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Pig preovulatory oocytes modulate cumulus cell protein and gene expression in vitro

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ABSTRACT

This study investigated the changes in protein and gene expression in oocytectomized cumulus cells (OOX) of medium-sized follicles from gilts, cultured with or without denuded oocytes isolated from large oestrogenic sow follicles. Proteomic analysis identified 14 proteins that were differentially expressed in OOX, of which the protein 14-3-3η, a signal transduction pathway modulator, was down-regulated in the presence of oocytes. Oocyte co-culture also down-regulated *FSHR* mRNA expression in OOX, as measured by real-time PCR, and *FSHR* and *14-3-3η* mRNA abundance were positively correlated. The oocyte also up-regulated *HSD3B* mRNA, suggesting an effect on cumulus cell progesterone synthesis. Together with data on gene expression in granulosa cells during the follicular phase of the sow oestrous cycle, this study suggests that modulation of the expression of steroidogenesis related proteins and genes in cumulus cells by the porcine preovulatory oocyte reflects the specific physiological requirements of the preovulatory follicle.

1. Introduction

Ovarian follicle development in large domestic animals occurs over a relatively long period of time (~100 days in the pig) and requires a series of well coordinated events. These result in the ovulation of a subset of one or more oocytes competent of undergoing fertilization and early embryonic development. Initially, the oocyte was considered to play a passive role in folliculogenesis, relying mainly on gonadotrophins and metabolic factors to stimulate the granulosa and theca cells, which then provided the necessary stimulus for oocyte growth and acquisition of developmental competence. However, more recently, the oocyte was reported to play an active role during follicle development by secreting soluble factors that act on the surrounding somatic cells to modulate its own microenvironment (Eppig, 2001; Gilchrist et al., 2008; Hunter and Paradis, 2009; Juengel and McNatty, 2005; Matzuk et al., 2002). Perhaps the most convincing demonstration of the oocyte's role in controlling follicle development came from Eppig et al. (2002), who showed that reaggregated ovaries prepared with the ovarian somatic cells of newborn mice, and either primary or secondary oocytes, showed dramatic differences in their rate of follicle growth.

The first evidence for a role for the oocyte in follicular function in fact came from an early study in the rabbit, demonstrating that removal of the oocyte from the follicle led to premature luteinization (el-Fouly et al., 1970). Subsequently, the oocyte has been clearly shown to modulate granulosa cell luteinization, as the removal of the oocyte from mouse, bovine and porcine cumulus–oocyte complexes leads to increased progesterone production by the remaining cumulus cells (Coskun et al., 1995; Li et al., 2000; Vanderhyden et al., 1993), while addition of denuded oocytes to mouse, bovine and porcine mural granulosa cells has the opposite effect (Brankin et al., 2003; Coskun et al., 1995; Gilchrist et al., 2008; Li et al., 2000). Furthermore, evidence suggests that the oocyte controls steroidogenesis in a more general sense. In mouse cumulus cells and pig mural granulosa cells, presence of the oocyte enhanced FSH-induced oestradiol production (Brankin et al., 2003; Vanderhyden et al., 1993), while in pig cumulus cells and in bovine mural granulosa cells presence of the oocyte suppressed oestradiol production (Coskun et al., 1995; Glister et al., 2003). In addition, murine, porcine and bovine oocyte derived factors also stimulate cell proliferation, as shown by thymidine incorporation studies (Gilchrist et al., 2003, 2006; Hickey et al., 2004, 2005). Addition of porcine oocytes to cultured granulosa and theca cells increases their viability (Brankin et al., 2003), while the presence of bovine oocytes reduce cumulus cell apoptosis and likely contribute to cell proliferation (Hussein et al., 2005). Mouse oocytes have been shown to control cumulus cell metabolism as revealed by the down-regulation of several glycolytic enzymes and an amino acid transporter in response to oocytectomy or oocyte removal

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(Sugiura et al., 2005; Eppig et al., 2005). Interestingly, most of the aforementioned studies have used oocytes isolated from small and medium antral follicles and the effects observed have been generalized to fully grown oocytes, irrespective of the maturational status of the follicles from which they were isolated. The study of Eppig et al. (2002) suggests that such assumptions need careful evaluation.

The objectives of this study were: (1) to determine the influence of factors secreted by the porcine preovulatory GV oocyte on cumulus cell protein expression, (2) to investigate changes in the expression of candidate genes in cumulus cells previously shown to be modulated by the oocyte, and (3) to use real-time PCR-based analysis of gene expression in granulosa cells recovered from similar sows at sequential stages of follicular development to confirm associations among functionally important genes. The experimental approach was firstly to use a well characterized pig model, involving the weaned primiparous sow, to recover *in vivo* matured GV-stage oocytes from follicles confirmed to be at the late follicular phase of development, but not yet exposed to the preovulatory LH surge (Foxcroft et al., 2007; Paradis et al., 2009). Secondly, to use the oocytectomy model to determine if factors secreted by these oocytes can change protein and gene expression in cumulus cells recovered from late prepubertal gilts, to confer a more preovulatory-like phenotype. In the context of this experiment, the use GV oocytes was important since previous studies have demonstrated that maturing oocytes lose some of their ability to modulate granulosa and cumulus cells functions (Gilchrist et al., 2001; Nagyova et al., 2000). To our knowledge, this is the first study to investigate the effect of the porcine oocyte on protein and gene

