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Differentially expressed genes and gene networks involved in pig ovarian follicular atresia

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[1]*GenPhySE, Université de Toulouse, INRA, INPT, ENVT, Castanet Tolosan, France;* [2]*INRA UMR 0085, CNRS UMR 7247, Université Francois Rabelais de Tours, IFCE, Physiologie de la Reproduction et des Comportements, Nouzilly, France;* [3]*INRA, UMR1348 Pegase, Saint-Gilles, France; and* [4]*AgroCampus-Ouest, UMR1348 Pegase, Saint-Gilles, France*

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Terenina E, Fabre S, Bonnet A, Monniaux D, Robert-Granié C, SanCristobal M, Sarry J, Vignoles F, Gondret F, Monget P, Tosser-Klopp G. Differentially expressed genes and gene networks involved in pig ovarian follicular atresia. *Physiol Genomics* 49: 67–80, 2017. First published December 9, 2016; doi:10.1152/physiolgenomics.00069.2016.—Ovarian folliculogenesis corresponds to the development of follicles leading to either ovulation or degeneration, this latter process being called atresia. Even if atresia involves apoptosis, its mechanism is not well understood. The objective of this study was to analyze global gene expression in pig granulosa cells of ovarian follicles during atresia. The transcriptome analysis was performed on a 9,216 cDNA microarray to identify gene networks and candidate genes involved in pig ovarian follicular atresia. We found 1,684 significantly regulated genes to be differentially regulated between small healthy follicles and small atretic follicles. Among them, 287 genes had a fold-change higher than two between the two follicle groups. Eleven genes (*DKK3, GADD45A, CAMTA2, CCDC80, DAPK2, ECSIT, MSMB, NUPR1, RUNX2, SAMD4A*, and *ZNF628*) having a fold-change higher than five between groups could likely serve as markers of follicular atresia. Moreover, automatic confrontation of deregulated genes with literature data highlighted 93 genes as regulatory candidates of pig granulosa cell atresia. Among these genes known to be inhibitors of apoptosis, stimulators of apoptosis, or tumor suppressors *INHBB, HNF4, CLU*, different interleukins (*IL5, IL24*), TNF-associated receptor (*TNFR1*), and cytochrome-c oxidase (*COX*) were suggested as playing an important role in porcine atresia. The present study also enlists key upstream regulators in follicle atresia based on our results and on a literature review. The novel gene candidates and gene networks identified in the current study lead to a better understanding of the molecular regulation of ovarian follicular atresia.

pig ovary; folliculogenesis; atresia; gene expression; cDNA microarray; biomarkers; upstream regulators; functional pathways

OVARIAN FOLLICULOGENESIS REFERS to the development of follicles leading to either ovulation or degeneration. This degeneration process is called atresia. In mammals, it has long been recognized that the majority of follicles present at birth become atretic, so that only <1% of follicles ovulate during ovarian follicular growth and development (53, 107). Thus, the most highly probable fate for a follicle is to degenerate by entering atresia. This process may be considered as physiologically normal, allowing the ovary to generate the ovulatory quota characteristic of the species, at each sexual cycle.

A number of studies in antral growing follicles have indicated that follicular atresia is initiated and caused by apoptosis (programmed cell death) of granulosa cells (53, 56, 71, 107). Two general pathways have been suggested to be involved in the physiological induction of apoptosis: negative induction by survival factor withdrawal and positive induction by the binding of a specific ligand, such as tumor necrosis factor-alpha or Fas ligand, to its membrane receptor (41). In follicles, apoptosis is triggered when endocrine and/or intra-follicular concentrations of survival factors, particularly gonadotropins and some growth factors, are inadequate (93). It is now recognized that a diverse spectrum of pro- and antiapoptosis susceptibility genes are thus expressed in germ cells and/or somatic cells of the ovary, including members of the BCL2 and caspase gene families (108). Many of these genes are regulated by gonadotropins and growth factors, and changes in the temporal pattern of cell death gene expression suggest that an intimate association exists between the products of these genes and the activation of apoptosis.

The biochemical characteristics of apoptosis have been investigated to discover causative factors and markers identifying follicles at the early stages of atresia. Results from studies of ovarian gene expression in vivo and in vitro have indicated that ovarian products, such as steroid hormones, transforming growth factor family members, insulin-like growth factor (IGF) 1, IGF-binding proteins (IGFBPs), and inhibins could play a role in atretic process (2, 42). However, most of this information has been acquired based on candidate-gene approaches.

In the present study, we propose a high-throughput transcriptomic approach to get a better understanding of the molecular actors, their regulatory genes, and physiological networks involved in pig follicle atresia. A specific microarray has been developed to identify genes expressed in granulosa cells along the terminal ovarian follicle growth in the pig (17). This represents a useful tool to study porcine ovarian follicular atresia. The aims of this study were *1*) to analyze global gene expression changes in pig granulosa cells of ovarian follicles during atresia and *2*) to identify the gene networks and propose new candidates genes that can

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further serve as targets to characterize and modulate atresia in granulosa cells.

MATERIAL AND METHODS

Collection of ovaries. Estrous cycles of 10 sows were synchronized by oral administration of 20 mg/day altrenogest (Regumate; Hoechst-Roussel, Paris, France) for 18 days. Ovaries were removed by laparotomy 24 or 96 h after the last altrenogest feeding. All procedures were approved by the Agricultural and Scientific Research agencies and conducted at an INRA experimental farm (animal experimentation authorization C37-175-2) in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Granulosa cell isolation and RNA extraction. Once ovaries were collected, all visible (>1 mm in diameter) antral follicles were isolated carefully under a binocular microscope. After dissection, follicle diameter was measured. Only follicles measuring 1–2 mm were kept, and granulosa cells were recovered from all individual follicles as described previously (78) and stored at −80°C until RNA extraction. For each follicle, a sample of granulosa cells was smeared on a histological slide and then stained by Feulgen reagent to determine follicular quality (76, 77). The quality of each follicle was assessed by microscopic examination of smears according to classical histological criteria; follicles were judged healthy (noted SHF for small healthy follicle), when having frequent mitosis and no pyknosis in granulosa cells, or atretic (noted SAF for small atretic follicle), when no mitosis and numerous pyknotic bodies in granulosa cells were observed. This method of classification has been previously demonstrated as particularly relevant since it very well reflects the level of expression of markers of follicular growth such as LH receptor, aromatase, and PAPP-A (73).

Within each sow, the follicles were pooled by quality classes, to generate SHF (small healthy follicle) and SAF (small atretic follicle) categories, respectively. Total RNA was extracted from SHF and SAF according to the technique described by Chomczynski and Sacchi (25) with minor modifications (46). The quality of each RNA sample was checked through the Bioanalyser Agilent 2100 (Agilent Technologies, Massy, France) and low-quality RNA preparations were discarded (RNA integrity number <8).

Microarray hybridization. After RNA quality check, 16 independent RNA samples were obtained, corresponding to 7 SHF samples and 9 SAF samples, respectively. A porcine microarray produced by CRB-GADIE (http://crb-gadie.inra.fr) and published in the Gene Expression Omnibus (GEO) database as GPL3729 (https://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GPL3729) was used to hybridize the RNA samples as previously described (17, 18). cDNA from luciferase was present on the array as positive control (193 spots), and water was included as negative control (64 spots). Microarrays were first hybridized with a ^{33}P-labeled oligonucleotide sequence present in all PCR products to control the quality of the spotting process and to evaluate the amount of DNA in each spot (26). After being stripped, the arrays were hybridized with ^{33}P-labeled complex probes synthesized from 5 μg of each RNA sample, using SuperScript II RNase H reverse transcriptase (Invitrogen, Cergy-Pontoise, France). Each complex probe was hybridized on membrane exposed for 1, 3, or 7 days to radioisotopic-sensitive imaging plates (BAS-2025, Fujifilm; Raytest, Courbevoie, France). The imaging plates were scanned thereafter with a phosphor imaging system at 25 μm resolution (BAS-5000, Fujifilm, Raytest). Hybridization images obtained from oligonucleotide and complex probes were quantified using the semiautomated AGScan software (22, 26).

