Gametes Alter the Oviductal Secretory Proteome*

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The mammalian oviduct provides an optimal environment for the maturation of gametes, fertilization, and early embryonic development. Secretory cells lining the lumen of the mammalian oviduct synthesize and secrete proteins that have been shown to interact with and influence the activities of gametes and embryos. We hypothesized that the presence of gametes in the oviduct alters the oviductal secretory proteomic profile. We used a combination of two-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry to identify oviductal protein secretions that were altered in response to the presence of gametes in the oviduct. The oviductal response to spermatozoa was different from its response to oocytes as verified by Western blotting. The presence of spermatozoa or oocytes in the oviduct altered the secretion of specific proteins. Most of these proteins are known to have an influence on gamete maturation, viability, and function, and there is evidence to suggest these proteins may prepare the oviductal environment for arrival of the zygote. Our findings suggest the presence of a gamete recognition system within the oviduct capable of distinguishing between spermatozoa and oocytes. Molecular & Cellular Proteomics 4:1785–1796, 2005.

The mammalian oviduct is the venue of important events leading to the establishment of pregnancy. These events include final maturation and transport of the female and male gametes, fertilization, cleavage-stage embryonic development, and transport of the embryo to the uterus. In mammals, the physiological interaction between gametes, embryos, and oviductal epithelia involves intimate and specific contact between the two cell types (1–6). During the estrous or menstrual cycle, the mammalian oviduct undergoes significant endocrine-induced morphological, biochemical, and physiological changes. These changes establish an essential microenvironment within the oviduct. Oviductal fluid is a crucial part of this milieu and consists of transudate from serum together with specific compounds synthesized by the luminal epithelium. The current dogma states that the oviductal environment and the composition of oviductal fluid are solely under the influence of the hormonal changes in the oviduct (7–9). However, in recent years, several investigations from our laboratory and others have challenged this view by providing evidence of transcriptional changes in the oviduct in response to gametes irrespective of oviductal hormonal status (10–12).

Although these data provide strong evidence in relation to the modulation of the oviductal environment by gametes, they lack information regarding changes to the oviductal proteomic profile, for example the secretory profile. In mammals, not all the changes in the transcriptome are translated into proteomic alterations due to post-translational modifications. Elvington et al. (13) and Thomas et al. (14) provide the only evidence that at least spermatozoa can influence the (secretory) proteomic profile of oviductal epithelial cells. These investigations have reported de novo protein synthesis in oviductal epithelial cell monolayers in response to spermatozoa in vitro. However, they failed to obtain the identity of the de novo synthesized proteins.

In the present investigation, we report alterations in the secretory oviductal proteome in response to oocytes and spermatozoa using quantitative 2D gel electrophoresis and mass spectrometry. Secretory oviductal proteomic changes specific to each gamete were defined. Furthermore the results obtained by 2D gel electrophoresis were verified in two candidate proteins using Western blot analysis. Bioinformatic analysis indicated that the majority of these proteins may have a role in maintenance and protection of gametes in the oviduct. This is the first report to demonstrate an oviduct-specific proteomic response to both of the gametes.

EXPERIMENTAL PROCEDURES

Semen Preparation—Boar semen obtained from JSR Healthbred Limited (Yorkshire, UK) was collected, then diluted, and stored for 24 h in Beltsville thawing solution (15). Immediately prior to use, diluted boar semen was washed three times with PBS by centrifugation and resuspension at 500 × g for 10 min. Sperm concentration

Received, April 27, 2005, and in revised form, August 16, 2005
Published, MCP Papers in Press, August 16, 2005, DOI 10.1074/mcp.M500119-MCP200

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This paper is available online at http://www.mcponline.org

Molecular & Cellular Proteomics 4.11

1 The abbreviations used are: 2D, two-dimensional; COC, cumulus-oocyte; IVM, in vitro maturation; LDH, lactate dehydrogenase; BCA, bicinchoninic acid; ROS, reactive oxygen species; redox, reduction and oxidation; SOD, superoxide dismutase; GPx, glutathione peroxidase; Trx, thioredoxin; HSP, heat shock protein; PHGPx, phospholipid-hydroperoxide glutathione peroxidase; PDX2, peroxiredoxin 2.
was measured using a hemocytometer, and the proportion of motile sperm was determined. The washed semen sample concentration was adjusted to $1 \times 10^6$ motile sperm/ml in PBS.

**Oocyte Isolation and Maturation**—Gilt reproductive tracts were obtained from a local abattoir on the day of slaughter. These were transported to the laboratory at room temperature in PBS supplemented with 100 units/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 0.25 μg/ml amphotericin B (Invitrogen). The ovaries did not show any signs of cyclicity such as follicular growth, ovulation, or corpora lutea. Oviducts were cut away from the ovary and the uterine horn and were used in experiments as described elsewhere.

