protein changes cannot be attributed to proteins that originated from gametes.

Among the oviductal fluid proteins identified, 20 proteins were regulated only by sperm, and one protein was regulated only by oocytes (Ig kappa light chain). Three proteins were commonly regulated by both sperm and oocytes (Complement Component C3, Ig kappa variable region, and haemoglobin beta chain), and one protein showed regulation by sperm and oocytes in opposing directions (Complement Component C3). Almost all of these regulated proteins have been previously

Table 1. Oviductal Protein Expression Measurement Using Isotope Coded Affinity Tag Technology (ICAT)[a]

			surgical control experiment[b]			sperm experiment[b]			oocyte experiment[b]		
accession	protein	role in reproduction	ave. H:L	SD	*n*	ave. H:L	SD	*n*	ave. H:L	SD	*n*
gil627688	alpha-1 acid glycoprotein	Sperm-binding protein;[33] may influence sperm-zona pellucida binding[21]	-			2.04	0.70	2	-		
gil231467	alpha-2-HS-glycoprotein precursor	Reduces zona pellucida 'hardening' during *in vitro* oocyte maturation[20,34]	1.19	0.18	2	3.02	0.93	4	1.03	0.04	2
gil47523122	antileukoproteinase	Involved in maternal-embryo 'communication'[35]	1.18	0.51	2	-			-		
gil11693172	calreticulin	Involved in signal transduction events during sperm-egg interactions at fertilization[36]	-			1.60	0.50	2	-		
gil1236646	ch4 and secrete domains of swine IgM	Binding to sperm head reduces fertilization ability[37]	1.20	0.19	20	4.23	5.64	25	1.51	0.21	24
gil2624793	chain A, Bm-40, FsEC DOMAIN PAIR	Unknown	0.96	0.18	3	-			-		
gil47522844	complement component C3	Involved in clearance of non-viable sperm;[38] sperm-oocyte binding;[23] early embryonic development[25]	0.99	0.30	13	5.23	3.46	19	0.62	0.50	5
gil1070458	ferroxidase precursor	Unknown	0.71		1	-			-		
gil1304179	fibrinogen A-alpha-chain	Binding to sperm may confer resistance against phagocytosis[18]	-			7.29	1.66	5	-		
gil1346006	fibrinogen beta chain precursor	Binding to sperm may confer resistance against phagocytosis[18]	1.23		1	20.3		1	-		
gil47522826	haptoglobin alpha 1S	Involved in oocyte development;[39] binds to early embryo[40]	0.93	0.16	13	2.06	0.32	18	1.16	0.13	11
gil122465	hemoglobin alpha chain	Nitric oxide scavenger which improves fertilization rate *in vitro*;[41] and embryo development[42]	-			3.94	0.47	2	-		
gil104966	hemoglobin beta chain	Nitric oxide scavenger which improves fertilization rate *in vitro*;[41] and embryo development[42]	1.83	0.13	3	3.40	0.38	4	3.94	0.24	4
gil47522736	hyaluronidase	Digests hyaluronic acid between the cells of the cumulus oophorus, facilitating fertilization[43]	1.51	0.25	2	2.07	0.45	17	1.59	0.13	2
gil2136513	Ig gamma 2a chain	Unknown	-			1.87	0.66	4	-		
gil2136514	Ig gamma 2b chain	Unknown	-			1.73	0.53	5	-		
gil125947	Ig lambda chain C	Unknown	1.00	0.09	10	1.60	0.23	8	-		
gil47523192	IgG heavy chain	Unknown	1.16	0.15	12	-			1.24	0.17	18
gil164507	Ig gamma-chain	Unknown	1.17	0.18	14	-			-		
gil21703157	Ig kappa light chain	Unknown	-			-			2.78	0.77	3
gil41323507	Ig kappa variable region	Unknown	1.14	0.22	7	2.67		1	2.09	0.41	10
gil48374067	inter-alpha-trypsin inhibitor	Covalently binds to cumulus oophorous; required for optimal oocyte development[44]	-			10.8	0.21	2	-		
gil12643979	metalloproteinase inhibitor 1 precursor	Mediates proliferation, differentiation, and apoptosis of cells throughout the reproductive tract during the female reproductive cycle[45]	1.25		1	-			-		