Microarray data management. The experimental design, its implementation, and the handling of data comply with MIAME standards (19), and all the experimental data were managed with BASE software (90), adapted by the SIGENAE bioinformatics platform (http://www.sigenae.org) to manage radioactive experiments.

Microarray data analysis. Raw data and normalized data were submitted to the GEO database as GSM596969 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM596969).

Data exploration and statistical analysis were performed using R software (http://www.r-project.org). The data coming from complex probes hybridization were analyzed on a natural logarithmic scale. As a first step, negative spots with >7 intensity values were checked on images and replaced by the median of negative spots in case of obvious overshining effect or hybridization stain. Then, luciferase-positive controls were removed, and the correlations of the gene expressions from each membrane were examined within condition. Hybridizations with a correlation coefficient <0.80 were discarded. Spots with low signal value (below the average of empty spots + 3 standard deviations) were considered as unexpressed and were excluded from the analysis. Finally, the negative control spots were removed and the remaining data were centered for each membrane.

The significance of the follicle status (SAF or SHF) on gene expression levels was assessed by a Student's *t*-test ($P < 0.05$), followed by the Benjamini-Hochberg procedure controlling false discovery rate (FDR 5%) for each cDNA (9).

The relevance of the selection procedure was further evaluated via an unsupervised hierarchical clustering using the Ward method and Euclidian distance with the R functions hclust and heatmap (24).

Sequence annotation. Each cDNA sequence was compared with Refseq_rna mammalian database using the National Center for Biotechnology Information's blastn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Blast results with an e-value <1e-3 were parsed and filtered to keep queries matching a gene or an mRNA or a CDS and possessing a global coverage of at least 70% of the query sequence. Resulting hits were sorted out according to their closeness to the pig genome, their coverage, and sequence identity. The selected cDNA sequences were submitted to the HUGO (Human Genome Organization) gene nomenclature committee, using their RefSeq IDs (http://www.genenames.org). Then, HUGO gene symbols were used as gene names.

Quantitative PCR analysis of target gene expression. Total RNA (1 μg) used in microarray experiments (SAF, $n = 5$; SHF, $n = 5$, among the samples used for array analysis) was reverse-transcribed as previously described (110). Primer sequences for genes were designed using Beacon Designer 7.0 software (Premier Biosoft Int., Palo Alto, CA) and are given in Table 1. The TCTP gene (translationally controlled tumor protein, accession number BX667045) and MT-CO3 gene (cytochrome C oxidase subunit III, accession number CT971556) were used as internal controls (17). Quantitative real-time PCR was performed using SYBR green fluorescence detection during amplification on a Roche LC480 system (Roche Diagnostics, Meylan, France) according to the manufacturer's recommendations. Duplicates of each template, consisting of 3 μl of a 1/10 dilution of the reverse transcription reaction, were loaded in 384-well plates with a 10 μl PCR mix SYBR green Power master mix (Applied Biosystems, Courtaboeuf, France), and 0.15 μM of forward and reverse primers (final volume 20 μl) were added. The PCR amplification conditions were as follows: 50°C for 30 min, initial denaturation at 95°C for 10 min, and 40 cycles (95°C for 15 s and 60°C for 1 min). The last cycle was followed by a dissociation step (ramping to 95°C). The real-time PCR amplification efficiency was calculated for each primer pair with five 1:2 dilution points of the calibrator sample (pool of the 10 cDNA samples) and was not lower than 1.8. After determination of the threshold cycle for each sample, the PFAFFL method was applied to calculate the relative changes of each mRNA in each sample (83). The relative expression was normalized by the corresponding geometric average of the two internal genes using geNorm v3.4 (113). The significance of differential expression between SHF and SAF was tested by Student's *t*-test.

Pathway analysis. A pathway is an interconnected arrangement of processes, representing the functional roles of genes in the genome. The biological processes into individual genes may participate were

Table 1. *Sequences of oligonucleotide primers tested by quantitative RT-PCR analysis*

Gene Name	Sense Primer (5′→3′)	Anti-sense Primer (5′→3′)
CDKN1B	ACGGGGTTAGCGGAGCAG	ACATACTCTTAATACGAGCAGTTTACG
CLU	GCAGATGGCGGAGAAGTTCAG	GGAGATAGTAGCGGTCGTCATTC
MT-CO3	CCTCGCCTCAGGAGTATCCA	CGCCTAGTGCAATGGTGATG
CSNK2B	GAGATTGAATTTGTCCTGGATGTAGTC	AGCCGCTGAAGTGAAGATGAG
GADD45A	GGGTTGCTGACTCGTAGGATG	GTGGTGCTGTGCCTGCTG
GSTA1	CCGAGGCAGAATGGAGTGTATC	TGGCGATGTAATTGAGGATGGC
HMOX1	AGGACACTAAGGACCAGAGACC	ACCGTTGCCACCAGAAAGC
LRP8	TCCTCCACCTCTAAGTTCTCCAG	GCCTCTCTACACCACCCTCTC
SH3GLB1	GCGTTGGCAGCAGGAAGG	TTGAGCAGTTCTAAGTAGGTGATGG
TCTP	AGATGCCATCAGTCCCACTTG	GGCTGTTGGGATCGGATCTA

approached using Gene Ontology (GO) database and AmiGO terms (http://amigo.geneontology.org/amigo). The significantly up- or downregulated genes involved in follicular atresia were assembled into networks using Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com). This application provides computational algorithms to identify the biological pathways, functions, and biological mechanisms of selected genes. Statistical significance of pathway representation was established with respect to a null distribution constructed by permutations. Functional analysis was done using all genes that compose the array as "set reference." A threshold network score of 40 as the highest score obtained with the help of the gene set reference was applied to select the highest significant networks for further analysis.

Search for upstream regulators. To propose upstream regulatory candidates that may have participated to differences in gene expression patterns between SAF and SHF, a recently developed web tool (http://keyregulatorfinder.genouest.org/) (13) was used. This methodology provides a reasonable number of upstream regulatory candidates from a submitted list of regulated genes, and success has been demonstrated for different case studies. In the present experiment, the differentially expressed genes were identified by their official gene symbol and submitted to analysis. The sign of variation was assigned to show the expression levels between SAF and SHF samples ("+" when upregulated in SAF vs. SHF, "−" when downregulated, and "?" when sign of variation between groups was not consistent for different probes of the same gene). The software output is a list of specific regulatory candidates. A postprioritization of the candidates was made by calculating the intersection with the differentially expressed genes in the input list, using Jvenn software (6).

RESULTS

Differentially expressed genes in follicle atresia. Follicles dissected from ovaries of cyclic sows were classified into two different categories based on individual diameter and quality traits assessed by histological examinations. Small healthy follicles (noted SHF) were then compared with small atretic follicles (noted SAF) for high throughput expression patterns. After normalization, 4,675 out of the 8,959 transcripts present on the pig cDNA array were found to be expressed in ovarian porcine follicles. Using the criterion of FDR $<$ 5%, we identified 1,684 transcript spots as significantly regulated between SHF and SAF (Supplemental Table S1). (The online version of this article contains supplemental material.)These transcripts included 1,339 known cDNAs and 345 unknown cDNAs (Supplemental Table S1). The 1,339 annotated transcripts corresponded to 1,223 unique HUGO genes. The unsupervised hierarchical clustering (Fig. 1) shows that the 1,684 differentially expressed cDNAs separated the two experimental groups corresponding to SAF and SHF status. Among the 1,684 cDNAs, 770 were upregulated in atretic follicles (SAF) and 914 in healthy follicles (SHF). When we used a cut-off of |2| in fold-change between conditions, 142 genes were upregulated in SAF group (Table 2), whereas 145 genes were downregulated in those follicles compared with SHF (Table 3). In addition, some genes have undergone a very high fold-change (up to 17-fold) between follicle conditions, indicating that their expression level was very sensitive to atresia. About 11 genes notably exhibited a higher than fivefold overexpression in SAF and very poor expression in SHF (defined as belonging to the group of 20% of the lower expressed genes on the microarray) and were thus considered potential biomarkers of atresia in porcine small follicles: *DKK3, GADD45A, CAMTA2, CCDC80, DAPK2, ECSIT, MSMB, NUPR1, RUNX2, SAMD4A*, and *ZNF628*.