Cumulus-oocyte complexes (COCs) were aspirated from medium-sized ovarian follicles (3–6-mm diameter) using a 19-gauge needle. Oocytes were washed three times in M199 (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) (Invitrogen), 100 units/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 0.25 μg/ml amphotericin B (Invitrogen). COCs were cultured in groups of 50/well in 4-well plates (Nalge Nunc International, Herford, UK) for up to 48 h at 37 °C with 5% CO₂ in air. COCs with an expanded cumulus were collected for inclusion in further experiments and washed three times in PBS. The nuclear status of oocytes after culture in this in vitro maturation (IVM) protocol was determined previously by 4,6-diamino-2-phenylindole staining described by Mori et al. (16). COCs were denuded by vortexing for 3 min and then fixed for 15 min in 2.5% (w/v) glutaraldehyde, washed with PBS, stained with 2.5% (w/v) 4,6-diamino-2-phenylindol (Sigma, Dorset, UK), and mounted on slides. The nuclear state of the stained oocytes was assessed under a fluorescence microscope. Our IVM protocol previously resulted in 76% of oocytes completing at least meiosis I stage of nuclear maturation after 48 h.

**Oviduct Preparation**—The oviducts were washed three times with PBS supplemented with 100 units/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen). Oviducts were sealed at one end using a piece of cotton thread and filled with 1 ml of PBS (Invitrogen) using a syringe. The other oviduct end was then closed, and the sealed PBS-filled oviducts were placed in glass beakers and covered with PBS. Beakers were incubated at 37 °C and 5% CO₂ in air for 2 h. The cotton thread was cut at both ends of the oviduct, and oviducts were flushed with ~2 ml of PBS, which was discarded. Washed oviduct pairs were utilized in subsequent co-incubation experiments with gametes.

**Sperm-Oviduct and Oocyte-Oviduct Co-incubation**—Each oviduct was sealed at one end using a piece of cotton thread. One of each oviduct pair (originating from the same animal) was filled with 1 ml of PBS before sealing the oviduct (control). The other oviduct from each pair was filled with 1 ml of $1 \times 10^6$ motile sperm/ml in PBS before sealing the oviduct (sperm, test) or PBS containing 10 IVM oocytes (oocyte, test). Sealed oviducts were placed in glass beakers and covered with PBS. Beakers were incubated at 37 °C and 5% CO₂ in air for 18 h. The 18-h incubation time was chosen to allow enough time for exposure of gametes to the oviduct while making the experimental design practical. An aliquot of washed semen sample diluted in PBS (1 $\times 10^6$ motile sperm/ml) was incubated at 37 °C and 5% CO₂ in air for 18 h to serve as the control sperm sample. Oocytes in PBS (10 IVM oocytes/ml) were incubated at 37 °C and 5% CO₂ in air for 18 h to serve as a control oocyte sample. After sperm-oviduct and oocyte-oviduct co-incubations, the cotton thread was cut at both ends of the oviduct, and oviducts were flushed with ~2 ml of PBS. All fluid was collected. Ten milliliters of the control sperm sample and 10 ml of the control oocyte sample were then added to the conditioned medium collected from PBS-filled oviducts whose oviduct counterparts had been incubated with sperm and oocyte samples, respectively. A protease inhibitor solution (PMSF, Sigma) was added to the collected media to a final concentration of 1 mM. All samples were immediately centrifuged at 2,000 × g for 10 min to remove spermatozoa, detached oviductal cells, or any other debris. The supernatant was collected and ultracentrifuged at 100,000 × g for 30 min at 4 °C. The clarified medium (conditioned medium) was collected and stored at −70 °C.

**Lactate Dehydrogenase Assay**—The lactate dehydrogenase (LDH) activity of all the collected oviductal fluid samples was examined using a colorimetric assay for cytotoxicity (Oxford Biomedical Research) according to the manufacturer’s instructions. Briefly 100 μl of conditioned medium was added to 100 μl of the kit substrate mixture and incubated at room temperature for 30 min in the dark. Fifty microliters of 1 M hydrochloric acid was added to terminate the reaction, and absorbance was read at 490 nm using a Benchmark 96-well plate reader (Bio-Rad).

**Two-dimensional Gel Electrophoresis**—Conditioned medium was concentrated using 3-kDa molecular mass cut off Centricron microconcentrators (Millipore). Control sperm- and oocyte-conditioned media supernatants (no oviduct) that had been incubated alongside the oviduct-gamete co-culture supernatants were concentrated 10 times further than the oviduct-gamete co-culture supernatants. A Plus-one 2D clean up kit (Amersham Biosciences) was used according to the manufacturer’s instructions to purify, desalt, and remove all impurities from the protein samples. The resulting protein pellet was dissolved in buffer A (8 M urea, 2% (w/v) CHAPS). The bicinchoninic acid (BCA) assay was performed as described previously by Smith et al. (17) to determine the protein concentration of all samples. Briefly 10 μl of each protein sample was added to 200 μl of 2% (v/v) copper sulfate 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).

