RESEARCH

Ovarian preantral follicles are responsive to FSH as early as the primary stage of development

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Abstract

Follicle-stimulating hormone (FSH) is required for ovarian antral folliculogenesis and steroidogenesis, and there is increasing evidence that it may play critical roles in preantral follicle development. We hypothesized that preantral follicles begin responding to FSH as early as the primary stage of development. Our objectives were to establish whether the FSH receptor (FSHR) was expressed in bovine preantral follicles and to determine the effects of FSH in these follicles and the surrounding ovarian tissue. Preantral follicles were isolated from bovine ovaries and subjected to immunolocalization of FSHR. Ovarian cortical strips were cultured with FSH or vehicle for 2 or 4 days and subjected to RNA sequencing, hematoxylin/eosin staining and immunostaining for p42/44 MAPK. Finally, cortical strips were cultured for 4 days with FSH before Western blot analysis of total and phosphorylated p42/44 MAPK and total aromatase. We found greater FSHR labeling intensity per cell in preantral follicles at the primary stage compared to other stages ($P < 0.05$). FSH upregulated genes involved in energy metabolism and MAPK signaling and downregulated genes related to phagosome and allograft rejection in the ovarian cortex. Preantral follicles cultured *in situ* with FSH had greater expression of total p42/44 MAPK ($P < 0.05$), but no difference was detected in whole tissue Western blot for phosphorylated p42/44 MAPK or aromatase. We conclude that the FSHR is expressed in preantral follicles as early as the primary stage of development, and that FSH upregulates cell metabolism and activates MAPK signaling pathways in preantral follicles.

Key Words

- ▶ FSH
- ▶ MAPK signaling
- ▶ preantral follicle
- ▶ folliculogenesis
- ▶ ovary

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Introduction

Ovarian antral follicles require gonadotropin stimulation for development and ovulation whereas preantral follicles seem capable of development in the absence of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), suggesting that gonadotropins are not required for preantral follicular development (Dufour *et al.* 1979, Kumar *et al.* 1997, Balla *et al.* 2003, Pakarainen *et al.* 2005). Although folliculogenesis arrest occurs at the late preantral/early antral stages in hypophysectomized mice

and ewes, a reduction in the number of small preantral follicles has also been observed, indicating that although not required, FSH may have a physiological role in preantral folliculogenesis (Dufour *et al.* 1979, Wang & Greenwald 1993). Additional evidence of a role for FSH in preantral follicle development comes from the findings of FSHR mRNA and protein in follicles as early as the primary stage in humans, mice, sheep, pigs, and rabbits (Tisdall *et al.* 1995, Rajapaksha *et al.* 1996, Oktay *et al.* 1997,

Durlej *et al.* 2011, Lan *et al.* 2014). Furthermore, *in vitro* studies demonstrate that presence of FSH improves long-term survival of preantral follicles in culture and promotes development of healthy oocytes (Roy & Treacy 1993, Cortvrindt *et al.* 1997, Wright *et al.* 1999, Mao *et al.* 2002, Adriaens *et al.* 2004, Hardy *et al.* 2017).

The canonical pathway stimulated by FSH in antral follicles results in activation of protein kinase A (PKA) and other regulatory proteins (Hunzicker-Dunn *et al.* 2012). The PKA pathway has multiple downstream targets including proteins of the MAPK family which directly and indirectly drive granulosa cell proliferation and differentiation (Shiota *et al.* 2003, Wayne *et al.* 2007, Puri *et al.* 2016), and ovulation of antral follicles (Schuermann *et al.* 2018). More specifically, phosphorylated p42/44 MAPK has been shown to contribute to the expression of genes required for development of the preovulatory follicle, such as *Cyp19a1*, *Lhcgr*, *Inha*, and *Inhb* (Kwintkiewicz *et al.* 2007).

Despite the presence of FSHR in preantral follicles and the knowledge that FSH improves preantral follicle development *in vitro*, there have been no studies to elucidate the global changes elicited by FSH or whether all preantral follicle stages can respond to it. Likewise, studies showing MAPK pathways as targets of FSH are limited to antral follicles and cultured granulosa cells (Kwintkiewicz *et al.* 2007, Schuermann *et al.* 2018). In this study, we tested the hypothesis that growing preantral follicles are responsive to FSH starting at the primary stage of development, the very first stage following activation of the quiescent primordial follicle. Our objectives were to (1) detect expression of the FSH receptor (FSHR) in growing bovine preantral follicles; (2) evaluate the global transcriptomic changes in the ovary cortex in response to FSH stimulation; and (3) examine whether FSH induced changes in p42/44 MAPK and CYP19A1 (aromatase) protein expression in preantral follicle granulosa cells.

Materials and methods

All materials were purchased from Fisher Scientific unless otherwise noted.

Preantral follicle isolation

Bovine ovaries were sourced from a local abattoir and transported in warm saline. Preantral follicles were mechanically isolated by homogenization followed by sequential filtration. Ovaries were homogenized (Omni Macro Homogenizer, Omni International, Kennesaw,

GA, USA) for 1 min at 3500 *g* and the homogenate was filtered through a cloth. The resulting filtrate was strained through a 200 μ m followed by a 40 μ m cell strainer. Material retained in the 40 μ m strainer was rinsed out with wash medium (TCM 199 containing 3 mg/mL BSA, 25 mM Hepes, 1 mM sodium pyruvate, 100 nM non-essential amino acids, 100 IU penicillin, 100 μ g/mL streptomycin) and preantral follicles were searched under a stereomicroscope. Follicles were selected based on morphology (absence of antrum, visible oocyte, and intact basement membrane) and placed in drops of wash medium. Next, follicles were incubated in 0.02% (v/v) trypan blue in PBS for 1 min to assess viability. After washing in PBS containing 0.1% (w/v) polyvinylpyrrolidone (PVP), follicles that remained unstained were considered viable and used for subsequent experiments. A total of 187 preantral follicles (49 primary, 78 early secondary, 60 secondary classified according to Candelaria & Denicol 2020) were obtained in 18 collection days.

