Transcriptome analyses of ovarian stroma: tunica albuginea, interstitium and theca interna

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Abstract

The ovary has specialised stromal compartments, including the tunica albuginea, interstitial stroma and theca interna, which develops concurrently with the follicular antrum. To characterise the molecular determinants of these compartments, stroma adjacent to preantral follicles (pre-theca), interstitium and tunica albuginea were laser microdissected ($n=4$ per group) and theca interna was dissected from bovine antral follicles ($n=6$). RNA microarray analysis showed minimal differences between interstitial stroma and pre-theca, and these were combined for some analyses and referred to as stroma. Genes significantly upregulated in theca interna compared to stroma included *INSL3*, *LHCGR*, *HSD3B1*, *CYP17A1*, *ALDH1A1*, *OGN*, *POSTN* and *ASPN*. Quantitative RT-PCR showed significantly greater expression of *OGN* and *LGALS1* in interstitial stroma and theca interna versus tunica and greater expression of *ACD* in tunica compared to theca interna. *PLN* was significantly higher in interstitial stroma compared to tunica and theca. Ingenuity pathway, network and upstream regulator analyses were undertaken. Cell survival was also upregulated in theca interna. The tunica albuginea was associated with GPCR and cAMP signalling, suggesting tunica contractility. It was also associated with TGF- β signalling and increased fibrous matrix. Western immunoblotting was positive for *OGN*, *LGALS1*, *ALDH1A1*, *ACD* and *PLN* with *PLN* and *OGN* highly expressed in tunica and interstitial stroma (each $n=6$), but not in theca interna from antral follicles ($n=24$). Immunohistochemistry localised *LGALS1* and *POSTN* to extracellular matrix and *PLN* to smooth muscle cells. These results have identified novel differences between the ovarian stromal compartments.

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Introduction

Ovarian function is important for female health and reproduction. Much is known about the endocrine and paracrine signalling of ovarian development and function, particularly the factors that regulate the important events of ovarian organogenesis (Pepling 2012, Liu *et al.* 2015, Suzuki *et al.* 2015), follicle activation, growth and ovulation (Edson *et al.* 2009, Scaramuzzi *et al.* 2011, Hsueh *et al.* 2015, Monniaux 2016) and corpus luteum formation and regression (Pate *et al.* 2012). In addition, physical changes to the internal environment of the ovary may also influence follicle dynamics (Woodruff & Shea 2011, Shea *et al.* 2014). Unlike the oocyte and membrana granulosa, which are anatomically distinct, the theca is a heterogeneous tissue compartment contiguous with the ovarian interstitial stroma. An important stage in follicle maturity is the vascularisation of the theca interna and acquisition of steroidogenic potential by thecal cells, coincident with formation of the serum-derived

fluid within the antral cavity. In contrast to the current understanding of oocyte and granulosa cell maturation (Khan *et al.* 2016), the dynamics of the organisation and differentiation of the theca are poorly understood (Braw-Tal & Roth 2005, Orisaka *et al.* 2006a,b, Honda *et al.* 2007, Young & McNeilly 2010, Liu *et al.* 2015). In addition, developmental studies undertaken in mice may not adequately reflect ovarian formation or growth as it occurs in larger mammals (Jimenez 2009) and the existence of stem or progenitor cells in the ovary is an area only now becoming the object of investigation (Honda *et al.* 2007, Lee *et al.* 2013, Hummitzsch *et al.* 2015a, Adib & Valojerdi 2017, Truman *et al.* 2017). Previous studies have found that granulosa cells influence the expression of thecal markers in cells harvested from whole neonatal mouse ovaries (Honda *et al.* 2007) and bovine ovarian cortex, but not medulla (Orisaka *et al.* 2006b), suggesting the presence of thecal progenitor cells in the cortex.

Laser capture microdissection (LCM) is a powerful new technology that allows the precise selection of

targeted groups of cells based upon their location and appearance in tissue sections. In organs such as the ovary, which contains various types of cells and follicles at different developmental stages, LCM provides unique capabilities for isolating specific cells and structures, following changes in space and time. It had been applied successfully to identify and collect specific cells to enable studies of the expression of genes in fetal (Bocca *et al.* 2008) and adult oocytes (Markholt *et al.* 2012) and granulosa cells (Simpson *et al.* 2001, Bonnet *et al.* 2011, 2013), as well as of follicles at different maturational time points, including comparisons of cells from wild-type and oestrogen receptor beta-null ovaries (Binder *et al.* 2013) and the ovarian surface epithelium (Bowen *et al.* 2009). Despite this progress, to date, the collection of RNA of sufficient quality for microarray from thecal cells has been problematic (Binder *et al.* 2013).

Although our previous transcriptomic studies of the bovine theca interna have examined changes during follicular growth from small-to-large antral sizes (Hatzirodos *et al.* 2014a) and atresia (Hatzirodos *et al.* 2014b) as well as thecal cells treated with LH and/or BMP6 (Glister *et al.* 2013); however, less is known about the other stromal compartments of the ovary. The initial objective of the present study was to identify the differences in gene expression indicative of the transition from preantral to antral stages during follicular development and in the tunica albuginea. Gene expression of cell samples from ovaries of cycling cows was compared following hybridisation of cDNA to Affymetrix bovine gene arrays as described previously (Hatzirodos *et al.* 2015). Samples obtained by LCM were collected from the stroma adjacent to preantral follicles (pre-theca), the interstitial stroma between follicles, and the tunica albuginea, while theca interna of small antral follicles were collected by microdissection. To confirm differential gene expression, quantitative real-time PCR was performed on samples of tunica albuginea, interstitial stroma and theca interna. Protein levels and/or localisation were estimated by Western immunoblot and immunohistochemistry, respectively. Due to similarity of expression by interstitium and the stroma surrounding preantral follicles these two groups were combined and compared to the tunica albuginea and theca interna.

Materials and methods

Tissues

Bovine ovaries (one per animal) were collected at a local abattoir (Thomas Foods International, Murray Bridge, SA, Australia) from non-pregnant *Bos taurus* cows, within 20 min of slaughter and transported to the laboratory on ice. Because preantral follicles are too small for accurate manual dissection, laser capture was used to harvest the pre-theca, tunica and interstitial stroma for the initial microarray analysis. In order to harvest sufficient tissue for qRT-PCR and Western immunoblot and be consistent with our previous studies (Hatzirodos *et al.*

2014b), the theca interna of antral follicles, interstitium and tunica albuginea were also collected by microdissection.

For laser capture and microarray analyses of pre-theca, interstitium and tunica albuginea, strips of ovarian cortex were cut from each ovary and placed surface-side down into cryomolds (Miles Inc., Elkhart, IN, USA), containing optimal cutting temperature (OCT) compound (ProSciTech, Thuringowa central, QLD, Australia), frozen on dry ice and stored at -80°C .

For isolation of RNA from dissected samples, bovine thecal interna from small antral healthy follicles ($<5\text{ mm}$; $n=7$ follicles from five animals for qRT-PCR and $n=6$ follicles from another four different animals for microarray analyses), interstitium between small follicles ($n=6-7$ follicles from 6 to 7 different animals) and tunica albuginea ($n=7-8$ follicles from 7 to 8 different animals) samples were dissected from the ovary. The RNA from dissected interstitium and tunica albuginea was subsequently used for qRT-PCR and the dissected theca interna was used for both microarray analyses and qRT-PCR.

Classification of follicles in sections

For the estimation of follicle dimensions, haematoxylin and eosin-stained $4\mu\text{m}$ paraffin or $10\mu\text{m}$ frozen serial sections were viewed with an Olympus BX50 microscope (Olympus) and a Spot RT digital camera and software used to measure the maximum follicle and oocyte diameter (Diagnostic Instruments, Sterling Heights, MI, USA).

Laser capture microdissection

Serial frozen sections of $10\mu\text{m}$ thick were cut using a CM1800 cryostat (Leica Microsystems). Cryosections used for laser capture microdissection (LCM) were placed onto room temperature PET (polyethylene terephthalate) membrane frame slides (Leica Microsystems) and stored individually with desiccant in 50 mL tubes. Slides were fixed in 70% ethanol in DEPC-treated water, stained in 1% cresyl violet acetate (pH 7.75) (ProSciTech) in 70% ethanol (Cumplings *et al.* 2011), washed for 30 s with 70, 90 and 100% ethanol, followed by 1 min in 100% ethanol and stored overnight at -80°C in a parafilm-sealed 50 mL tube with desiccant. Slides were thawed for 15 min at -20°C followed by 15 min at 4°C immediately prior to LCM (LMD AS, Leica Microsystems). Excised samples were collected into $25\mu\text{L}$ of Ambion lysis solution for RNA isolation (Invitrogen). Pre-thecal tissue, measuring approximately 0.038 mm^2 per follicle, was collected from 11 preantral follicles showing a visible oocyte and 2–3 layers of granulosa cells and a diameter $<400\mu\text{m}$. In each case, ovarian stromal tissue adjacent to the follicle was collected separately, as well as tissue from the tunica albuginea. Four samples per tissue type collected from 1 to 3 animals per sample (12 samples in total, from 11 animals) were used for RNA isolation (Table 1) for microarray analyses.

RNA isolation and microarray hybridisation

In addition to the samples collected by LCM, RNA was also isolated from six thecal tissue samples dissected from healthy antral follicles $<5\text{ mm}$ in diameter (six samples in total, from

Table 1 Bovine ovarian samples used for microarray analysis.