Quantitative PCR validation. Eight of these genes, chosen as being representative for differential expression levels in microarray between SHF and SAF, were further examined by quantitative (q)PCR (Fig. 2). As expected, higher expression levels for GADD45A, CLU, and HMOX1 were confirmed in SAF compared with SHF, whereas LRP8, GSTA1, and CSNK2B exhibited decreased expression in the former compared with SHF. Notably, fold-change in expression levels for CLU, LRP8, and GSTA1 between SHF and SAF categories was largely higher for qPCR than for microarray analysis (9.3 vs. 3.6, −26.9 vs. −2.7, and −42.3 vs. −10.5, respectively). The qPCR analysis also confirmed a trend to an increased expression level ($P = 0.1$) for CDKNB1 in SAF compared with SHF. Finally, SH3GLB1, which was found as a nondifferentially expressed gene between experimental groups in microarray, did not differ between those groups in qPCR analysis. Thus, we confirm the reliability of the microarray approach for a set of genes with various expression ranges and profiles.

Gene ontology. Using IPA we carried out a network analysis on the differentially regulated genes (FDR $<$ 5%, fold-change $>$ |2|) between SAF and SHF categories. A total of 210 genes out of 287 were grouped in several relevant functional networks. Three highly significant biological networks with direct relationships between the differently expressed genes were identified. Genes participating in these three networks are shown in Supplemental Table S2. The first network relates to endocrine system disorders and reproductive system diseases and involves 71 genes, with a majority of them, 46 out of 71, being downregulated under atresia. The second network is related to cancer and to cell death and survival; it involves 78 genes, with 44 being upregulated and 34 being downregulated under atresia. The last significant network is related to cell

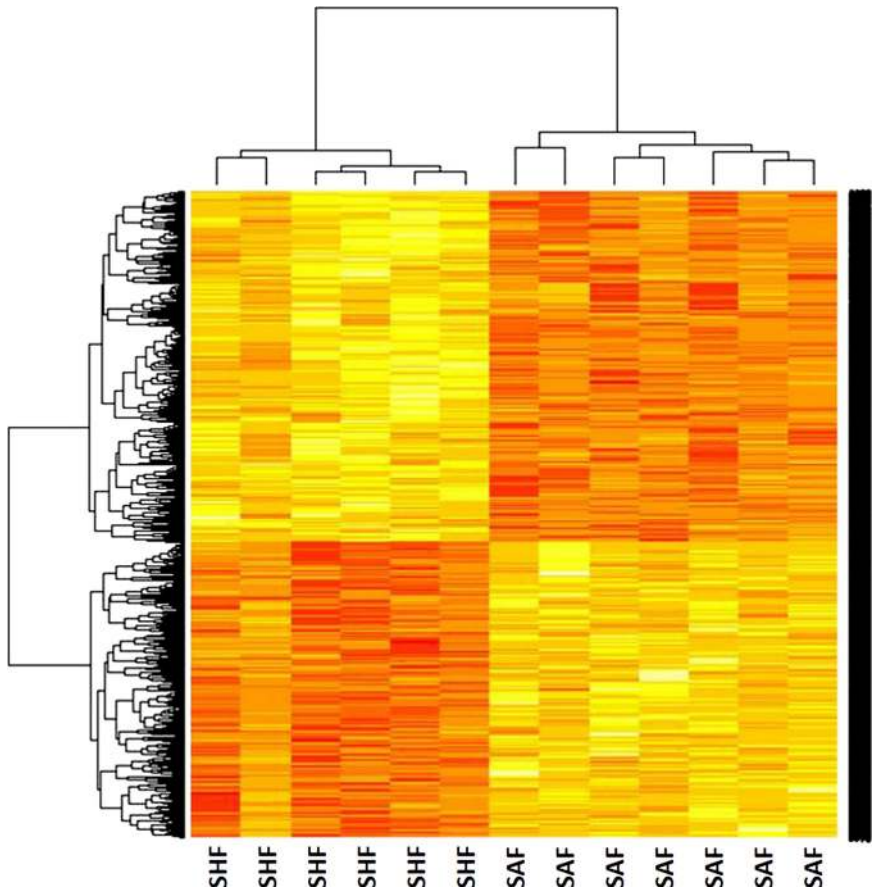

Fig. 1. Heat map display of unsupervised hierarchical clustering of 1,684 up- or downregulated cDNAs identified in this study (in yellow or in red, respectively). The cDNAs are displayed in lines and microarrays in columns. SAF, small atretic follicles; SHF, small healthy follicles.

death and survival, cell morphology, and reproductive system development and function and involves 61 genes (35 genes were upregulated and 26 genes were downregulated under atresia). Figure 3 shows nodes and edges of the first regulatory network related to endocrine system disorder, pointing on *TP53* and *N3RC1* genes as the central nodes.

Search for upstream regulators. The 287 differentially expressed genes (FDR $< 5\%$, fold-change $> |2|$ between SAF and SHF) were automatically confronted with the literature data on mammalian cell signaling to hierarchize upstream regulatory candidates. A dedicated web tool algorithm (http://keyregulatorfinder.genouest.org) was used, and a postprioritization of these candidates was made by comparing the list of candidates with the expression levels of these genes in SAH and SHF groups in the microarrays. Figure 4 shows the intersections of both lists; the detailed list of corresponding genes is in Supplemental Table S3. Among those regulatory candidates, 44 were included in the list of upregulated genes with fold-change >2 and 49 as downregulated genes with fold-change <2 between SAF and SHF conditions. Two genes, *ESR1* (fold-change $= -1.9$) and *NFKB1* (fold-change $= -1.3$), were present in the list of differentially expressed genes having a fold-change <2 between conditions. Nineteen genes were proposed as potential regulators, without being stated as differentially expressed by the microarray experiment. Among those 19 genes, only four genes (*CSRP3, JUN, NEF2L2*, and *STAT1*) were present on the pig GPL3729 microarray; however, their signal intensities were below the cut-offs; this does not preclude a regulatory role via posttranslational processes. The remaining 15 genes (*ADGRA3, ADGRE4P, ADGRG1, AP2A1, ATF2, BHLHE41, FOS, GST, IFRD1, IRF9, NFYA, RUNX1, SPI1, USF1*, and *USF2*) were absent from the microarrays.

DISCUSSION

We used a comprehensive gene expression profiling by means of microarray analysis to identify groups of genes differently expressed by follicular status in pig ovaries. Similar microarray approaches have been used previously to characterize sheep granulosa cells and oocytes during early follicular development (16), growing healthy antral pig follicles (18), changes in granulosa cells gene expression associated with growth, plateau, and atretic phases in medium bovine follicles (29), and granulosa cells in the theca interna from bovine ovarian follicles during atresia (47–49). However, the present study is the first gene array analysis investigating ovarian follicular atresia in pigs. A large number of cDNA were found as differential between quality categories of follicles (healthy SHF vs. atretic SAF), and 287 differentially expressed annotated genes having a large fold-change between the two conditions were highlighted.