Immunolocalization of FSHR in preantral follicles and cumulus-oocyte complexes (COCs)

Fresh preantral follicles and COCs were placed in drops of 4% (v/v) paraformaldehyde (PFA) for 15 min, washed in wash buffer (PBS containing 0.1% (v/v) Tween 20 and 0.1% (w/v) BSA) and permeabilized (PBS containing 0.3% (v/v) Triton x-100 and 0.1% (w/v) BSA) for 10 min at room temperature (RT). Next, follicles were placed in 3% (v/v) avidin and 3% (v/v) biotin blocking solutions (SP2001, Vector Laboratories) for 15 min each at RT. Follicles were washed and incubated in blocking solution (5% (v/v) normal donkey serum in PBS) for 1 h at RT, followed by overnight incubation in 2 μ g/mL rabbit anti-mouse FSH receptor antibody (FSHR, ab113421, Abcam) at 4°C. Bovine COCs were used as a positive control to validate specificity of antibody binding to the FSHR. Follicles were subsequently incubated with 3 μ g/mL biotinylated donkey-anti-rabbit IgG secondary antibody (711-065-152, Jackson Laboratories) for 1 h at RT. Finally, follicles were placed in drops of 5 μ g/mL avidin-conjugated FITC for 1 h at RT in the dark followed by 10 min with 1 μ g/mL Hoechst 33342 to label DNA. Follicles were imaged with an inverted epifluorescence microscope (Revolve, Echo Labs, San Diego, CA, USA) under FITC and DAPI channels using fixed exposure time and settings. Every replicate included negative control follicles by omitting the primary antibody and replacing it with rabbit isotype IgG (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article). Expression of

FSHR was quantified in the FITC channel in arbitrary fluorescence units by signal intensity using ImageJ software (NIH). Two readings were taken for each follicle and averaged. The average intensity reading from the negative control follicles for each replicate was subtracted from the average intensity for each positive follicle to control for background fluorescence. Cell number was determined for each individual follicle by counting cell nuclei. The final calculated FSHR signal intensity was then divided by the number of cells of each individual follicle to generate FSH staining intensity per cell.

Immunolocalization of FSHR in the ovarian cortex

Ovarian cortex was fixed in 4% PFA overnight and placed in 30% (w/v) sucrose until the sample sunk to the bottom of the jar. Next, cortex tissue was embedded in OCT compound, frozen at -80°C , sectioned in $10\text{-}\mu\text{m}$ slices and placed on positively charged slides. Slides containing sections were washed in TBS to remove OCT before antigen retrieval. Slides were incubated with citric acid-based antigen-retrieval solution (pH 6) (H-3300, Vector Laboratories) and placed in a steamer at 200°C for 10 min. After cooling, slides were washed (twice for 5 min) and blocked with 3% biotin and avidin solution and with blocking solution (10% donkey serum, 1% BSA, 0.3 mM glycine). Sections were incubated with $10\text{ }\mu\text{g/mL}$ of anti-FSHR antibody overnight in a humidified chamber at 4°C or with blocking solution only or a rabbit isotype IgG (negative controls). After washing (twice for 5 min), donkey-anti-rabbit biotinylated secondary antibody was added at $3\text{ }\mu\text{g/mL}$ for 1 h, washed, and followed by incubation of $5\text{ }\mu\text{g/mL}$ avidin-conjugated FITC for 1 h at RT in the dark and 10-min incubation with $1\text{ }\mu\text{g/mL}$ Hoechst 33342. After mounting, slides were imaged to assess labeling of follicles.

Ovarian cortical strip culture

Ovarian cortex was cut into strips of $5 \times 10 \times 1\text{ mm}$ using a sterile scalpel blade and rinsed in wash medium. Before culture, cortical strips were carefully examined under a stereomicroscope for removal of visible antral follicles and luteal tissue. In experiment 1, cortical strips were washed in PBS and randomly assigned to be flash frozen in liquid nitrogen and immediately stored at -80°C until RNA isolation, or fixed in 4% PFA overnight (day 0 control), or cultured for 2 or 4 days in cortical strip medium (MEM- α glutamax containing 3 mg/mL BSA, 10 mg/L insulin, 5.5 mg/L transferrin, 6.7 $\mu\text{g/L}$

selenious acid, 100 $\mu\text{g/mL}$ penicillin, 100 IU streptomycin, 100 nM non-essential amino acids, 50 $\mu\text{g/mL}$ ascorbic acid) supplemented with 100 ng/mL of human recombinant FSH (hrFSH; RayBiotech, Peachtree Corners, GA, USA) or the equivalent volume of sterile MilliQ water (vehicle control). After 4 days, strips were harvested and either fixed in 4% PFA for hematoxylin and eosin (H&E) or immunostaining following cryosectioning, or flash frozen. In experiment 2, cortical strips were processed as previously described and cultured for 4 days with 100 ng/mL hrFSH or vehicle. At the end of culture, cortical strips were flash frozen and immediately stored at -80°C until processed for Western blotting. In both experiments, cortical strips were cultured in well insert membranes inside six-well plates containing 2 mL of culture medium in a humidified chamber at 38.5°C and 5% CO_2 in air. Medium was completely changed every day. Each experiment was performed in three replicates.

Immunolocalization of p42/44 MAPK

After 4 days of culture, cortical strips were processed for cryosectioning as previously described. Tissue sections were incubated with 0.5 $\mu\text{g/mL}$ of rabbit polyclonal antibody against p44/42 MAPK (4695, Cell Signaling Technology) or a rabbit isotype IgG overnight in a humidified chamber at 4°C . After washing, 1 $\mu\text{g/mL}$ FITC-conjugated donkey anti-rabbit secondary antibody was added for 1 h at RT, followed by 10 min incubation with 1 $\mu\text{g/mL}$ Hoechst 33342. Slides were imaged and preantral follicles showing the oocyte were included for analysis. Intensity of the fluorescence signal in the FITC channel was measured for each follicle using ImageJ software.

Western blot

Ovarian cortex was flash frozen and homogenized over liquid nitrogen. For extraction of membrane-bound FSHR, homogenate was placed in a tube containing membrane extraction buffer (25 mM Tris, 1mM EDTA, 0.1% β -mercaptoethanol, 0.25M sucrose, pH 7.4, protease inhibitor (Roche)) and chilled on ice for 15 min. The solution was centrifuged at 11,000 g for 20 min at 4°C . The supernatant was subjected to ultracentrifugation at 100,000 g for 1 h at 4°C . The sample was frozen/thawed to dislodge the membrane protein pellet and reconstituted with 25 mM Tris buffer. Protein concentration was quantified with a Bradford assay. For extraction of cytoplasmic p42/44 MAPK and aromatase, homogenized tissue was incubated in RIPA buffer for 2 h at 4°C and

centrifuged for 3 min at 300 *g*. Protein was denatured and reduced with Laemmli buffer (Bio-Rad) with 1M of DTT or 10% β -mercaptoethanol and the solution was boiled for 5 min. Thirty micrograms of protein and ladder were loaded into each lane of a 10% polyacrylamide gel (Bio-Rad) for electrophoresis. After the protein was transferred to a PVDF membrane, the membrane was blocked with 5% dry milk and 0.1% tween for 1 h at RT. The membranes were incubated with FSHR (1 μ g/mL), p44/42 MAPK (0.084 μ g/mL), phosphorylated p42/44 MAPK (0.2 μ g/mL, 9101, Cell Signaling Technology), aromatase (0.5 μ g/mL; ab18995, Abcam), rabbit isotype control (1 μ g/mL), and loading control histone 3 antibodies (0.2 μ g/mL; ab1791, Abcam) overnight at 4°C. Membranes were washed and incubated with HRP-conjugated donkey anti-rabbit secondary antibody (Jackson Laboratories) for 1 h at RT. After washing, colorimetric detection was performed using Ultra TMB solution. Densitometric quantification of bands was done using ImageJ software.