Tissue origin/sample number	Animal number	Concentration (pg/ μ L)	RNA integrity number
Stroma close to follicle			
S1	1	140	6.1
S2	2	126	6.2
S3	3	144	4.9
S4	4, 5, 6	133	5.6
Interstitial stroma			
IS1	6, 7	146	4.8
IS2	4, 5, 8	153	6.5
IS3	9	160	5.5
IS4	10, 11	132	5.9
Tunica albuginea			
T1	6, 7	89	5.0
T2	4, 5, 8	129	5.9
T3	9	190	5.5
T4	10, 11	127	6.3
Theca interna from small antral follicles (<5 mm)			
T10	12	200	>8.0
T11	13	200	>8.0
T12	14	200	>8.0
T16	12	200	>8.0
T17	15	200	>8.0
T18	16	200	>8.0

four animals) and subjected to microarray analyses. Total RNA was extracted from thecal samples using RNeasy mini kits (Qiagen). The concentration of RNA was determined by spectrophotometric measurement at 260nm. For each preparation, 5 μ g of RNA was incubated with 1 μ L of DNase I (2 U/ μ L Ambion) at 37°C for 20min, and then treated with DNase inactivation reagent (Ambion) according to manufacturers' instructions.

Total RNA was extracted from the laser-dissected samples using the RNAqueous®-Micro Kit (Cat# AM1931, Thermo Fisher Scientific) procedure for LCM. The RNA derived from the laser-dissected samples (2–4 ng) was DNase treated similarly as above.

RNA integrity was assessed with the Experion™ automated electrophoresis system (Bio-Rad) (Table 1). All samples were then amplified and cDNA generated with the Ovation® Pico WTA System V2 (NuGEN, San Carlos, CA, USA) from an original amount of 5 μ L each of 100–200 pg/ μ L per sample prior to labelling for hybridisation at the Ramaciotti Centre for Genomics (The University of New South Wales, NSW, Australia).

Following confirmation of the quality of the RNA and cDNA synthesis, hybridisation to bovine gene 1.0 ST Arrays (Affymetrix) and scanning were performed according to Affymetrix protocols at the Ramaciotti Centre for Genomics, briefly described below. cDNA was fragmented and labelled using the Encore Biotin Module kit (NuGEN) and used to hybridise against each array. The arrays were then washed and stained with streptavidin-phycoerythrin (final concentration 10 μ g/mL). The signal was amplified by biotinylated anti-streptavidin antibody, and arrays were scanned according to the manufacturer's instructions. The scanned images were inspected for the presence of any defects on the arrays.

Data normalisation and analyses

Affymetrix CEL files were imported into Partek Genomics Suite Software version 6.5 (Partek Incorporated, St Louis, MO, USA) and all 18 microarrays passed the quality assessment procedure. Raw expression data was normalised using pre-background adjustment for GC content, RMA background correction (Robust Multi-array Average) with quantile normalisation, log base 2 transformation and mean probe set summarisation with adjustment for GC content. For initial statistical analysis, the data were first subjected to Principal Component Analysis (PCA, based on the method of Jolliffe (Jolliffe 1986)) using the correlation method for the dispersion matrix, and the normalised method for Eigenvector scaling. The overall gene expression in pre-thecal samples and interstitial stroma was very similar by PCA analysis, so these two groups were combined for subsequent analyses and termed stroma.

The expression data were analysed by one-way ANOVA using the Method of Moments estimation (Eisenhart 1947) with *post hoc* FDR or Bonferroni test for multiple comparisons as implemented by Partek. The fold change in expression for each gene was based on the ratio of signal intensity of the base 2 antilog mean values. Fisher's least significant difference (LSD) contrast method was used to compare theca interna versus stroma and tunica albuginea versus stroma. Differentially expressed gene data sets were imported into Ingenuity Pathway Analysis (IPA; Qiagen) and subjected to core analyses focussing on direct and indirect relationships pertaining to canonical pathways (metabolic and cell signalling), upstream regulators and network generation of differentially expressed gene interactions with other molecules within the Ingenuity Knowledge Base.

Quantitative real-time PCR (qRT-PCR) for validation of microarray analyses

RNA was extracted from dissected bovine theca interna from small antral healthy follicles (<5 mm; $n=7$ from five animals), interstitial stroma between small follicles ($n=6-7$ from 6 to 7 different animals) and tunica albuginea ($n=7-8$ from 7 to 8 different animals). Follicle health was assessed as previously described (Hatzirodos *et al.* 2014a), based upon the absence of degenerative cells in a sample of the follicle wall. Samples were homogenised in 0.5 mL or 1 mL of TRIzol (Cat # 15596-026, Thermo Fisher Scientific), each for two 40 s cycles at 3500 rpm in a PowerLyzer® 24 Bench Top Bead-Based Homogeniser (MoBio, Carlsbad, CA, USA). The samples were then processed according to the standard TRIzol protocol and resuspended in 20 μ L of nuclease-free H₂O. Ten micrograms or less of each sample was treated with 2 U of DNase I for 20 min at 37°C and the enzyme removed using DNase inactivation reagent (Thermo Fisher Scientific). Two hundred nanograms of DNase-treated RNA was used for reverse transcription reactions with or without Superscript RT III (Thermo Fisher Scientific) to generate cDNA or a negative control to detect genomic contaminant respectively.

Quantitative real-time PCR of the cell samples for the target genes and the housekeeping genes *GAPDH*, *RPL19* and *RPL32* was performed using a Rotor-Gene 6000 series 1.7 thermal cycler (Corbett Life Science, Concord, NSW, Australia). cDNA

dilutions were amplified in 10 µL reactions containing 5 µL of Power SYBRTM Green PCR Master Mix (Applied Biosystems/Life Technologies), 0.2 µL or 0.3 µL each of reverse and forward primers (Geneworks; Table 2), respectively, 2 µL of the 1/10 cDNA dilution and 2.6 µL or 2.4 µL of DEPC-treated water. PCR amplification of the cDNA samples was carried out in duplicate at 95°C for 15 s, followed by 60°C for 60 s for a total of 40 cycles. The Rotor-Gene 6000 software (Q Series, Qiagen) was used to determine the cycle threshold (Ct) values at a threshold of 0.05 normalised fluorescence units. Gene expression was determined by the mean of $2^{-\Delta Ct}$, where ΔCt represents the target gene Ct – (Mean of *GAPDH* and *RPL19* or *RPL32*) Ct.

Immunohistochemistry

Portions of ovaries embedded in OCT compound or paraffin were used for antigen localisation or co-localisation using indirect immunofluorescence. Ten micrometer sections were cut from each of the frozen ovaries using a CM1800 Leica cryostat (Leica Microsystems) and collected on Superfrost glass slides (HD Scientific Supplies, Glengala, Victoria, Australia) and stored at –20°C until use. Unfixed sections were dried under vacuum for 5 min followed by fixation in 10% buffered formalin. After fixation, sections were rinsed in three 5 min changes of PBS (10 mM sodium/potassium phosphate with 0.274 M NaCl, 5 mM KCl; pH 7.2; hPBS) before treatment with blocking solution (10% normal donkey serum (D-9663); Sigma Chemical Co.) in antibody diluent containing 0.55 M NaCl and 10 mM sodium phosphate (pH 7.1) for 20 min at RT. Paraffin-embedded ovaries were sectioned at 4 µm on a CM1850 V2.2 Leica microtome, dewaxed and subjected to a pressure cooker antigen retrieval method (2100 retriever, Prestige Medical Ltd; Blackburn, United Kingdom) for 20 min in 10 mM citrate buffer (pH 6.0). Table 3 summarises details of the antibodies used, the fixation conditions and incubation conditions in primary antisera that were performed overnight at 4°C. Sections were also treated with 3 µM 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) solution (Molecular Probes) to identify cell nuclei. Sections were mounted in fluorescence mounting medium (S302380, Dako Australia Pty Ltd) and viewed with an Olympus BX63F microscope with epifluorescence attachment (Olympus Australia). Negative controls included those with no primary antiserum or non-immune mouse or rabbit sera (Dako). No staining was observed with these controls.

Western immunoblotting

Total protein was extracted from tunica albuginea ($n=6$ follicles from six animals), interstitial stroma between small follicles ($n=6$ from 6 animals) and theca interna samples ($n=16-20$ follicles with a diameter <5 mm from seven animals) using RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 0.15 M sodium chloride, 50 mM Tris-hydrochloric acid and 1 mM EDTA) containing protease inhibitor cocktail (#P9599, Sigma-Aldrich). All samples were homogenised separately for two 20 s cycles at 3500 rpm in a PowerLyzer[®] 24 Bench Top Bead-Based Homogeniser (MoBio).

Proteins were quantified using the Bradford method (Bio-Rad Laboratories) and 20 µg of protein for each sample were separated on SDS-gels (Any kDTM Mini-PROTEAN[®] TGXTM Precast Protein Gels, #4569035, Bio-Rad Laboratories), and then transferred overnight at 4°C to PVDF transfer membrane (Amersham HybondTM-P, GE Healthcare), at a constant voltage (33 V). After protein visualisation with Ponceau red staining, the PVDF membrane was washed three times for 10 min at room temperature with Tris-buffered saline (TBS; 50 mM Tris-hydrochloride, 100 mM sodium chloride, pH 7.5) containing 0.05% Tween-20 (Sigma-Aldrich). Non-specific binding was inhibited by incubating with blocking buffer (10% skim milk in TBS containing 0.5% Tween-20) for 1 h at RT. Membranes were then incubated with the primary antibodies (Table 3) for 2 h in blocking buffer. After incubation, membranes were washed three times for 10 min each with TBS containing 0.1% Tween-20 at RT. The membranes were then incubated for 1 h at room temperature with the appropriate secondary antibody: peroxidase-conjugated goat anti-rabbit IgG (diluted 1:10,000; #AP132P; Merck Millipore) or peroxidase-conjugated goat anti-mouse IgG (diluted 1:10,000; #A0168; Sigma-Aldrich), diluted in blocking buffer. After incubation with the secondary antibody, the membranes were washed three times for 10 min each with TBS containing 0.1% Tween-20 and subsequently twice for 10 min each in TBS containing 0.5% Tween-20 and 0.1% SDS. For visualisation, membranes were incubated with ECL AdvanceTM Western blotting detection reagent (GE Healthcare) for 5 min and exposed to Amersham Hyperfilm ECL and then scanned and quantified using image software Multi Gauge V3.0 (Fujifilm Life Sciences, Tokyo, Japan). Beta-actin was used as a loading control.

