Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle

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Abstract

Microarray analysis was used to compare the gene expression of granulosa cells from dominant follicles with that of those after superstimulatory treatment. Cows were allocated randomly to two groups (superstimulation and control, $n=6/\text{group}$). A new follicular wave was induced by ablation of follicles ≥ 5 mm in diameter, and a progesterone-releasing device controlled internal drug release (CIDR) was placed in the vagina. The superstimulation group was given eight doses of 25 mg FSH at 12-h intervals starting from the day of wave emergence (day 0), whereas the control group was not given FSH treatment. Both groups were given prostaglandin $F_{2\alpha}$ twice, 12 h apart, on day 3 and the CIDR was removed at the second injection; 25 mg porcine luteinizing hormone (pLH) was given 24 h after CIDR removal, and cows were ovariectomized 24 h later. Granulosa cells were collected for RNA extraction, amplification, and microarray hybridization. A total of 190 genes were downregulated and 280 genes were upregulated. To validate the microarray results, five genes were selected for real-time PCR (*NTS*, *FOS*, *THBS1*, *FN1*, and *IGF2*). Expression of four genes increased significantly in the three different animals tested (*NTS*, *FOS*, *THBS1*, and *FN1*). The upregulated genes are related to matrix remodeling (i.e. tissue proliferation), disturbance of angiogenesis, apoptosis, and oxidative stress response. We conclude that superstimulation treatment i) results in granulosa cells that lag behind in maturation and differentiation (most of the upregulated genes are markers of the follicular growth stage), ii) activates genes involved with the NFE2L2 oxidative stress response and endoplasmic reticulum stress response, and iii) disturbs angiogenesis.

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Introduction

The primary action of follicle-stimulating hormone (FSH) in the female is to promote follicular development (Adams *et al.* 1992). A group of follicles (wave or cohort) emerges after an increase in peripheral FSH concentrations (Adams *et al.* 1992, 2008, Jaiswal *et al.* 2004). Follicular products from the growing cohort, particularly the dominant follicle, are responsible for suppressing FSH concentrations (Adams 1994, Berfelt *et al.* 1994). When circulating FSH begins to decline, subordinate follicles stop growing and become atretic (Adams *et al.* 1993a, 1993b, Berfelt *et al.* 1994, Ginther *et al.* 1999). However, the dominant follicle acquires luteinizing hormone (LH) receptors on its granulosa cells (Adams 1994) and is, therefore, no longer dependent on FSH (Bao & Garverick 1998, Ginther *et al.* 1999). Maintenance of elevated circulating concentrations of FSH rescues the subordinate follicles within the cohort from regression and thereby delays or prevents selection of a single dominant follicle, i.e. results in multiple dominant follicles capable of ovulating (superstimulatory/

superovulatory response) (Scanlon *et al.* 1968, Wildt *et al.* 1975, Adams *et al.* 1993b, Mapletoft *et al.* 2002).

Superstimulation is a technique that has been used widely in animal breeding programs, both for commercial and research purposes. One of the major limitations of superovulation is the extreme variability in the response to treatments. In one study (Looney 1986), 30% of 2048 cows produced 70% of the total embryos collected, whereas 24% of cows failed to produce an embryo. Major determinants of the superstimulatory response are the number of follicles available at wave emergence (Singh *et al.* 2004) and the timing of the onset of treatments in relation to wave emergence (Nasser *et al.* 1993, Adams *et al.* 1994, Adams 1998). The reason for the high variation among individuals in the number of follicles recruited into a wave and the proportion of follicles that fail to ovulate after superovulatory treatment remains unknown. The effect of superstimulation treatment on the follicular environment and oocyte quality is also not well understood. Previous studies suggest that superstimulation can trigger genes related to the oxidative stress response of embryos of mice

(Rossignol *et al.* 2006, Fauque *et al.* 2007, Sato *et al.* 2007) and cattle (Mundim *et al.* 2009). However, these studies involved analysis of the genetic status of embryos and tested only a few genes. Microarray technology allows a better understanding of molecular status by evaluating the expression levels of thousands of genes at the same time.

Current parameters used for determining oocyte competence are based on oocyte morphology and the ability to fertilize and to develop to the blastocyst stage. However, the molecular status of follicles and oocytes subsequent to superstimulation is not known. Therefore, there is a need to determine how gene expression of a follicle is affected by preventing follicular selection and whether alterations in intracellular molecular pathways can explain the extensive individual variability in the response to superstimulation treatment.

The objective was to determine the effect of superstimulation treatment on major molecular and cellular pathways, as evidenced by gene expression of granulosa cells. We tested the hypothesis that, given an equivalent growing phase, the molecular pathways related to cell differentiation are altered in granulosa cells from superstimulated follicles compared with those of single (unstimulated) pre-ovulatory follicles.