RNA isolation and 3'-Tag RNA sequencing

Cortical strips frozen at day 0 or after 2 and 4 days of culture were homogenized over liquid nitrogen. Total RNA was extracted using Trizol Plus RNA Purification Kit following manufacturer instructions. Genomic DNA was removed with an on-column procedure. Concentration and quality of RNA were assessed using Nanodrop and 2100 Bioanalyzer (Agilent Technologies) or Caliper LabChip GX Analyzer. RNA samples were submitted to the DNA Technologies and Expression Analysis Core at UC Davis. Gene expression profiling was carried out using a 3'-Tag-RNA-Seq protocol. Barcoded sequencing libraries were prepared using the QuantSeq FWD kit (Lexogen, Vienna, Austria) for multiplexed sequencing according to the recommendations of the manufacturer using UDI-adapter and UMI Second- Strand Synthesis modules (Lexogen). The fragment size distribution of the libraries was verified via micro-capillary gel electrophoresis on a LabChip GX system (PerkinElmer). The libraries were quantified by fluorometry on a Qubit fluorometer (Life Technologies) and pooled in equimolar ratios. Libraries were sequenced in one lane of the HiSeq 4000 sequencer (Illumina) with single-end 100 bp reads.

Bioinformatic analysis

The raw FASTQ files were quality-checked using FastQC (v. 0.11.2) (Leggett *et al.* 2013), and Trimmomatic software was employed for quality trimming and adapters clipping

(Bolger *et al.* 2014). The remaining reads were aligned to the bovine reference genome (*Bos taurus* UMD3.1) and the read counts estimated at gene level, performing these steps with the Rsubread package for the R software (Liao *et al.* 2013). As unsupervised analysis, a multidimensional scaling (MDS) plot was made to establish relatedness between samples and identify any outliers with the Glimma package (Su *et al.* 2017). The remaining steps were performed with the edgeR package (Robinson *et al.* 2010). First, low-expression genes were filtered out if there were less than 1 count per million mapped reads in at least three of the samples. Next, between-sample normalization was performed using Trimmed Mean of M (TMM) and a robust estimate of the negative binomial dispersion parameter was applied to each gene by using observation weights. Finally, a negative binomial generalized log-linear model was fit to read counts for each gene (McCarthy *et al.* 2012). A regression-based approach was used to find those genes with significant temporal expression changes and significant differences between FSH-treated and control groups. This analysis was performed with the maSigPro package (Nueda *et al.* 2014). Genes whose change in expression between groups was associated with FDR < 0.05 were considered differentially expressed genes (DEG). DEG determined from the time-course analysis were clustered with Cluster 3.0 (de Hoon *et al.* 2004), according to their normalized expression level profile, using Spearman Rank Correlation as similarity metric and Centroid Linkage as the clustering method. Gene expressions were transformed and normalized to construct the hierarchical cluster and the heatmap for the expression levels. The clusters' tree and normalized values were plotted with Java Treeview (Saldanha 2004) in order to visualize the gene responses to FSH overtime. NetworkAnalyst software (www.networkanalyst.ca) (Xia *et al.* 2015) was used to identify relevant biological functions represented by differentially expressed genes. Biological processes, molecular functions, and cellular components with a *P*-value < 0.05 were considered significantly enriched. Sequencing data have been deposited in NCBI's Gene Expression Omnibus (GEO) under accession number GSE147176.

Statistical analysis

Intensity of FSHR, p42/44 MAPK staining and Western blot data were analyzed by ANOVA using the PROC GLM procedure of SAS version 9.4. When appropriate, Tukey was used as a post hoc test. Models included the effects of treatment, stage of development (where applicable)

and replicate. A linear regression analysis was performed using the stats and jtools packages for R to evaluate the relationship between cell number and intensity of FSHR staining within each developmental stage evaluated. Analysis of RNA sequencing data was performed using R software. For each cluster, the mean gene expression levels within day between treatments were compared by *t*-test using the SAS software. Expression levels of *HDAC* transcripts within day were also analyzed by *t*-test. No outliers were excluded from final data analyses. Comparisons associated with $P < 0.05$ were considered significant.

Results

Expression of FSHR in preantral follicles

Binding specificity of the FSHR antibody was confirmed by immunolocalization and Western blot. We observed consistent signal for FSHR in cumulus cells but not in the oocyte in isolated COCs (Fig. 1A) and in cryosections of bovine ovary where we observed staining of granulosa cells of preantral and antral follicles (Fig. 1B and C). Western blot analysis of bovine ovary containing antral follicles resulted in a strong band of approximately 70 kDa (Fig. 1D). While the full-length bovine FSHR is a 78 kDa protein, a 70 kDa FSHR has been identified and suggested to be an alternative-spliced protein whose transcript was shortened in the transmembrane and intracellular regions, but not the extracellular region which the antibody used

in this study was raised against (O'Shaughnessy *et al.* 1996, Rajapaksha *et al.* 1996).

Preantral follicles were isolated via mechanical manipulation due to concerns that enzymatic digestion could potentially damage the membrane-bound FSHR. All preantral follicle stages examined expressed the FSHR (Fig. 2A). Some isolated follicles retained ovarian cells outside the basement membrane, and no signal was detected in those cells. The average number and range of cells were 57 (24–88) for primary, 104 (76–174) for early secondary and 182 (112–327) for secondary follicles. In a per follicle basis, there was no difference in the intensity of FSHR labeling between primary, early secondary and secondary stage follicles. When the intensity value was divided by the number of cells in each follicle, primary follicles had the highest expression of FSHR per cell, followed by early and secondary follicles ($P < 0.001$; Fig. 2B). Taking into consideration that immunolabeling per cell may not increase proportionally with the number of cells if there were no differences in receptor abundance, we subjected the data to linear regression analysis to verify our findings. The intensity of FSHR staining increased as the number of cells increased in primary and early secondary ($P < 0.05$), but not in secondary follicles ($P = 0.65$; Supplementary Fig. 2). Interestingly, when predicting the FSHR intensity in each stage while holding a constant number of cells (the overall cell number average of all stages), the predicted staining intensity was higher for primary follicles.

Figure 1

Validation of FSHR antibody specificity for the bovine receptor. (A) Cumulus-oocyte complex (COC) under transmitted light (TL), FITC channel showing FSHR expression, and merged image with DAPI labeling cell nuclei. Insert in FITC channel is a zoomed image of the region demarcated by the rectangle and shows peripheral localization of FSHR labeling. Scale bar = 100 μ m. (B) Preantral follicle and (C) antral follicle cryosections labeled with FSHR. In C, arrow points to mural granulosa cells. TC, theca cells; AC, antral cavity. Scale bar = 50 μ m. (D) Western blot of bovine ovarian tissue showing FSHR band around 70 kDa and isotype control. Loading control antibody used was anti-histone 3A (H3A). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0126>.