Table 1 (Continued)

			surgical control experiment[b]			sperm experiment[b]			oocyte experiment[b]		
accession	protein	role in reproduction	ave. H:L	SD	*n*	ave. H:L	SD	*n*	ave. H:L	SD	*n*
gil38044106	oviductin protease	Unknown	0.76		1	-			-		
gil2493679	oviduct-specific glycoprotein	Sustains sperm motility and viability;[46] increases fertilization rate and embyo development *in vitro*[47]	1.03	0.19	3	3.31	1.58	4	-		
gil47523160	porcine inhibitor of carbonic anhydrase	May play a role in pH regulation during sperm capacitation and fertilization[48]	-			1.91	0.30	12	-		
gil2914422	retinol-binding protein	Involved in retinol delivery to oocytes[49] sperm,[50] and embryos[51]	-			2.63		1	-		
gil136192	Serotransferrin	Correlation exists between high transferrin concentration and high sperm motility[52]	1.48	0.28	37	2.05	0.46	105	1.43	0.62	46
gil113578	serum albumin precursor	Involved in protein tyrosine phosphorylation in sperm;[53] generally involved in calcium, fatty acid, amino acid and hormone transport to cells[54]	1.07	0.17	95	2.25	0.95	73	1.24	0.25	92
gil12621074	triosephosphate isomerase 1	Unknown	-			1.95	0.61	3	-		
gil139641	vitamin D-binding protein precursor	May influence sperm function[55]	-			2.08	0.16	2	-		

[a] ICAT was used to compare oviductal fluid from diluent versus no diluent oviducts (surgical-control experiment, $n = 5$), sperm versus no sperm oviducts (sperm experiment, $n = 5$), and oocyte versus no oocyte oviducts (oocyte experiment, $n = 5$). The relative heavy (H):light (L) expression measurement, standard deviation (SD), and number of peptides quantified (*n*) for each matching protein are displayed. The roles that the identified proteins have been reported to play in reproduction are briefly summarized. In cases where there is no data in the table (-), the ion scores of peptides matching that protein was below our threshold for inclusion. [b] Ave. H:L, mean fold change quantified on the basis of the relative abundance of heavy to light peptide pairs that originated from a particular protein; SD, standard deviation; *n*, number of peptide pairs used to calculate mean fold change.

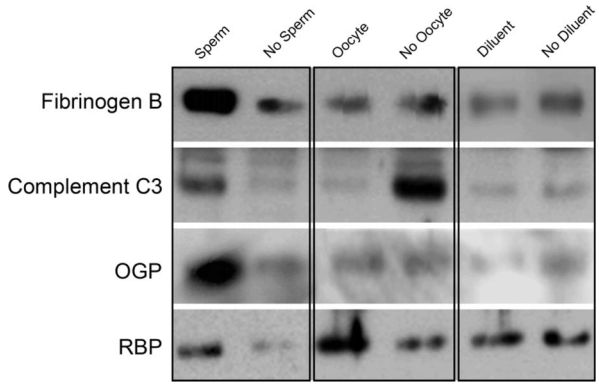

Figure 3. Validation of ICAT findings by immunoblotting. Antibodies against fibrinogen B, complement C3, OGP, and RBP were used to compare oviductal protein expression in oviducts that had been in the presence of sperm versus no sperm, oocytes versus no oocytes, and diluent versus no diluent. Immunoblotting was undertaken three times for each antibody, each time providing the same result. Sperm presence in the oviduct resulted in increased complement C3, OGP, and RBP protein secretion into oviductal fluid. Oocyte presence in the oviduct resulted in decreased oviductal C3 protein secretion and increased oviductal RBP expression. Presence or absence of diluent resulted in no differences in expression of any of these proteins.