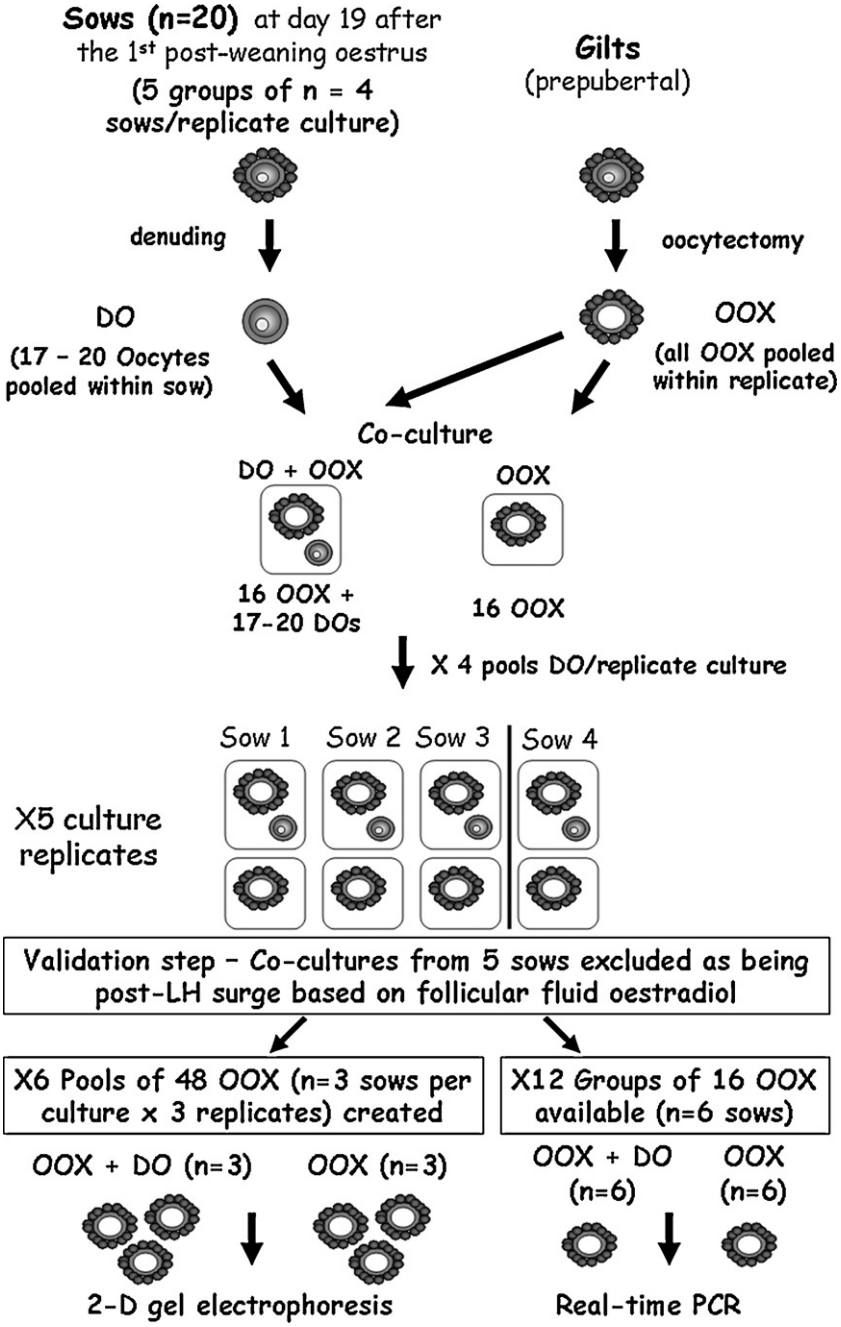

Fig. 1. Schematic outline of the use of animals and tissues, and associated analytical procedures, for the co-incubation experiment. Groups of oocytectomized cumulus–oocyte complexes (OOX) prepared from prepubertal gilt ovaries were cultured with or without denuded oocytes (DOs) prepared from replicate groups of weaned sows. Critical retrospective validation of the origin of DO as coming from large oestrogenic follicles determined the OOX ultimately used for proteomic and real-time PCR analysis.

expression in the surrounding cumulus cells, and the first study in any species to utilize oocytes isolated from large oestrogenic preovulatory follicles prior to the LH surge.

2. Materials and methods

2.1. Chemicals and media

Unless otherwise stated all chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). The media used for washing the COCs and OOX was modified Tyrode lactate (TL)-HEPES medium supplemented with 0.1% (w/v) polyvinyl alcohol (PVA) (Funahashi et al., 1997), 50 U/ml of penicillin and 50 μg/ml of streptomycin (Invitrogen, #15070-063, Burlington, ON, Canada). The phosphate-buffered saline (PBS) pH 7.4 was composed of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4. The modified M-199 (mM-199) media used for co-culture was Medium 199 with Earle's salts (Invitrogen # 31100-035) supplemented with 26 mM $NaHCO_3$, 0.91 mM sodium pyruvate, 0.1% PVA, 0.57 mM cysteine, 5 μg/ml insulin, 50 U/ml of penicillin and 50 μg/ml of streptomycin.

2.2. Animals

This experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care and with the approval of the University of Alberta, Faculty Animal Policy and Welfare Committee (Protocol #2005-40B). A total of 18 primiparous F1 sows (Large White × Landrace, Hypor, Regina, SK, Canada) were used for the temporal analysis of gene expression in the granulosa cells. These sows and the granulosa cell collection and processing were previously described in Paradis et al. (2009). Briefly, the sows were euthanized and their granulosa cells were collected by dissection at specific phases of follicular development corresponding to the period of recruitment of a growing cohort of follicles, the mid-selection phase where follicles either keep growing or, if not selected, start to undergo atresia and the final selection of the preovulatory follicle population before and after the preovulatory LH surge. A further 20 primiparous sows, managed in the same way, were used for the co-incubation experiment (see Fig. 1 for detailed experimental design). On each of five occasions, groups of four sows were euthanized on day 19 ± 1 after the 1st post-weaning oestrus. At this point a population of around 20 preovulatory follicles has been established (Grant et al., 1989) and the oocytes are fully competent to undergo meiotic resumption, fertilization and embryo development. The sows used in the experiment were euthanized on-site in a purpose-built necropsy facility according to the Swine Research and Technology Centre standard operating procedures. Prior to euthanasia, a single blood sample was collected into a 10 ml heparinized Vacutainer™ (Becton Dickinson, Franklin Lakes, NJ) by jugular puncture, centrifuged at 1700 × *g* for 15 min at room temperature and the plasma was then stored at −20 °C until assayed for plasma oestradiol concentration. As described previously (Paradis et al., 2009), follicular fluid and plasma oestradiol concentrations (see procedure below) were subsequently used to confirm that follicles had not been exposed to the LH surge and resumed meiosis constituting the validation step shown in Fig. 1.

2.3. Sow oocyte collection and culture

Within 1 h after euthanasia, the sows' ovaries were transported to the laboratory in 0.9% (w/v) warm saline supplemented with antibiotic and antimycotic. Before processing the ovaries, the number and size of all visible follicles was recorded and the follicular fluid from the largest follicle(s) of the D19 animals was collected for assay of oestradiol concentrations. Cumulus–oocyte complexes (COCs) were collected from both ovaries by aspiration and the COCs from each sow were processed as a group. The COCs were selected and washed three times in warm PVA-TL-HEPES to remove any cellular debris. The oocytes were then denuded by vortexing at low speed for 5 min in 200 μl of PBS in a 1.5 ml microcentrifuge tube. The denuded oocytes (DOs) were observed under a dissecting microscope to ensure that they were intact and free of cumulus cells and then washed three times in 5 ml of warm PVA-TL-HEPES to remove free cumulus cells. Groups of 17–20 DOs from individual animals were then transferred to 36 μl droplets of culture media under mineral oil for a final concentration of ~0.5 DOs/μl (Fig. 1). The culture media used was composed of mM199 (see Section 2.1) supplemented with 10 μg/ml of pFSH and pLH (Folltropin-V and Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario Canada) and 20 μM of the phosphodiesterase PDE3 inhibitor cilostamide (BIOMOL International, L.P., # PD-125, Plymouth Meeting, PA, USA). Cilostamide was chosen to prevent oocyte meiotic resumption because (1) it is very efficient at relatively low concentrations, (2) its effects are fully reversible, and (3) its specificity for PDE3 minimizes adverse effects on oocytes. Preventing oocyte maturation was essential since maturing oocyte lose some of their ability to modulate cumulus cells functions (Gilchrist et al., 2001; Nagyova et al., 2000). The denuded oocytes were then cultured for an initial 6-h period at 5% CO_2 in a humidified atmosphere at 38.5 °C to condition the culture media with oocyte-secreted factors while awaiting the preparation of OOX.