Figure 2

FSHR expression in growing bovine preantral follicles. (A) Primary, early secondary, and secondary follicles showing FSHR expression (FITC) and DNA labelling (DAPI). Scale bar = 50 μ m. (B) Quantification of fluorescent signal intensity per cell for each follicular stage. Intensity for each follicle measured was divided by its number of granulosa cells to determine staining intensity per cell. Primary follicles showed higher signal intensity per cell (* $P < 0.001$) compared with early secondary and secondary follicles. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0126>.

Ovarian follicle count and RNA sequencing of the ovarian cortex

Upon finding ubiquitous expression of the FSHR in bovine preantral follicles, our next steps were to investigate whether FSH was altering the developmental dynamics of these follicles, and the global gene expression changes in the ovary enclosing preantral follicles in response to FSH.

Analysis of follicle count after H&E staining showed a tendency for follicles exposed to FSH to be more advanced in development compared to vehicle control. The proportion of primordial follicles was 16.4 ± 2.4 and $3.3 \pm 2.4\%$ in vehicle and FSH-treated, respectively ($P=0.06$); proportion of primary follicles was 55.4 ± 5.5 and $65.8 \pm 5.5\%$, and secondary follicles, 19.9 ± 2 and $24.4 \pm 2\%$ in vehicle and FSH-treated, respectively ($P=NS$).

The MDS plot of gene expression showed clear clustering of samples between FSH- and vehicle-treated at days 2 and 4 of culture (Fig. 3A). As time was the major driver of transcriptomic changes, samples at day 0 were excluded from the MDS in order to have a better discernment of the relationship between the other samples. All samples contained similar expression levels of germ cell markers such as *ZP1*, *ZP2*, *ZP3*, *GDF9*, *DDX4* and *DAZL*, indicating that oocytes were present in the cultured ovarian strips and that their numbers were likely similar across samples. The gene *B4GALT2*, reported as a granulosa cell marker (Malcuit *et al.* 2009), was upregulated in FSH-treated samples at day 4. The follicular marker *RAB23* (Malcuit *et al.* 2009) was expressed in all samples and not changed by FSH.

Time-course gene expression analysis identified 252 genes that changed over time in FSH-compared to the vehicle-treated ovary (Table 1 and Supplementary Table 1). Examining the cluster tree and heat map, we identified five gene clusters that responded similarly to FSH and that corresponded to the majority of the 252 DEGs (Fig. 3B, C, D, E and F). Mean expression profiles for each cluster revealed that clusters 1, 2 and 5 represented genes that responded positively to FSH either at day 2 or day 4 of culture (Fig. 3B, C and F). Cluster 3 represented genes that were downregulated by FSH (Fig. 3D). Genes grouped in cluster 4 were downregulated by FSH at day 2 and rescued at day 4, resulting in a subtle, although significant, upregulation compared to vehicle control. Clusters 1, 2 and 5, which contained genes upregulated by FSH, were combined for downstream analysis. The top three biological processes enriched by upregulated genes included glycolysis/gluconeogenesis, carbon metabolism, and biosynthesis of amino acids (Table 2); top three biological processes enriched by downregulated genes resulting from individual analysis of clusters 3 and 4 included phagosome, allograft rejection, and circadian rhythm (Table 3).

Finally, we searched for transcripts related to epigenetic changes that could be occurring in response to FSH. Nine members of the histone deacetylase family were expressed (*HDAC1*, *HDAC2*, *HDAC3*, *HDAC5*, *HDAC6*, *HDAC7*, *HDAC8*, *HDAC10* and *HDAC11*), and three of them, *HDAC6*, *HDAC8* and *HDAC11* were downregulated by FSH (Fig. 4A, B and C).

Protein expression of p42/44 MAPK and aromatase

MAPK pathways have critical roles in cell proliferation and have been identified as targets of FSH signaling in

Figure 3

Transcriptomic profile of bovine ovarian cortical tissue after no treatment (day 0) or treatment with FSH or vehicle in the culture media for 2 and 4 days. (A) Multidimensional scaling (MDS) plot showing distinct clustering of samples according to day and treatment. (B, C, D, E and F) Clusters of genes found by time-course analysis to be differentially expressed between treatments over time are represented as a heatmap and line plot. Legend for heat map describes colors for highly upregulated genes (red) through highly downregulated genes (green). Asterisks denote significant differences between treatments within the same day: * $P < 0.01$; ** $P < 0.001$.

antral follicles. We used ovarian cortical strips cultured in parallel to the ones used for RNA sequencing to confirm our gene expression finding that MAPK signaling is a target of FSH in the ovary and to investigate whether FSH was stimulating MAPK in follicles, ovarian stromal cells,

or both. Cortical strips harvested after 4 days of culture were immunolabeled with an antibody against total p42/44 MAPK. Agreeing with our previous observation, the majority of the preantral follicles found in the cortical tissue after 4 days of culture were in the primary stage

Table 1 Transcripts identified in the time-course analysis as differentially expressed in response to FSH in the bovine ovarian cortex.