Deglycosylation of osteoglycin with N-Glycosidase F (PNGase F)

After denaturation in 50 mM Tris-HCl, pH 6.8, containing 1% SDS and 1.4% beta-mercaptoethanol for 5 min at 95°C, 20 µg of total protein from interstitium ($n=4$, described in previous paragraph) were incubated with 2.5 U/mL PNGase F containing 15% Triton-X100 for 3 h at 37°C. The reaction was stopped by incubation for 5 min at 95°C. The samples were then separated by SDS-PAGE as described in the previous paragraph.

Statistical analysis for qRT-PCR and Western blots

All statistical calculations for qRT-PCR and Western blots were performed using Microsoft Office Excel 2010 (Microsoft), GraphPad Prism version 6.00 (GraphPad Software Inc.) and SPSS version 21 (IBM Australia). One-way ANOVA followed by Tukey's *post hoc* test were applied to look for significant differences between the different cell and tissue types. Values of $P<0.05$ were considered significant.

Results

Follicle characterisation for LCM

In order to aid the identification of preantral follicles, size was determined from 4 µm serial sections of paraffin-embedded ovaries. The maximum oocyte and

Table 2 Primer sequences used for qRT-PCR.

Gene name	Gene symbol	GenBank accession no.	Primer sequences (5'-3')		Primer concentration (μM)	Product size (bp)
			Forward	Reverse		
Adrenocortical dysplasia homolog	<i>ACD</i>	XM_010823608.1	AAGGACAAACACCAAGGG	AAGGCAGAACCATCACGATG	0.3	99
Aldehyde dehydrogenase 1 family, member 1	<i>ALDH1A1</i>	NM_174239.2	GCGGAAACACAGTGGTGTG	GAGAAAGATGGCTGCCCT	0.2	150
Androgen receptor	<i>AR</i>	NM_001244127.1	TGCCCTGACCTGGTTTC	TCGGACACACTGGCTGTACATC	0.3	67
Asporin	<i>ASPN</i>	NM_001034309.2	AAGGACATGGAAGACGAAGG	GGGAAGAAGGGTTAATTGG	0.3	80
Brain expressed X-linked 2	<i>BEX2</i>	NM_001077087.1	AGATGCCAACACGAGGAATG	ATTCACTAGCACCCGAAAGG	0.2	92
Centromere protein U (alias MF11P)	<i>CENPU</i>	XM_870192.4	TGTACAGTTCGGACACTTCTG	ATTGTCACCTGATGGGCTTG	0.3	103
Cytochrome P450 family 17 subfamily A polypeptide 1	<i>CYP17A1</i>	NM_174304.2	ACCATCAGAGAAAGTGCTCCGA	CCACAACGTCTGTGCCTTGT	0.2	115
Glutathione S-transferase alpha 3	<i>GSTA3</i>	NM_001077112.1	CGGAGAGGATTTTCTGTTG	AGCAGAGGGAAATCGGAAAG	0.2	114
Glyceraldehyde 3-phosphate dehydrogenase	<i>GAPDH</i>	XR_027767	ACCACCTTGGCATCGTGGAG	GGGCCATCCACAGTCTTCTG	0.2	76
Insulin-like factor 3	<i>INSL3</i>	AM711877	GGCGACCGTGAGCTCT	ACTGGCCATCAGCCCATG	0.2	62
Interleukin 17 receptor D	<i>IL17RD</i>	NM_001205427.1	ACGTGCGCAATTTGCAACATG	CGTTTAAACCAAGCCCGAGTC	0.3	141
Lectin, galactoside-binding, soluble, 1	<i>LGALS1</i>	NM_175782.1	AATCATGGCTTGTGCTCTGG	AGGTTGTTCTGCTCTTTGCC	0.3	129
Microfibrillar-associated protein 3	<i>MFAP3</i>	NM_001166519.1	AACCTCAGCAGATTCCCACTC	ATCCAGCTGCTGCTCTTTTG	0.3	137
Myotrophin	<i>MTPN</i>	NM_203362.2	AGCAGATTGTGGACAGCTTG	ACGCAGGAAACATGACCTTC	0.3	126
Nuclear receptor subfamily 5, group A, member 1	<i>NR5A1</i>	NM_174403.2	CAGACCTTCATCTCCATCTG	CTTGCCATGCTGAATCTGAC	0.2	147
Osteoglycin	<i>OCN</i>	NM_173946	TGCAAGGCTAATGACACCAG	GATGTTTTCCAGGATGACG	0.2	85
Phospholamban	<i>PLN</i>	NM_001103319.1	AATGCCCTCAACAGCACGTC	TGGGATTGCAGCAGAACTTC	0.3	121
Perostin	<i>POSTN</i>	NM_001040479.1	ATTGGAGGCAACAGCTCAG	TGGACCAATTTCTCTTTCG	0.3	100
Regulator of G-protein signalling 5	<i>RG55</i>	NM_001034707.2	GCCATTGACCTTGTCATTC	TGTTCTGCAGGAGCTTGTG	0.2	119
Relaxin/Insulin-like family peptide receptor 2 (alias LGR8)	<i>RXFP2</i>	NM_001304277.1	CAC TGCAAGCTCTCCATCCA	CGATCTAAAGTACCGAGGGCAGTA	0.2	62
Ribosomal Protein L19	<i>RPL19</i>	NM_001040516.1	GATCCGGAAGCTGATCAAAG	TACCCATATGCCTGCCTTTC	0.2	113
Ribosomal Protein L32	<i>RPL32</i>	NM_001034783.2	GCCATCAGAATCACCAATCC	AAATGTGCACACGAGCTGTC	0.1	73
Sterol regulatory element-binding protein 2	<i>SREBF2</i>	NM_001205600.1	CTGATGCCAAGATGCACAAG	GGCCGAGTTATGATTGACC	0.2	87
Transforming growth factor beta 1 induced transcript 1	<i>TGFB11</i>	NM_001035313.1	TCCCTGTTCTTCCCAAAGC	GGCCTGAGGCTGGAAGATG	0.3	109

Table 3 Summary of primary antibodies used for Western blot and immunohistochemistry.

Antigen (species)	Host species, code/clone number, epitope sequence, source	Western blot (concentration or dilution)	IHC fixation, concentration or dilution	Secondary antibody* (catalogue number)/ conjugated fluorophores (catalogue number)
Actin (chicken)	Mouse, ab3280/ACTN05 (C4), from chicken gizzard, Abcam	0.1 µg/mL	10% BFS, 2 µg/mL	Biotin-SP-conjugated AffiniPure donkey anti-mouse IgG (715-066-151)/Cy3-conjugated streptavidin (016-160-084)
ACD/PTOP (human)	Rabbit, LS-C108067/70127, aa476-516, LifeSpan BioSciences	1 µg/mL		
ALDH1A1 (monkey)	Rabbit, ab194588, aa202-501 (C terminal), Abcam	1.75 µg/mL		
LGALS1 (bovine)	Rabbit, LS-C293992/71149, aa1-135, LifeSpan BioSciences	0.5 µg/mL	10% BFS, 2 µg/mL	Biotin-SP-conjugated AffiniPure donkey anti-rabbit IgG (711-066-152)/ Cy3-conjugated streptavidin (016-160-084)
OGN (human)	Rabbit, MBS92306, synthetic peptide conjugated to KLH within residues 246-276 (C-terminus), MyBioSource	1:1000		
PLN (human)	Mouse, sc-398631/ F-10, amino acids 537-836 (C-terminus), Santa Cruz Biotechnology Inc		10% BFS, 2 µg/mL	Biotin-SP-conjugated AffiniPure donkey anti-rabbit IgG (711-066-152)/ Cy3-conjugated streptavidin (016-160-084)
PLN (human)	Rabbit, MBS9206238, synthetic peptide conjugated to KLH within residues 1-22 (N-terminus), MyBioSource	0.03 µg/mL	10% BFS, 1.25 µg/mL	Biotin-SP-conjugated AffiniPure donkey anti-rabbit IgG (711-066-152)/ Cy3-conjugated streptavidin (016-160-084)
Perlecan* (mouse)	Rat, MAB1948/ A7L6, heparin sulphate proteoglycan from EHS mouse tumour, Millipore		10% BFS, 10 µg/mL	FITC-conjugated AffiniPure donkey anti-rat (712-096-153)
Smooth muscle actin (human)	Mouse, M0951/ 1A4, synthetic peptide conjugated to KLH (N-terminal), Dako		10% BFS, 1:100	FITC-conjugated AffiniPure donkey anti-mouse (715-096-151)
Lectin	From <i>Bandeiraea simplicifolia</i> , L9381, Sigma		10% BFS, 10 µg/mL	FITC conjugate

10% BFS is 10% formalin in phosphate buffered saline.

*All secondary antibodies and fluorophores for immunohistochemistry were sourced from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) and used at 1:100. *Perlecan was used as a basement membrane marker (McArthur *et al.* 2000). IHC, immunohistochemistry.

follicle diameter was measured for preantral ($n=117$), early antral ($n=12$) and antral follicles ($n=56$). The diameter of follicles identified as preantral ($377 \pm 78 \mu\text{m}$) was significantly less than early antral ($526 \pm 98 \mu\text{m}$) and antral ($606 \pm 29 \mu\text{m}$) ($P < 0.001$, Fig. 1A), consistent with the dimensions previously reported for bovine follicles (Aerts & Bols 2010). Oocytes of antral follicles were significantly larger than those of early antral ($P < 0.01$) or preantral follicles ($P < 0.001$) (Fig. 1A). The effect of formalin-fixed cryosections was assessed by comparing the cross-sectional area of primordial, primary, primary-secondary (transitional), secondary and preantral follicles to formalin-fixed paraffin-embedded ovarian sections (Fig. 1B). Cryosectioned ovaries had significantly smaller transitional and secondary follicles ($P < 0.01$ and $P < 0.001$, respectively) compared to paraffin sections. On the basis of these results a follicle diameter of $< 400 \mu\text{m}$ was chosen to identify preantral follicles when conducting the LCM.