Samples were diluted to a concentration of 1.7 μg of protein/μl in buffer A after which 0.5% (v/v) IPG buffer pH 4–7 (Amersham Biosciences) and 0.02% (w/v) bromphenol blue were added. DTT (Amersham Biosciences) was then added to give a final concentration of 40 mM DTT. Two to eight hundred micrograms of protein were used to rehydrate 18-cm, pH 4–7 IPG strips (Amersham Biosciences). Proteins were resolved in the first dimension by IEF for a total of 33,500 V-h using the IPGphor isoelectric focusing system (Amersham Biosciences). The IEF program started with 500 V for 500 V-h followed by a step-and-hold increase to 1000 V for 1000 V-h and finally to a step-and-hold increase to 8000 V for 32,000 V-h. After focusing, IPG strips were equilibrated to reduce protein-disulfide bonds in 10 ml of equilibrating solution per strip (6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris-HCl, pH 8.8, 0.25% (w/v) bromphenol blue, and 1% (w/v) DTT) with gentle rocking for 15 min. The free cysteine residues of proteins were then alkylated to prevent reformation of disulfide bonds by rocking each strip for 15 min in 10 ml of solution containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris-HCl, pH 8.8, 0.25% (w/v) bromphenol blue, and 2.5% (w/v) iodoacetamide. These strips were then affixed onto homogeneous 12.5% SDS-polyacrylamide slab gels (25 × 2100 × 1 mm). The second dimension was performed in the EttanDalt vertical system (Amersham Biosciences) at 25 °C.

**Image Analysis and Quantitation**—The 2D gels were fixed overnight in 7% (v/v) acetic acid in 40% (v/v) methanol at room temperature. Gels were then rinsed with water and stained with colloidal Coomassie Brilliant Blue G250 (Sigma) for 2 h at room temperature. Gels were destained in 10% acetic acid in 25% (v/v) methanol for 1 min, washed, and stored in 25% (v/v) methanol at room temperature. Images were captured by scanning using an Image Scanner II flat bed scanner (Amersham Biosciences) and LabScan software (Amersham Biosciences). Gel images were calibrated and normalized using ImageMaster 2D Platinum image analysis software (Amersham Bio-
sciences) to allow for quantitative comparison between gels. To assess the reproducibility of 2D PAGE, gels were prepared in triplicate. The staining intensity of each spot was normalized against the sum total of intensities of all detectable spots in the 2D gel; this normalization performed by ImageMaster 2D Platinum software corrects for any minor differences in protein loading among replicate gels. The triplicate normalized gels were then subjected to triplicate curation in which the gel with the most spots (detected using 2D Platinum software) was chosen as the reference gel (designated gel I), and the other two gels were called gel II and gel III, respectively. First, gel I was curated against the reference gel, and then gel III was curated against it. Next gel II was curated against gel III. Gels were then analyzed for differential protein expression by using 2D Platinum software to calculate and compare the volume of each spot on every gel. Spot volume was calculated as the volume above the spot border situated at 75% of the spot height (measured from the peak of the spot). Finally, gels were checked manually to check that protein spots expressed differentially in the reference gel were also different in the other two gels within a triplicate. Only protein spots that changed more than 2-fold in magnitude in at least one experimental day (up or down) and were observed to be altered in the same direction in all three experimental days were considered to be altered.

**Tryptic Digestion**—Differentially expressed proteins were excised from the gel, washed in 25% (v/v) methanol, and then incubated for 1 h at 37 °C in Coomassie destain solution consisting of 40% (v/v) acetonitrile (VWR International Ltd., Leicester, UK) in 200 mM ammonium bicarbonate. The destain solution was removed, and spots were incubated with ACN for 15 min at room temperature. ACN was subsequently removed, and the spots were dried in a vacuum centrifuge. Dried spots were stored at 4 °C until required. Proteins were digested with 20 ng/μl sequencing grade modified trypsin (Promega, Southampton, UK) in 50 mM ammonium bicarbonate at 37 °C for 12 h. The supernatant from the trypsin digest was transferred to a silica-coated microcentrifuge tube. Peptides were sequentially extracted three times by incubation with peptide extraction solution consisting of 25 mM ammonium bicarbonate (10 min at room temperature), 5% formic acid (15 min at 37 °C), and ACN (15 min at 37 °C). Each extraction was followed by centrifugation and removal of supernatants. The original supernatant and the supernatants from the three sequential extractions were combined and dried in a vacuum centrifuge for 4–6 h. The dried peptides were dissolved in 70% acetonitrile (15 min at 37 °C), and ACN (15 min at 37 °C). Subsequently, the dried peptides were dissolved in 70% formic acid in 3% (v/v) ACN in water. Samples were centrifuged for 5 min at 12,000 g, and the supernatants were subjected to LC-ESI-MS/MS.

**LC-ESI-MS/MS**—Liquid chromatographic separations of the tryptic digests were performed using a reverse phase CapLC™ system (Waters, Manchester, UK). Peptides were desalted by a PepMap C18 microguard column (300-μm internal diameter x 1 mm) (LC-Dionex, Leeds, UK) and were then transferred to the analytical column (PepMap C18, 75-μm internal diameter x 15-cm column (LC-Dionex)). The peptides were eluted in a 60-min gradient. The compositions of the hydrophilic and hydrophobic solvents were 5% ACN, 0.1% formic acid and 95% ACN, 0.1% formic acid. The column eluent was sprayed directly into the nano-ESI source of a Q-TOF microcolumn (Waters). An initial MS scan was performed, and selection of ions for CID was automated by Mass Lynx software (Waters), CID selection criteria were set for 2+ and 3+ ions within the range of 400–2000 m/z above 10 ion counts.