Gene symbol	Gene ID	Gene name	Cluster	Gene symbol	Gene ID	Gene name	Cluster
AASS	520865	aminoadipate-semialdehyde synthase	5	MTMR10	615443	myotubularin related protein 10	4
ABCF1	281589	ATP binding cassette subfamily F member 1	1	MYOF	513597	myoferlin	5
ACBD3	783375	acyl-CoA binding domain containing 3	1	NARF	618340	nuclear prelamin A recognition factor	1
ACVRL1	534536	activin A receptor like type 1	2	NDUFB7	338065	NADH:ubiquinone oxidoreductase subunit B7	1
ADAM12	407229	ADAM metalloproteinase domain 12	2	NEK6	515816	NIMA related kinase 6	2
ADGB	528523	androglobin	5	NFYB	614382	nuclear transcription factor Y subunit beta	5
ADM	280713	adrenomedullin	2	NKIRAS1	522875	NFKB inhibitor interacting Ras like 1	5
AKR1A1	618607	aldo-keto reductase family 1 member A1	2	NTNG1	787473	netrin G1	4
ALDOA	509566	aldolase, fructose-bisphosphate A	1	NXPE3	532838	neuraxophilin and PC-esterase domain family member 3	5
ANKRD13D	505682	ankyrin repeat domain 13D	4	OMA1	506223	OMA1 zinc metalloproteinase	4
ANKRD34B	529927	ankyrin repeat domain 34B	4	ORAI3	515971	ORAI calcium release-activated calcium modulator 3	2
AP1AR	533664	adaptor related protein complex 1 associated regulatory protein	5	OS9	504832	OS9, endoplasmic reticulum lectin	3
AP2B1	282183	adaptor-related protein complex 2, beta 1 subunit	1	OSBPL11	538576	oxysterol binding protein like 11	3
APAF1	537782	apoptotic peptidase activating factor 1	3	OTOR	613456	otoraplin	1
ARHGDI4	338054	Rho GDP dissociation inhibitor alpha	1	PAICS	404170	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	5
ARMC8	539591	armadillo repeat containing 8	4	PARP1	286764	poly (ADP-ribose) polymerase 1	3
ARMCX2	767841	armadillo repeat containing, X-linked 2	3	PATZ1	532416	POZ/BTB and AT hook containing zinc finger 1	5
ARRDC4	538517	arrestin domain containing 4	3	PDCD1	613842	programmed cell death 1	1
ASB5	516722	ankyrin repeat and SOCS box containing 5	5	PDE1A	524446	phosphodiesterase 11A	4
ASPM	534849	abnormal spindle microtubule assembly	3	PDE2A	281971	phosphodiesterase 2A	3
ATG10	787344	autophagy related 10	4	PFKL	508683	phosphofructokinase, liver type	2
ATOH8	616225	atonal bHLH transcription factor 8	4	PFKP	507119	phosphofructokinase, platelet	2
BAMBI	530147	BMP and activin membrane bound inhibitor	4	P116	507058	peptidase inhibitor 16	4
BAZ1B	508442	bromodomain adjacent to zinc finger domain 1B	5	PJA1	786612	praja ring finger ubiquitin ligase 1	2
C16H1orf105	616962	chromosome 16 open reading frame, human C1orf105	4	PKM	512571	pyruvate kinase, muscle	2
C7H19orf66	539087	repressor of yield of DENV protein homolog	2	PLBD2	514347	phospholipase B domain containing 2	1
CAND2	533826	cullin associated and neddylation dissociated 2 (putative)	3	PLEKHA8	538919	pleckstrin homology domain containing A8	1
CAVIN1	538457	caveolae associated protein 1	3	PNRC2	767836	proline rich nuclear receptor coactivator 2	5
CCDC126	782956	coiled-coil domain containing 126	5	POLR3F	529410	RNA polymerase III subunit F	5
CCDC174	512697	coiled-coil domain containing 174	1	POU6F2	517861	POU class 6 homeobox 2	1
CCDC82	511627	coiled-coil domain containing 82	4	PPIF	414346	peptidylprolyl isomerase F	1
CD59	505574	CD59 molecule	5	PPM1D	514005	protein phosphatase, Mg2+/Mn2+ dependent 1D	5
CD86	414345	CD86 molecule	3	PPP1R1B	282459	protein phosphatase 1 regulatory inhibitor subunit 1B	3
CDC123	514926	cell division cycle 123	5	PPP1R9A	539311	protein phosphatase 1 regulatory subunit 9A	4
CENPC	450214	centromere protein C	5	PRCP	534927	prolylcarboxypeptidase	3
CHID1	504781	chitinase domain containing 1	1	PRDM10	507760	PR/SET domain 10	2

<i>CHRNA7</i>	282178	cholinergic receptor, nicotinic, alpha 7	4	<i>PROX1</i>	515324	prospero homeobox 1	4
<i>CLECTA</i>	532865	C-type lectin domain family 7 member A	3	<i>PRR7</i>	538641	proline rich 7, synaptic	2
<i>CLIP3</i>	522965	CAP-Gly domain containing linker protein 3	5	<i>PRSS35</i>	614940	protease, serine 35	4
<i>CLU</i>	280750	Clusterin	3	<i>PSTPIP2</i>	523223	proline-serine-threonine phosphatase interacting protein 2	4
<i>CNNM2</i>	504632	cyclin and CBS domain divalent metal cation transport mediator 2	3	<i>PTPN18</i>	617215	protein tyrosine phosphatase, non-receptor type 18	3
<i>COL6A2</i>	282194	collagen type VI alpha 2 chain	3	<i>PYGL</i>	505472	phosphorylase, glycogen, liver	2
<i>COMMD6</i>	616226	COMM domain containing 6	1	<i>RBM20</i>	525419	RNA binding motif protein 20	5
<i>CRLF3</i>	528033	cytokine receptor like factor 3	3	<i>RBM24</i>	524220	RNA binding motif protein 24	4
<i>CYBA</i>	281111	cytochrome b-245, alpha polypeptide	3	<i>RBM47</i>	540831	RNA binding motif protein 47	4
<i>CYBB</i>	281112	cytochrome b-245, beta polypeptide	4	<i>RBMX2</i>	613705	RNA binding motif protein, X-linked 2	5
<i>DDAH1</i>	537391	dimethylarginine dimethylaminohydrolase 1	3	<i>RGS7</i>	281452	regulator of G-protein signaling 7	4
<i>DDX10</i>	528483	DEAD-box helicase 10	1	<i>RGS7BP</i>	613327	regulator of G-protein signaling 7 binding protein	4
<i>DDX41</i>	505276	DEAD-box helicase 41	2	<i>RNA5EH2B</i>	509258	ribonuclease H2 subunit B	4
<i>DES</i>	280765	Desmin	5	<i>RNF10</i>	520757	ring finger protein 10	1
<i>DHODH</i>	533873	dihydroorotate dehydrogenase (quinone)	2	<i>RNPS1</i>	504506	RNA binding protein with serine rich domain 1	1
<i>DNAJC24</i>	768227	DnaJ heat shock protein family (Hsp40) member C24	4	<i>RORB</i>	528382	RAR related orphan receptor B	4
<i>DNM2</i>	511691	dynamitin 2	2	<i>RORC</i>	527470	RAR related orphan receptor C	4
<i>DRAM1</i>	533992	DNA damage regulated autophagy modulator 1	2	<i>RPRD1A</i>	504355	regulation of nuclear pre-mRNA domain containing 1A	5
<i>DRG1</i>	540161	developmentally regulated GTP binding protein 1	1	<i>RRAGB</i>	506754	Ras related GTP binding B	4
<i>DTX2</i>	532767	deltex E3 ubiquitin ligase 2	4	<i>S100BPB</i>	539983	S100P binding protein	4
<i>DUSP1</i>	539175	dual specificity phosphatase 1	4	<i>SAMM50</i>	618777	SAMM50 sorting and assembly machinery component	5
<i>DYNLL2</i>	540369	dynein light chain LC8-type 2	3	<i>SAR1A</i>	517171	secretion associated Ras related GTPase 1A	2
<i>DYNLT1</i>	282380	dynein light chain Tctex-type 1	1	<i>SARS</i>	281476	seryl-tRNA synthetase	1
<i>ECI1</i>	535174	enoyl-CoA delta isomerase 1	2	<i>SCAI</i>	521431	suppressor of cancer cell invasion	4
<i>EGFLAM</i>	534427	EGF-like, fibronectin type III and laminin G domains	3	<i>SCAP</i>	507878	SREBF chaperone	4
<i>EIF2A</i>	614746	eukaryotic translation initiation factor 2A, 65kDa	5	<i>SEC13</i>	512530	SEC13 homolog, nuclear pore and COPII coat complex component	1
<i>ELK4</i>	540309	ELK4, ETS transcription factor	1	<i>SEC23A</i>	533994	Sec23 homolog A, coat complex II component	5
<i>ELOC</i>	540859	elongin C	1	<i>SEC24C</i>	509488	SEC24 homolog C, COPII coat complex component	2
<i>ELP3</i>	784720	elongator acetyltransferase complex subunit 3	4	<i>SELENOF</i>	614360	selenoprotein F	1
<i>ENG</i>	615844	endoglin	3	<i>SFSWAP</i>	512326	splicing factor SWAP homolog	5
<i>ENPP2</i>	532663	ectonucleotide pyrophosphatase/phosphodiesterase 2	4	<i>SH3KBP1</i>	510489	SH3 domain containing kinase binding protein 1	3
<i>ERCC4</i>	516371	ERCC excision repair 4, endonuclease catalytic subunit	4	<i>SHISA9</i>	1E+08	shisa family member 9	4
<i>ETV1</i>	540846	ETS variant 1	1	<i>SHMT2</i>	507197	serine hydroxymethyltransferase 2	1
<i>F13A1</i>	617881	coagulation factor XIII A chain	4	<i>SLC16A3</i>	510085	solute carrier family 16 member 3	2
<i>FADD</i>	493720	Fas associated via death domain	1	<i>SLC20A1</i>	514255	solute carrier family 20 member 1	1
<i>FBXL2</i>	616212	F-box and leucine rich repeat protein 2	1	<i>SLC29A3</i>	504503	solute carrier family 29 (nucleoside transporters), member 3	3