Statistical analyses of the microarray data

Principal component analysis (PCA) for the first three components (Fig. 2) of all microarrays in this study was conducted. Interstitial stroma and pre-theca were

similar, and therefore, they were subsequently treated as a single group, called 'stroma' ($n=8$), for further analysis. PCA clearly separated the stroma, the theca interna and the tunica albuginea into three separate clusters (Fig. 2). Stroma was compared to the theca interna and the tunica albuginea. The array intensities for all genes from the microarray analysis are shown in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Comparing theca interna from small antral follicles to stroma, there were 654 differentially expressed probe IDs (arbitrary fold change threshold of 3, Bonferroni correction for multiple testing, $P < 0.05$), with 534 of these mapped to Entrez gene IDs with three sets of duplicate probes, leaving 279 upregulated genes (Table 4) and 252 downregulated genes (Table 5) in the theca interna.

Comparing tunica albuginea to stroma, there were 365 differentially expressed probe IDs (arbitrary fold change threshold of 2, FDR correction for multiple testing, $q < 0.05$ – i.e. less stringent cut-off points than for the theca interna versus stroma comparison analyses), with 295 of these mapped to Entrez gene IDs with one set of duplicate probes, leaving 174 upregulated genes (Table 6) and 120 downregulated genes (Table 7) in the tunica albuginea.

Figure 1 Follicle characterisation. (A) In H&E-stained paraffin sections, the diameter of oocyte and follicle was measured for preantral ($n=117$), early antral ($n=12$) and antral ($n=56$) follicles. Significantly different results between follicle types were determined by one-way ANOVA with Tukey's *post hoc* test. $**P<0.01$, $***P<0.001$. (B) Comparisons of formalin fixation with paraffin embedding versus formalin fixation with OCT embedding on follicle diameter is shown for primordial ($n=65$ and $n=14$, respectively), primary ($n=29$ and $n=15$, respectively), primary to secondary ($n=7$ and $n=8$, respectively), secondary ($n=8$) and preantral ($n=5$) follicles. Significantly different results between paraffin and frozen were determined by independent *t* test. $**P<0.01$, $***P<0.001$.

Validation of microarray analysis by qRT-PCR

To confirm changes in the expression of genes identified by microarray analysis, qRT-PCR analyses was performed on dissected samples of tunica albuginea, interstitial stroma and theca interna (Fig. 3). Microarray analysis showed three thecal-specific genes (*CYP17A1*, *INSL3* and its receptor *RFXP2*) were highly expressed in the theca interna compared to the other compartments and the same pattern was observed in the qRT-PCR results (Fig. 3A, B, C and D). One gene, *ALDH1A1*, was found significantly highly expressed in the theca in the microarray but was not significantly different as assessed by qRT-PCR. Two genes, *BEX2* and *SRBF2*,

Figure 2 Unsupervised PCA of arrays from interstitial stroma ($n=4$), pre-theca ($n=4$), theca interna ($n=6$) and tunica albuginea ($n=4$). Each point represents a microarray chip and are separated by tissue type. The graph is a scatter plot of the values for the first (X-axis), second (Y-axis) and third (Z-axis) principal components based on the Pearson correlation matrix of the total normalised array intensity data.

which showed no differential expression between the four compartments in the microarray analysis, were also unchanged between theca interna, interstitial stroma and tunica albuginea in the qRT-PCR (Fig. 3E and F).

Pathway, network and upstream regulator analyses

The canonical pathways indicated by IPA most likely to be affected by differential gene expression in the theca interna of small antral follicles compared to stroma was an upregulation of genes associated with steroid hormone (androgen and glucocorticoid biosynthesis) and cholesterol synthesis (Fig. 4A). On the other hand, genes in our dataset associated with hepatic fibrosis/hepatic stellate activation, including TGF β signalling and regulation of epithelial-mesenchymal transition, were downregulated in the theca interna. For the tunica albuginea versus stroma dataset, the top canonical pathways were associated mainly with hepatic fibrosis/hepatic stellate activation and GPCR cAMP signalling (Fig. 4B). Furthermore, most of these genes were upregulated in the tunica albuginea compared to stroma. The proportion of genes altered in these pathways is found in Fig. 4 and individual genes from our datasets which are involved in these canonical pathways are presented in Supplementary Table 2 for theca interna versus stroma, and in Supplementary Table 3 for tunica albuginea versus stroma.

The top three networks generated by IPA from the theca interna versus stroma dataset are shown in Fig. 5

Table 4 Top 100 genes differentially upregulated in theca interna from small antral follicles compared to stroma (interstitial stroma and pre-theca).

Gene symbol	Fold change	Gene symbol	Fold change	Gene symbols	Fold change
<i>INSL3</i>	157.8	<i>PIK3R3</i>	13.4	<i>PTCH1</i>	8.8
<i>LHCGR</i>	85.3	<i>CD36</i>	13.2	<i>CTSC</i>	8.8
<i>HSD3B1</i>	71.9	<i>HHIP</i>	12.0	<i>IQGAP2</i>	8.6
<i>CXCL14</i>	52.5	<i>OMD</i>	11.9	<i>DFNA5</i>	8.5
<i>LOC104968612</i>	49.8	<i>DHCR24</i>	11.7	<i>PTPRB</i>	8.4
<i>GSTA3</i>	46.5	<i>CENPA</i>	11.5	<i>TMEM254</i>	8.4
<i>MT1E</i>	38.0	<i>CEP55</i>	11.5	<i>FAM129A</i>	8.3
<i>KCNIP1</i>	29.3	<i>ARHGAP11A</i>	11.3	<i>DIAPH3</i>	8.3
<i>S100A2</i>	27.0	<i>FBLN5</i>	11.2	<i>ANLN</i>	8.2
<i>ADAMDEC1</i>	26.4	<i>TPX2</i>	11.1	<i>GPM6A</i>	8.0
<i>APOA1</i>	26.2	<i>ENTPD1</i>	11.0	<i>CLEC3B</i>	8.0
<i>TOP2A</i>	25.4	<i>CENPE</i>	11.0	<i>LOC508666</i>	7.7
<i>MAL2</i>	25.3	<i>FST</i>	10.4	<i>MS4A8</i>	7.7
<i>LOC104968446</i>	23.3	<i>UBE2C</i>	10.3	<i>RAN</i>	7.6
<i>ITGBL1</i>	23.1	<i>CKAP2</i>	10.3	<i>ZMAT4</i>	7.3
<i>HPGD</i>	22.7	<i>RRM2</i>	10.3	<i>KIAA0101</i>	7.3
<i>LOC104975684</i>	20.4	<i>LIPG</i>	10.1	<i>ERP27</i>	7.2
<i>ALDH1A1</i>	19.1	<i>CENPF</i>	9.8	<i>TM4SF18</i>	6.9
<i>ASPN</i>	18.0	<i>CCL14</i>	9.8	<i>KCNE4</i>	6.9
<i>PLD1</i>	17.8	<i>PRR11</i>	9.8	<i>INHA</i>	6.8
<i>SLC16A7</i>	16.8	<i>RAB38</i>	9.8	<i>PSMB9</i>	6.8
<i>KIF11</i>	16.3	<i>IPO11</i>	9.5	<i>NDC80</i>	6.7
<i>CATHL5</i>	16.3	<i>SCG2</i>	9.5	<i>IDH1</i>	6.7
<i>GSTA5</i>	16.1	<i>INHBA</i>	9.4	<i>THSD7A</i>	6.6
<i>MKI67</i>	15.7	<i>ASS1</i>	9.3	<i>NCAPG</i>	6.5
<i>OGN</i>	15.1	<i>ANGPTL5</i>	9.3	<i>GRHL1</i>	6.4
<i>GRB14</i>	15.0	<i>DEPDC1</i>	9.3	<i>CAV1</i>	6.3
<i>CAPN6</i>	14.8	<i>XDH</i>	9.2	<i>CHODL</i>	6.3
<i>SLCO2A1</i>	14.8	<i>POSTN</i>	9.1	<i>CMKLR1</i>	6.3
<i>SPC24</i>	14.5	<i>LOC514257</i>	9.0	<i>GJB5</i>	6.3
<i>TNNI3</i>	13.9	<i>ASPM</i>	8.9	<i>ADK</i>	6.3
<i>LOC527388</i>	13.7	<i>STC1</i>	8.9	<i>FICF</i>	6.3
<i>SLC25A5</i>	13.6	<i>SECTM1A</i>	8.9		
<i>PAPSS2</i>	13.5	<i>DCTPP1</i>	8.8		

(Benjamini–Hochberg *post hoc* test for multiple corrections following one-way ANOVA). The fold change is the ratio of signal intensity of combined pre-theca and interstitial stroma to theca interna from microarray analyses. Differentially regulated genes (>-3 fold, $P<0.05$) were annotated based on the Entrez Gene database. Genes are listed in descending order of fold change.

and the genes and their degree of change of expression are also indicated. The first relates to cellular assembly and organisation, the cell cycle and DNA functions and has *ESR1* (oestrogen receptor alpha) at its centre and involves downregulation of *NR1D1* (transcriptional repressor Rev-erb α) and upregulation of *CITED1* (Cbp/p300-interacting transactivator 1) in the theca interna. The second pathway is cell cycle and neurological diseases and contains cell death genes like cytochrome C and caspase and protective or cell survival genes like *SOD1*, Hsp70, prohibitin (*PHB*) and *NUPR1/RELB/IER3* which is downregulated in theca interna. The third pathway has *ERK1/2* at its centre and also involves the upregulation of *INSL3*, *LOX* and *FBL5*.

The top three networks for tunica albuginea versus stroma are shown in Fig. 6 and the genes and their degree of change of expression are also indicated. The first network of embryonic development has *ERK1/2* at its centre but also contains upregulated calcium

and potassium channels (*CACNA1G*, T-type calcium channel, *KCND2*) and prostaglandin-related genes *PDGES* (PGE2 synthase) and *EPX1* (Epoxide hydrolase 1 that can synthesise arachidonic acid). The second network on cell morphology contains a number of upregulated extracellular matrix molecules (*LAMB1*, collagens type III and IV, *COL1A2*) and enzymes that process extracellular matrix (elastase, gelatinase, *PCOLCE*) in the tunica albuginea. The third network is organ morphology and contains a number of upregulated growth factors including VEGF, NDP (norrin) and HGF, and transcription factor in Wnt signalling (TCF/LEF) and a considerable number of downregulated genes.