Alternatively, LC separations of the tryptic digests were performed on a PepMap C18 reverse phase capillary column (LC-Dionex) and eluted in a 30-min gradient via an LC Packings Ultimate nano-LC directly into the mass spectrometer. The compositions of the hydrophilic and hydrophobic solvents were 5% ACN, 0.1% formic acid and 95% ACN, 0.1% formic acid. An Applied Biosystems QStarXL electrospray ionization quadrupole time-of-flight tandem mass spectrometer (ESI-qQ-TOF) was used for mass spectrometric analysis. Analyst QS software (Applied Biosystems) was used for data acquisition and data analysis. The data acquisition on the mass spectrometer was performed using information-dependent acquisition. After each TOF-MS scan, two peaks with charge states 2 or 3 were selected for tandem mass spectrometry.

Spectra were searched against the Mass Spectrometry Data Base (MSDB) in a sequence query search using MASCOT 2.0 software (www.matrixscience.com). The taxonomy was limited to filter for only mammalian matches, and trypsin was used as the cleavage enzyme with one missed cleavage site allowed. The peptide tolerance was set to 1.0 Da, and the MS/MS tolerance was set to 0.3 Da. Carbamidomethyl modification of cysteine and oxidized methionine were set as variable modifications. Matches were considered valid if MS/MS data for multiple unique peptides per protein were identified, each with ions scores above the threshold of statistical significance (values generated by MASCOT). Manual examination of MS/MS data was performed for single peptide matches. In these cases, a continuous stretch of peptide sequence covered by either the y- or b-ion series was required for the protein match to be considered valid.

**Western Blot Analysis**—Conditioned medium proteins (30 μg of protein) were separated by one-dimensional gel electrophoresis using self-cast homogeneous 12.5% SDS-polyacrylamide gels. Gels were run at 25 mA for ~2 h using a Mini-Protean II™ gel electrophoresis system (Bio-Rad). Resolved proteins were transferred to a PVDF Immobilon P™ transfer membrane (0.2-μm pore size) (Millipore) using a Bio-Rad Mini Trans-blot electrotransfer protein cell. Following transfer, membranes were blocked with 5% (w/v) nonfat 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: mouse anti-heat shock 70-kDa protein (HSP70) monoclonal antibody (Stressgen Biotechnologies Corp., Victoria, Canada) or rabbit anti-peroxiredoxin II polyclonal antibody (LabFron-tier, Seoul, Korea). Antibodies were diluted 1:1000 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, UK) and exposure to Hyperfilm ECL high performance chemiluminescent film (Amersham Biosciences).

**Experimental Design**—Experiments were designed to compare the oviductal secretory protein profile of oviduct pairs in response to gametes. Three experimental groups were established. In the first experimental group, one oviduct was incubated with spermatozoa, and its counterpart was incubated with PBS. In the second experimental group, one oviduct was incubated with oocytes, and its counterpart was incubated with PBS. Finally in the third experimental group, one oviduct was incubated with spermatozoa, and its counterpart was incubated with oocytes. A total of 10 oviductal pairs were used in each experimental group. All three experiments were undertaken on three separate occasions. The reproducibility of the 2D electrophoresis technique was assessed by creating two 2D electrophoresis gels using oviductal proteins obtained from the first experimental group. An altered oviductal secretory protein profile was examined across the three experimental repetitions (three experimental days). Any proteins showing greater than 2-fold alteration in expression level in at least one repetition and the rest of repetitions showing changes in agreement with that were considered as differentially expressed/produced in response to gametes. These proteins were selected for further examination using LC-ESI-MS/MS.
RESULTS

2D Gel Analysis—Proteins present in oviductal fluid after 18 h of oviductal co-culture with PBS or sperm were loaded onto 2D gels at 200, 600, and 800 μg of total protein. The images of the resulting Coomassie-stained gels were analyzed using 2D Platinum software to assess the effect of...
protein loading on the ability to detect regulatory differences between PBS- and sperm-incubated oviducts. Both the total number of protein spots and detectable regulatory differences increased when the gels were loaded with 600 μg compared with 200 μg of total protein (data not shown). Loading the gels with 800 μg resulted in poor quality isoelectric focusing and,
as a consequence, resulted in the detection of fewer total proteins and fewer regulatory differences. Therefore, 600 μg of total protein loading was considered optimal for this particular experimental program.

Each gel was found to comprise more than 500 protein spots. Representative 2D gels for PBS- and sperm- or oocyte-incubated oviducts are shown in Figs. 1 and 2. The gels were highly reproducible among each triplicate, showing 94–97% homology (Table I).

Triplicate independent oviductal fluid proteins prepared on three independent experimental days were also examined for reproducibility. Homology was found to be 73–79% (Table II), indicating a biological variation in the precise composition of oviductal fluid dependent upon the biological specimen examined. Therefore, gels from each experimental day were compared with each other to account for this biological variation. Any proteins showing greater than 2-fold alteration in expression level in at least one biological batch repetition and the rest of the biological batch repetitions showing changes in agreement with that were considered as differentially expressed in response to gametes.