2.4. Gilt COC collection and oocytectomy

Gilt ovaries were collected from a commercial slaughterhouse and transported to the laboratory within 2 h after collection in a thermoflask containing 0.9% (w/v) warm saline supplemented with antibiotic and antimycotic. The cumulus–oocyte complexes (COCs) were collected by aspiration of 2–6 mm follicles into a 50 ml conical tube. The COCs were washed three times by sedimentation in warm PVA-TL-HEPES and then transferred into a Petri dish containing 15 ml of warm PVA-TL-HEPES. COCs with at least two layers of compact cumulus cells completely surrounding the oocyte were selected and washed an additional three times in PVA-TL-HEPES. The oocytes were microsurgically removed from the COC as described by Buccione et al. (1990) with the following modification. The oocyte cytoplasm was removed by aspiration with an enucleation pipet rather than by suction through the holding pipet, as this technique was found to cause less damage to the porcine cumulus cells following oocytectomy. The resulting oocytectomized cumulus cells (OOX) with less than 5% of oocyte cytoplasm remaining were used in the subsequent co-incubations. The OOX were then washed three times in mM199, pre-incubated at 5% CO_2 in a humidified atmosphere at 38.5 °C, transferred in groups of 16 into the 36 μl droplets of culture media under mineral oil, with or without oocytes, and incubated for 22 h at 5% CO_2 in a humidified atmosphere at 38.5 °C (Fig. 1). The 22 h maturation period corresponds to the first stage of porcine oocyte in vitro maturation during which the oocytes were maintained in meiotic arrest, allowing sufficient time for the OOX to be exposed to oocyte derived factors. A total of eight droplets were used per replicate culture, four with and four without oocytes from an individual sow (see Figs. 1 and 2). The co-cultures were repeated on five separate occasions using the same conditions.

After 22 h of culture, the OOX from each individual culture droplet were recovered, washed twice in warm PBS, transferred into their own 1.5 ml microcentrifuge tube containing 1 ml of warm PBS, centrifuged for 5 min at 8000 × *g* at room temperature and the OOX pellet was snap frozen on dry ice and stored at −80 °C. Denuded oocytes were fixed for at least 24 h in ethanol:acetic acid (3:1) before being stained for 15 min with a solution of 25 μg/ml of Hoechst 33258 in 100% ethanol, then destained for approximately 10 min in a solution of 20% (v/v) glycerol and their nuclear status evaluated under a microscope to ensure that the oocytes remained arrested at the germinal vesicle (GV) stage during culture.

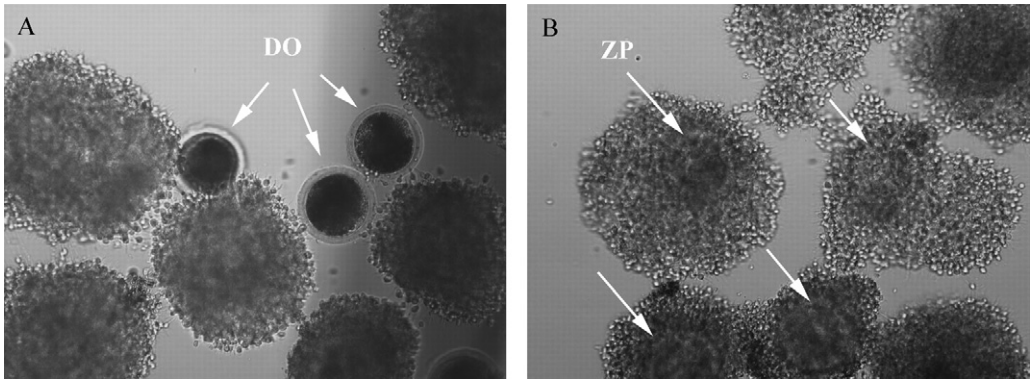

Fig. 2. Morphological appearance of the oocytectomized cumulus cells (OOX) after 22 h of culture with (A) and without (B) denuded oocytes (DOs) isolated from large oestrogenic sow follicles prior to the LH surge. In the focal plane used to capture image (B), the zona pellucidae (ZP) from which the oocyte has been aspirated are clearly visible. In image (A), in which the focal plane captures clear images of the DOs, the empty ZP are less obvious.

2.5. Radioimmunoassay

2.5.1. Plasma oestradiol

Oestradiol concentrations were determined in all plasma samples in triplicate in a single RIA using the method of Yang et al. (2000). Extraction efficiency was 72.4 ± 0.6% and estimated potencies were not corrected for recovery. Assay sensitivity, defined as 86% of total binding, was 0.35 pg/ml. The intra-assay CV was 5%

2.5.2. Follicular fluid oestradiol

Follicle fluid (FF) oestradiol concentrations were quantified in a single RIA using a double antibody kit (Diagnostic Products Corporation # KE2D1, Diagnostic Products Corporation, Los Angeles, CA, USA) without extraction using the method of Paradis et al. (2009). The intra-assay CV for the single assay run was 5.7%. Sensitivity estimated at 91% of total binding was 0.1 pg/tube, equivalent to 2.4 ng/ml. The recovery of a known amount of oestradiol when added to a sample of known potency was 94.5 ± 2.7%

2.6. 2D gel electrophoresis and data analysis

Using follicle size and follicular fluid oestradiol concentration as a retrospective measure of sow follicle maturity (large preovulatory follicle prior to the LH surge), OOX were selected from three of the five replicate cultures to be resolved by two-dimensional gel electrophoresis (Fig. 1). The remaining two replicates had less than two sows (groups of DOs) that satisfied the above criteria and could not be used. Six Immobiline™ DryStrips pH 3-10, 7 cm were rehydrated overnight with 150 μl of rehydration solution (7 M urea, 2 M thiourea, 4% Chaps, 1.2% (v/v) DeStreak reagent (GE Healthcare, Piscataway, NJ) and 0.8% (v/v) IPG buffer pH 3–10 (GE Healthcare)). The three groups of 16 OOX from each of the three selected replicate cultures incubated with or without DOs were solubilized in 35 μl of sample buffer (7 M urea, 2 M thiourea, 4% Chaps, 20 mM TCEP and 0.8% (v/v) IPG buffer pH 3–10 (GE Healthcare)) for 2 h at room temperature, vortexing occasionally. Within each replicate culture, all 48 OOX incubated alone or with DOs were then pooled (Fig. 1) and the samples centrifuged for 5 min at 14,000 × *g* at room temperature. The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube without disturbing the pellets and the volume was adjusted to 125 μl with sample buffer containing a trace of bromophenol blue.

The 6 pooled samples were simultaneously applied to the Immobiline™ DryStrips by anodic cup loading and the isoelectric focusing was performed for 13,500 Vh on an Ettan IPGphor apparatus (GE Healthcare). After focusing, the Immobiline™ DryStrips were equilibrated for 15 min in SDS equilibration buffer (6 M urea, 75 mM Tris–HCl pH 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS and a trace of bromophenol blue) with 10 mg/ml of dithiothreitol followed by 15 min in SDS equilibration buffer with 25 mg/ml of iodoacetamide. The equilibrated strips were loaded onto 10% (w/v) SDS-PAGE gels (8 cm × 10 cm), subjected to electrophoresis and the gels were fixed overnight in 50% (v/v) methanol. The gels were visualized using SYPRO Ruby protein stain according to the manufacturer's instructions (BioRad, Hercules, CA) and the images captured on a Typhoon Trio (GE Healthcare) at 550 V, high sensitivity and 100 μm resolution. From the total of six gels, only the gels from two replicates were submitted to the final analysis, as both gels from the third replicate showed incomplete isoelectric focusing. Gel images were processed and analyzed using the Progenesis SameSpot software (Nonlinear dynamics, Newcastle Upon Tyne, UK) and each spot was manually verified to ensure proper matching. A total of >600 spots were matched across the four gels and the individual spot intensities were normalized with the total spot volume from the gel of origin.