For theca interna versus stroma upstream transcriptional regulators analyses revealed 14 activated, including PTGER2 (prostaglandin E2), CDKN1B and RABL6 (cell cycle), NR5A1 (SF1), FSH and MTOR, and 24 inhibited upstream regulators, including NUPR1 (cell survival), IL1 and TNF α , in the theca interna relative to stroma (Supplementary Table 4).

Table 5 Top 100 genes differentially downregulated in theca interna from small antral follicles compared to stroma (interstitial stroma and pre-theca).

Gene symbol	Fold change	Gene symbol	Fold change	Gene symbols	Fold change
<i>SFRP4</i>	-45.8	<i>MOB3B</i>	-8.5	<i>ADRA1B</i>	-5.9
<i>NUPR1</i>	-30.1	<i>PID1</i>	-8.3	<i>MFAP4</i>	-5.9
<i>TUBA3E</i>	-28.1	<i>TMOD1</i>	-8.2	<i>ALDH1L2</i>	-5.9
<i>MUSTN1</i>	-26.2	<i>MIR29C</i>	-8.0	<i>PLTP</i>	-5.8
<i>ENPEP</i>	-26.2	<i>HTRA3</i>	-7.9	<i>TMOD2</i>	-5.8
<i>FAIM2</i>	-24.5	<i>FBLN1</i>	-7.9	<i>ASTN1</i>	-5.7
<i>ADAD1</i>	-19.2	<i>MXRA8</i>	-7.8	<i>MITF</i>	-5.7
<i>OPCML</i>	-18.5	<i>SMOC1</i>	-7.8	<i>C23H6orf25</i>	-5.7
<i>GTSF1</i>	-17.9	<i>BEX4</i>	-7.7	<i>GABRB2</i>	-5.7
<i>HOPX</i>	-17.6	<i>FGL2</i>	-7.7	<i>ITIH4</i>	-5.6
<i>NR4A1</i>	-16.9	<i>CIART</i>	-7.6	<i>NR3C2</i>	-5.6
<i>MIR99A</i>	-14.5	<i>CHL1</i>	-7.2	<i>ANK3</i>	-5.5
<i>MDGA2</i>	-14.4	<i>SETBP1</i>	-7.2	<i>MET</i>	-5.4
<i>MIRLET7C</i>	-14.1	<i>EGR1</i>	-7.1	<i>MAOB</i>	-5.4
<i>LOC100337251</i>	-12.1	<i>BCL2</i>	-7.0	<i>IGFBP5</i>	-5.4
<i>GNAO1</i>	-12.1	<i>DAZL</i>	-7.0	<i>LIMCH1</i>	-5.4
<i>C1S</i>	-11.7	<i>FBXO32</i>	-7.0	<i>LOC505383</i>	-5.3
<i>NR1D1</i>	-11.4	<i>RUNX1</i>	-6.9	<i>SMOC2</i>	-5.3
<i>COCH</i>	-11.2	<i>ROR1</i>	-6.9	<i>TMEM100</i>	-5.2
<i>CPEB1</i>	-11.1	<i>PCSK2</i>	-6.9	<i>MYOCD</i>	-5.2
<i>NTRK2</i>	-10.5	<i>UNC13C</i>	-6.9	<i>MYH11</i>	-5.1
<i>RGS2</i>	-10.4	<i>EQTN</i>	-6.8	<i>FAT4</i>	-5.1
<i>CDH6</i>	-10.1	<i>ID2</i>	-6.7	<i>CNN1</i>	-5.0
<i>RASGEF1B</i>	-9.4	<i>HGF</i>	-6.7	<i>CHAD</i>	-5.0
<i>SEMA3D</i>	-9.4	<i>WNT5A</i>	-6.6	<i>MDK</i>	-5.0
<i>PRELP</i>	-9.4	<i>LOC505383</i>	-6.6	<i>C1R</i>	-4.9
<i>ITIH5</i>	-9.4	<i>SDC4</i>	-6.6	<i>LMCD1</i>	-4.9
<i>PTGES</i>	-9.3	<i>NR4A3</i>	-6.5	<i>SEMA6A</i>	-4.9
<i>SCUBE3</i>	-9.2	<i>ATP1B2</i>	-6.5	<i>PROCA1</i>	-4.9
<i>FABP7</i>	-9.1	<i>ITGB8</i>	-6.5	<i>SNCAIP</i>	-4.9
<i>GRM8</i>	-9.0	<i>GXYLT2</i>	-6.3	<i>CNTN1</i>	-4.8
<i>TMEFF2</i>	-8.9	<i>NR4A2</i>	-6.3	<i>BAMBI</i>	-4.8
<i>CHI3L1</i>	-8.7	<i>AMY2B</i>	-6.2	<i>WASF3</i>	-4.8
<i>THRB</i>	-8.6	<i>FGF10</i>	-6.1	<i>AKR1C4</i>	-4.8

(Benjamini–Hochberg *post hoc* test for multiple corrections following one-way ANOVA). The fold change is the ratio of signal intensity of combined pre-theca and interstitial stroma to theca interna from microarray analyses. Differentially regulated genes (>3 fold, $P < 0.05$) were annotated based on the Entrez Gene database. Genes are listed in descending order of fold change.

For tunica albuginea versus stroma, 26 upstream regulators were activated and 16 inhibited, such as SP600125 (JNK inhibitor), docosa-hexaenoic acid (which can be converted to epoxides and arachidonic acid), DHEA and ESR1 (Supplementary Table 5). The activated upstream regulators were mainly associated with TGF β signalling (TGFB1-3, TGFB1) and the immune system/response (lipopolysaccharide, IL1A, IL10RA, CXCL12, TLR4).

Differentially expressed genes in theca interna compared to other stromal compartments

Microarray analysis showed significant upregulation of *OGN* and *LGALS1* in theca interna versus tunica albuginea, interstitium and pre-theca (Fig. 7A and B), whereas qRT-PCR indicted mRNAs for both genes were significantly upregulated in both theca interna and interstitium versus tunica albuginea (Fig. 7A and B). By microarray *POSTN* was found to be significantly

upregulated in the theca interna (Fig. 7C); however, this was not confirmed by qRT-PCR ($P > 0.07$). *ASP*N was strongly upregulated in theca interna compared to tunica albuginea, interstitium and pre-theca in microarray analysis and qRT-PCR (Fig. 7D). qRT-PCR did not confirm the finding by microarray of upregulation of *RGS5* in theca interna (Fig. 7E). However, the significant upregulation of the gene in interstitium compared to tunica albuginea was observed by both techniques.

Microarray analysis revealed a significant increase in expression of *MFAP3* in theca interna, and a significant decrease in pre-theca compared to tunica albuginea and interstitium (Fig. 7F). However qRT-PCR showed a significant upregulation only in theca interna versus tunica albuginea. In contrast to the before mentioned genes, where the expression was upregulated in the theca interna, the expression of *PLN* was significantly downregulated in theca interna versus pre-theca by microarray analysis and versus interstitium by qRT-PCR (Fig. 7G). There was also a significant difference

Table 6 Top 100 genes differentially upregulated in tunica albuginea compared to stroma (interstitial stroma and pre-theca).

Gene symbol	Fold change	Gene symbol	Fold change	Gene symbols	Fold change
ADAM22	21.9	TMEM132D	3.3	KHDRBS3	2.7
TNC	7.5	GATA5	3.3	SRPX2	2.6
STRA6	7.0	NCAM1	3.2	CTPS1	2.6
HTR1D	6.7	GALNT3	3.2	MKX	2.6
PENK	6.3	ARSL	3.2	PRSS12	2.6
PPP1R14C	6.0	FAM129A	3.1	ALDH1A1	2.6
COL8A2	5.5	ETV5	3.1	CEACAM1	2.6
EPHX1	5.5	STC1	3.1	LOC101902670	2.6
BGN	5.1	CYR61	3.0	CHRM2	2.6
TRHDE	5.1	MXRA5	3.0	TBC1D9	2.6
LOC781347	4.9	KCNS3	2.9	SYTL2	2.5
RAMP3	4.8	COL8A1	2.9	DOCK4	2.5
ENPP6	4.7	CERS6	2.9	PTN	2.5
HOPX	4.7	MOXD1	2.9	ITIH5	2.5
ATP8A1	4.5	DYSF	2.9	PDLIM1	2.5
SHISA9	4.3	PDE2A	2.8	LOC534200	2.5
LOC100337183	4.3	LOC104968548	2.8	PPARG-TSEN2	2.5
KCNRG	4.1	KCNA4	2.8	CD9	2.5
PCOLCE2	3.9	NDP	2.8	C1QTNF1	2.5
IQGAP2	3.8	KCTD8	2.8	LOXL2	2.5
PTGES	3.8	VEGFC	2.8	GLB1	2.5
ADAMTSL4	3.8	RARRES1	2.8	FAM163A	2.5
CTGF	3.7	FBN2	2.8	PHACTR1	2.5
IL18	3.7	CXCL16	2.8	MRAP2	2.5
PALMD	3.7	NEDD9	2.8	CX3CL1	2.5
PIEZO2	3.6	TNFRSF11B	2.8	STK17B	2.5
COL1A1	3.5	XDH	2.7	CACNA1G	2.5
FAM26E	3.4	HGF	2.7	ECM1	2.4
FAT1	3.4	DPYSL3	2.7	DAB2	2.4
THBS1	3.4	OPCML	2.7	LOC101903503	2.4
SRGAP3	3.4	LOC101908685	2.7	KCND2	2.4
RGS16	3.3	AEBP1	2.7	ENC1	2.4
PTGER2	3.3	NRIP3	2.7		
CNTNAP3	3.3	COL1A2	2.7		

(Benjamini–Hochberg *post hoc* test for multiple corrections following one-way ANOVA). The fold change is the ratio of signal intensity of combined pre-theca and interstitial stroma to theca interna from microarray analyses. Differentially regulated genes (>2 fold, $P < 0.05$) were annotated based on the Entrez Gene database. Genes are listed in descending order of fold change.

in the expression of *PLN* between interstitium and tunica albuginea by qRT-PCR. *ACD* was significantly downregulated in tunica, interstitium and theca interna compared to pre-theca by microarray analysis and significantly downregulated in theca interna compared to tunica albuginea in qRT-PCR (Fig. 7H). Microarray analysis showed significant downregulation of *IL17RD* in tunica albuginea, interstitium and theca interna compared to pre-theca (Fig. 7I); however, qRT-PCR did not reveal any differences in gene expression between the stromal compartments. *MLF1IP* (Fig. 7J) was significantly downregulated in tunica albuginea and theca interna compared to pre-theca; however, qRT-PCR revealed no differences for this gene between the stromal compartments.