Oviductal Secretory Protein Profile of Oviduct Pairs in Response to Gametes—Identification of the oviductal secretory proteome changes in response to the presence of gametes in the oviduct was based on the comparison of oviductal fluid collected from multiple independent biological repetitions. The comparison of oviductal fluid proteins from oviducts incubated with PBS versus sperm identified 20 spots that were reproducibly differentially regulated across all three biological batches of which nine were up- and 11 were down-regulated (shown in Table III). Five spots were reproducibly differentially regulated by the presence of oocytes in the oviduct; three were up- and two were down-regulated. Only one of these protein spots, superoxide dismutase (SOD), was commonly regulated by both the sperm and oocyte presence in the oviduct. The direct comparison of oviductal fluid proteins from oviducts incubated with sperm versus oocytes identified nine spots that were reproducibly differentially regulated of which six were up- and three were down-regulated. Three of these protein spots, heat shock 70-kDa protein 1A, triose-phosphate isomerase, and ribonuclease UK114, confirm the up-regulatory changes identified in the comparison of oviductal fluid proteins from oviducts incubated with PBS versus sperm. There was no detectable protein presence in any of controls taken during experiments as determined by the BCA assay.

LDH activity was nearly the same in all the oviductal sam-

### TABLE I

| Reference gel | Gel II | Gel III |
|---------------|--------|--------|
| Sperm         | 100    | 96     | 96     |
| PBS           | 100    | 94     | 97     |

Note that homology refers to how closely the volume of each protein spot matches the volume of that spot in the reference gel.

### TABLE II

Note that homology between gels obtained on three different experimental days

| Reference gel | Experiment II | Experiment III |
|---------------|---------------|----------------|
| Sperm         | 100           | 74             | 73             |
| PBS           | 100           | 76             | 78             |
| Oocyte        | 100           | 74             | 73             |
| PBS           | 100           | 76             | 78             |
| Oocyte        | 100           | 77             | 72             |
| Sperm         | 100           | 70             | 73             |

From all the proteins identified by searching the MSDB database, 16 proteins were identified as pig proteins, whereas 10 were matched to human, four were matched to rat, two were matched to goat, and two were matched to cow proteins. Typically good quality MS/MS data for multiple unique peptides were matched resulting in high protein coverage of these non-pig proteins, suggesting that the porcine homologues of the human, rat, goat, and cow proteins are yet to be identified.

**DISCUSSION**

We applied high resolution 2D gel electrophoresis and identification of proteins by mass spectrometry to the analysis of changes in the protein composition of oviductal fluid resulting from the presence of gametes in the oviduct. We took several measures to ensure that the observed changes could not be attributed to anything other than differentially regulated oviductal protein secretions. A BCA assay was performed to determine the protein content of control sperm- and oocyte-conditioned media supernatants (no oviduct) that had been incubated and prepared for 2D PAGE alongside the oviduct-gamete co-culture supernatants. No protein was detected in these supernatants even when concentrated in excess of what would have been present in the co-culture experiment. Furthermore when all of the available supernatant was subjected to 2D PAGE, no spots were observed on the resulting Coomassie-stained gels. It was concluded that sperm and oocyte proteins did not contribute to the protein spots observed on the gels and therefore did not have any influence on the determination of differential protein expression.

The appearance of proteins in oviductal secretions cannot be explained by cellular decay or intracellular leakage into the extracellular space either. Using LDH as a marker for cellular
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Table III

Oviductal proteins regulated by the presence of sperm and oocytes

+ indicates an increase in protein expression, − indicates a decrease in protein expression, and numbers in parentheses indicate how many experimental repetitions that particular protein was found to be regulated over 2-fold. Experimental molecular weight and pl are presented. MASCOT Mowse scores are displayed. p, pig; h, human; r, rat; g, goat; c, cow. Functional categories are abbreviated as follows: PMR, protein production, maintenance, and repair; Ant, antioxidant and free radical scavengers; Met, metabolism; Misc, miscellaneous. Peptide sequence data are provided in table footnotes for protein matches based on a single peptide sequence, including the experimental mi/z transformed to a relative molecular mass (expt), calculated relative molecular mass of the matched peptide (calc), difference between the experimental and calculated masses (Δ), and the peptide charge.