2.7. Preparative 2D gel for protein identification

Cumulus cells originating from 500 intact COCs, obtained from prepubertal gilts and cultured under the same conditions as the OOX, were resolved on two-dimensional gel electrophoresis to obtain a preparative gel for protein excision and sequencing. The 2D electrophoresis was performed as for the analytical gels (Section 2.6), however the sample was loaded by rehydration loading and a larger gel format was used to accommodate the higher protein load. Modifications were as follows: the cumulus cells were solubilised in 75 μl of sample buffer (7 M urea, 2 M thiourea, 4% Chaps, 5 mM TCEP) for 2 h at room temperature, vortexing occasionally. The sample was then centrifuged for 10 min at 18,000 × *g* and the supernatant transferred to a fresh tube containing 265 μl of rehydration solution (7 M urea, 2 M thiourea, 4% Chaps and a trace of bromophenol blue) supplemented with DeStreak reagent and IPG buffer pH 3-10 (GE Healthcare) to obtain a final concentration of 1.2% (v/v) and 0.8% (v/v), respectively. An Immobiline™ DryStrip pH 3–10, 18 cm was rehydrated overnight with 340 μl of sample and subjected to isoelectric focusing for a total of 46,000 Vh on an Ettan IPGphor apparatus (GE Healthcare). After focusing the Immobiline™ DryStrips were equilibrated as previously described and loaded onto 10% (w/v) SDS-PAGE gels (16 cm × 20 cm), subjected to electrophoresis and the gels were fixed overnight in 50% (v/v) methanol. The gels were visualized using Colloidal Blue protein stain according to manufacturer's instructions (GE Healthcare) and the image was captured on a 14-bit scanner (Imagescanner, GE Healthcare). The image from the preparative gel was matched to the images from the analytical gels. The proteins found to be differentially expressed and showing ≥1.2-fold changes were manually excised from the preparative and analytical gels and sent to a mass spectrometry facility for further processing and identification (Centre Genomique du Quebec, Sainte-Foy, Canada). All of the procedures for sample preparation, tryptic digestion, mass spectrometry and database searches by the Centre Genomique du Quebec are described in detail by Novak et al. (2009).

2.8. Criteria for protein identification

Scaffold (version Scaffold_2.1.03, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two unique identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.9. RNA isolation and real-time RT-PCR

Total RNA was extracted from 6 remaining pairs of 16 OOX incubated alone or with DOs (Fig. 1) using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA) following manufacturer's instructions. The DOs originated from follicles similar in size and estradiol content to those used for the 2D gel analysis. All samples were DNase treated as suggested in the manufacturer's protocol; the RNA was eluted in 11 μl and quantified using the spectrophotometer ND-1000 to ensure adequate recovery (NanoDrop, Wilmington, DE, USA). OOX total RNA was reverse transcribed with Superscript III reverse transcriptase (Invitrogen) according to manufacturer's instruction, using a combination of 5 μM oligo dT and 5 ng/μl of random hexamer. RNaseOUT (Invitrogen) was also added to the reaction at a concentration of 2 U/μl. cDNA synthesis was performed using approximately 200 ng of OOX total RNA and following the reverse transcription, the cDNA was diluted to the equivalent of 1 ng/μl with nuclease-free H_2O (Ambion).

Real-time PCR was performed in duplicate using 1 ng of cDNA from OOX or from 20 ng of cDNA from granulosa cells isolated during the recruitment, mid-selection and final selection phase before and after the preovulatory LH surge (described in detail in Paradis et al., 2009) in 96-well fast plates using the Taqman® Fast Universal PCR Master Mix and the ABI 7900HT thermocycler (Applied Biosystems, Foster City, CA, USA). The primers and Taqman-MGB probes (Table 1) were designed using the Primer Express® software v3.0 (Applied Biosystems) using sequences found on GENBANK or ordered from the porcine TaqMan® Gene Expression Assays library (Applied Biosystems). The amplification efficiency for each gene, determined using serial dilution of ovarian cDNA, was found to be ≥90% for all genes and the slopes for all 9 genes were found to be identical (data not shown). Cyclophilin was used as the endogenous control to correct for RNA extraction and reverse transcription efficiency and its transcript abundance was found to be stable between treatments, confirming its validity as an endogenous control.

2.10. Statistical analysis

Real-time PCR data for the genes of interest (GOI) were normalized against their respective means for cyclophilin using the ΔCt method ($\Delta Ct = Ct_{GOI} - Ct_{cyclophilin}$). The cycle threshold (Ct) is defined as the PCR cycle where the fluorescence reaches a determined threshold. Consequently, the Ct and corrected Ct (ΔCt) value are inversely related to the copy number of the targeted gene initially present in the sample. For the analysis of mRNA abundance between OOX cultured alone or with DO, the ΔCt values for all GOI were normally distributed and the individual ΔCt for each GOI were analyzed using the MIXED procedure of SAS. The model for the experiment included treatment as the independent variables, and sow as the random variable. Differences between means were analyzed using a Least Significant Difference (LSD) test at a 95% confidence level. For the analysis of the temporal changes in FSHR and HSD3B mRNA abundance in granulosa cells, the ΔCt values for the GOI were RANK transformed for normality and the analysis was performed on the transformed data. The individual ΔCt for each GOI were analyzed using the MIXED procedure of SAS. The model for the experiment included phase as the independent variable. Differences between means were analyzed using a least significant difference (LSD) test at a 95% confidence level. For ease of interpretation of the expression profiles the data were converted using the formula $2^{-(\Delta Ct - \Delta Ct_{calibrator})}$ and are expressed as relative mRNA abundance ± SEM. The highest expression (lowest ΔCt) for each GOI was used as the calibrator value and the corresponding ΔCt is expressed as a relative abundance to that value. Finally, correlation analysis was performed to determine the nature of the relationships between FSHR mRNA abundance and that of YWHAH mRNA, one of the proteins identified by LC–MS/MS.

3. Results

3.1. Phenotypic characteristics of OOX after culture

Visual assessment of the appearance of the OOX following 22 h of culture in the presence or absence of denuded preovulatory

Table 1
Details of primers and probes used for real-time PCR.

Gene	AC number	Primer	Sequence 5′ → 3′ or assay ID	Product size (bp)	Annealing/extension temperature (°C)
BAX	AJ606301	Forward Reverse Probe	CCCCCGAGAAGTCTTTTTCC TGAAGTTGCCGTCAGCAAAC AGTGGCGGCCGAAA	56	62
BCL2	AB271960	Forward Reverse Probe	CGCTGGGAGAACAGGGTATG TGCGACAGCTTATAGTGGATGTACT AACCGGGAAATAGTG	66	60
CCND2	*NM_214088*	ABI	Ss03382534_s1	97	62
CYP19A1	NM_214429	Forward Reverse Probe	GTGCCTTTTGCCAGCATTG AATTCCAAACCAAGAGAAGAAAGC AGTCCTGCTGCTCACT	61	60
FSHR	NM_214386	Forward Reverse Probe	TCACAGTGAGGAACCCCAACA GCCATACGCTTGGCGATCT TGTCCTCCTCTAGTGACACC	64	60
HSD3B1	NM_001004049	Forward Reverse Probe	CGTCCTGACACACAACTCCAA CCACGTTGCCGACGTAGAC TTCTCCAGAGTCAACCC	59	60
LDHA	SDU07178	Forward Reverse Probe	TCACAAACAGGTGGTGGACAGT CCCAGGACGTGTAGCCTTTC CTTATGAGGTGATCAAAC	62	62
LHCGR	NM_214449	Forward Reverse Probe	TGGAGCTGAAGGAGAATGCA CCCTCGGAAGGCGTCAT ACCTGAAGAAGATGCAC	56	60
PFKP	AK238519	Forward Reverse Probe	AGGCTCCATTCTTGGGACAA CCGCAATGTCCTCCAGGTACT ACGCACGCTTCCT	59	60
PPIA	AY266299	Forward Reverse Probe	AATGCTGGCCCCAACACA TCAGTCTTGGCAGTGCAAATG ACGGTTCCCAGTTTT	56	60
STAR	NM_213755	Forward Reverse Probe	GGGACGAGGTGCTGAGTAAAGT CACCTCCAGCCGGAACAC ATCCCAGATGTGGGCAA	59	60
YWHAH	XM_001928076	Forward Reverse Probe	GGGCGATTACTACCGCTACCT TCAACCACGCTGTTTTTCTTCTC CCGAGGTGGCTTC	63	62