Materials and methods

Animals and treatments

The experiment was conducted on 12 cross-bred beef cows, weighing 515–795 kg, maintained in outdoor pens at the University of Saskatchewan during October to December. Procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

To synchronize estrus and ovulation, the cows were given two luteolytic doses of prostaglandin ($\text{PGF}_{2\alpha}$; 500 μg

cloprostenol i.m.; Estrumate, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada) 14 days apart. Emergence of a new follicular wave was synchronized by transvaginal ultrasound-guided ablation of follicles ≥ 5 mm in diameter 5–8 days after ovulation. A new follicular wave was expected to emerge 1 day after ablation (Berfelt *et al.* 1994). An intravaginal, progesterone-releasing device (controlled internal drug release-bovine (CIDR-B) Pfizer Canada, Inc., Brandon, Manitoba Canada) was placed in the vagina immediately after follicle ablation. The cows were then allocated randomly to two groups: i) superstimulation group ($n=6$) and ii) control group (no superstimulation, $n=6$; Fig. 1). Starting 1 day after follicle ablation, i.e. on the day of wave emergence (day 0), cows in the superstimulation group were administered eight doses of FSH i.m. (Folltropin-V; Bioniche Animal Health, Belleville, ON, Canada; each equivalent to 25 mg NIH-FSH-P1) at 12-h intervals over 4 days. The control group was not given any FSH treatment. On day 3, cows in both groups were given 2 i.m. doses of 25 mg $\text{PGF}_{2\alpha}$ 12 h apart, and the CIDR was removed at the time of the second $\text{PGF}_{2\alpha}$ treatment. Cows were given 25 mg porcine LH (pLH) i.m. (Lutropin-V, Bioniche Animal Health) 24 h after CIDR removal and were ovariectomized 24 h after pLH treatment.

Tissue collection

Ovariectomies were performed using a colpotomy approach, as described by Singh *et al.* (1998). Briefly, caudal epidural anesthesia was induced with 5–10 ml lidocaine (Lidocaine HCl 2%, Catalog # 1LID009P, Bimeda-MTC Animal Health, Inc., Lavaltrie, QC, Canada). The perineum was disinfected using an iodine-based detergent solution. A small incision was made in the dorsolateral aspect of the vaginal fornix. The peritoneum was ruptured manually, allowing direct access to and palpation of the reproductive tract. Local anesthesia was manually applied to the ovarian pedicle using a gauze soaked with lidocaine. A plastic clip was applied to the ovarian pedicle to minimize hemorrhage. The chain of an ecraseur was looped

Figure 1 Experimental protocol used to test the effect of ovarian superstimulation on gene expression in granulosa cells. At 5 to 8 days after ovulation, follicles ≥ 5 mm were ablated and a progesterone-releasing device (CIDR) was placed intravaginally. FSH treatment was initiated at wave emergence (day 0). Cows in the superstimulation group were given eight doses of FSH at 12-h intervals, and cows in the control group were not treated with FSH. On day 3, cows in both groups were given $\text{PGF}_{2\alpha}$ and the CIDR was removed. LH was given 24 h after CIDR removal, and cows were ovariectomized 24 h later.

around the ovarian pedicle and slowly tightened until the ovarian attachments were severed. The ovaries were placed in polyethylene bags, kept on ice, and transported to the laboratory within 5 min after collection. The number of follicles in both ovaries was counted and the dominant follicle (control group) or the three largest follicles (superstimulated group) were identified by visual assessment and confirmed by measuring the diameter after follicles were opened. The goal was to collect antral and mural granulosa cells. Antral granulosa cells were those floating freely in the follicular fluid, while the mural granulosa cells were those that were attached to the inner wall of the follicle. Antral granulosa cells were collected by aspiration of the follicular antrum using a 20-gauge needle and syringe. Follicles were flushed three times with Dulbecco's PBS (Invitrogen Corporation, Catalog # 14190-144). The cumulus-oocyte complex was then identified and separated from the aspirate. The follicular fluid was centrifuged and the pellet of antral granulosa cells was harvested. The collapsed follicles were then cut in half using a scalpel blade and the inner follicular wall was scraped with a microbiology culture loop (LightLabs, Catalog # PD104, Dallas, TX, USA) to remove the mural layer of granulosa cells. The mural and antral granulosa cells were pooled for each animal, snap frozen in liquid nitrogen and kept at -80°C for later microarray analysis.

RNA extraction and amplification

Total RNA was extracted using the Trizol extraction method according to the manufacturer's instructions (Invitrogen Life Technology) and resuspended in 50 μl nuclease-free water. RNA was purified using the Arcturus *PicoPure* RNA Isolation and Purification Kit (Catalog # KIT0204, Applied Biosystem) following the manufacturer's protocol. The purification process includes DNase treatment to remove DNA and final pure RNA was recovered in 15 μl elution buffer. RNA quality was evaluated using Bioanalyzer-2100 (Agilent Technologies, Palo Alto, CA, USA) with the RNA NanoLab Chip (Catalog # 5067-1511, Agilent Technologies). Only RNA samples with RNA integrity number > 5 were used for microarray hybridizations.