| Predicted protein | Functional category | UniProt accession no. | Molecular mass | Peptides | Coverage | Mowse score | Average -fold change ± S.E. |
|------------------|---------------------|-----------------------|----------------|---------|----------|------------|--------------------------|
| Oviductal proteins regulated by presence of spermatozoa in the oviduct (n = 6) | | |
| Nucleophosmin (h) | Misc | Q6V962 | 16.1 | 4.4 | 9 | 19 | 472 | Unique to sperm (3) |
| CCBT protein (h) | PMR | Q7Z759 | 30.1 | 5.2 | 4 | 8 | 211 | +3.1 ± 0.1 (3) |
| Cytoskeleton-associated protein 1a (p) | Misc | Q8HXL4 | 28.6 | 5.1 | 1 | 6 | 99 | +2.5 ± 0.9 (1) |
| Triose-phosphate isomerase (r) | Met | P48500 | 23.9 | 6.8 | 4 | 26 | 206 | +2.2 ± 0.3 (2) |
| Heat shock 70-kDa protein 1A (p) | PMR | P34930 | 30.9 | 4.7 | 3 | 5 | 154 | +1.8 ± 0.1 (0) |
| Elongation factor 1-B (p) | Misc | P29412 | 26.4 | 4.5 | 1 | 8 | 77 | +1.7 ± 0.3 (2) |
| Heat shock 70-kDa protein 1A (p) | PMR | P34930 | 31.5 | 4.6 | 5 | 5 | 263 | +1.6 ± 0.5 (1) |
| Protein Cua precursor (h) | Misc | Q9NYQ9 | 14.0 | 4.9 | 3 | 20 | 148 | +1.4 ± 0.4 (1) |
| Thioredoxin (p) | Ant | P82460 | 11.8 | 5.1 | 3 | 31 | 167 | +1.4 ± 0.5 (2) |
| λ chain C (p) | Misc | P01846 | 23.9 | 6.7 | 9 | 51 | 453 | −1.5 ± 0.4 (1) |
| Osmotic stress protein 94 (h) | PMR | Q95757 | 29.8 | 5.0 | 3 | 3 | 139 | −1.6 ± 0.4 (1) |
| Phosphoglycerate mutase (h) | Met | P18669 | 24.8 | 6.5 | 3 | 18 | 182 | −1.6 ± 0.4 (1) |
| α enolase (r) | Met | P04764 | 47.1 | 6.5 | 2 | 2 | 102 | −1.6 ± 0.6 (1) |
| 40-kDa peptideyl-propyl cis-trans isomerase (c) | PMR | P26682 | 39.0 | 6.8 | 11 | 23 | 638 | −1.7 ± 0.8 (1) |
| Lamin A/C (h) | Misc | P02545 | 46.4 | 5.5 | 8 | 14 | 406 | −2.0 ± 0.5 (2) |
| Cathepsin D (p) | PMR | P00795 | 26.5 | 5.6 | 2 | 2 | 112 | −2.0 ± 0.7 (1) |
| Osteoclast-stimulating factor (p) | PMR | Q8MJ49 | 24.9 | 5.6 | 3 | 14 | 171 | −2.2 ± 0.1 (2) |
| Non-selenium phospholipid-hydroperoxide glutathione peroxidase (p) | Ant | Q9TSX9 | 25.0 | 5.4 | 6 | 25 | 321 | −2.3 ± 0.8 (2) |
| Ribonuclease UK114 (g) | PMR | P80601 | 13.0 | 5.3 | 1 | 11 | 62 | −2.4 ± 0.1 (3) |
| Superoxide dismutase (Cu,Zn) (p) | Ant | P04178 | 17.8 | 6.5 | 4 | 17 | 204 | Unique to PBS (3) |

Oviductal proteins regulated by presence of oocytes in the oviduct (n = 3)

| Apolipoprotein A-I precursor (p) | Met | P18648 | 22.1 | 5.0 | 4 | 16 | 231 | +3.3 ± 3.5 (1) |
| Haptoglobin precursor (p) | Misc | Q8SSP7 | 11.7 | 5.2 | 6 | 19 | 352 | +2.7 ± 0.3 (3) |
| Dimethylarginine dimethylaminohydrolase 2 (f) | Ant | Q8MG60 | 26.8 | 5.8 | 7 | 27 | 321 | +2.7 ± 0.5 (3) |
| Transgelin 2 (h) | Misc | Q9BUH5 | 17.8 | 5.7 | 3 | 10 | 164 | −2.1 ± 0.7 (1) |
| Superoxide dismutase (Cu,Zn) (p) | Ant | P04178 | 17.9 | 6.4 | 14 | 22 | 764 | −4.3 ± 0.2 (3) |

Oviductal proteins regulated by presence of sperm compared with oviductal proteins regulated by presence of oocytes (n = 3)

| Heat shock 70-kDa protein 1A (p) | PMR | P34930 | 31.4 | 4.5 | 5 | 15 | 165 | +5.4 ± 0.0 (3) |
| Esterase D (p) | Met | Q9GM98 | 13.0 | 6.9 | 11 | 29 | 580 | +5.2 ± 2.3 (3) |
| Lamin B1 (h) | Misc | P20700 | 22.3 | 5.1 | 3 | 5 | 156 | +2.9 ± 1.6 (2) |
| Triose-phosphate isomerase (r) | Met | P48500 | 23.2 | 6.8 | 4 | 26 | 218 | +2.5 ± 0.6 (3) |
| Protein carboxyl-O-methyltransferase (c) | PMR | P15246 | 23.0 | 6.4 | 1 | 4 | 62 | +2.4 ± 0.8 (2) |
| Ribonuclease UK114 (g) | PMR | P80601 | 11.6 | 5.2 | 3 | 27 | 157 | +1.9 ± 1.6 (1) |
| FAM10A5 (h) | PMR | Q8NFI4 | 49.0 | 5.1 | 8 | 11 | 406 | −1.6 ± 0.5 (1) |
| FAM10A5 (h) | PMR | Q8NFI4 | 49.2 | 5.2 | 7 | 11 | 360 | −2.0 ± 0.8 (1) |
| Peroxiredoxin 2 (p) | Ant | P52552 | 21.1 | 5.2 | 3 | 13 | 159 | −2.7 ± 1.0 (2) |