It has been previously proposed that apoptosis is the main biological process involved in follicular atresia (105, 106). This coordinated process involves many posttranslational processes, particularly the activation of a group of caspases. However, as illustrated by Fig. 5, a number of genes proposed by others as triggering apoptosis are not included in the list of

Table 2. *List of upregulated genes under atresia*

HUGO Name	Fold-Change	Position	HUGO Name	Fold-Change	Position	HUGO Name	Fold-Change	Position	HUGO Name	Fold-Change	Position
MSMB	17.1	4246	*SOX4*	3.2*	5430	*KIF13A*	2.5	6980	*MYL1*	2.2	2025
NUPR1	14.6	1811	*PTPRT*	3.2	4245	*SMG8*	2.5	8517	*NIN*	2.2	7882
CAMTA2	7.5	6196	*LRP1B*	3.1	4234	*MARCKSL1*	2.5	6131	*UMOD*	2.2	2500
ZNF628	7.2	5462	*PLIN2*	3.0	2919	*TAGLN*	2.5	3380	*CXXC5*	2.2	1823
RUNX2	6.9	3814	*TMSB4*	2.9*	2550	*TFEB*	2.5	2952	*LRRC4C*	2.2	4965
ECSIT	6.2	5450	*BHLHE40*	2.9	7754	*ABLIM1*	2.4	7664	*MEF2A*	2.2	648
ARHGEF25	6.1	9097	*LCN6*	2.9	7809	*MX2*	2.4	5245	*AKR1B1*	2.1	5438
GADD45A	5.5	6663	*FTL*	2.9	9172	*PRKD1*	2.4	4357	*SESN3*	2.1	7366
SAMD4A	5.3	5745	*EGR1*	2.9	5683	*VMA21*	2.4	8136	*ACTG1*	2.1*	8058
CCDC80	5.2*	1752	*VMP1*	2.9	8243	*COMMD10*	2.4	2928	*EFNA4*	2.1	6793
DKK3	5.1	5863	*PPP1R10*	2.8	6207	*KIAA1394*	2.4	7067	*ACTA1*	2.1*	8714
DAPK2	5.0	5449	*NDUFA1*	2.8	6195	*CRTC1*	2.4	4989	*ACTN1*	2.1	276
RASSF8	4.8	6052	*MITF*	2.8*	1574	*KLF9*	2.4	4258	*KLF6*	2.1	7993
SAFB2	4.5	3661	*COMT*	2.7	1810	*SORT1*	2.4	4392	*POLQ*	2.1	4987
BCAS1	4.4	3908	*EPHA4*	2.7	1082	*ZNF335*	2.4	1679	*TP53INP2*	2.1	8410
EMR4P	4.0	3290	*HMOX1*	2.7	1000	*IDH2*	2.4	8549	*ANXA5*	2.1	7998
GPCR39	4.0	3146	*CHI3L1*	2.7	7540	*ZNF582*	2.4	4372	*B2M*	2.1	3716
CD24	4.0	6225	*NACC2*	2.7	5734	*AKT2*	2.3	3071	*CGA*	2.1	6206
RND3	4.0	2099	*ISG15*	2.7	8484	*ALDH16A1*	2.3	5451	*IFI6*	2.1	7132
CAPG	3.8	4918	*JUP*	2.7	4092	*FAM8A1*	2.3	8152	*RNASEL*	2.1	2966
SPP1	3.8	5559	*FLT1*	2.6	5437	*IRS2*	2.3	6759	*SLC39A6*	2.1	4020
MMP2	3.7	6136	*GPX3*	2.6	8010	*UVRAG*	2.3	8085	*ACTG2*	2.0	2188
ZNF282	3.7	3897	*ZNF395*	2.6	3082	*MTM1*	2.3	8050	*ARPC3*	2.0	2150
ZNF514	3.7	6208	*LMF1*	2.6	5757	*MYO5B*	2.3	4396	*LTBP3*	2.0	7034
CLU	3.6*	2586	*ALCAM*	2.6	4018	*PLSCR3*	2.3	9090	*PIK3IP1*	2.0	6312
CEBPD	3.5	8794	*CCDC152*	2.6	3903	*PRDX4*	2.3	3142	*SSBP2*	2.0	755
ZER1	3.5	7814	*METTL7B*	2.6	3279	*RREB1*	2.3	2423	*STAT3*	2.0	6760
FBXO32	3.5	4996	*THBS1*	2.6	5215	*CXCR4*	2.3	2461	*CMTM6*	2.0	3454
FOSL2	3.5*	1184	*ZNF609*	2.6	4175	*MDK*	2.3	4262	*DLGAP4*	2.0	4288
THAP11	3.4	4977	*EFEMP2*	2.5	3069	*UBE3B*	2.3	3725	*HNF4A*	2.0	5439
VAT1	3.4	771	*CTNNA1*	2.5	958	*S100A6*	2.2	2921	*IGHMBP2*	2.0	8541
VRK3	3.3	7801	*EGF*	2.5	3058	*SLC39A14*	2.2	5759	*KIAA1671*	2.0	2127
IGFBP3	3.3	2032	*ITM2B*	2.5	2549	*GOLGA3*	2.2	3896	*PALLD*	2.0	7559
FAM134B	3.3*	2672	*PLAT*	2.5	2268	*OAS1*	2.2	4549	*UBE2O*	2.0	7740
PRRC2C	3.2	5758	*PLEKHO1*	2.5	818	*ZNF644*	2.2	7838			
LGALS3	3.2	7363	*PSMC2*	2.5	8490	*MAF*	2.2	3982			

Only fold-changes >2 between small atretic follicle (SAF) and small healthy follicle (SHF) categories are retained in this list. Position relative to the Gene Expression Omnibus (GEO) accession number GPL3729. *Means of several positions with the same annotation.

differentially expressed genes between SAF and SHF conditions in the present experiment, although they were present in the microarray design. It is important to note that previous studies did not necessarily evaluate the presence of those genes in a similar context. In comparison between bovine follicles in the plateau phase and atresia stage (29) classical granulosa apoptosis markers such as FAS (TNF receptor superfamily, member 6) and BCL2-associated X protein (BAX) are not significantly different in their expression. This result is supported by the findings of (47), showing that none of the caspases or Fas genes were differentially expressed in granulosa cells during atresia of bovine ovarian follicles. As previously shown (29), since follicle-stimulating hormone is thought to modulate the dynamics of the growth, plateau, and atretic phases in bovine follicles, this corresponds to the steady BAX levels studied phases. As reviewed in Hatzirodos et al. (47), most studies have been performed on antral follicles of a larger size (30, 54, 65) than those used in this experiment. It is possible that cell death mechanisms that operate in larger follicles are different from those at an earlier stage, where cells are under different hormonal control, LH in addition to FSH, and with focimatrix present (54). An alternative explication is that only those granulosa cells in the atretic follicle with elevated expression of antiapoptotic genes are capable of surviving longer during the process of apoptosis (47, 88).

Functional networks involved in pig ovarian follicular atresia. In the present study, three networks of co-regulated differentially expressed genes were mainly involved in the molecular differences between SAF and SHF groups. These networks included both down- and upregulated genes, suggesting that cellular changes (notably related to "cancer" and "reproductive system disease") may have occurred to counteract degenerative effects of other genes.