Samples (5 ng) of purified RNA were used for amplification. For the superstimulation group, equal amounts of RNA from the three largest follicles were pooled and a total of 5 ng RNA from the pooled sample was used. For the control group, the same amount of RNA was obtained from the single dominant follicle. The amplification process was chosen with the intent of increasing the amount of genetic material used for microarrays. A linear amplification was performed using two 6-h rounds of T7 RNA polymerase (RiboAmp HS^{Plus} RNA Amplification Kit; Molecular Devices, Sunnyvale, CA, USA) following the manufacturer's directions. The antisense RNA (aRNA) output was measured using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

Sample labeling, hybridization, and microarray scanning

For each sample, 2.5 μg aRNA were labeled using DY-547/647 (Red – CY5 and Green CY3) fluorescent dyes from a Universal

Linkage System (ULS) Labeling Kit (EA-006, Kreatech Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's protocol. With the intent of removing all non-reacted ULS-labeled material, another round of purification was performed on the labeled aRNA, also using the Pico-Pure RNA Isolation Kit but without DNase I treatment. Pure labeled aRNA was eluted with 11 μl elution buffer. Labeling efficiency was measured using NanoDrop ND-1000. A minimum of 30 pmol/ μg (dye concentration/aRNA concentration) of labeling signal was required to proceed with hybridization. A hybridization mixture was prepared using 825 ng of each cyanine (Cy3 and Cy5) labeled amplified aRNA, Agilent and tomato spikes, nuclease-free water, 10 $\times$ blocking agent, and a 25 $\times$ fragmentation buffer, in a total volume of 55 μl , which was pipetted onto the hybridization slides. Three biological replicates in each group (superstimulation vs control) were used in the experimental design, in a dye-swap setup. Overall, six hybridizations were performed using a custom-built bovine oligo-array slide (EmbryoGENE EMBV3 manufactured by Agilent; Design ID: 028298, GEO accession # GPL13226). The slide contained a total of 45 220 oligonucleotide probes. Each probe had a duplicate and the slide also included Agilent's positive and negative controls in 4x44K format. Oligo sequences were taken from the Oligo Microarray Consortium database (BOMC, <http://www.bovineoligo.org>).

Hybridizations were performed using 2 $\times$ GEx Hybridization Buffer HI-RPM (Agilent Technologies, Wilmington, DE, USA) at 65 $^{\circ}\text{C}$ in a preheated oven for 17 h with a rotator speed of 0.05 g. Slides were washed with two buffers from the gene expression wash buffer kit (Agilent technologies, Inc., Catalog # 5188-5327) according to the manufacturer's protocol. Later, slides were dipped in 100% acetonitrile for 10 s at room temperature and washed with stabilization and drying solution for 30 s at room temperature.

The slides were scanned immediately and visualized using a Power Scanner (Tecan US, Inc., Durham, NC, USA). After image acquisition, scanned images were analyzed and quantified using Array-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

Data normalization and statistical analysis

Raw signal intensity files were uploaded to the EmbryoGENE laboratory information management system and microarray analysis platform. Quality of hybridization was evaluated using Gydle software (<http://www.gydle.com/>). Signal intensity data were analyzed using the FlexArray software, version 1.6.1 (Blazejczyk *et al.* 2007). The intensity of the background signal was subtracted from the median gray-scale value of the spot in question to obtain corrected signal intensity. If the background intensity was higher than the signal intensity for a spot, the negative value was replaced with 0.5 as a default. Data were normalized within and between arrays using Loess and Quantile normalization methodology (GEO #: GSE45381) respectively (Bolstad *et al.* 2003). Linear Models for Microarray (Limma) data analysis was performed (implemented in FlexArray software; <http://genomequebec.mcgill.ca/FlexArray/license.php>) to obtain differentially expressed genes in the superstimulation group compared with the reference

(unstimulated control) group (Smyth 2004, 2005) using a fold change of ≥ 2 and a P value of ≤ 0.05 as a threshold. To identify true positive gene changes, a false discovery rate analysis was done using the Benjamini–Hochberg method (Benjamini & Hochberg 1995) with a fold change of ≥ 2 and a P value of ≤ 0.05 .

Functional annotation and pathway analysis

The list of differentially expressed gene, generated after Limma analysis, was uploaded into Ingenuity Pathways Analysis software (IPA; Ingenuity Systems, www.ingenuity.com) to identify gene networks. Gene networks were used to identify likely biological functions, molecular processes and disorders, and pathways most related to the gene list. IPA analyses are based on human and mouse studies.

Real-time PCR

Based on the results of microarray data, five genes (neurotensin (*NTS*), FBJ murine osteosarcoma viral oncogene homolog (*FOS*), thrombospondin 1 (*THBS1*), fibronectin 1 (*FN1*), and insulin-like growth factor 2 (*IGF2*)) were selected for validation with real-time PCR. The selected genes were involved in the hypotheses generated on the basis of the results of the microarray analysis. Primers were designed using Primer3 v.0.4.0 website (<http://frodo.wi.mit.edu/primer3/>) and analyzed using IDT PrimerQuest tool – Oligo Analyzer (<http://scitools.idtdna.com/analyzer/Applications/OligoAnalyzer/>), and BLAST analysis was performed using NCBI database (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome). Selected primers were specific to the gene of interest and met the following criteria: 20–24 bp; 55–65 °C melting temperature; 40–60% CG content; and no hairpin, self-dimer, or hetero-dimer formation. Nucleotide sequences of selected forward and reverse primers are presented in Table 1 and primers were synthesized by a commercial company (Integrated DNA Technologies (IDT), Inc., Coralville, IA, USA). Primers were tested by performing real-time PCR of cDNA from pooled granulosa cell samples. The amplicon was separated by electrophoresis on a standard 1% agarose gel to determine the size of the band. The band was cut and eluted using a QIAquick Gel Extraction kit (Catalog # 28704, Qiagen), quantified using NanoDrop ND-100, and sequenced using an ABI 3730x1 DNA Analyzer. Primers were used only if the amplicon sequence matched the desired gene of interest. The amplicon was used for generating a standard curve. The range of the standard curve was 10^{-2} – 10^{-11} ng/ μ l. Real-time PCR was performed on a Stratagene Mx3005P fast thermal cycler (Applied Bioscience) using SYBR Green master mix (Applied Bioscience). The cycle threshold was recorded, and expression of the gene of interest was normalized to a geometric mean of three reference genes (*UBE2D2*, *EIF2B2*, and *SF3A1*) using the Relative Expression Software Tool (REST 2009, Qiagen).