| a | R.LIEEAQASASAIYPGSR.C; precursor mass, 1668.79 (expt), 1668.87 (calc); Δ, −0.08 Da; charge, 2+. |
| — | K.SPAGLQLNDYLDK.S; precursor mass, 1602.89 (expt), 1602.83 (calc); Δ, 0.07 Da; charge, 2+. |
| — | K.PAPAAGPSQAYLVD.R; precursor mass, 1627.16 (expt), 1626.87 (calc); Δ, 0.29 Da; charge, 2+. |
| — | K.ELVDDSINVR.K; precursor mass, 1272.63 (expt), 1272.63 (calc); Δ, 0.00 Da; charge, 2+. |

decay, an LDH assay was performed on all oviductal fluid samples to assess and compare the extent of cellular decay within the oviducts during the course of the experiments. The results were the same for all samples, indicating that the amount of cellular decay was not greater in any one sample relative to another. Despite this, oviductal proteins were ob-
We identified 19 proteins that were regulated by only sperm, four proteins that were regulated by only oocytes, and one protein that was commonly regulated by both sperm and oocytes. Most of these regulated proteins were either molecular chaperones and regulators of protein folding and stability or antioxidant and free radical scavenger proteins. We have attempted to organize the differentially expressed proteins into functional categories of (i) protein production, maintenance, and repair; (ii) antioxidant and free radical scavengers; (iii) metabolism; and (iv) miscellaneous (Fig. 3).

**Protein Production, Maintenance, and Repair**—Most of the oviductal secretory proteins altered by gametes were regulators of protein folding and stability, such as various chaperones, protein isomerases, and proteolytic enzymes. Most of these identified proteins were uniquely regulated by either sperm or oocyte presence in the oviduct. A number of proteins were identified in different 2D gel spots of approximately the same molecular weight but with different pl. These are most likely post-translationally modified isoforms of the same protein. For example, heat shock 70-kDa protein 1A was detected in two different spots on the same 2D gels, both of which were up-regulated by sperm when compared with the PBS control oviduct, and one of these heat shock 70-kDa protein 1A spots was again detected as up-regulated by sperm when compared directly with the oocyte oviduct. Another molecular chaperone, FAM10A5, was also identified in two different spots, both of which were up-regulated by the presence of oocytes in the oviduct when compared directly with the sperm in the oviduct. The presence of two isoforms might indicate that these proteins are subject to post-translational modification upon gamete arrival in the oviduct. Further experiments are being designed to characterize these modifications.

The classical understanding of mammalian heat shock proteins characterizes them as intracellular molecules that are only released into the extracellular environment upon cell necrosis. However, it has been demonstrated more recently that heat shock proteins can be released from a variety of viable (non-necrotic) cell types, and both HSP60 and HSP70 have been shown to be present in the peripheral circulation and serum of normal individuals (for a review, see Ref. 18). Before this, HSP70 has been reported to be present in oviductal fluid (19). The mechanism(s) leading to the release of heat shock proteins is unknown, but secretion via exosomes (20–22) or lipid rafts (23) has been suggested.

Western blots for HSP70 confirmed that the presence of sperm in the oviduct up-regulated expression of HSP70, whereas the presence of oocytes had no observable effect on HSP70 expression (Fig. 4). Interestingly human semen has been shown to induce transcription of HSP70 in cervical epithelial cells isolated from women following intercourse (24).

Although the influence of these heat shock/chaperone proteins and enzymes on oviductal function is unknown, the well researched functions of exogenous heat shock proteins in vitro make it possible to propose a beneficial influence on gamete development. Exogenous heat shock proteins are able to act as inflammatory activators of innate and adaptive immunity via interaction and influence on a number of different immune cell receptors (18, 25), including those of the Toll-like receptor family (26). In addition, exogenous heat shock proteins have also been shown to have non-immunological, cytoprotective effects on a variety of cells types (27–29). The capacity of glial cells to secrete HSP70 and of HSP70 to protect stressed neural cells has been demonstrated, supporting the theory that heat shock protein release is an altruistic response on the part of one cell that is aimed at the protection of its more vulnerable neighbors (30). We hypothesize that the up-regulated release of heat shock proteins by the oviduct in response to sperm is aimed at the maintenance of viability and function of spermatozoa in the oviduct. Further experiments examining the effects of HSP70 on sperm can test the validity of this hypothesis.

**Antioxidant and Free Radical Scavengers**—It has been suggested that reactive oxygen species (ROS; H₂O₂, O₂·, OH, and NO), generated through normal cell metabolism, may influence gametes and embryos in the oviduct and diminish their overall ability to achieve fertilization and pregnancy. The negative effects of ROS on maintenance of sperm viability (31–34), in vitro oocyte maturation (35, 36), in vitro fertilization, and in vitro embryonic development (37, 38) have been studied extensively. Because the oviduct is the venue for all these events in vivo, it is likely that a system for control of ROS levels is present in the oviduct. Oviductal control of ROS generation could be a determining factor in successful fertilization.
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Mammalian redox pathways (77). Reactive oxygen species are generated in cells during normal metabolism. SOD converts O$_2^-$ to H$_2$O$_2$, which is subsequently degraded to H$_2$O by GPx. Trx has H$_2$O$_2$ reducing activity as well as a function in refolding oxidized proteins.