The first functional network is related to endocrine system disorders and reproductive system diseases. In this network, a decrease in the expression of some genes has been previously found to be associated with the atretic process. Particularly, we observed decreased expression levels of *INHA*, *INHBA*, and *INHBB* in SAF vs. SHF groups that is consistent with roles of inhibin family genes in atresia of granulosa cells. Inhibin inhibits pituitary secretion of follicle-stimulating hormone through negative feedback regulation (3, 14). Amounts of inhibin precursors increase initially in the largest follicles, but intrafollicular amounts of the large-molecular-weight forms do not change further with differentiation to dominance (3). Inhibins have a role in controlling follicular development, through either regulation of systemic gonadotropins or local intraovarian modulation of the effects of the gonadotropins (87). Previous research has shown that growing follicles contain increased total amounts of inhibin, whereas atretic and

Table 3. *List of downregulated genes under atresia*

HUGO Name	Fold-Change	Position	HUGO Name	Fold-Change	Position	HUGO Name	Fold-Change	Position
INHA	−25.0	37	*HSPA9*	−2.5	2415	*ANP32E*	−2.1	4266
INHBA	−15.6	7746	*PVRL2*	−2.5	8909	*EZR*	−2.1	2432
HSD17B1	−11.4	3017	*DTYMK*	−2.5	1479	*MRPS30*	−2.1	426
GSTA1	−10.5*	29	*SPC24*	−2.5	5998	*MTX1*	−2.1	3163
GSTA5	−8.7	5178	*CTSL*	−2.5	9210	*RPS2*	−2.1	2874
INHBB	−7.8	6733	*FOXC1*	−2.5	738	*SNRPF*	−2.1	4700
JMJD1C	−7.8	570	*P4HB*	−2.5	83	*ADAMTS12*	−2.1	3061
HIF1A	−6.4	558	*DAG1*	−2.5	802	*GZMH*	−2.1	6333
CHEK1	−6.0	5166	*SSR4*	−2.5	3588	*SERPINA3*	−2.1	7493
ANLN	−5.5	4602	*FARS2*	−2.4	6478	*SMTNL2*	−2.1	4122
NR5A2	−5.3	2958	*TPM3L2*	−2.4	4450	*TRM112L*	−2.1	8475
AKR1CL1	−5.3*	8106	*ENO1*	−2.4	225	*EPC2*	−2.1	7614
POR	−5.3	3466	*PTPRU*	−2.4	7686	*ASRGL1*	−2.1	511
AKR1C4	−4.8*	522	*PDIA3*	−2.4	194	*DBI*	−2.1	5961
UBE3A	−4.8	438	*SQLE*	−2.4	5167	*ISCU*	−2.1	5189
FDXR	−4.6	46	*DDT*	−2.4	2674	*LSMD1*	−2.1	5767
PPARG	−4.4*	5154	*MLEC*	−2.4	2009	*MYBL2*	−2.1	8014
HMGB2	−4.4	7338	*LSM4*	−2.4	7083	*TMED4*	−2.1	935
RGS3	−4.4	2727	*MAP1A*	−2.4	8006	*CKS2*	−2.1	138
REXO2	−4.3	1712	*TMEM120A*	−2.4	3014	*CNN2*	−2.1	7134
TMEM130	−4.2	6322	*H1FX*	−2.3	6605	*LAMB2*	−2.1	5804
PPP1R18	−4.1	49	*SIVA1*	−2.3	4689	*RPL19*	−2.1	593
GPR56	−4.0	313	*PPIA*	−2.3	7182	*RPL24*	−2.1	7551
CYB5A	−3.9	5091	*PPIB*	−2.3	786	*RPS8*	−2.1	5935
GSTA4	−3.9	2394	*ACTN1*	−2.3	3087	*SEC61A2*	−2.1	7743
CYP19A1	−3.8*	2530	*RPS15*	−2.3	7492	*SLC25A39*	−2.1	89
UBE2C	−3.7	706	*GPI*	−2.3	1443	*ALAS1*	−2.1	3976
MIF	−3.5	8687	*HSF4*	−2.3	8899	*BANF1*	−2.1	1423
AFM	−3.2	7566	*LGI3*	−2.3	4810	*CITED1*	−2.1	1643
DENND5B	−3.2	6803	*SORL1*	−2.2	7745	*LMAN2*	−2.1	7683
ATP5C1	−3.1	7361	*COX4I2*	−2.2	7763	*RPL41*	−2.1	1085
PDIA6	−3.1	607	*RNASEH2C*	−2.2	1438	*RPS12*	−2.1	3186
BMP1	−3.1*	5121	*RPL18*	−2.2	3593	*RPS14*	−2.1	28
RPN2	−3.0	5779	*RPL37A*	−2.2	4699	*RPS6*	−2.1	9029
STAT2	−3.0	25	*TIE1*	−2.2	1850	*SPOCK3*	−2.1	7866
DUT	−2.9	5259	*MRPS6*	−2.2	5435	*SNRNP25*	−2.0	8711
GRB14	−2.9	5754	*RPL26L1*	−2.2	4174	*COPS6*	−2.0	3580
HDC	−2.9	7339	*PTGS2*	−2.2	8430	*PABPC1*	−2.9	6929
HIST1H2AG	−2.9	2616	*EML4*	−2.2	2862	*PRPF3*	−2.0	4591
MRPL22	−2.6	7326	*GNB2L1*	−2.2	5541	*KANSL2*	−2.0	5310
RPL22	−2.9	7458	*HINT1*	−2.2	7637	*COLGALT1*	−2.0	1472
TNS3	−2.8	38	*RPL35*	−2.2	5210	*RIP5*	−2.0	3657
CYP19A3	−2.8	4254	*CCDC167*	−2.2	5866	*TMEM97*	−2.0	3974
EFCAB2	−2.7	1162	*MRPL17*	−2.2	5394	*USP10*	−2.0	702
LRP8	−2.7*	282	*PLEKHO1*	−2.2	8911	*RPS3A*	−2.0	2750
GPR125	−2.6	8987	*PRDX5*	−2.2	711	*PCNX*	−2.0	7530
RPS4Y1	−2.6	6988	*RPL29*	−2.2	5238	*IFLTD1*	−2.0	5610
CALM1	−2.6	1291	*TTLL12*	−2.2	4648			
FZD5	−2.6	3476	*ERC1*	−2.2	7166			

Only fold-changes >2 between SAF and SHF categories are retained in this list. Ratio is inversed and preceded by a minus sign when value is <1. Position relative to the GEO accession number GPL3729. *Means of several positions with the same annotation.

subordinate follicles contain decreased amounts of the largest precursor forms. Further studies are required to elucidate this mechanism. Follicles destined to become atretic are characterized by loss of capacity to produce estradiol and enhanced production of low-molecular-weight IGFBPs. These changes not only precede atresia but also occur before the cessation of follicular growth and changes in intrafollicular inhibins (101).

Downregulation of these genes as well as the *CYP19A1* gene (second network) was described to be an accurate indicator of follicular health and showed an expression pattern being significantly different in growth, plateau, and atretic phases in medium bovine follicles (29). Bonnet et al. (18) previously showed overexpression of the genes implicated in lipid metabolism network in granulosa cells during pig terminal follicular development. This finding is in agreement with differentiation mechanisms leading to fully steroidogenic granulosa cells in large antral follicles attested to by the overexpression of *CYP19A1*. This first functional network also includes upregulation of the transcription factor *HNF4A* (hepatocyte nuclear factor 4 alpha). In addition, there is a large increase in expression level of *MSMB* (beta-microseminoprotein) in SAF compared with the SHF group. This latter gene was rather downregulated in prostate carcinoma (27); downregulation of *MSMB* has been related to uncontrolled cell growth and enhance tumorigenesis (85, 102). Alterations in *MSMB* gene expression are associated with the development of prostate cancer (45). Upregulation of *NUPR1* (nuclear protein 1) was also observed in SAF; this gene encodes proteins that inhibit apoptosis,