Table 1 Primers used for real-time PCR.

Genes	Strand	Primer sequence	Annealing temperature (°C)
<i>EIF2B2</i>	Forward	5'-CATGAGATGGCAGT-CAATTTGT-3'	53.6
	Reverse	5'-CTTGAACATAGGAGCACA-GACG-3'	55.5
<i>SF3A1</i>	Forward	5'-TGTGTCCCTCTTGCTG-AGTTT-3'	56.4
	Reverse	5'-ATTCCTGGTTT-CACGTCCTCTA-3'	55.5
<i>UBE2D2</i>	Forward	5'-TGGACTCAGAAGTATGC-GATGT-3'	55.8
	Reverse	5'-CTTCTGCTAGGAGG-CAATGT-3'	56.6
<i>NTS</i>	Forward	5'-AGTGTTCCTCTTG-GAAAATGA-3'	60
	Reverse	5'-TCTTCCTGAATCAACTCC-CAGT-3'	60.1
<i>FOS</i>	Forward	5'-AGGTAGAACAGTTGTCCC-CAGA-3'	60
	Reverse	5'-CAAAGCCGACTTCT-CATCTTCT-3'	60
<i>THBS1</i>	Forward	5'-TCGACTGTGAGAAGATG-GAGAA-3'	59.9
	Reverse	5'-GTTGTCAAGGGTGA-CAAAGACA-3'	60
<i>FN1</i>	Forward	5'-AGAAGAGTGAGCCTTT-GATTGG-3'	59.9
	Reverse	5'-AGATCATTTGTTGCCCAA-GACT-3'	60
<i>IGF2</i>	Forward	5'-GCAACACCAGAAAAG-CAAAC-3'	59.4
	Reverse	5'-CAGATCCAAAGGAAC-GAGAAG-3'	60.2

RIA

Estradiol (E_2) and progesterone (P_4) concentrations were measured in samples of antral fluid aspirated from super-stimulated follicles and from dominant and subordinate follicles in the control group by RIAs. Slaughterhouse ovaries were used to obtain a charcoal-extracted pool of follicular fluid, which was used to prepare the standards and dilute follicular fluid samples. The standard curve ranged from 5 to 1000 pg/ml for E_2 and 0.1 to 40 ng/ml for P_4 . Samples were diluted using the charcoal-extracted pooled follicular fluid so that hormone concentrations fell within the limits of the standard curve and samples were assayed in duplicates. E_2 was measured with a modified human double-antibody RIA kit (Catalog # KE2D1, Coat-A-Count; Siemens Healthcare Diagnostics, Inc., Mississauga, ON, Canada), dilutions ranged from 1:25 to 1:500. P_4 was measured using a commercial RIA kit (Catalog # TKOP1, Coat-A-Count; Siemens Healthcare Diagnostics, Inc.) and all samples were diluted 1:10. All samples for P_4 analysis were analyzed in a single assay only and the intra-assay coefficient of variation was 5.6%. E_2 was measured in two different assays and the intra-assay coefficient of variation was 11%, while the interassay coefficient of variation was 8.1%. Hormone data were analyzed by ANOVA using a general linear model procedure (GLM; SAS Learning Edition 4.1; SAS Institute, Cary, NC, USA) to compare follicular fluid from the

superstimulation group vs dominant follicle from non-superstimulated (control group) vs the subordinate follicles from non-superstimulated (control group) animals.

Results

Differential gene expression profile

A total of 470 genes were differentially expressed in granulosa cells from superstimulated cows compared with those of untreated control cows. Of these, 190 genes had significantly lower expression in the superstimulated group (i.e. downregulated genes), and 280 genes had significantly higher expression in the superstimulated group (i.e. upregulated genes) compared with controls (Fig. 2). The ten most upregulated and downregulated genes in the superstimulation vs control group are listed in Table 2.

Function, network, and pathway analyses

Cellular functions most affected by superstimulation treatment were cellular growth and development (Fig. 3A). Regarding disease and disorders, superstimulation activated genes related to cancer, genetic disorders, and disorders of the cardiovascular system (Fig. 3B). Network analysis identified a network of processes involving matrix-remodeling, more specifically genes involved in proliferation of cells, apoptosis, and angiogenesis (Fig. 4). The most significant canonical pathways identified by Ingenuity pathway analysis were those related to *IGF1* (*IGF*) and *MAPK7* (*ERK5*) signaling, oxidative stress response, and inhibition of angiogenesis (Fig. 5).