Protein expression in tunica albuginea, interstitium and theca interna

In contrast to the results of microarray and qRT-PCR procedures, the expression of osteoglycin by Western

blot was found to be significantly upregulated in tunica albuginea and interstitium extracts compared to theca interna (Fig. 8A and A'). Deglycosylation of osteoglycin in tunica albuginea and interstitium extracts with PNGase F, resulted in a molecular weight size reduction from 34 kDa to approximately 25 kDa (Fig. 8A''). Galectin-1 (Fig. 8B and B'), aldehyde dehydrogenase 1 family member A1 (Fig. 8C and C') and adrenocortical dysplasia homolog (Fig. 8D and D') showed no differences in the expression between the three tissue types. Phospholamban was significantly downregulated in the theca interna compared to tunica albuginea and interstitium (Fig. 8E and E'). The results for the negative controls are shown in Supplementary Fig. 1.

Localisation of galectin-1, phospholamban and periostin in bovine adult ovaries

Galectin-1 was localised in the extracellular matrix of the theca (Fig. 9A) and interstitium (Fig. 9B). Phospholamban showed a peri-nuclear localised pattern

Table 7 Top 100 genes differentially downregulated in tunica albuginea compared to stroma (interstitial stroma and pre-theca).

Gene symbol	Fold change	Gene symbol	Fold change	Gene symbols	Fold change
<i>TUBA3E</i>	-17.8	<i>CKS1B</i>	-3.7	<i>GSTM3</i>	-2.6
<i>GTSF1</i>	-10.7	<i>ZP3</i>	-3.6	<i>SOHLH2</i>	-2.6
<i>ADAD1</i>	-9.3	<i>EPCAM</i>	-3.6	<i>CSRP2</i>	-2.6
<i>PTTG1</i>	-7.9	<i>GDF3</i>	-3.5	<i>ODC1</i>	-2.6
<i>CENPW</i>	-7.7	<i>SMC1B</i>	-3.5	<i>NOSTRIN</i>	-2.6
<i>GSTA3</i>	-6.9	<i>CKS2</i>	-3.4	<i>LAMA2</i>	-2.5
<i>AURKA</i>	-6.4	<i>MGC133636</i>	-3.4	<i>FATE1</i>	-2.5
<i>LOC104976349</i>	-5.8	<i>KPNA2</i>	-3.3	<i>LOC783811</i>	-2.5
<i>RUNDC3B</i>	-5.8	<i>KCNT2</i>	-3.2	<i>TMC5</i>	-2.5
<i>IGF1</i>	-5.4	<i>CCNB3</i>	-3.2	<i>TNNI3</i>	-2.5
<i>FOXR1</i>	-5.2	<i>NEB</i>	-3.2	<i>NNAT</i>	-2.5
<i>DAZL</i>	-5.1	<i>KDR</i>	-3.1	<i>CENPV</i>	-2.5
<i>OSR2</i>	-5.0	<i>LHX8</i>	-3.1	<i>LOC783613</i>	-2.5
<i>PHYHIP</i>	-5.0	<i>HEY2</i>	-3.1	<i>ZNF423</i>	-2.4
<i>PCSK2</i>	-4.9	<i>HES1</i>	-3.0	<i>SALL1</i>	-2.4
<i>MUC13</i>	-4.7	<i>EBF1</i>	-3.0	<i>CPM</i>	-2.4
<i>CCDC181</i>	-4.6	<i>C23H6orf25</i>	-3.0	<i>CCBE1</i>	-2.4
<i>CHL1</i>	-4.6	<i>LOC537655</i>	-2.9	<i>FKBP5</i>	-2.4
<i>LOC101904481</i>	-4.5	<i>EDNRA</i>	-2.9	<i>MET</i>	-2.4
<i>SKA1</i>	-4.5	<i>TAC3</i>	-2.9	<i>CLDN5</i>	-2.3
<i>UNC13C</i>	-4.4	<i>ART4</i>	-2.9	<i>PECAM1</i>	-2.3
<i>HIST2H2BF</i>	-4.4	<i>PHACTR3</i>	-2.9	<i>CIART</i>	-2.3
<i>TSPAN13</i>	-4.4	<i>CD93</i>	-2.9	<i>AGTR1</i>	-2.3
<i>NPM2</i>	-4.2	<i>CDH7</i>	-2.8	<i>EPAS1</i>	-2.3
<i>CHRNA3</i>	-4.2	<i>LOC504995</i>	-2.8	<i>FBXO15</i>	-2.3
<i>RGS5</i>	-4.1	<i>CENPU</i>	-2.7	<i>TESC</i>	-2.3
<i>MT3</i>	-4.1	<i>ICA1L</i>	-2.7	<i>SYCP3</i>	-2.3
<i>SFRP2</i>	-3.9	<i>ABCC9</i>	-2.7	<i>LOC101902172</i>	-2.3
<i>TNFSF18</i>	-3.9	<i>GNAO1</i>	-2.7	<i>SLC16A10</i>	-2.3
<i>LMO3</i>	-3.8	<i>SGK1</i>	-2.6	<i>EDNRB</i>	-2.3
<i>LHX9</i>	-3.8	<i>CXADR</i>	-2.6	<i>WBSCR22</i>	-2.2
<i>SAXO1</i>	-3.7	<i>TGM2</i>	-2.6	<i>TGFB3</i>	-2.2
<i>LOC100297621</i>	-3.7	<i>ALX1</i>	-2.6		
<i>LOC519417</i>	-3.7	<i>ABCB1</i>	-2.6		

(Benjamini–Hochberg *post hoc* test for multiple corrections following one-way ANOVA). The fold change is the ratio of signal intensity of combined pre-theca and interstitial stroma to theca interna from microarray analyses. Differentially regulated genes (>2 fold, $P < 0.05$) were annotated based on the Entrez Gene database. Genes are listed in descending order of fold change.

Figure 3 Microarray and qRT-PCR validation data for differentially expressed genes (A–F). The microarray values are signal intensities (normalised, but not log transformed) for tunica albuginea (tunica), interstitial stroma (interstitium), pre-theca and theca interna (theca). qRT-PCR gene expression values were determined from the mean of the ratio of the ΔC_t for the target genes to ribosomal protein L19 (*RPL19*) or L32 (*RPL32*) and glyceraldehyde phosphate dehydrogenase (*GAPDH*) and the data are mean $\pm$ S.E.M. Significantly different results for qRT-PCR were determined by one-way ANOVA with Tukey's *post hoc* test. The microarray signal intensity data were analysed by ANOVA with corrections for multiple testing using the FDR. Number of samples are shown below the graphs in brackets. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 4 Top canonical pathways identified by IPA for the submitted differentially expressed genes for theca interna versus stroma (A) and tunica albuginea versus stroma (B). The bar chart represents the percentage of genes from the data set that map to each canonical pathway showing those which are upregulated (in red) and downregulated (in blue) in theca interna with respect to stroma (A) or tunica albuginea with respect to stroma (B). The line chart ranks these pathways derived for the same data set, from the highest to lowest degree of association based on the value of a right-tailed Fisher's exact t test. The number of differentially expressed genes in the data set and the total number of genes in the canonical pathway are indicated for each pathway on the right hand side.

(Fig. 9C) within vascular smooth muscle cells (Fig. 9D). Periostin localised to the peri-vascular connective tissue sheath of blood vessels within the interstitium (Fig. 9D), as well as the peri-follicular connective tissue (Fig. 9E).

Discussion

This study focused on the different stromal compartments within the ovary. Four separate areas of stroma were examined but when the principal component analyses revealed that the stroma adjacent to the preantral follicles was similar to interstitial stroma these were combined into one group called 'stroma' for some additional analyses. The major comparisons we made were of stroma versus tunica albuginea and stroma versus theca interna. This is inherently logical as stroma first penetrates the ovary primordium and tunica albuginea develops from that stroma (Hummitzsch

A Cellular Assembly and Organization, Cell Cycle, DNA Replication, Recombination and Repair

B Cell Cycle, Neurological Disease, Cellular Assembly and Organization

C Cell Morphology, Cellular Development, Tissue Development

Figure 5 The most significant networks generated by IPA based on the 531 differentially expressed genes between theca interna and stroma (A–C). These networks were generated in IPA using triangular connectivity based on focus genes (those present in our data set) and built up according to the number of interactions between a single prospective gene and others in the existing network, and the number of interactions the prospective genes have outside this network with other genes as determined by IPA (Jolliffe 1986). Interactions between molecules, and the degree and direction of regulation are indicated with upregulation (red) or downregulation (blue) in theca interna and increasing colour intensity with degree of fold change.

et al. 2013, Hummitzsch *et al.* 2015b). Theca interna differentiates in the stroma area surrounding preantral follicles (Hummitzsch *et al.* 2015b), suggesting that both tunica albuginea and theca interna are derived at least in part from the stroma. The comparisons we undertook

A Embryonic Development

B Cell Morphology

C Organ Morphology

Figure 6 The most significant networks generated by IPA based on the 294 differentially expressed genes between tunica albuginea and stroma (A–C). The networks were generated in IPA using triangular connectivity based on focus genes (those present in our data set) and built up according to the number of interactions between a single prospective gene and others in the existing network, and the number of interactions the prospective genes have outside this network with other genes as determined by IPA (Jolliffe 1986). Interactions between molecules and the degree and direction of regulation are indicated with upregulation (red) or downregulation (blue) in tunica albuginea and increasing colour intensity with degree of fold change.

included principal component analyses, ANOVA analyses with *post hoc* tests for multiple comparisons to identify differentially expressed genes, and then using subsets of highly differentially expressed genes pathway, network and upstream regulator analyses.