oocytes did not reveal any particular phenotype (Fig. 2) and OOX diameter, used as a measure of expansion, was identical between OOX cultured alone and with oocytes (data not shown). On a scale from 0 to 4, where 0 is no expansion and 4 is full expansion, both sets of OOX scored 1 after 22 h of culture (Fig. 2). Moreover, the number of potentially atretic OOX, as assessed by morphological characteristics including cell attachment to the dish and appearance/colour of the cytoplasm, did not indicate any differences between the two groups (Fig. 2). The nuclear status of the denuded oocytes used for the co-culture was also assessed after 22 h of culture using Hoechst 33258 staining to ensure that the oocyte remained arrested at the germinal vesicle stage and did not resume meiosis. Evaluation of oocyte nuclear maturation confirmed that more than 95% of the oocytes remained arrested at the germinal vesicle stage (GV) (data not shown), validating the effectiveness of cilostamide in preventing meiotic resumption and confirming that the effects observed were emanating from fully grown preovulatory GV oocytes. Finally, follicular fluid oestradiol and plasma oestradiol, as well as the average size of the three largest follicles, were used to determine that the sow oocytes used in the experiment were derived from large oestrogenic preovulatory follicles before exposure to the endogenous preovulatory LH surge (Table 2).

3.2. Protein profiling and identification

More than 600 protein spots were matched across the 4 gels, of which 14 proteins were found to be differentially expressed by OOX incubated with and without denuded oocytes. Interestingly, 9 of the 14 differentially expressed proteins were up-regulated in OOX incubated without DOs (Fig. 3A, B and Table 3). The magnitude of the up-regulation ranged from 1.2-fold for protein spot 20 to 2.7-fold for protein spot 2 (Fig. 3B and Table 3). Conversely, only 5 of

Table 2
Characteristics of the fifteen sows used for the recovery of preovulatory follicles (POF) from which oocytes were isolated.

Sow weight (kg)	Nos. POF per sow	Average size of the three largest follicles (mm)	Plasma E2[a] concentration (pg/ml)	Follicular fluid E2[a] concentration (ng/ml)
170 ± 10	21 ± 2	8.4 ± 0.9	25 ± 12	141 ± 67

Data are expressed as means ± SD.
[a] Oestradiol.

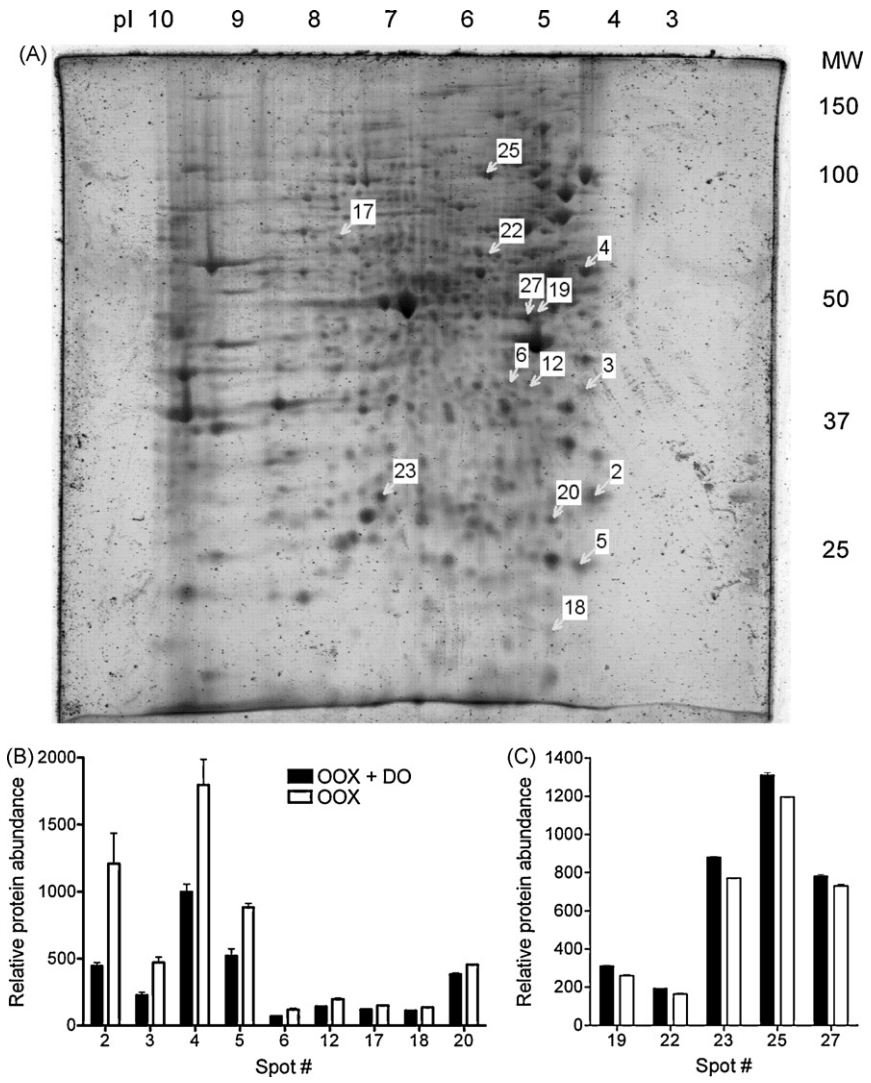

Fig. 3. Representative two-dimensional gel of proteins from a pool of 48 OOX incubated without oocytes. Protein spot numbers identify proteins differentially expressed between treatments (A). The proteins found to be up- and down-regulated in OOX culture alone are shown in (B) and (C), respectively.

Table 3
Proteins identified by LC–MS/MS that were differentially expressed between oocytectomized cumulus cells (OOX) incubated alone or with denuded oocytes (DOs) isolated from large oestrogenic sow follicles prior to the LH surge.