Figure 2 Venn diagram summarizing microarray analysis of granulosa cells of follicles from superstimulated vs untreated control cows. Detected signals were those that were ≥ 2 s.d. above the background (mean $\pm$ s.d. of background, 129.8 ± 14.9). Linear Models for Microarray Data (Limma) was used for statistical analysis, and upregulated and downregulated genes were determined using a fold change of ≥ 2 and a P value of ≤ 0.05 .

Table 2 Top ten upregulated and downregulated genes in granulosa cells of beef cows after ovarian superstimulation treatment compared with untreated controls ($n=3$ cows per group).

Genes	Description	Fold change	P value
Upregulated			
<i>NTS</i>	Neurotensin	9.987	9.13×10^{-7}
<i>S100B</i>	S100 calcium binding protein B	8.345	8.43×10^{-3}
<i>IGFBP1</i>	IGF binding protein 1	7.797	2.03×10^{-3}
<i>GFPT2</i>	Glutamine-fructose-6-phosphate transaminase 2	7.777	8.23×10^{-3}
<i>SLC39A8</i>	Solute carrier family 39 (zinc transporter), member 8	7.775	4.53×10^{-4}
<i>COBL1</i>	Cordon-bleu protein-like 1	7.35	7.07×10^{-3}
<i>FOS</i>	FBJ murine osteosarcoma viral oncogene homolog	6.86	1.27×10^{-5}
<i>LUM</i>	Lumican	6.006	5.27×10^{-4}
<i>RCAN1</i>	Regulator of calcineurin 1	5.892	2.76×10^{-4}
<i>THBS1</i>	Thrombospondin 1	5.685	1.71×10^{-3}
Downregulated			
<i>FES</i>	Feline sarcoma oncogene	7.638	1.85×10^{-3}
<i>NRP1</i>	Neuropilin 1	6.482	2.16×10^{-5}
<i>SOWAHA</i> (<i>ANKRD43</i>)	Ankyrin repeat domain 43	5.517	3.52×10^{-4}
<i>CNIH3</i>	Cornichon homolog 3 (<i>Drosophila</i>)	4.944	1.89×10^{-4}
<i>MGST2</i>	Microsomal glutathione S-transferase 2	4.687	4.36×10^{-4}
<i>ANGPT2</i>	Angiopoietin 2	4.53	3.69×10^{-2}
<i>LTF</i>	Lactotransferrin	4.524	1.35×10^{-4}
<i>TRIB2</i>	Tribbles homolog 2 (<i>Drosophila</i>)	4.472	8.34×10^{-3}
<i>SYNE1</i>	Spectrin repeat containing, nuclear envelope 1	4.467	7.13×10^{-3}
<i>MXRA8</i>	Matrix-remodeling associated 8	4.289	7.35×10^{-3}

Real-time PCR validation

Based on microarray data and function analysis, five genes were selected for validation with real-time PCR (i.e. *NTS*, *FOS*, *FN1*, *THBS1*, and *IGF2*). After quantification in three independent biological replicates from superstimulation and control groups, differential expression was validated for four of the five genes (90% CI; $P \leq 0.1$; Fig. 6).

Hormone levels in follicular fluid

Follicular fluid from superstimulated follicles and from dominant follicles of the control group had a higher E_2 levels compared with subordinate follicles of the control group (153.8 ± 32.7 , 160.4 ± 64.9 , and 0.05 ± 0.02 ng/ml; mean $\pm$ s.e.m. respectively; $P=0.01$). Follicular fluid P_4 levels did not differ among groups (99.8 ± 19.7 , 74.0 ± 18.5 , and 108.6 ± 62.7 ; $P=0.8$ ng/ml; mean $\pm$ s.e.m. respectively; $P=0.8$). $E_2:P_4$ ratio was greater in follicular fluid from superstimulated follicles and from dominant follicles of the control group compared with subordinate follicles of the control group (3.5 ± 0.8 , 2.2 ± 1.2 , and 0.004 ± 0.002 ; mean $\pm$ s.e.m. respectively; $P=0.03$); however, the ratio did not differ between the superstimulated follicles and the dominant follicle.

Figure 3 Function analysis of gene expression of granulosa cells after superstimulation treatment based on log *P* value. The higher the log *P* value (taller bars), the more significant the function is. Numbers at the top of the bars indicate number of genes involved in each function. The top ten molecular and cellular functions (A) and top ten diseases and roles in physiological system development and function (B) are illustrated.

Discussion

Ovarian superstimulation protocols are used widely in monovular species as a component of assisted reproductive technologies. Superstimulation involves the use of FSH to stimulate continued growth of a follicular cohort to a pre-ovulatory stage. The effect of superstimulation on follicular dynamics in cattle is well known (Adams *et al.* 1992, Nasser *et al.* 1993, Adams 1998, Mapletoft *et al.* 2002); however, the genomic response of bovine follicles to gonadotropin stimulation has not been established. Results of this study indicate that molecular pathways related to granulosa cell differentiation are altered in superstimulated follicles compared with dominant follicles of unstimulated follicular waves at an equivalent stage of development (that is, identical durations from wave emergence to P₄ withdrawal and exogenous LH treatment and identical post-LH period). The results provide a rationale for three hypotheses: compared with the granulosa cells of single (natural) dominant follicles, superstimulation treatment

i) results in granulosa cells that lag behind in maturation and differentiation (most of the upregulated genes are markers of the follicular growth stage), ii) activates genes involved with the oxidative stress response, and iii) disturbs angiogenesis.