(Continued)

Table 1 Continued.

Gene symbol	Gene ID	Gene name	Cluster	Gene symbol	Gene ID	Gene name	Cluster
<i>FKBP1A</i>	614795	FK506 binding protein 1A	2	<i>SRP72</i>	509430	signal recognition particle 72	1
<i>FXYD5</i>	505584	FXYD domain containing ion transport regulator 5	1	<i>SSFA2</i>	538826	sperm specific antigen 2	4
<i>GOS2</i>	507436	G0/G1 switch 2	1	<i>SSX2IP</i>	534692	SSX family member 2 interacting protein	4
<i>GGCT</i>	533374	gamma-glutamylcyclotransferase	3	<i>STXBP2</i>	515618	syntaxin binding protein 2	3
<i>GJC1</i>	511119	gap junction protein gamma 1	5	<i>SUPT16H</i>	514989	SPT16 homolog, facilitates chromatin remodeling subunit	4
<i>GK5</i>	616031	glycerol kinase 5 (putative)	4	<i>TBCE</i>	505066	tubulin folding cofactor E	3
<i>GLRX2</i>	513762	glutaredoxin 2	1	<i>TBRG1</i>	507460	transforming growth factor beta regulator 1	1
<i>GON4L</i>	526125	gon-4-like (C. elegans)	2	<i>TERF1</i>	519474	telomeric repeat binding factor 1	5
<i>GPATCH3</i>	514771	G-patch domain containing 3	2	<i>TFE3</i>	520800	transcription factor binding to IGDM enhancer 3	1
<i>GPRC5B</i>	516334	G protein-coupled receptor class C group 5 member B	4	<i>TGFB3</i>	538957	transforming growth factor beta 3	2
<i>GTF2B</i>	513231	general transcription factor IIB	4	<i>TMM44</i>	528165	translocase of inner mitochondrial membrane 44	2
<i>GUCA1A</i>	282243	guanylate cyclase activator 1A	2	<i>TIMP4</i>	317694	TIMP metalloproteinase inhibitor 4	3
<i>HAUS1</i>	528378	HAUS augmin like complex subunit 1	3	<i>TIPARP</i>	540975	TCDD inducible poly (ADP-ribose) polymerase	2
<i>HDAC11</i>	519899	histone deacetylase 11	3	<i>TMCO1</i>	614715	transmembrane and coiled-coil domains 1	1
<i>HDAC6</i>	513602	histone deacetylase 6	4	<i>TMEM169</i>	617045	transmembrane protein 169	5
<i>HFM1</i>	505814	HFM1, ATP dependent DNA helicase homolog	4	<i>TMEM2</i>	515491	transmembrane protein 2	1
<i>HSPH1</i>	507165	heat shock protein family H (Hsp110) member 1	5	<i>TMEM214</i>	514683	transmembrane protein 214	1
<i>IFT74</i>	514024	intraflagellar transport 74	5	<i>TMEM251</i>	768076	transmembrane protein 251	2
<i>IGFBP3</i>	282261	insulin like growth factor binding protein 3	2	<i>TMLHE</i>	535630	trimethyllysine hydroxylase, epsilon	4
<i>IKZF1</i>	541154	IKAROS family zinc finger 1	4	<i>TMOD2</i>	532085	tropomodulin 2	4
<i>IL4R</i>	404154	interleukin 4 receptor	1	<i>TMTC1</i>	533191	transmembrane and tetraicopeptide repeat containing 1	4
<i>IMPDH1</i>	504305	inosine monophosphate dehydrogenase 1	3	<i>TOX</i>	525888	thymocyte selection associated high mobility group box	4
<i>IPO7</i>	513707	importin 7	1	<i>TPRG1L</i>	616333	tumor protein p63 regulated 1 like	4
<i>KCNMB4</i>	520448	potassium calcium-activated channel subfamily M regulatory beta subunit 4	4	<i>TRAF5</i>	507234	TNF receptor associated factor 5	3
<i>LBR</i>	509348	lamin B receptor	3	<i>TRAPPC3L</i>	783172	trafficking protein particle complex 3-like	3
<i>LDLRAP1</i>	511199	low density lipoprotein receptor adaptor protein 1	1	<i>TRMT6</i>	505816	tRNA methyltransferase 6	1
<i>LEF1</i>	535399	lymphoid enhancer binding factor 1	5	<i>TTC33</i>	531278	tetratricopeptide repeat domain 33	3
<i>LITAF</i>	520564	lipopolysaccharide induced TNF factor	5	<i>TXN</i>	280950	thioredoxin	1
<i>LMBRD1</i>	510495	LMBR1 domain containing 1	5	<i>TXNDC9</i>	539143	thioredoxin domain containing 9	1
<i>LMO3</i>	532870	LIM domain only 3	4	<i>UBE2D1</i>	535287	ubiquitin conjugating enzyme E2 D1	4
<i>LOC511175</i>	511175	uncharacterized LOC511175	4	<i>UBE2Z</i>	1E+08	ubiquitin conjugating enzyme E2 Z	2
<i>LOXL4</i>	281904	lysyl oxidase like 4	2	<i>UNC119</i>	538501	unc-119 lipid binding chaperone	3
<i>LRRC17</i>	777690	leucine rich repeat containing 17	4	<i>UNC93B1</i>	508878	unc-93 homolog B1 (C. elegans)	3
<i>LYRM1</i>	508417	LYR motif containing 1	5	<i>VAMP4</i>	616923	vesicle associated membrane protein 4	4
<i>MAP4K2</i>	520058	mitogen-activated protein kinase kinase kinase 2	3	<i>VPS4A</i>	618168	vacuolar protein sorting 4 homolog A	3