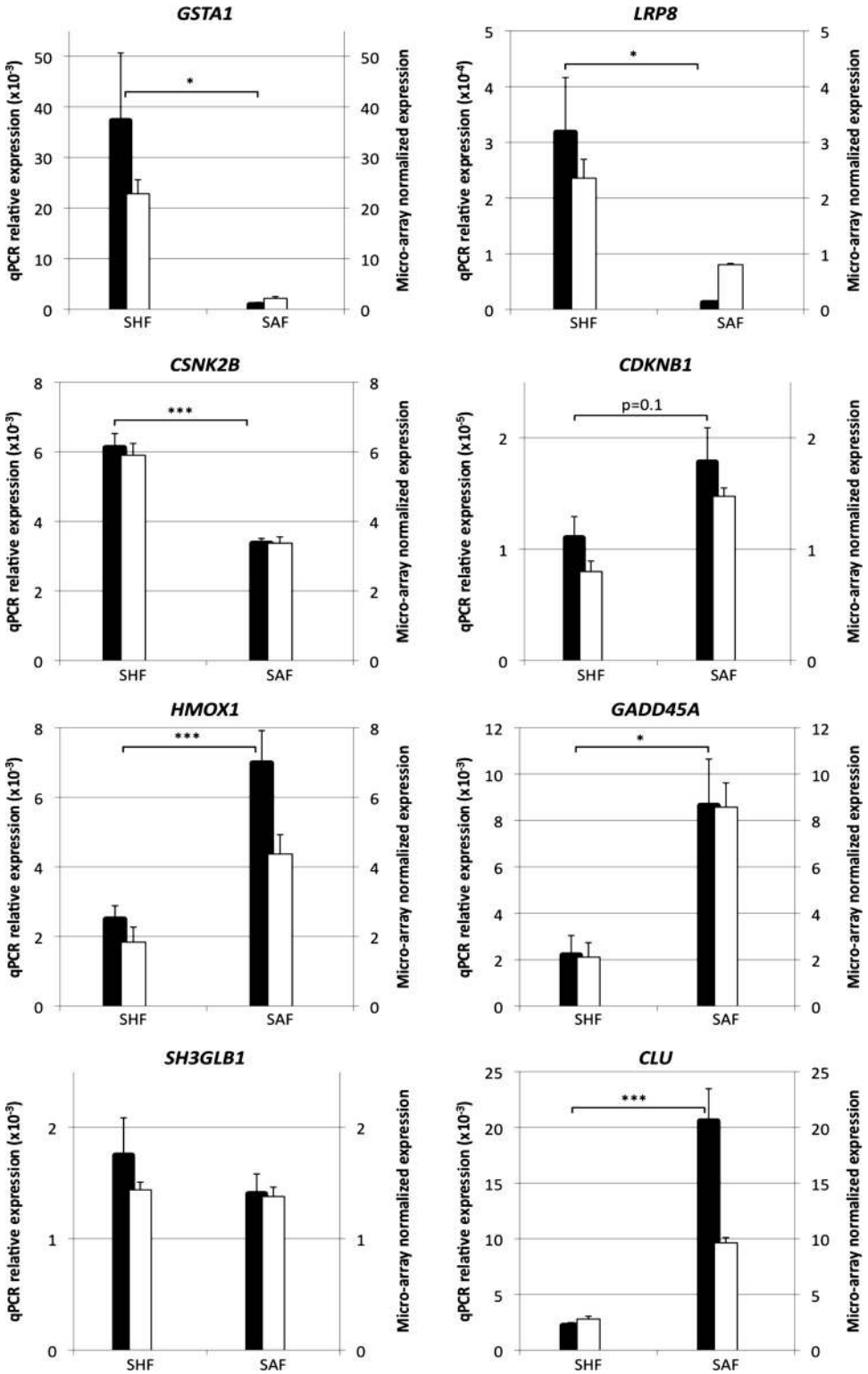

Fig. 2. Real-time quantitative (q)PCR validation of expression levels of target genes found differentially expressed between SHF and SAF by microarray analyses. Total RNA from pig granulosa cells of small healthy follicles (SHF, $n = 5$) and small atretic follicles (SAF, $n = 6$) were reverse-transcribed and submitted to real-time qPCR analysis for quantification of *SH3GLB1, CLU, GADD45A, HMOX1, CDKNB1, CSNK2B, LRP8*, and *GSTA1* gene expression levels. Quantitative qPCR data (black histogram) are means ± SE of relative expression to the reference genes *TCTP* and *MT-CO3*. PCR data are compared with normalized microarray data (white histogram) where *SH3GLB1* was chosen as a nondifferentially expressed gene. Significant difference in qPCR analysis between means from SHF and SAF were obtained after a Student's *t*-test (after Welch correction for unequal variance): $*P < 0.05$, $***P < 0.001$.

promotes pancreatic cancer development, and protects cells from stress (44), raising the question of its role in atretic follicles. A role for *AKT*2, coding for kinases controlling cell proliferation mechanism, has been also previously observed in cancers (7, 118). The *ADAMTS1* gene family is related to the cleavage of extramembranous domains (92). The expression of this gene is increased significantly during follicular growth and atresia in small but not large follicles, and more in preovulatory follicles of older than younger cows (57).

The second network is related to cancer and to cell death and survival. Many of these genes participate to the control of the actin microfilament network, including myosin-related genes. This may reflect changes in cell shape that generally accompany the atresia process in follicles. Actin is associated with

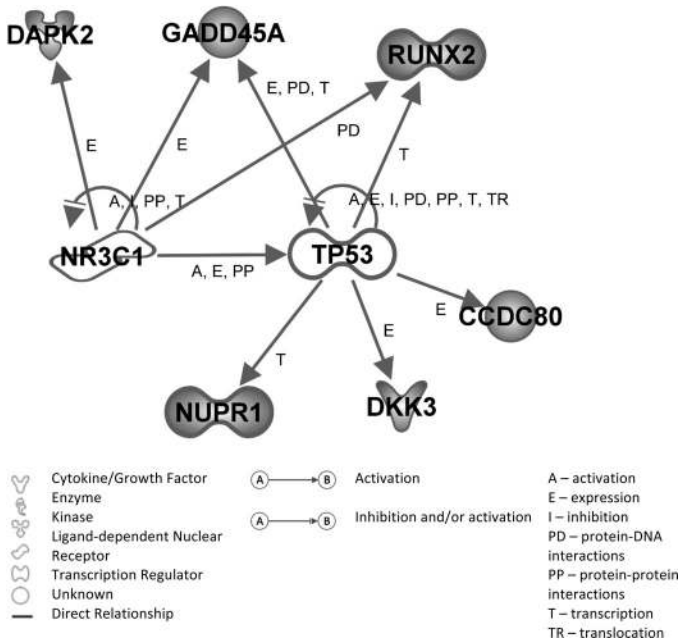

Fig. 3. Atresia biomarker-transcription factor connections.

granulosa cell shape changes during antral folliculogenesis (18) that may consequently affect steroid synthesis and proliferation. This network also includes two upregulated genes in the SAF category, *CEBPD* and *CLU*, which are involved in cell death, tumor progression, and neurodegenerative disorders (55, 120). Among others, the growth arrest and DNA damage-inducible, alpha (*GADD45A*) gene has been recently reported as an indicator of late atresia (29). Indeed, *GADD45A* activity is required for the progression of apoptotic cell death in follicular atresia, as it controls cell cycle arrest, apoptosis induction, and DNA damage repair in response to DNA-damaging agents (119). It has also been shown that transfection of a *GADD45A* expression vector has induced apoptosis, reportedly by interacting MEKK4/MTK1 and activating JNK/p38 signaling, which induces apoptosis (103). Douville and Sirard (29) have suggested that *GADD45A* activity is increasingly needed for the progression of apoptotic cell death in follicular atresia. These results highlight the fact that a particular gene expression pattern per stage reflects follicle growth or atresia.