The array data were initially confirmed using qRT-PCR of six genes using RNA from the laser captured three stromal groups and the dissected theca interna

for comparison with RNA isolated from dissected tunica albuginea, interstitial stroma and theca interna (Fig. 3). Excellent agreement was found with tunica albuginea and theca interna comparisons, but less so with interstitial stroma where only five of the six genes examined agreed. A further ten genes were analysed by qRT-PCR and again the differences between tunica albuginea and theca interna were in general agreement with the microarray analyses; however, the results for seven genes in the interstitium were not (Fig. 7). Microdissected interstitium could contain microscopic areas of regressed follicles and corpora lutea, which are easily avoided when undertaking laser capture with the aid of a microscopy but not a visual dissection of the ovary. Thus, the microarray analyses of the arrays are likely to be correct based on the results from the tunica albuginea and the theca interna.

The canonical pathways for theca interna of small antral follicles compared to stroma indicated an upregulation of genes associated with steroid hormone (androgen and glucocorticoid biosynthesis) and cholesterol synthesis with hepatic fibrosis/ hepatic stellate activation and a downregulation of TGF β signalling and the epithelial-mesenchymal transition. These are inherently expected as the theca interna is more steroidogenic and less fibrous than the stroma. The pathway of hepatic fibrosis/hepatic stellate activation contains genes indicative of less TGF β activity but also upregulation of collagens type IV and XXI which might be associated with increased vascularisation of the theca relative to the stroma. The networks identified include cellular assembly and organisation, the cell cycle and DNA functions and has *ESR1* at its centre and involves downregulation of *NR1D1* (transcriptional repressor Rev-erba) and upregulation of *CITED1* (Cbp/p300-interacting transactivator 1). The second pathway was cell cycle and neurological diseases and contained cell death genes like cytochrome C and caspase and protective or cell survival genes like superoxide dismutase 1 (*SOD1*), heat shock protein 70 (*HSP70*), prohibitin (*PHB*) and nuclear protein 1 (*NUPR1/RELB/IER3*), which are downregulated in theca interna. Upstream regulators were predicted by IPA analyses (Supplementary Table 4). These identified regulators indicate potential pathways and biological processes that could occur in different tissues and cells. They should not be interpreted literally as other related molecules could be the real regulator of these processes; for example, the FSH pathway could also indicate the LH pathway. Analysing upstream transcriptional regulators revealed 14 activated regulators, including PTGER2 (prostaglandin E receptor 2), CDKN1B (cyclin-dependent kinase inhibitor 1B) and RABL6 (RAS Oncogene Family Like 6) involved in cell cycle, NR5A1 (nuclear receptor subfamily 5 group A member 1; SF1), FSH and MTOR (mechanistic target of rapamycin), and 24 inhibited upstream regulators,

Figure 7 Microarray and qRT-PCR data for differentially expressed genes of interest (A–J). The microarray values are signal intensities (normalised, but not log transformed). qRT-PCR gene expression values were determined from the mean of the ratio of the ΔC_t for the target genes to ribosomal protein L19 (*RPL19*) or L32 (*RPL32*), and glyceraldehyde phosphate dehydrogenase (*GAPDH*) and the data are mean $\pm$ S.E.M. Significantly different results for qRT-PCR were determined by one-way ANOVA with Tukey's *post hoc* test. The microarray signal intensity data were analysed by ANOVA with corrections for multiple testing using the FDR. The number samples are shown below the graphs in brackets. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

including NUPR1 (nuclear protein 1; involved in cell survival), IL1 (interleukin 1) and TNF (tumour necrosis factor), in the theca interna relative to stroma. These networks and upstream regulators suggest that the theca interna is subject to cell survival pressures. This is not unexpected either as the theca interna undergoes considerable changes during follicular atresia (Clark *et al.* 2004, Hatzirodos *et al.* 2014b), whereas the stroma is not a target of atresia.

For the tunica albuginea versus stroma the top conical pathway was associated mainly with hepatic fibrosis/hepatic stellate activation. The second network on cell morphology contained a number of upregulated extracellular matrix molecules (*LAMB1*, collagens type III and IV, *COL1A2*) and enzymes that process extracellular matrix (elastase, gelatinase, *PCOLCE*) in the tunica albuginea. The activated upstream regulators were mainly associated with TGF β signalling (TGF β 1-3, TGF β RI) and the immune system/response (lipopolysaccharide, IL1A, IL10RA, CXCL12, TLR4). This is not unexpected as the tunica albuginea is exceedingly

fibrous with much structural collagen, and collagen synthesis is stimulated by TGF β (Govinden & Bhoola 2003, Verrecchia & Mauviel 2004).

For the tunica albuginea versus stroma dataset, another top canonical pathway included GPCR and cAMP signalling. Furthermore, based on microarray analysis, it is suggested that most of these genes were upregulated in the tunica albuginea compared to stroma. The first network of embryonic development has *ERK1/2* at its centre but also contains upregulated calcium and potassium channels (*CACNA1G*, T-type calcium channel, *KCND2*) and prostaglandin-related genes *PDGES* (PGE2 synthase) and *EPX1* (Epoxide hydrolase 1 that can synthesise arachidonic acid). For tunica albuginea versus stroma based upon IPA analyses, 26 upstream regulators were activated and 16 inhibited, such as SP600125 (JNK inhibitor), docosa-hexaenoic acid (which can be converted to epoxides and arachidonic acid), the steroid hormone DHEA and ESR1. The upregulation of GPCRs including adrenergic (*ADRA2A*) and cholinergic receptors (*CHRM2*), cAMP

signalling, such as that regulated by prostaglandins, and ion channels suggesting that perhaps the bovine tunica albuginea could be contractile as it is in fish ovarian tunica (Piccinno *et al.* 2014) and in mammalian testis tunica albuginea (Middendorff *et al.* 2002). In addition, granulosa cells of preovulatory follicles express endothelin 2 (Ko *et al.* 2006) causing ovarian contraction (Ko *et al.* 2006, Bridges *et al.* 2010).

This study also revealed major differences in the expression levels of genes associated with extracellular matrix proteins and vascular markers between stromal compartments that might be linked to follicle growth, in general, and the preantral-to-antral transition, in particular. Along with biglycan, asporin and decorin are members of the small leucine-rich repeat proteoglycan/protein (SLRP) class I family (Schaefer & Iozzo 2008). Decorin (DCN) regulates collagen fibrillogenesis

(Iozzo 1997, Geng *et al.* 2006, Chen & Birk 2013) and protects collagen fibrils from cleavage by matrix metalloproteinases (Geng *et al.* 2006). Microarray analyses are consistent with expression of decorin throughout the ovary, whereas collagen type I was more highly expressed in the theca interna and tunica than the interstitium or pre-theca (Supplementary Table 1). Previously, asporin mRNA has been detected in the testis (Henry *et al.* 2001) and ovary (Lorenzo *et al.* 2001, Hatziridos *et al.* 2015) and microarray analysis has shown it to be downregulated in cells from the theca following the LH surge (Christenson *et al.* 2013) and FGF9 treatment (Schutz *et al.* 2018). Asporin also binds to collagen type I and inhibits fibrillogenesis, having higher affinity for collagen than decorin (Kalamajski *et al.* 2009). It is expressed by cancer-associated fibroblasts and enhances cell invasion through collagen

Figure 9 Localisation of galectin-1, phospholamban and periostin in adult bovine ovaries. Galectin-1 (red) is localised to the theca interna (A) and interstitial stroma (B). Arrows in A indicate the follicular basal lamina labelled with an antibody to perlecan (green) (McArthur *et al.* 2000). Phospholamban (red) showed perinuclear localisation (C) in smooth muscle cells (D) labelled with an antibody to smooth muscle actin (green). Periostin (red) localised to the peri-vascular connective tissue (E) and the theca interna (F), and the endothelium is labelled with lectin (green). Nuclei and labelled with DAPI throughout. Scale bars: A, C and D=20 μ m, B, E and F=50 μ m.

type I *in vitro* (Simkova *et al.* 2016). We found asporin expression to be negligible except within the theca interna. In the ovary, the spatial distribution of SLRPs may facilitate plasticity of the thecal stroma to accommodate the enlargement of growing antral follicles.

Osteoglycin is a member of the SLRP class III family (Schaefer & Iozzo 2008) with functions in several tissues, including bone, skin, hypothalamus and pituitary. Like DCN and MFAP2 (microfibril-associated protein 2), OGN was found by microarray to be overexpressed in thecal cells of dominant bovine follicles compared to granulosa cells (Hatzirodos *et al.* 2015) or small or large luteal cells (Romereim *et al.* 2017). Osteoglycin is positively or negatively regulated by a variety of factors, and different mRNA or glycosylated protein variants exist (Deckx *et al.* 2016). Along with ASPN, FGF9 treatment also downregulated OGN expression in bovine thecal cells (Schutz *et al.* 2018). Extracellular, osteoglycin binds to collagen type I and conditioned media from myoblastic cells overexpressing osteoglycin increased COL1 expression in osteoblast cells, while underexpression decreases COL1 expression (Tanaka *et al.* 2012). Osteoglycin has also been localised to endothelial cells and shown to negatively regulate angiogenesis via association with vascular endothelial growth factor receptor 2 (Wu *et al.* 2017). In contrast to the mRNA levels, osteoglycin protein was not detected in the theca. At this point, we have no explanation for the lack of protein in the presence of a high level of mRNA expression. Absence of osteoglycin from the theca interna may facilitate angiogenesis in the growing antral follicle (Fraser 2006). Osteoglycin and other SLRP family members are regulated by homeobox (HOX) and Runx genes (Tasheva *et al.* 2004), and HOXA7 (Ota *et al.* 2006) and RUNX1 (Jo & Curry 2006) are both expressed by the thecal cells of antral follicles. Our microarray data showed RUNX1, HOXC4 and HOXC8 to be downregulated in the theca compared to the interstitial stroma (see Supplementary Table 1).