MAPKAPK3	615215	mitogen-activated protein kinase-activated protein kinase 3	1	WDFV4	527790	WDFY family member 4	4
MB21D2	519376	Mab-21 domain containing 2	5	WDR70	540724	WD repeat domain 70	5
MCM3	281302	minichromosome maintenance complex component 3	3	XPO1	510568	exportin 1	3
MKNK2	538519	MAP kinase interacting serine/threonine kinase 2	2	XPR1	536550	xenotropic and polytropic retrovirus receptor 1	4
MMD	513155	monocyte to macrophage differentiation associated	1	YIPF6	540347	Yip1 domain family member 6	1
MRPL15	510347	mitochondrial ribosomal protein L15	1	ZC4H2	768236	zinc finger C4H2-type containing	3
MRPS36	613835	mitochondrial ribosomal protein S36	1	ZDHHC4	514338	zinc finger DHHC-type containing 4	1
MRT04	509932	MRT4 homolog, ribosome maturation factor	1	ZNF165	506398	zinc finger protein 165	1
MSH3	616744	mutS homolog 3	5	ZNF608	509992	zinc finger protein 608	4
MSR1	281311	macrophage scavenger receptor 1	3	ZNF814	613566	zinc finger protein 814	3
MTERF4	512153	mitochondrial transcription termination factor 4	5				

Clusters 1–5 represent 235 of the 252 genes found to be differentially expressed using FDR < 0.05.

(84% of the vehicle- and 62% of the FSH-treated follicles), with a small proportion of primordial (2% of vehicle and 4% of FSH), early secondary (2% of vehicle and 26% of FSH) and secondary stages (11% of vehicle and 8% of FSH). Exposure to FSH increased expression of p42/44 MAPK in preantral follicles ($P < 0.01$; Fig. 5A and B), and there was no difference in labeling intensity according to stage of development ($P=0.20$). Labeling was localized to the granulosa cells within the follicles, and only faintly detected in parts of the ovarian stroma. Finally, we investigated whether FSH promoted phosphorylation of p42/44 MAPK using whole tissue Western blot analysis. Ovarian cortical strips were cultured for 4 days with FSH or vehicle, and expression of phospho- and total p42/44 MAPK were examined. We also evaluated the expression of aromatase, a known target of FSH signaling in antral follicles, but whose transcript abundance was not affected by FSH by RNA sequencing. There was clear protein expression of both total and phosphorylated p42/44 MAPK in the presence of FSH and a numerical, although not significant, decrease in expression in the vehicle-exposed tissue ($P=0.26$; Fig. 6A and B). The variability observed in MAPK expression may have resulted from variation in the number of follicles present within the fragments examined. Western blot analysis of aromatase expression revealed a clear band corresponding to the expected protein and no difference between treatments ($P=0.87$; Fig. 6C and D), confirming the RNA sequencing results.

Discussion

Here we provide evidence that the FSH receptor is expressed in bovine growing preantral follicles as early as the primary stage of development. Moreover, we demonstrate for the first time that preantral follicles respond to FSH by upregulating specific cellular functions and pathways. Although expression of the FSHR in growing preantral follicles has been demonstrated in several species (Kristensen *et al.* 2015, Scarlet *et al.* 2016) including bovine (Marelli *et al.* 2018), we felt that it was critical to confirm expression of the receptor before moving on to evaluate possible effects of FSH in preantral follicles. Our main hypothesis that FSH plays a role in preantral follicle development comes from the accumulating evidence that preantral follicles benefit from FSH *in vitro* (Cortvrindt *et al.* 1997, Adriaens *et al.* 2004, Wu *et al.* 2007, Zhang *et al.* 2013, Aguiar *et al.* 2016, Bizarro-Silva *et al.* 2018) even though they may not have an absolute requirement

Table 2 Biological processes enriched by the genes upregulated after exposure of the bovine ovarian cortex to FSH for 2 or 4 days.

Biological process	Genes	P-value
Glycolysis/gluconeogenesis	<i>PFKL, PKM, AKR1A1, PFKP, ALDOA</i>	9.71E–06
Carbon metabolism	<i>PFKL, PKM, PFKP, ALDOA, SHMT2, ACADM</i>	1.04E–05
Biosynthesis of amino acids	<i>PFKL, PKM, PFKP, ALDOA, SHMT2</i>	1.86E–05
Central carbon metabolism in cancer	<i>PFKL, PKM, PFKP, SLC16A3</i>	0.000163
Pentose phosphate pathway	<i>ALDOA, PFKL, PFKP</i>	0.000269
Fructose and mannose metabolism	<i>ALDOA, PFKL, PFKP</i>	0.000483
Galactose metabolism	<i>PFKL, PFKP</i>	0.00875
Fatty acid degradation	<i>ECI1, ACADM</i>	0.0157
Parkinson's disease	<i>PPIF, NDUFB7, PINK1</i>	0.0309
Mitophagy - animal	<i>TFE3, PINK1</i>	0.0365
Inflammatory bowel disease (IBD)	<i>IL4R, TGFB3</i>	0.0386
Protein processing in endoplasmic reticulum	<i>SEC13, SEC24C, SAR1A</i>	0.0393
Renal cell carcinoma	<i>TFE3, TGFB3</i>	0.0417
MAPK signaling pathway	<i>MAPKAPK3, ELK4, MKNK2, TGFB3</i>	0.0436
PPAR signaling pathway	<i>CYP27A1, ACADM</i>	0.0494

Clusters 1, 2 and 5 were combined and the analysis performed using Network Analyst software.

on the hormone, as demonstrated by the apparent normal growth to the late secondary stage in FSHR-knockout mice (Abel *et al.* 2000). The work of Adriaens *et al.* (2004) is of particular relevance in making the case for a role of FSH in preantral follicles by demonstrating that exposure to FSH during preantral development *in vitro* improved survival of mouse follicles, enhancing oocyte developmental competence and preimplantation embryonic development. Likewise, administration of exogenous FSH rescued preantral follicle development when juvenile rats were hypophysectomized or given a GnRH antagonist (McGee *et al.* 1997).