In addition, the *HIF1A* gene was found to be downregulated in atretic follicles. It encodes the alpha subunit of transcription factor hypoxia-inducible factor-1 and plays an essential role in embryonic vascularization, tumor angiogenesis, and pathophysiology of ischemic disease. *HIF1A* induction is mediated by *IGF1* in cells grown under normal oxygen concentrations, thus uncoupling *HIF1A* induction from oxygen deprivation (34). Proapoptotic and antitumorigenic effects of *HIF1A* have also been reported (4). Finally, mutations in *RUNX2* (runt-related transcription factor 2), a gene that was upregulated in SAF vs. SHF categories, have been correlated with cleidocranial dysplasia (40) and associated with a prostate cancer (84). Upregulation in *DKK3* induces apoptosis in cisplatin-resistant lung adenocarcinoma cells via the Wnt/β-catenin pathway (115). Another important gene in this network may be *DAPK2*, a member of the death-associated protein kinase (DAPK) family and related to apoptosis pathways (8). This gene was also reported to be a modulator of TRAIL [tumor necrosis factor (TNF)-related apoptosis-inducing ligand] signaling (5). Therefore, upregulated *DAPK2* gene in atretic follicles suggests a DAPK2-mediated role of apoptosis process (36) in atresia.

The third network concerns cell death and survival, cell morphology, and reproductive system development and function. Among these genes, two are particularly interesting. The ECSIT signaling integrator was identified as a mitochondrial protein that has a neuroapoptotic role in Alzheimer's disease pathogenesis (99). *CCDC80* (coiled-coil domain containing 80) plays a role in cell organization and apoptosis, and inhibition of *CCDC80* expression may notably be an important event in the development of colorectal and pancreatic cancers (15, 39).

Our results are in concordance with our previous study where we described gene sets whose expression increases during the follicular development and decreases with atresia. The atresia process requires fewer new genes than the growth process (98). Moreover, our finding corresponds to differentially expressed genes along the terminal follicular growth described by Bonnet and colleagues (18). This study showed in particular downregulation of ribosomal protein, cell morphology, and ion-binding genes. Numerous genes whose expression increased during the terminal follicular growth

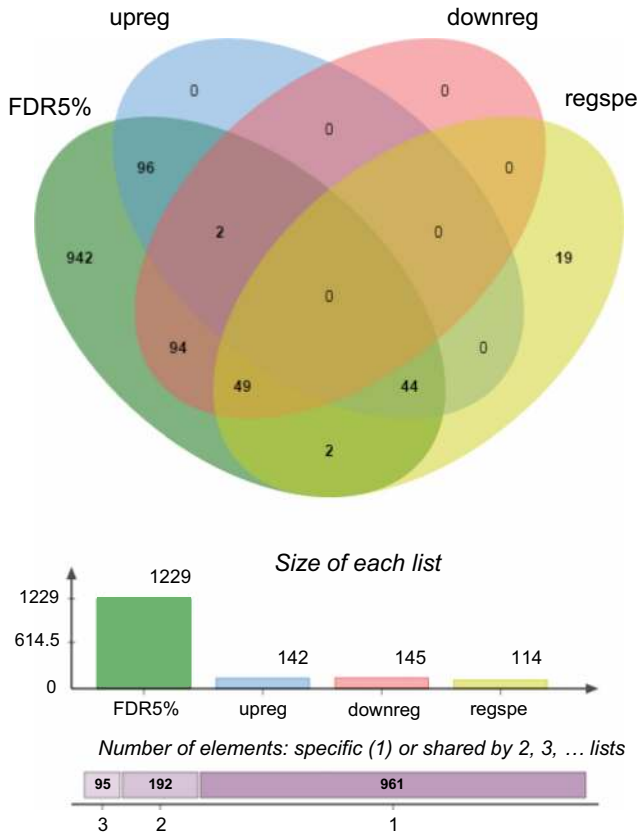

Fig. 4. Graphic representation of the various gene lists. Differentially expressed genes [false discovery rate (FDR) 5%], either upregulated genes [fold-change (FC) >2], or downregulated between SHF and SAF groups (FC >−2), and specific regulatory upstream candidates as suggested by automatic confrontation with literature data (KeyRegulatorFinder web tool) are colored in green, blue, red, and yellow respectively. The number of common genes is indicated for any of the intersection between these 4 lists.

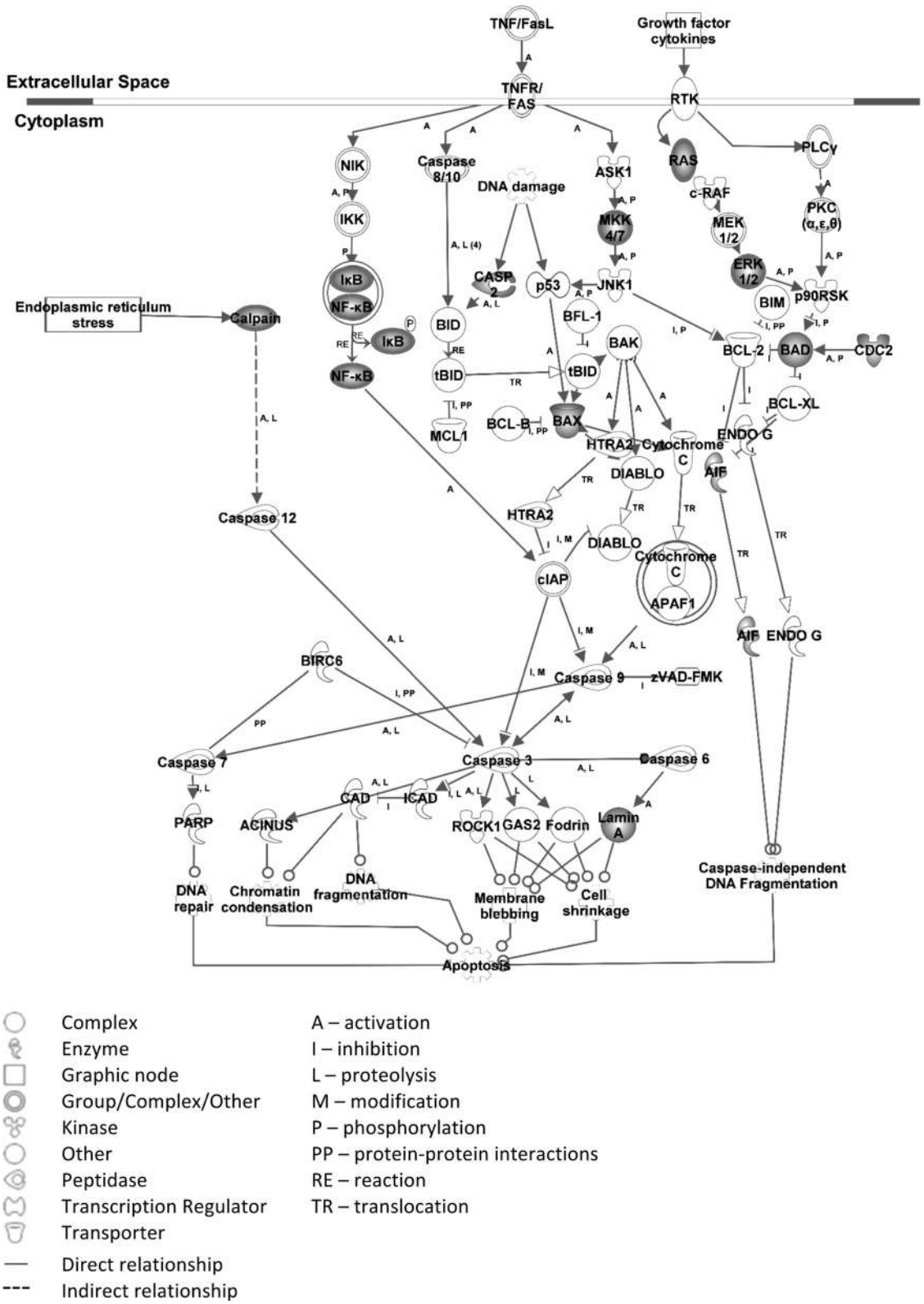

Fig. 5. Apoptosis signaling pathway that highlights the key molecular events involved in triggering apoptosis. Genes in gray correspond to the genes present on the used array but not showing as differentially expressed in our study.

were implicated in glutathione metabolism (*GSTA1*) and lipid metabolism (*CYP19A*). One of predictors is actin, which is associated with granulosa cell shape changes along antral folliculogenesis that may consequently affect steroid synthesis and proliferation.