A recent microarray study, examining bovine follicle activation *in vitro*, identified LGALS1 upregulation in primary follicles in comparison to primordial follicles, possibly indicating expansion of the surrounding connective tissue necessary to accommodate the growing follicle (Yang & Fortune 2015). Galectin-1 is suggested to have a role in luteal regression. In the murine ovary, it is expressed by fibroblasts within corpora lutea undergoing structural regression (Nio-Kobayashi & Iwanaga 2010), while in the human, it is also expressed by granulosa lutein cells of the mid and late stage corpus luteum (Nio-Kobayashi *et al.* 2014). However, in the bovine galectin-1 is localised to the large luteal cells of the healthy bovine corpus luteum, binds to N-glycans on vascular endothelial growth factor receptor-2 and is a survival factor for luteal cells *in vitro* (Sano *et al.* 2015). Furthermore, galectin-1 has been shown to inhibit the FSH-stimulated progesterone production of porcine

granulosa cells *in vitro* (Walzel *et al.* 2004). We found expression of galectin-1 in all compartments measured, with slightly higher mRNA levels in the theca compared to the tunica ($P < 0.05$), but no difference in protein level as measured by immunoblot. Immunohistochemistry revealed that galectin-1 was localised to the extracellular matrix within the interstitium and theca interna, where it may have a role in regulating growth via growth factor binding.

Periostin is another matricellular protein involved in collagen I crosslinking and has been implicated in a number of fibrotic diseases (Kudo 2011) and also has a role in angiogenesis (Kim *et al.* 2014). It is localised to the epidermal proliferative unit associated with hair follicles important in wound healing (Nishiyama *et al.* 2011) and is expressed by mesenchymal stromal cells (Coutu *et al.* 2008). Periostin has also been identified as highly expressed in the stroma of the chicken ovary (Nepomuceno *et al.* 2015) and upregulated in some ovarian tumours (Tian *et al.* 2011). We found periostin to be localised to the peri-vascular connective tissue within the interstitium and discontinuously to the peri-follicular connective tissue of some antral follicles, suggesting roles in angiogenesis and follicle growth.

Regulator of G-protein signalling 5 (RGS5), a member of the family of RGS molecules that regulate the signal transduction of G-protein-coupled receptors (GPCRs) and G-proteins, is expressed by pericytes and vascular smooth muscle cells (Bondjers *et al.* 2003, Cho *et al.* 2003, Berger *et al.* 2005). RGS5 has been found to downregulate proliferation of vascular smooth muscle cells *in vivo* and *in vitro* (Daniel *et al.* 2016). Induced expression of RGS5 significantly reduced cell proliferation of ovarian cancer tumour cells *in vitro* and increased the vascular density in tumours *in vivo* (Altman *et al.* 2012). The relatively high expression of RGS5 in the ovarian interstitial tissue found in this study may reflect a role in arteriolar homeostasis in this region. A previous study using LCM and a proteomic approach has identified pathways involved in cardiac muscle contraction as unique to the stromal compartment of the avian ovary (Nepomuceno *et al.* 2015).

ALDH1A1 (aldehyde dehydrogenase 1 family member A1) provides a source of retinol in the fetal ovary that regulates germ cell meiosis (Bowles *et al.* 2016) and contributes to follicle development (Kawai *et al.* 2012, Liu *et al.* 2018). Granulosa cell expression of ALDH1A1 is upregulated by FSH (Liu *et al.* 2018); however, it is more highly expressed in the theca interna than the membrana granulosa in follicles from both mice (Kawai *et al.* 2016) and cows (Hatzirodos *et al.* 2015). The regulation of ALDH1A1 in thecal cells and contribution to follicular development is yet to be determined.

The transcriptional co-activator CITED1 (Cbp/P300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 1) has been previously shown to be increased in bovine granulosa cells from large antral

follicles compared with granulosa cells from small follicles (Hatzirodos *et al.* 2015). *Cited1* is induced by FSH in granulosa cells from human preovulatory follicles (Perلمان *et al.* 2006) and via the progesterone receptor isoform A (PGR-A) in rat and mouse granulosa cells and appears to play a role in the neovascularisation of follicles at the time of ovulation (Sriraman *et al.* 2010). In our dataset, we observed upregulation of *CITED1* in the theca interna relative to stroma and it therefore might be involved in increased vascularisation of the theca interna during follicle growth. Treatment of NIH3T3 cells with TGF β results in a transcription-enhancing effect of *CITED1* on Smad molecules (Shioda *et al.* 1998).

CITED1 interacts with the oestrogen receptor α (ESR1) in an oestrogen-dependent manner (Yahata *et al.* 2001) and *ESR1* is mainly expressed in thecal, interstitial and surface epithelial cells (Lee *et al.* 2009). Specific knockout of *ESR1* in the theca interna results in fewer corpora lutea, more antral follicles and more thecal cells and haemorrhage (Lee *et al.* 2009). *ESR1* acts on nuclear receptor subfamily 1 group D members, NR1D1 and NR1D2. Therefore, the downregulation of *ESR1* in theca interna in our data set could be responsible for the observed downregulation of both transcription factors in the theca interna. NR1D1 and NR1D2 are negative regulators of core clock proteins (e.g. ARNTL) and play roles in circadian rhythms, lipid and carbohydrate metabolism. NR1D1 modulates the circadian expression of the plasminogen activator inhibitor type I (PA-I), regulates malate dehydrogenase (MDH1) linking glycolysis and fatty acid synthesis (Wang *et al.* 2006) and plays a role in adipocyte differentiation (Laitinen *et al.* 2005). NR1D1 itself is regulated by inflammation and oxidative stress (Yang *et al.* 2014) and under the latter condition increases oxidative phosphorylation, mitochondrial biogenesis, anti-oxidative defence and mitochondrial mass by decreasing mitophagy (Sengupta *et al.* 2016) and thus it could have been expected that *NR1D1* would be upregulated in the theca interna and not downregulated as observed.

Prohibitin (PHB) is a member of the highly conserved ubiquitinous protein family controlling the cell cycle, differentiation and senescence. Networks generated by IPA revealed upregulation of *PHB* in the theca interna compared to stroma. Mitochondrial PHB has been shown to be a granulosa cell survival factor regulating anti-apoptotic gene expression (Chowdhury *et al.* 2016a) and steroidogenesis (Chowdhury *et al.* 2016b). Overexpression of *PHB* in adipocytes results in obesity and is associated with elevated serum oestradiol and anovulation in mice (Ande *et al.* 2017). *PHB* has been shown to be expressed by theca cells in a stage-dependent manner (Thompson *et al.* 2004), but its specific role in androgen synthesis and cell survival is yet to be investigated in these cells.

Nuclear protein 1 (NUPR1), implicated in the progression of several tumour types, may regulate

the expression of many target genes by binding to various transcription factors. Its expression has been shown to be regulated by intracellular Ca²⁺ (Lee *et al.* 2015). The granulosa cells of atretic porcine follicles have shown upregulated expression of *NUPR1* in comparison to healthy follicles (Terenina *et al.* 2017). In addition, inactivation of *NUPR1* in cancer cells causes mitochondrial dysfunction and is associated with apoptosis and programmed necrosis (Santofimia-Castano *et al.* 2018). Our IPA network analysis revealed that *NUPR1* was downregulated in theca interna compared to stroma, suggesting that it may contribute to the atresia of thecal cells.

MLF1IP, *ACD*, *IL17RD* and *PLN* were identified by microarray as potential markers of the pre-theca; however, these genes were not differentially expressed between stromal compartments. Myeloid leukaemia factor 1-interacting protein MLF1IP (also known as CENPU) is associated with a variety of cancers including ovarian cancer (Li *et al.* 2018) and was previously found to be expressed in the bovine oocytes (Vallee *et al.* 2005) and murine follicles and corpora lutea (Wang *et al.* 2013). Adrenocortical dysplasia protein homolog (ACD) is involved in the maintenance of telomere length and its expression is associated with various tumours (Polito *et al.* 2018). Mutation of the *ACD* gene results in reduced follicular development and fertility (Keegan *et al.* 2005). Similar expression to FGF (SEF or IL17RD) has been localised to the membrana granulosa and theca interna of preovulatory human ovaries (Lutwak *et al.* 2014). IL17RD inhibits FGF signalling (Tsang *et al.* 2002), and it is downregulated in cancerous tissues. It has been shown to regulate the epithelial to mesenchyme transition (He *et al.* 2016), while overexpression in prostate cancer cells inhibits proliferation (Darby *et al.* 2009). Together, these genes may have a role in the regulation of cell growth in the ovary. Phospholamban (PLN), a calcium-regulating transmembrane sarcoplasmic reticulum phosphoprotein, controls muscle contraction via regulation of sarco(endo)plasmic reticulum calcium ATPase (SERCA) (Gorski *et al.* 2017). In addition to cardiomyocytes, PLN is found in skeletal (Damiani *et al.* 2000, Vangheluwe *et al.* 2005) and smooth muscle (Kim *et al.* 2008) and endothelial cells where it affects vessel relaxation (Sutliff *et al.* 1999). PLN also binds to the anti-apoptotic mitochondrial protein HS-1-associated protein X-1 (HAX-1) and may be important in cell survival (Vafiadaki *et al.* 2009). The role of PLN in the ovary is yet to be elucidated.

In summary, these analyses are in line with known differences between stromal compartments. It is known that the most structural collagen occurs in the tunica albuginea, then the stroma and then the theca interna and our results are in agreement with this. Our results also indicated that the theca interna is steroidogenic as is well known. However, they have also highlighted that the theca interna is regulated by genes associated with

cell survival and death as this stromal compartment also degenerates when the follicle undergoes atresia.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0323>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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