Spot #	↑↓ in OOX w/o DOs (fold change)	Protein name (symbol)	Accession #	Observed MW (kDa)/pI	Theoretical MW (kDa)/pI	# of unique Peptides (% coverage)
2	↑ (2.7)	14-3-3 protein eta (Protein AS1) (YWHAH)	UPI0000EB0531	28/4	28/4.8	21 (60)
3	↑ (2.1)	Nucleophosmin (NPM1)	P06748	38/4	33/4.6	10 (39)
4	↑ (1.8)	Prolyl 4-hydroxylase, beta subunit (P4HB)	P05307	60/4.5	57/4.7	15 (32)
5	↑ (1.7)	Translationally controlled tumor protein (TCTP)	P13693	23/4.5	20/4.8	6 (31)
6.1	↑ (1.7)	Eukaryotic translation initiation factor 3 subunit I	Q9QZD9	38/5	36/5.4	15 (53)
6.2		Calponin-3	Q32L92	38/5.5	36/5.7	15 (43)
12.1	↑ (1.4)	Eukaryotic translation initiation factor 3 subunit I	Q9QZD9	38/5	36/5.4	11 (49)
12.2		Calponin-3	Q32L92	38/5.5	36/5.7	11 (37)
17	↑ (1.3)	Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q)	O60506	70/7.5	60-68/8.5	10 (22)
19.1	↓ (1.2)	Eukaryotic initiation factor 4A-I (EIF4A1)	P60842	45/5	46/5.3	10 (31)
19.2		cAMP-dependent protein kinase type I-alpha regulatory subunit	P07802	45/5	43/5.3	7 (24)
20	↑ (1.2)	Rho GDP-dissociation inhibitor 1 (ARHGDIA)	P19803	26/5	23/5	8 (43)
22.1	↓ (1.2)	Highly similar to T-complex protein 1 subunit epsilon	UPI00015601FB	62/5.7	59/5.5	25 (48)
22.2		Procollagen-proline 2-oxoglutarate-4-dioxygenase	A1X898	62/5.7	61/5.7	19 (40)
23	↓ (1.15)	Phosphoglycerate mutase 1 (PGAM1)	P18669	27/7	29/6.7	20 (78)
25	↓ (1.1)	Neutral alpha-glucosidase AB (GANAB)	P79403	110/5.7	107/5.5	46 (56)
27.1	↓ (1.1)	Eukaryotic initiation factor 4A-I (EIF4A1)	P60842	43/5	46/5.3	16 (42)
27.2		cAMP-dependent protein kinase type I-alpha regulatory subunit	P07802	43/5	43/5.3	6 (22)

Protein spots #6, 12, 19, 22 and 27 could not be effectively matched to a single protein and may have contained a mixture of proteins. The identities of the two most probable identifications are therefore provided.

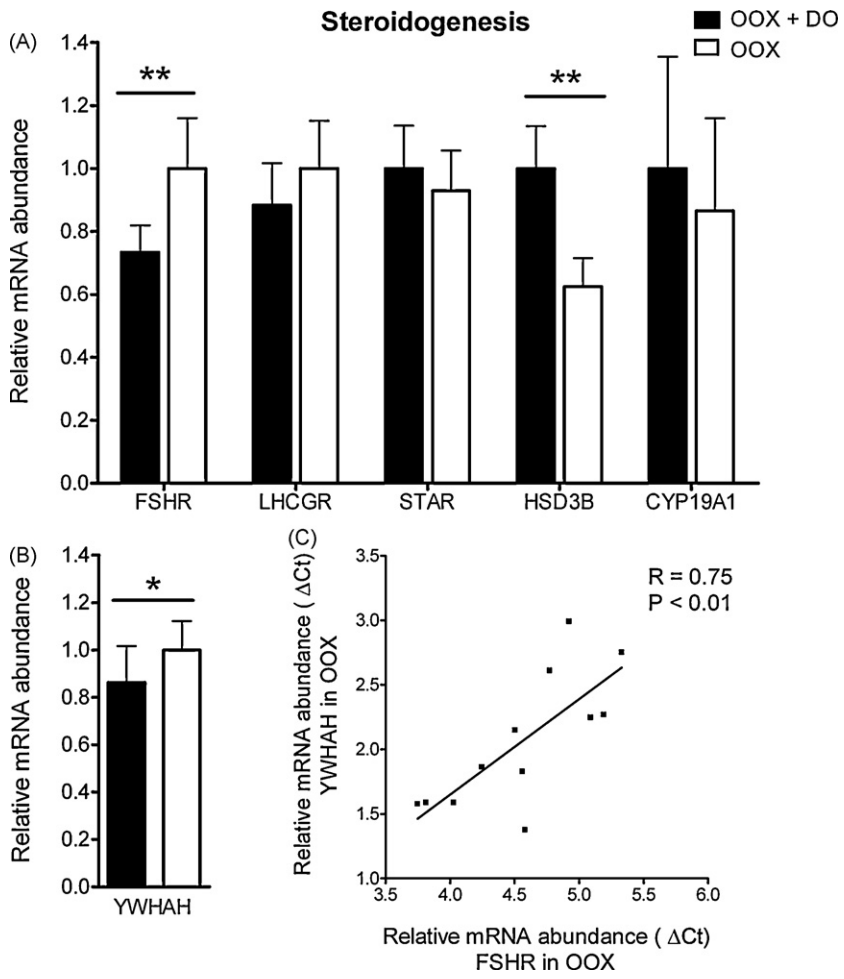

Fig. 4. Quantification via real-time RT-PCR of (A) the mRNA of genes involved in steroidogenesis and (B) YWHAH mRNA in pig oocytectomized cumulus cells after 22 h of culture with ($n = 6$) and without ($n = 6$) denuded oocytes isolated from large oestrogenic sow follicles prior to the LH surge. Data are expressed as relative mRNA abundance ± SEM. The asterisks indicates differences within gene between treatment (**$P \leq 0.05$ and *$P \leq 0.1$). (C) Correlation analysis between FSHR and YWHAH mRNA abundance (ΔCt).

the 14 proteins were down-regulated in OOX incubated without oocytes and the magnitude of the down-regulation observed was generally lower, ranging between 1.1- and 1.2-fold changes (Fig. 3A and C and Table 3).

To determine the identity of the differentially expressed proteins, the spots were excised from the gel and sent for identification by LC–MS/MS. The identity of all the proteins spots, with the exception of protein spot #18, was successfully determined (Table 3). Protein spot #18 could not be effectively visualized on the analytical gel for excision and could not be accurately matched to the preparative gel and therefore was not sent for identification. In addition, after analysis of the peptides identified for each protein spot, five proteins could not be effectively matched to a single protein and may have contained a mixture of proteins. Consequently, the identities of the two most probable identifications are provided (Table 3: spots #6, 12, 19, 22, 27). The likelihood that these protein spots correspond to specific proteins was assessed based on the number of unique peptides identified, the percentage of protein coverage, and the comparison between the observed and the predicted molecular weight and isoelectric point.

3.3. Real-time PCR analysis

3.3.1. Co-incubation experiment

In parallel with the protein analysis, real-time PCR was performed on selected candidate genes. Culturing the OOX with or without oocytes did not affect mRNA abundance for the apoptosis related gene BCL2-associated X protein (*BAX*) and B-cell CLL/lymphoma 2 (*BCL2*), the cell proliferation marker cyclin D2 (*CCND2*), or the phosphofructokinase (*PFKP*) and lactate dehydrogenase A (*LDHA*) genes, known for their involvement in metabolism (data not shown). The mRNA abundance for several genes involved in steroidogenesis was also evaluated (Fig. 4A). Although, luteinizing hormone receptor (*LHCGR*), steroidogenic acute regulatory protein (*STAR*) and cytochrome P450 aromatase (*CYP19A1*) mRNA abundance was not affected by the co-culture of OOX with oocyte, follicle stimulating hormone receptor (*FSHR*) mRNA decreased by more than 25% ($P < 0.05$) and 3β-hydroxysteroid dehydrogenase (*HSD3B*) mRNA abundance increased by almost 40% ($P < 0.05$) in the presence of denuded oocytes. Interestingly, *14-3-3η* (*YWHAH*) mRNA expression also tended ($P = 0.08$) to decrease in OOX cultured with oocytes in a similar way to the protein and a strong positive correlation between YWHAH mRNA and *FSHR* mRNA abundance was found (Fig. 4B and C; $R = 0.75$, $P < 0.01$).