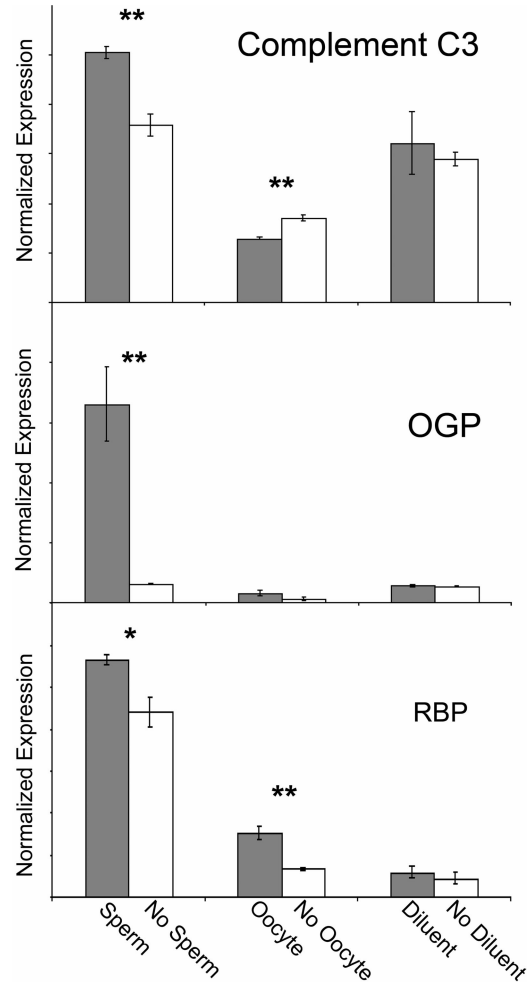

Figure 4. Real-time PCR analysis of oviductal mRNA expression. Expression values (normalized based on β-actin expression values) in the oviduct at *oestrous*, in the presence of sperm versus no sperm, oocytes versus no oocytes, and diluent versus no diluent. Real-time PCR was performed three times for each comparison. Graph displays mean value, and error bars display the standard deviation. Sperm presence in the oviduct resulted in significant increases in oviductal gene expression values of complement C3, OGP, and RBP genes. Oocyte presence in the oviduct resulted in a significant decrease in oviductal C3 expression and a significant increase in oviductal RBP expression. Presence or absence of diluent resulted in no significant differences in expression of any of these genes. $^{**}P < 0.01$, $^{*}P < 0.03$.

reported to play direct roles in reproduction, and their roles have been briefly summarized in Table 1. Our present study indicates, for the first time, that many of these oviductal secretory proteins are under regulation by gametes *in vivo*.

Proteins were grouped into general categories dependent on where they exert their influence during the reproductive process (Figure 5). A number of regulated proteins are known to influence sperm function. For example, fibrinogen binding to spermatozoa has been suggested to play a role in the protection of spermatozoa against phagocytosis *in vivo*.[18] Binding of a fibrinogen-related protein to defective sperm has also been implicated in the disposal of defective sperm in the hamster.[19] Therefore, fibrinogen may be involved in the selective clearance of non-viable spermatozoa and simultaneous protection of viable spermatozoa. Proteins that are known to influence oocyte function were also identified as being regulated. Alpha-2-HS-glycoprotein has been found to reduce the incidence of spontaneous zona pellucida 'hardening' (ZPH) that occurs during *in vitro* oocyte maturation, which inhibits sperm penetration and prevents fertilization.[20] Another group of regulated proteins were those involved in the fertilization process. For example, alpha-1 acid glycoprotein, which was up-regulated by sperm presence, is thought to bind to the zona pellucida, and influence sperm-zona pellucida binding.[21]

Most of the regulated proteins exert their influence on multiple processes during the establishment of pregnancy. For example, OGP has been shown to exert numerous beneficial affects on both of the gametes and on embryos.[22] OGP up-regulation in response to sperm was confirmed by Western blotting and real-time PCR. Presence of oocytes in the oviduct had no observable effect on OGP protein or gene expression. C3 is directly involved in the selective clearance of nonviable spermatozoa from the female reproductive tract,[23–25] sperm-oocyte binding,[23] and early embryonic development.[25] In spite of the potential importance of C3 in achieving pregnancy, little is known about the activation and regulation of the C3 throughout the female genital tract. Our current investigations identified C3 relative gene- and protein-expression to be up-regulated by the oviduct in response to spermatozoa, and down-regulated in response to oocytes. This may represent a system designed to increase exposure of sperm to C3, and reduce exposure of oocytes and early embryos to C3. This suggestion is supported by the finding that C3 synthesis and secretion into porcine oviductal fluid is highest at oestrus (time of mating) and has been shown to steadily reduce to undetectable levels during the first 5 days of pregnancy.[24] Future experiments examining the direct effects of C3 on oocyte maturation, fertilization, and embryo development may help explain why its secretion into oviductal fluid is regulated in the manner identified in the present study.