Compared with a single dominant follicle of a naturally occurring follicular wave, ovarian superstimulatory treatment delays selection and results in development of multiple dominant follicles (Adams *et al.* 1993b). However, results of this study suggest that the multiple pre-ovulatory follicles that develop after superstimulation are not typical dominant follicles. The list of upregulated genes in the superstimulation group in this study is similar to those of other studies (Gilbert *et al.* 2011, 2012) from dominant follicles before the LH surge at day 14 of the estrous cycle. It is important to note that the granulosa cells from our superstimulation group were collected 24 h after exogenous LH surge (i.e. close to the expected time of ovulation). The principal molecular functions altered by ovarian superstimulation were those associated with cell growth and proliferation, providing a rationale for the hypothesis that superstimulation delays granulosa cell differentiation. That is, the granulosa layers of single pre-ovulatory follicles at the time of collection have differentiated beyond the growing stage by downregulating genes associated with cell growth and proliferation and/or perhaps responding better to LH signaling compared with the granulosa cells from superstimulated follicles. It would be interesting to determine whether delaying the LH surge after superstimulation treatment would result in follicular maturation and a better fertility outcome. It is worth noting that not all functions are equally affected, e.g. steroidogenic function (intrafollicular E₂ concentration, E₂:P₄ ratio) was very similar between the dominant and superstimulated follicles.

The extracellular matrix plays a prominent role in ovarian function by participating in processes such as cell migration, proliferation, growth, and development of follicles (Berkholtz *et al.* 2006a, 2006b). In this study, upregulated genes involved with remodeling of the extracellular matrix included *NTS*, *FOS*, *THBS1*, *FN1*, *ADAMTS1*, connective tissue growth factor (*CTGF*), genes from the IGF family, and those involved in collagen formation. A specific network was built to show how extracellular matrix-remodeling genes interact with each other and how superstimulation affected this process (Fig. 4).

The *NTS* gene encodes a tridecapeptide, *NTS*, found in the hypothalamus. *NTS* is known to mediate the positive feedback of E₂ on the gonadotrophin-releasing hormone (GNRH) neurons responsible for inducing the pre-ovulatory LH surge (Smith & Jennes 2001). *NTS* was thought to be localized exclusively in the nervous system, but results of recent studies document the presence of *NTS* mRNA in the ovaries (Hernandez-Gonzalez *et al.* 2006, Kerr *et al.* 2009, Gilbert *et al.* 2011).

Figure 4 Network of genes upregulated or downregulated in granulosa cells after ovarian superstimulation in cattle. All genes involved in this network are part of the matrix-remodeling network; more specifically, cellular proliferation ($n=16$ genes), apoptosis ($n=15$ genes), and angiogenesis ($n=11$ genes). Genes are arranged into four horizontal compartments (nucleus, cytoplasm, plasma membrane, and extracellular space) based on the subcellular location of their gene products. The differences in color intensity of molecules show the degree of up- (red) or down- (green) regulation and connecting lines indicate a known relationship between molecules. The genes found to be upregulated were early growth response 1 (*EGR1*), FBJ murine osteosarcoma viral oncogene homolog (*FOS*), progesterone receptor (*PGR*), syndecan 4 (*SDC4*), heparanase (*HPSE*), fibronectin 1 (*FN1*), insulin-like growth factor binding protein 1 (*IGFBP1*), insulin-like growth factor 2 (*IGF2*), plasminogen activator tissue (*PLAT*), a disintegrin and metalloprotease metalloproteinase 1 (*ADAMTS1*), neurotensin (*NTS*), thrombospondin 1 (*THBS1*), collagens, fibroblast growth factor 2 (*FGF2*), serpin peptidase inhibitor class E member 1 (*SERPINE1*), metalloproteinase inhibitor 1 (*TIMP1*) and connective tissue growth factor (*CTGF*). The only downregulated gene was vascular endothelial growth factor (*VEGF*). The genes included transcription regulators (*EGFR* and *FOS*), ligand-dependent nuclear receptors (*PGR*), cytokine or growth factors (*FGF2*, *CTGF* and *IGF2*), peptidases (*ADAMTS1* and *PLAT*), enzymes (*HPSE* and *FN1*) or other (*VEGF*, *SERPINE*, *SDC4*, *TIMP1*, *NTS*, *THBS1*, collagens, and *IGFBP1*) categories.