Fig. 4. Validation of 2D gels using one-dimensional Western blots. Antibodies against HSP70 and PDX2 were used to semiquantitatively compare oviductal fluid from PBS-filled oviducts with sperm-filled oviducts, PBS-filled oviducts with oocyte-filled oviducts, and sperm-filled oviducts with oocyte-filled oviducts.

Fig. 5. Mammalian redox pathways (77). Reactive oxygen species are generated in cells during normal metabolism. SOD converts O$_2^-$ to H$_2$O$_2$, which is subsequently degraded to H$_2$O by GPx. Trx has H$_2$O$_2$ reducing activity as well as a function in refolding oxidized proteins.

**Superoxide dismutase**

2O$_2^-$ + 2H^+ → 2H$_2$O$_2$  

**Glutathione peroxidase**

2H$_2$O$_2$ + 2GSH → 4H$_2$O + 2GSSG  

**Thioredoxin**

2GSSG + 2H$_2$O → 4H$_2$O + 2GSH  

**Peroxiredoxins**

4H$_2$O + 2H$_2$O$_2$ → 3O$_2$ + 6H$_2$O
proteins, such as ATP synthase, NADH dehydrogenase, and cytochrome c oxidase, suggesting that ATP-generating mitochondrial proteins might have novel functions in the plasma membrane (66). Therefore, the intracellular metabolism-associated proteins identified in our present study may not necessarily be involved in metabolic activities extracellularly. For example, ATP synthase complex that is usually involved in intracellular ATP generation at the surface of the cell has the ability of recruiting different ligands such as angiotatin, endothelial and monocyte-activating polypeptide II, and apolipoprotein A-I (67–70).

Miscellaneous—We categorized proteins that did not fit in the other categories into this section. For example, two proteins, apolipoprotein A-I precursor and haptoglobin precursor, were found to be up-regulated only in oviducts in the presence of oocytes. Apolipoprotein is thought to play an important role in lipoprotein transport into oocytes (71). Its secretion into the uterus was increased during sheep pregnancy, suggesting its importance in embryo lipoprotein transport (72). Haptoglobin has been identified previously in cow and rabbit oviductal fluid. In rabbits, the highest expression of mRNA was detected at the time of ovulation (73). The extramembranous matrix of preimplantation embryos and blastocyst fluid were found to contain haptoglobin, although the embryo itself does not express haptoglobin. This implies an uptake of maternal haptoglobin into the extramembranous matrices during the passage of the embryo through the oviduct. Haptoglobin has also been shown to be transported into human ovarian follicles and bind to apolipoprotein, forming apolipoprotein-haptoglobin protein complexes that are thought to exert an influence on oocyte maturation (74). Apolipoprotein-haptoglobin complexes have been isolated previously from the oviduct and shown to have positive effects on sperm motility (75).

In general, most of the proteins we identified as being regulated by gametes are known to have influence on gamete maturation, viability, and function. There is also evidence to suggest these proteins may prepare the oviductal environment for arrival of the zygote. The question arises how the oviduct is able to recognize the presence of gametes and alter its environment in response to them. This question becomes more complex, taking into consideration that sperm itself is a non-self entity for the female reproductive tract and as such should initiate an immune response. In the current investigation, the response presented to gametes can be regarded as a favorable response to maintain their viability and facilitate their function. It would be interesting to see whether the oviductal response to microorganisms, such as bacteria, or other cells from somatic origin would be the same. One expects a distinct difference between oviductal reaction to gametes and pathogenic microorganisms. Nevertheless our findings suggest the presence of a gamete recognition system, able to distinguish between spermatozoa and oocytes, within the oviduct. If such a system did exist, then it would most likely be conserved in different species that reproduce utilizing internal fertilization. Such a system for recognition of gametes in the female reproductive tract can be compared with Toll like receptors for recognition of non-self entities by the innate immune system (76). We may further speculate that a special form of Toll like receptor molecule may exist in the female reproductive tract for recognition of spermatozoa that has yet to be discovered.

At the time of ovulation and mating, pigs are in estrus, and therefore their oviducts are under dramatic hormonal influence. The present study was performed in vitro and not at the time of estrus to avoid the potential influence that hormones can have on the oviductal secretory profile. In future investigations, we aim to study alterations of the oviductal proteomic profile in vivo at the time of estrus. Our other previous study using an in vivo model at the time of mating/estrus under the influence of reproductive hormones (10) has clearly demonstrated female reproductive tract responses to gametes at the transcriptomic level. Although we do not expect the principle facts (alteration of the proteomic profile by gametes) to change, it is possible that under hormonal influence proteins other than the ones reported here will be altered by gametes.

In conclusion, the present investigation, for the first time, demonstrates specific alteration of the oviductal secretory proteomic profile in response to both gametes. These changes seem to provide a favorable microenvironment for gametes and prepare the oviduct milieu for the arrival of the embryo.

Acknowledgments—We thank Professor A. G. Pockley and Dr. C. Allum for donation of antibodies, C. Bruce and S. Elliott for technical assistance, and Dr. L. R. Abeydeera for critical discussion during the course of this study.

* This work was supported by grants from the Biotechnology and Biological Sciences Research Council, Engineering and Physical Sciences Research Council, and Sygen PLC. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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