Upon confirmation of the presence of the receptor in all stages of growing follicles, we examined the global transcriptomic changes elicited by FSH in the preantral follicle-containing ovary to test the hypothesis that FSH would upregulate genes within pathways involved in cell proliferation and metabolism. Notably, the transcriptomic profile of the ovary in response to FSH included upregulation

of genes related to MAPK pathways, a complex signaling system that utilizes substrates and targets depending on cell context, but is generally involved in regulation of cell proliferation, survival and differentiation (Zhang & Liu 2002). FSH has been shown to stimulate MAPK pathways in antral follicle granulosa cells (Yu *et al.* 2005, Puri *et al.* 2016, Law *et al.* 2017). To our knowledge, this is the first report of MAPK stimulation by FSH in preantral follicles, shedding light into at least one specific role of FSH signaling in early follicle development. With the finding that p42/44 MAPK protein is increased in preantral follicles of all stages, we confirmed that FSH is indeed signaling in primary preantral follicles, likely mediated by the FSHR, which we found to be abundant in this follicular stage. It must be noted, however, that our RNA sequencing results include transcripts from cells found throughout the tissue, not solely follicular cells. We acknowledge the possibility that changes in gene expression observed here include indirect effects of FSH-stimulated granulosa cells in the surrounding

Table 3 Biological processes enriched by the genes downregulated in the bovine ovarian cortex after exposure to FSH for 2 or 4 days.

Biological Process – Cluster 3	Genes	P-value
Phagosome	<i>MSR1, CLEC7A, BoLA, CYBA</i>	0.001
Allograft rejection	<i>BoLA, CD86</i>	0.009
Type-I diabetes mellitus	<i>BoLA, CD86</i>	0.01
Necroptosis	<i>TRAF5, PARP1, VPS4A</i>	0.01
Viral carcinogenesis	<i>BoLA, TRAF5, HDAC11</i>	0.02
Endocytosis	<i>BoLA, VPS4A, SH3KBP1</i>	0.03
Protein digestion and absorption	<i>COL6A2, PRCP</i>	0.04
Biological Process – Cluster 4		
Circadian rhythm	<i>RORC, RORB</i>	0.003
Autophagy – animal	<i>RRGB, ATG10</i>	0.04

Clusters 3 and 4 represented genes that were downregulated by FSH after 2 days of culture; cluster 4 shows a recovery of expression at day 4 in response to FSH, while cluster 3 genes remain downregulated for the entire culture period. Analyses were performed using Network Analyst software.

Figure 4

Expression of the histone deacetylases *HDAC6* (A), *HDAC8* (B), *HDAC11* (C) in the ovarian cortex was affected by FSH. Overall, FSH had an inhibitory effect on expression of the three transcripts, with inhibition of *HDAC6* being evident at day 2 and *HDAC8* and *HDAC11* at day 4 of culture.

a,b,c $P < 0.01$; A,B $P = 0.07$.

stromal cells. However, the demonstration of FSHR and p42/44 MAPK protein localization to the preantral follicles lead us to believe that the results we observed are indeed representative of changes within the follicles. Future transcriptomic studies should ideally examine isolated preantral follicles of specific stages in parallel with ovarian cortex for a better resolution of direct and indirect effects of FSH in this tissue.

Analysis of the ovarian cortex transcriptomic profile revealed decreased expression of the histone deacetylases

HDAC6, *HDAC8* and *HDAC11* in response to FSH at either day 2 or day 4 of culture. Histone acetylation is generally associated with a state of open, transcriptionally active chromatin, while deacetylated, or poorly acetylated histones generally indicate low transcriptional activity. HDACs associate with different proteins in co-repressor complexes to remove acetylation marks from histones, silencing expression of target genes (Verdin *et al.* 2003). Although the possible roles of HDACs as regulators of gene transcription in response to FSH during early folliculogenesis are largely unknown, evidence from adrenocortical cell culture experiments show that suppression of HDACs by chemical inhibitors suppresses steroidogenesis by targeting NR5A1 (steroidogenic factor 1, SF1) to ubiquitination (Chen *et al.* 2007). Moreover, exposure of bovine granulosa and theca cells to HDAC

Figure 5

Expression of total p42/44 MAPK increases in preantral follicles exposed to FSH *in situ* (i.e. cultured within the ovarian cortex). (A) Expression of total p42/44 MAPK as assessed by fluorescence signal intensity, was stronger in preantral follicles exposed to FSH (100 ng/mL) compared to vehicle-treated tissue. Scale bar = 50 μ m. (B) Quantification of staining intensity for total p42/44 MAPK in preantral follicles within tissue treated with FSH or vehicle (* $P < 0.01$). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0126>

Figure 6

Expression of total and phosphorylated p42/44 MAPK and total aromatase in bovine ovarian cortical tissue exposed to FSH or vehicle for 4 days. (A) Ratio of phosphorylated:total p42/44 MAPK in FSH- (100 ng/mL) and vehicle-treated tissue. (B) Representative Western blot with histone 3A loading control. (C) Quantification of Western blot band density for total aromatase expression in ovarian cortical tissue treated with FSH. (D) Representative Western blot with histone 3A loading control.

inhibitors reduced steroid secretion dramatically, even in the presence of FSH and LH (Glister *et al.* 2012). Expression of *HSD17B1* and *CYP19A1*, the genes encoding the steroidogenic enzymes 17 β -dehydrogenase and aromatase, respectively, was suppressed by HDAC inhibitors in the presence of FSH (Glister *et al.* 2012). The reduction of *HDACs* expression by FSH observed here could be explained as a possible mechanism of the cell context-dependent role of FSH in regulating folliculogenesis, involving suppression of steroidogenic activity and stimulation of cell proliferation in preantral follicles. In this context, the suppressed expression of *HDACs* could be responsible for the lack of increase in aromatase expression observed in response to FSH. Supporting this hypothesis, it has been demonstrated that HDAC11 directly inactivates Cdc25A, a Cdk/cyclin complex phosphatase that promotes progression of the cell cycle (Lozada *et al.* 2016). Clearly, specific experiments are required to confirm whether the regulation of transcription of *HDACs* by FSH in the ovarian cortex is involved in modulation of folliculogenesis.

In conclusion, we demonstrate the presence of the FSHR in preantral follicles from the primary stage onwards, and moreover, that these follicles respond to FSH stimulation. Our findings on individual, freshly isolated follicles support the conclusion that expression of the FSH receptor may be more abundant in preantral follicles of the primary stage. In the ovarian cortex containing only preantral follicles, FSH upregulates genes important for energy metabolism and MAPK signaling which may relate to proliferative effects of FSH in preantral follicles. More research is required to dissect whether the effects observed in the present study result from direct activation of FSHR. Based on our findings, we propose that FSH plays a regulatory role in early preantral folliculogenesis.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0126>.

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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