Little is known about the role of *NTS* in ovarian cells, but expression of *NTS* mRNA in granulosa and cumulus cells is high before the LH surge and decreases thereafter as ovulation approaches (Hernandez-Gonzalez *et al.* 2006, Gilbert *et al.* 2011). Based on the RT-PCR analysis, *NTS* was upregulated 7.6-fold (10-fold in microarrays data) after ovarian superstimulation, suggesting that treatment is associated with delay in cell differentiation and/or delayed follicular response to the LH surge. *FOS* (6.9-fold in microarray data and 8.1-fold in RT-PCR) and *FOSL1* (2.1-fold in microarrays) transcriptions were also upregulated, and both activate *NTS* synthesis (Evers *et al.* 1995). Expression of *FOS* has been associated with cell proliferation and development (Delidow *et al.* 1990) and FSH rapidly increases *FOS* expression in immature rat granulosa cells (Delidow *et al.* 1992). Expression of *FOS* decreases with luteinization (Rusovici & LaVoie 2003) and is already low in bovine granulosa cells by 6 h after the LH surge (Gilbert *et al.* 2011). Therefore, we conclude that granulosa cells of superstimulated follicles were either unable to shut down *NTS*, *FOS*, and *FOSL1* transcription or at least were slower to respond to exogenous LH.

CTGF has also been implicated in tissue remodeling. *CTGF* mRNA is expressed abundantly in granulosa cells of pre-antral and early antral follicles in rats (Harlow *et al.* 2002) and pigs (Wandji *et al.* 2000), but its expression is downregulated in pre-ovulatory follicles in

both species. Expression of *CTGF* is influenced by local estrogen, which in turn is modulated by the effect of FSH on granulosa cells (Harlow *et al.* 2007). Perhaps expression of *CTGF* will be a useful marker for granulosa cell maturity as many studies have reported an inverse relationship between *CTGF* expression and granulosa cell differentiation (Wandji *et al.* 2000, Harlow & Hillier 2002, Harlow *et al.* 2002, Liu *et al.* 2002, Schindler *et al.* 2010). Although the follicular fluid concentrations of E_2 were similar between the two groups in this study, *CTGF* was upregulated in the granulosa cells of superstimulated follicles, again showing a delay in follicular maturation. Further, expression of three other genes involved in matrix remodeling, *SERPINE*, *FN1*, and *IGF2* was also elevated in the superstimulation group. *SERPINE* is downregulated in bovine granulosa cells after LH treatment (Gilbert *et al.* 2012) and *FN1* expression is reported to be inversely related to follicle maturation (Colman-Lerner *et al.* 1999, Yasuda *et al.* 2005, Berkholtz *et al.* 2006b). IGF proteins are produced by the granulosa cells and have been shown to have a synergistic effect with FSH to induce cellular growth and proliferation (Hammond *et al.* 1985). The expression of *IGF2* is increased in follicles collected on day 5 ($D0$ = ovulation) and decreased in follicles at day 8 (de la Sota *et al.* 1996). The increased expression of the genes described earlier reflect the fact that matrix remodeling is active, probably due to follicular growth.

Figure 5 Canonical pathway analyses of gene expression of granulosa cells after superstimulation treatment using Ingenuity Pathway Analysis software (IPA). (A) Top ten pathway analyses based on $-\log_{10} P$ value. (B) Top ten pathway analyses based on a score ratio. The score ratio was calculated by IPA and is the number of differentially expressed molecules in the gene list/number of genes known to be involved in the pathway.

Matrix remodeling is not only important in follicle growth and development but also during ovulation and CL formation (Berkholtz *et al.* 2006a). In this study, some upregulated genes in the matrix-remodeling network (*HPSE*, early growth response 1 (*EGR1*), tissue inhibitor metalloproteinase 1 (*TIMP1*), and plasminogen activator (*PLAT*)) are markers of the initiation of ovulatory process (Gilbert *et al.* 2011, 2012). *HPSE* encodes heparanase that cleaves heparan sulfate (one of the tissue glycosaminoglycans) during matrix remodeling and is highly expressed in bovine granulosa cells 12 h after GnRH treatment. Heparanase was suggested to be a novel member of the LH-induced ECM-degrading enzyme family involved with follicular rupture (Klipper *et al.* 2009). Likewise, *PLAT* creates functionally redundant mechanisms for plasmin formation during ovulation (Sayasith *et al.* 2006) and is highly expressed in granulosa cells of pre-ovulatory follicles in rats (Galway *et al.* 1990, Leonardsson *et al.* 1995). The expression of

Timp1 is increased up to 4 h after hCG treatment in granulosa cells of immature PMSG-primed rat ovaries and gradually decreases afterward (Li & Curry 2009). It is interesting to note that *HPSE*, *PLAT*, and *TIMP1* expression was higher in superstimulated follicles. In contrast, *EGR1* expression is expected to decrease near the time of ovulation (Sayasith *et al.* 2006) but failed to be downregulated in superstimulated follicles. Overexpression of *EGR1* stimulated the expression of many genes in the prostaglandin biosynthesis pathway and increased expression of LH receptor mRNA (Sayasith *et al.* 2006). Its expression in superstimulated follicles from women was associated with increased oocyte competence (Hamel *et al.* 2008). It is apparent from our results that expressions of some but not all genes involved in the ovulatory cascade are affected by superstimulation. In this study, LH was given 24 h after the end of superstimulation treatment. Perhaps the exogenous LH is driving follicles that are not fully matured to reach the pre-ovulatory molecular stage and ovulate. However, this hypothesis needs to be further investigated.