Altogether, these genes could form the basis for a future study to develop a panel of tissue indicators with the purpose of prognosis of this phenomenon.

Regulators and markers of porcine follicle atresia. If the genes are coexpressed during follicular atresia, it is highly probable that they are co-regulated, so a better knowledge of upstream actors responsible for the regulation of gene coexpression modules may provide new insights into molecular mechanisms controlling atresia. A study of upstream transcriptional regulators may provide additional information that can explain the observed gene expression changes in the list of studied genes. Such an investigation is made arduous by the fact that the key upstream regulator may be not included in the list of genes suggested as differentially expressed according to a threshold probability (13). This approach was successfully used in our previous study to propose upstream transcriptional regulators that may participate in molecular flexibility in porcine adipose tissues in response to diet (38). A large number of differentially expressed genes in our study encoded transcription factors. Moreover, using academic knowledge on reactions and regulations included in TRANSPATH database (59) and a dedicated algorithm to search and sort upstream regulatory molecular actors (13), we were able to propose upstream regulators to different genes and biological processes. Figure 4 summarizes the differentially expressed genes including upregulated genes (fold-change >2) and downregulated genes (fold-change >-2) between noted SHF and SAF, together with potential specific candidates acting as upstream regulators of these genes. This analysis notably highlights 93 genes as possible regulatory candidates of pig granulosa cell atresia, for which expression changes larger than twofold between conditions were also experimentally observed between SHF and SAF groups. Whereas most of them have not yet been described in follicular atresia, they are generally known as inhibitors of apoptosis, stimulators of apoptosis, or tumor suppressors. Among them, *INHBB, HNF4*, and *CLU* were again in evidence. In addition, different interleukins (*IL5, IL24*), TNF-associated receptor (*TNFR1*), and cytochrome-c oxidase (*COX*) may have played a key role in porcine atresia. Table 4 summarizes key

Table 4. *Key upstream regulators in follicle atresia*

HUGO Name	Description	Reference No.
	Inhibitors of apoptosis	
IMPDH2	inosine 5′-phosphate dehydrogenase 2	(61)
GATA4	GATA binding protein 4	(60)
MEF2	myocyte enhancer factor 2	(1)
GSTA1	glutathione S-transferase alpha 1	(16, 31, 89, 111)
GADD45A	growth arrest and DNA-damage-inducible, alpha	(50, 119)
BOC	cell adhesion associated, oncogene regulated	(63)
CTSL	cathepsin L	(116)
NF-YA	nuclear factor Y-box A	(20, 35, 80)
IL5	interleukin 5	(43, 68, 69, 72)
IL24	interleukin 6	
IL6	interleukin 24	
IL6R	interleukin 24	
Nfkb1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	(10)
	Stimulators of apoptosis	
COX	cytochrome C	(52, 71, 96)
ID1	inhibitor of DNA binding 1	(91)
INHBB	inhibin beta B	(64, 112)
CD147	transmembrane glycoprotein	(67)
NDUFS6	NADH dehydrogenase (ubiquinone) Fe-S protein 6	
PPAR isotypes	peroxisome proliferator-activated receptors	(121)
RPN2	ribophorin II	(33)
SORT1	sortilin 1	(37)
TNFR1	TNF receptor 1	(58)
CALM1	calmodulin 1	(100)
MTX1	metaxin 1	(21)
SDHB	succinate dehydrogenase complex, subunit B	(23)
TSPO	translocator protein	(86)
HNF4a	hepatocyte nuclear factor 4, alpha	(62)
FDPS	farnesyl diphosphate synthase	(66)
CLU	clusterin	(12, 55, 70, 79, 104, 117, 120)
CEBP	CCAAT/enhancer binding protein	
MAP1A, MAP1B	microtubule-associated protein	(32)
	Tumor suppressor	
HINT1	histidine triad nucleotide binding protein 1	(11)
HNF1A	HNF1 homeobox A	(51)
ZNF350	zinc finger protein	(28)
GADD45A	growth arrest and DNA-damage-inducible, alpha	
CD82	transmembrane glycoprotein	(81, 94, 109)
SOX4	SRY (sex determining region Y)-box 4	(122)
ARF	ADP-ribosylation factor	(97, 114)

upstream regulators in follicle atresia based on our results and on a literature review.

Besides the upstream regulatory actors of pig follicle atresia, the present study also highlights 11 genes (*DKK3, GADD45A, CAMTA2, CCDC80, DAPK2, ECSIT, MSMB, NUPR1, RUNX2, SAMD4A, ZNF628*) with very sensitive expression to atresia (fold-change >5 in atretic vs. healthy follicles). All these genes belong to networks identified above and are linked to molecular functions related to proliferation of tumor cell lines and transcription regulation. It is thus proposed that they could further serve in a panel of tissue prognosis indicators of porcine follicle atresia. Six genes (*GADD45A, RUNX2, CCDC80, DKK3, NUPR1, DAPK2*) among these 11 genes and described above are functionally connected by two other transcription factors (*NR3C1* and *TP53*) as illustrated in Fig. 3. The *NR3C1* (nuclear receptor subfamily 3, group C, member 1) gene encodes a glucocorticoid receptor, which can function both as a transcription factor that binds to glucocorticoid response elements in the promoters of glucocorticoid-responsive genes and as a regulator of other transcription factors. *NR3C1* expression has been previously demonstrated to have importance in cancer (82). As previously described (47), we suggest an association between atretic status and genes that are influenced by the p53 transcription factor. *TP53* encodes the tumor protein p53, which responds to diverse cellular stresses to regulate target genes inducing cell cycle arrest, activation of apoptosis, senescence, DNA repair, or changes in metabolism (74). The tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family of proteins (95); the expression of both Bcl-2 and Bax is regulated by *TP53* (75).

Conclusions

In the present study, we describe and analyze differentially expressed genes during atresia of pig antral follicles to generate regulatory networks and key genes in this process. Three functional networks were elicited, and they are helpful to elucidate critical biological processes leading to atresia. The present study also enlists key upstream regulators in follicle atresia based on our results and on a literature review. Identification of 93 upstream regulatory candidate genes, which are known in literature to be inhibitors of apoptosis, stimulators of apoptosis, or tumor suppressors, confirms their roles in porcine atresia. Eleven new markers of follicular atresia in granulosa cells are also proposed: *DKK3, GADD45A, CAMTA2, CCDC80, DAPK2, ECSIT, MSMB, NUPR1, RUNX2, SAMD4A*, and *ZNF628*. These genes could further serve in a panel of tissue prognosis indicators of porcine follicle atresia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

GTK, PM, SF, AB, ET and DM discussed the aims of the study and the data retrieval. AB, JS, FV contributed to the data collection and data retrieval. MSC and CRG developed and performed all the statistics. FG performed the upstream regulators study. ET, GTK, PM, SF, AB, MSC, CRG and DM contributed to the interpretation and discussion of the results. ET drafted the manuscript and all authors contributed to this manuscript in its final version. All authors read and approved the final manuscript.

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