3.3.2. Granulosa cells of developing antral follicles

Abundance of *FSHR* mRNA was higher ($P < 0.05$) in granulosa cells from smaller follicles (3–6 mm), taken during the recruitment and mid-selection phase than in large preovulatory follicles recovered before and after the LH surge (Fig. 5A). The expression of *HSD3B* in the granulosa cells showed the opposite pattern; mRNA abundance was low in smaller follicles during the recruitment and

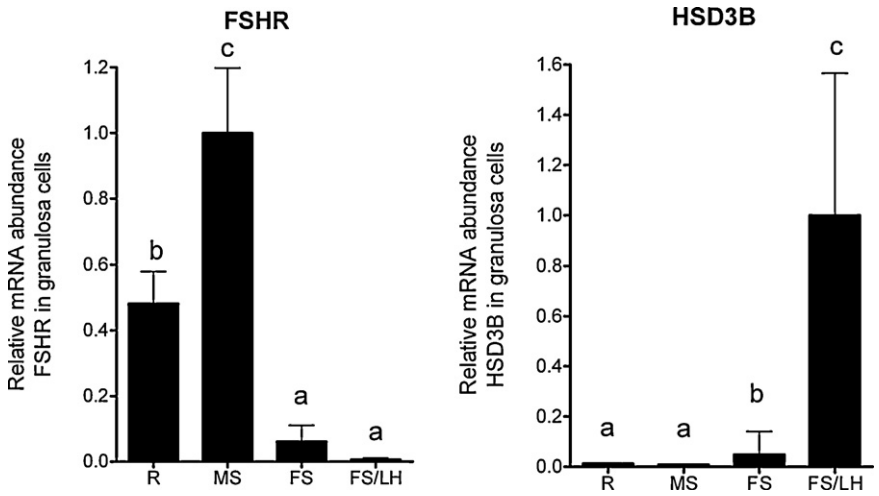

Fig. 5. Quantification via real-time RT-PCR of (A) *FSHR* mRNA, (B) *HSD3B* mRNA abundance in pig granulosa cells during follicle recruitment (R; $n = 6$), mid-selection (MS; $n = 6$), final selection (FS; $n = 3$) and final selection post-LH surge (FS/LH; $n = 3$). Data are expressed as relative mRNA abundance ± SEM for each phase, irrespective of cycle. Different letters within gene represent significant difference among phases ($^{a,b,c}P \leq 0.05$).

mid-selection phase but substantially increased ($P < 0.05$) during the periovulatory period prior and after the LH surge (Fig. 5B).

4. Discussion

The influence of oocyte secreted factors on neighbouring somatic cells is well established (Gilchrist et al., 2008; Juengel and McNatty, 2005). However, information on the role of the porcine oocyte and the follicular environment from which it originates remains limited. The present study described the effects of pig preovulatory oocytes on cumulus cells and demonstrates that the oocyte modulates steroidogenic-related genes and proteins in a way that reflects the specific physiological requirements of the preovulatory follicle.

Morphological evaluation of the OOX after 22 h of culture revealed no obvious differences in their diameter. Although the current experimental conditions are not optimal for evaluating cumulus expansion, our results are consistent with the lack of an effect of porcine oocytes on cumulus cell expansion and hyaluronic acid synthesis reported by others (Prochazka et al., 1991; Singh et al., 1993; Nagyova et al., 1999). Moreover, the overall morphological appearance of the OOX, including attachment to the culture dish and appearance and colour of the cytoplasm, did not suggest a higher incidence of atresia in the OOX incubated in the absence of denuded oocytes. This was reflected in the real-time PCR analysis of *BAX* (pro-apoptotic) and *BCL2* (anti-apoptotic) mRNA abundance, which were not affected by culture with or without denuded oocytes. In contrast, Hussein et al. (2005) reported that bovine oocytes prevent apoptosis in OOX and that this effect is achieved by modulation of BAX and BCL2 expression in the cumulus cells.

Based on the 2D gel analysis, 14 proteins were found to be differentially expressed between the OOX incubated with and without denuded oocytes. Given that over 600 protein spots were matched across the gels, less than 2% of the proteins appear to be affected, suggesting that the effects on the OOX were specific and do not reflect global changes in protein expression. This result is also consistent with that reported by Eppig et al. (1997), who showed that few differences were observed in the global pattern of protein synthesis between mural granulosa cells cultured with and without oocytes. Interestingly, 9 of the 14 proteins identified in this study were up-regulated in the OOX cultured without oocytes and the extent of the up-regulation was more substantial than the down-regulation observed in the remaining 5 proteins. These results suggest that the oocyte has a predominantly suppressive effect on protein expression in the cumulus cells. This observation is in accordance with the current concept in the mouse suggesting that the oocyte is responsible for maintaining the cumulus cell phenotype and that removal of the oocyte from the cumulus oocyte complexes leads to up-regulation of several mural granulosa cells markers in the cumulus cells, such as LHCGR, CYP11A1 and CD34 (Diaz et al., 2007; Eppig et al., 1997). It could also be suggested that the differences observed between the suppressive and stimulatory effects of the oocyte simply reflect the differences in the time required for protein synthesis, compared to that for protein turnover. However, irrespective of the mechanisms responsible for the observed differences in protein up- and down-regulation following co-incubation with oocytes, our results are in accordance with the proposition that oocytes are essential for maintaining the cumulus cell phenotype.

The protein showing the largest degree of up-regulation in the OOX cultured without denuded oocytes was identified by LC–MS/MS as 14-3-3 protein eta (14-3-3η or YWHAH). The 14-3-3 proteins are part of a large family of highly conserved acidic proteins shown to be involved in cell division, apoptosis, cell signaling and in the modulation of signal transduction pathways involving various receptors (van Hemert et al., 2001). In the context of the present study, it is interesting that the 14-3-3 protein family (14-3-3τ) was shown to interact with and inhibit FSHR (Cohen et al., 2004). These observations are particularly interesting since correlation analysis in the present study revealed a strong positive relationship between *FSHR* and *14-3-3η* mRNA abundance, suggesting that the expression of these two genes may be closely linked. Together with the observed down-regulation of *FSHR* mRNA in OOX incubated with oocytes, these results suggest that preovulatory porcine oocytes act on cumulus cells to modulate their responsiveness to FSH.