In this study, genes related to the stress response (Fig. 5) were activated in granulosa cells from superstimulated follicles. Our results are supported by similar findings in embryos from superstimulated donors (Mundim *et al.* 2009), where genes related to oxidative stress response tended to be activated. In this study, the NFE2L2 oxidative stress response pathway was one of the most activated pathways in the superstimulation group. NFE2L2 interacts with other transcription factors within the nucleus, such as *CREB*, *ATF4*, and *FOS*, to

Figure 6 Quantification ($\log_2$ of fold change; mean $\pm$ S.E.M.) of the mRNA profile of granulosa cells from cows after superstimulation treatment compared with negative control (no superstimulation) using real-time PCR ($n=3$ cows per group). Light gray bars represent the differential level of expression of transcripts detected in the microarray experiment, while dark gray bars represent the differential level of expression of the same transcripts obtained by real-time PCR. *Values are greater in the superstimulation group than in the untreated control group ($P \leq 0.1$).

activate antioxidant response elements (Gudi *et al.* 2000) to balance the oxidation level of the intracellular environment (Huang *et al.* 2000). The endoplasmic reticulum (ER) stress pathway was also activated in granulosa cells of superstimulated follicles in this study. Many diseases are associated with cellular stress responses, one of which is cancer (Cerutti 1989), which was identified in the top disease and disorder functions list. Perhaps the oxidative stress response comes from there being too many follicles to be supported by the ovarian vasculature. However, this hypothesis should be further tested.

Some of the upregulated genes in this study such as *FOS*, *FN1*, *EGR1*, *SDC4*, and *THBS1* have been demonstrated to induce the expression of pro-apoptotic proteins (Delidow *et al.* 1990, Sakata *et al.* 2000, Yasuda *et al.* 2005, Hou *et al.* 2008, Garside *et al.* 2010a). *THBS1* encodes a glycoprotein, thrombospondin 1, that is a component of the extracellular matrix. Thrombospondin mainly acts as anti-angiogenic factor; however, it also promotes atresia of rat granulosa cells *in vitro* (Garside *et al.* 2010a, 2010b). *THBS1* is highly expressed in small follicles from bovine ovaries (Greenaway *et al.* 2005) as well as in small and atretic follicles of many other species (Thomas *et al.* 2008, Garside *et al.* 2010b, Zalman *et al.* 2012). *Igf1* inhibits *Thbs1* transcription in cultured rat granulosa cells and its expression is further lowered when FSH is added in culture (Dreyfus *et al.* 1992, McGray *et al.* 2011). In contrast, thrombospondin 1 is highly expressed in bovine granulosa cells when FSH is added in culture but LH had no effect (Greenaway *et al.* 2005). *THBS1* expression was upregulated (4.1-fold according to RT-PCR and 5.7-fold in microarrays) in superstimulated follicles. Perhaps expression of this group of genes indicates that even though superstimulation rescued follicles from atresia, some molecules involved with that pathway are still being triggered. In contrast, other known anti-atresia markers (*ADAMTS1* and *TIMP*) were also upregulated after superstimulation. The protein encoded by *Adams1* is known to prevent atresia as mice with *Adams1*-null ovaries had many unusual atretic follicles (Shozu *et al.* 2005), demonstrating the importance of this gene in granulosa cell health. Proteins from the TIMP gene family are also known to be stimulated by FSH and to prevent atresia (Goldman *et al.* 1997). Therefore, superstimulation prevents atresia by increasing expression of some, but not all, anti-apoptotic genes.

Superstimulation treatment in this study influenced genes involved in angiogenesis, i.e. VEGF family, angiopoietin 2, and *THBS1*. Expression of angiogenic factors has been reported to be stage-dependent in granulosa cells, increasing during pre-ovulatory stage (Christenson & Stouffer 1997, Laitinen *et al.* 1997, Hazzard *et al.* 1999, Schams *et al.* 2001, Reisinger *et al.* 2007, Shimizu *et al.* 2007, Berisha *et al.* 2008), but the molecular mechanisms involved are not fully

known. Angiopoietin 2 destabilizes vascular structures and induces vascular remodeling (Yancopoulos *et al.* 2000). *THBS1* also encodes an anti-angiogenic protein (Greenaway *et al.* 2005). *THBS1* inhibits VEGF levels in the ovary directly via the low-density lipoprotein receptor-related protein-1 (*LRP1*; Greenaway *et al.* 2007). Inhibition of expression of angiogenic factor (VEGF) and increased expression of anti-angiogenic factor (*THBS1*) was consistent with the results of pathway and function analysis wherein cardiovascular system development was identified as one of the top disease and disorder functions affected. Results suggest that angiogenesis may be inhibited, and perhaps corpus luteum formation may be disturbed after superstimulation treatment.

In conclusion, ovarian superstimulation activates genes involved in the NFE2L2 oxidative stress response, and ER stress response, apoptosis, disturbance of angiogenesis and matrix remodeling. Moreover, ovarian superstimulation was associated with upregulation of growth-related genes in follicular granulosa cells. The results are consistent with the hypothesis that follicles that undergo superstimulation lag behind in maturational development and response to LH.

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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