The observation that both of the gametes alter the concentration of proteins such as immunoglobulins normally involved

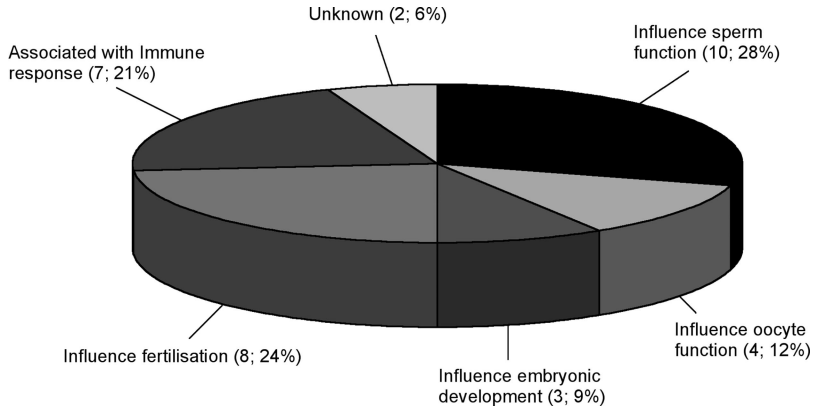

Figure 5. Oviductal proteins regulated by gametes organized into functional categories. Proteins that are known to exert their influence on sperm function, oocyte function, embryonic development, fertilization, or are associated with an immune response are grouped into general categories. Proteins may be present in more than one category. Only proteins that were more than 1.5-fold altered by either gamete are included. (Number of proteins; percentage of proteins in each category.) More information regarding individual proteins can be found in Table 1.

in immune responses implies that these proteins may play a role in reproduction different from their roles in the clearance of pathogens with which they are typically associated. Spermatozoa are non-self entities for females, but oocytes can be considered as self entities and, as such, would not be expected to induce an immune response. It would be interesting to see if the oviductal response to microorganisms, such as bacteria, or other cells from somatic origin would be the same. Nevertheless, generally, the oviductal responses presented to gametes can be regarded as a favorable response to maintain their viability and facilitate their function.

On the basis of these current results, we propose that gametes are able to regulate their own environment within the oviduct and coordinate the events preceding and during fertilization and early embryonic development. Previous studies using either sperm or oocytes *in vivo*, but not both, indicated that gametes alter the oviductal transcriptome.[26–28] No previous information existed regarding oviductal response to either of gametes at the proteomic level *in vivo*. In addition, the *in vitro* models used to investigate gamete−oviduct interactions so far have suffered from two main limitations. First, the oviducts or oviductal epithelial cell cultures used in previous studies were not under the hormonal influences that the female reproductive tract faces at the time of ovulation and mating in most mammals.[15] Second, no conclusive information exists regarding the number of spermatozoa that traverse the oviduct following natural mating. It is estimated that, from the billions of spermatozoa that are deposited into the female reproductive tract after natural mating, only tens to hundreds of sperm reach the upper regions of the female reproductive tract.[29] In previous studies, oviducts or epithelial cells were exposed to far more than the physiological number of spermatozoa at the site of fertiliszation after natural mating (10^6 to 10^7 sperm/mL[15,30]). Finally, there is always a need for caution when attempting to extrapolate *in vitro* observations to *in vivo* systems.[31] This may explain discrepancy between oviductal secretory proteins found to be altered in response to gametes in *in vitro* and *in vivo* based experimental systems.

Here, we have clearly demonstrated that, *in vivo*, the arrival of either gamete activates a cell-type-specific signalling pathway within the oviduct, which leads directly to specific alterations in oviductal fluid composition. This indicates the existence of a gamete recognition system in the oviduct that can alert females to the presence of either of the gametes in their reproductive tract. Such a system for the recognition of gametes is comparable to that of Toll-like receptors (TLR) for recognition of non-self entities.[32] We speculate that a comparable system exists in the female reproductive tract for recognition of gametes that has yet to be discovered.

The notion that gametes themselves can modulate the oviductal environment extends the concept of regulatory mechanisms within the female reproductive tract beyond hormonal regulatory systems. It presents a mechanism by which gametes have immediate and local control of their environment. Presence of a gamete recognition system may provide explanations behind the oviduct's ability to tolerate spermatozoa while remaining intolerant to pathogens. It also provides a mechanism for post-mating sexual selection. This notion profoundly influences our understanding of the physiology of events leading to conception and the bearing of offspring.

In conclusion, we have identified a number of oviductal proteins that are regulated by sperm or oocyte presence in the oviduct *in vivo*. This regulation generally seems to provide a favorable microenvironment for gametes and prepares the oviduct milieu for the arrival of the embryo. All of the altered proteins identified are well-known to influence gamete maturation, viability, and function; yet, this is the first report to demonstrate their regulation by gametes. This work conclusively demonstrates the existence of a gamete recognition mechanism within the female reproductive tract.

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Supporting Information Available: Extensive peptide details information of different experiments are provided in the form of an excel file. This file contains complete information about peptides quantified in different experiments and it is organized in three different tabs; surgical control, sperm, and oocyte experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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