Perhaps the most intriguing finding of this study is the antagonistic effect of the porcine preovulatory oocyte on *FSHR* (down-regulation) and *HSD3B* (up-regulation) mRNA abundance in the oocytectomized cumulus cells. Interestingly, no effects were observed on *LHCGR*, *STAR* or *CYP19A1* mRNA abundance. These observations are somewhat puzzling since these effects have either not been previously reported in the literature or contradict results from most existing studies. First, *LHCGR* mRNA was shown to be up-regulated in FSH-stimulated mouse oocytectomized cumulus cells, while addition of denuded oocytes to mural granulosa cells had the opposite effect (Eppig et al., 1997). In our study, the presence or absence of oocytes did not affect *LHCGR* mRNA abundance in

OOX, but rather modulated *FSHR* mRNA abundance and also potentially altered its activity via regulation of the 14-3-3 proteins. One could suggest that the effects on LHCGR observed in the study by Eppig et al. (1997) resulted from the indirect modulation of FSHR by the oocyte, since FSH is known to induce LHCGR. However, the authors showed that this effect occurred downstream of FSHR activation and cAMP production (Eppig et al., 1998). However, since FSHR signal transduction can occur through various protein kinases pathways including PKC (Chen et al., 2008; Downs et al., 2001; Downs and Chen, 2008; Fan et al., 2004; Jin et al., 2006; Lu et al., 2001; Su et al., 1999), the possibility exists that the mouse oocyte also modulates the activity of FSHR. On the other hand, Otsuka et al. (2001) reported that BMP15, a known oocyte-derived ligand, suppressed FSHR in rat granulosa cells isolated from small antral follicles. This observation clearly indicates that, as observed in our experiment, the rat oocyte can also modulate FSHR activity. In addition, our findings suggest that the preovulatory porcine oocyte increases *HSD3B* mRNA abundance. Since HSD3B is the enzyme responsible for the conversion of pregnenolone into progesterone and the porcine granulosa cells do not possess the enzyme CYP17A1 necessary to convert progesterone to androgen, the increase in *HSD3B* mRNA observed in the cumulus cells is likely contributing to increased progesterone production. This observation contrasts previous studies in mouse, bovine and porcine granulosa cells in which the oocyte has generally been shown to have a suppressive effect on progesterone production (Brankin et al., 2003; Coskun et al., 1995; Gilchrist et al., 2008; Li et al., 2000; Vanderhyden et al., 1993). However, all of the above studies have used oocytes from small and medium (3–6 mm) antral follicles in contrast to the preovulatory oocytes used in the present study.

In order to better comprehend the physiological relevance of these changes in *FSHR* and *HSD3B* mRNA abundance, and that of 14-3-3η protein and mRNA observed in the oocytectomized cumulus cells, we evaluated the temporal expression pattern of *FSHR* and *HSD3B* mRNA in porcine granulosa cells during the follicular phase. Our results clearly showed that *FSHR* mRNA abundance is high during the recruitment and mid-selection phase when only small and medium antral follicles are present on the ovary, and its expression was dramatically reduced during the final selection phase before and after the LH surge, at a time where the preovulatory population has been established and comprises only large oestrogenic follicles (Grant et al., 1989). In contrast, *HSD3B* mRNA abundance was found to be very low in small- and medium-sized antral follicles but increased substantially in the large preovulatory follicle population before and after the LH surge. Our results are consistent with conclusions from the existing literature that show *FSHR* mRNA decreasing as the follicle is growing and transitioning from FSH to LH dependence at the same time as *HSD3B* mRNA is increasing in preparation for ovulation (Yuan et al., 1996). Interestingly, the oocytectomized cumulus cells used in the current study were isolated from small–medium antral follicles from prepubertal gilt ovaries that are still dependent on FSHR expression and are not likely to express HSD3B. By comparison, the oocytes originated from large oestrogenic preovulatory sow follicles in which FSHR has normally been down-regulated and HSD3B expression has increased in preparation for ovulation. Interestingly, addition of progesterone to the culture media has been shown to improve meiotic resumption of porcine oocytes (Yamashita et al., 2003), while the use of progesterone receptor antagonist reduced oocyte developmental competence (Shimada et al., 2004), suggesting that progesterone signaling is essential in the preovulatory follicle. Collectively, these results strongly suggest that oocyte modulation of cumulus cell steroidogenesis is developmentally regulated in the pig, allowing the oocyte to change the follicular environment to adapt for the specific requirements of each stage of follicle growth.

There are already several lines of evidence to suggest that the influence of the oocyte on the surrounding somatic cell changes as the follicle develops. For example, Eppig et al. (2002) showed that primary and secondary oocytes affect the rate of follicle development differently. Moreover, mouse oocytes progressing to metaphase II gradually lose their mitogenic inducing ability on the granulosa cells (Gilchrist et al., 2001) while maturing porcine oocytes lose their ability to induce cumulus expansion and retention of hyaluronic acid (Nagyova et al., 2000). Similarly, Eppig et al. (1997) have shown that the ability of the oocyte to suppress LHCGR mRNA abundance in the mouse granulosa cell is developmentally regulated, since fully grown oocytes potently suppress LHCGR mRNA, while growing oocytes isolated from preantral follicles and maturing oocytes were less capable of suppressing LHCGR expression. Nevertheless, this is the first study to suggest that developmental regulation also occurs within fully grown oocytes and depends on the size and maturational status of the follicle from which they are isolated.

The up-regulation of phosphoglycerate mutase (PGAM1) in OOX cultured with denuded oocytes represents another compelling finding. PGAM1 is a glycolytic enzyme that catalyzes the interconversion of 2- and 3-phosphoglycerate and changes in its abundance and/or activity could potentially modulate levels of glycolysis. Mouse oocytes have also been shown to positively regulate cumulus cell metabolism by modulating several glycolytic enzymes (Sugiura et al., 2005; Eppig et al., 2005). In the current experiment, mRNA abundance for phosphofructokinase (PFKP) and lactate dehydrogenase A (LDHA), two essential enzymes in metabolism, were not differentially regulated in OOX suggesting that the glycolytic ability of the OOX is not regulated by the oocyte. However, changes in the activity of this enzyme could also modify the quantity of glucose 6-phosphate available for the pentose–phosphate pathway (PPP): this is indispensable for maintaining the levels of NADPH in the cell, necessary for downstream biosynthetic pathways such as steroid synthesis, and also for protecting the cells against oxidative damage by maintaining the level of reduced glutathione (Shalom-Barak and Knaus, 2002). Unfortunately, it was not possible to design an efficient primer–probe set to evaluate glucose 6-phosphate dehydrogenase (G6PD) mRNA abundance, which is the rate limiting enzyme in the pentose–phosphate pathway.

The role of many of the other proteins found to be differentially expressed in the OOX remains largely unknown in the ovary, while others have the potential to affect a very broad range of functions. It is, therefore, very difficult to speculate as to their possible functions in the context of the present study. Generally, the biological relevance of proteins found to be differentially expressed by as little as 1.1 fold remains to be established. However, it could be argued that a 10% change in a protein involved in a rate limiting step could have an important impact on the cell.

In conclusion, the findings of the present study clearly demonstrate that the porcine preovulatory oocyte secretes soluble factors that act on the surrounding cumulus cells to modulate protein and gene expression. Our results suggest that the effect of the fully grown GV oocyte is developmentally regulated, allowing the oocyte to modulate the follicular environment to adapt to the specific requirements of each stage of follicle growth. To our knowledge, this is the first study to investigate the role of the porcine oocyte on the cumulus cell global protein expression and it is the first study investigating the role of the preovulatory oocyte on mammalian cumulus cell functions. Additional studies are needed to further elucidate the nature of the interaction between FSHR and 14-3-3 protein and to assess the potential roles of other proteins identified in this study.

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