Lack of FSH support enhances LIF–STAT3 signaling in granulosa cells of atretic follicles in cattle

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Abstract

Subordinate follicles (SFs) of bovine follicular waves undergo atresia due to declining FSH concentrations; however, the signalling mechanisms have not been fully deciphered. We used an FSH-induced co-dominance model to determine the effect of FSH on signalling pathways in granulosa cells of the second-largest follicles (SF in control cows and co-dominant follicle (co-DF2) in FSH-treated cows). The SF was smaller than DF in control cows while diameters of co-DF1 and co-DF2 in FSH-treated cows were similar. The presence of cleaved CASP3 protein confirmed that granulosa cells of SFs, but not of DFs and co-DFs, were apoptotic. To determine the effect of FSH on molecular characteristics of the second-largest follicles, we generated relative variables for the second largest follicle in each cow. For this, variables of SF or co-DF2 were divided by the variables of the largest follicle DF or co-DF1 in each cow. There was higher transcript abundance of *MAPK1/3* and *AKT1/2/3* but lower abundance of phosphorylated *MAPK3/1* in SF than co-DF2 granulosa cells. Abundance of mRNA and phosphorylated protein of STAT3 was higher in granulosa cells of control SF than FSH-treated co-DF2. SF granulosa cells had higher levels of *LIFR* and *IL6ST* transcripts, the two receptors involved in STAT3 activation. Further, lower transcript abundance of interleukin 6 receptor (*IL6R*), another receptor involved in STAT3 activation, indicated that STAT3 activation in SF granulosa cells could be mainly due to leukemia inhibitory factor (LIF) signalling. These results indicate that atresia due to lack of FSH is associated with activated LIF–STAT3 signalling in SF granulosa cells, as FSH treatment reversed such activation.

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Introduction

Even though bovine ovaries contain thousands of follicles, only ~1% of them ovulate and contribute to the reproductive performance of a cow. As atresia is the most common outcome of follicular development, aberrant atresia will have a significant impact on fertility.

During the follicular wave, small follicles begin to grow together in response to a transient peak in circulating follicle-stimulating hormone (FSH) concentrations (Adams 1999, Adams *et al.* 2008). But only one follicle, selected to be dominant, continues to growth despite FSH levels reaching nadir while the others undergo atresia (Ginther *et al.* 1999). Follicular atresia is initiated with apoptosis of granulosa cells (Matsuda *et al.* 2012), which has been attributed to the depletion of survival factors, including FSH. In addition, follicular atresia requires a stimulatory component such as

pro-apoptotic signals, because apoptosis must be localized to the atretic but not all other follicles.

Several studies have examined the role of protein kinase B (AKT) and MAPKs in granulosa cells, mostly using *in vitro* culture models (Seger *et al.* 2001, Ryan *et al.* 2008). Gonadotropins have been shown to activate AKT and MAPK signalling in granulosa cells (Babu *et al.* 2000, Zeleznik *et al.* 2003) and these pathways are considered important for dominant follicle (DF) selection (Evans & Martin 2000, Gasperin *et al.* 2014). STAT is a family of proteins known to mediate signalling of cytokines and stimulate cell proliferation, differentiation and apoptosis (Levy & Darnell 2002, Rawlings *et al.* 2004). The role of the STAT signalling pathway in mammalian folliculogenesis remains relatively uncharacterized (Sobinoff *et al.* 2013). Our recent study demonstrated increased phosphorylation of STAT3 in granulosa cells of atretic follicles collected on day 4 of

the follicular wave, and proposed that this pathway may be involved in granulosa cell apoptosis during atresia (Gasperin *et al.* 2014). However, that study did not establish a causal relationship between declines in FSH concentrations and the activation of STAT3. Additionally, the identity of a stimulatory signal that may activate STAT3 when the FSH concentrations are low remains to be established. Some factors such as the Fas ligand (FASLG) have been proposed to be involved in granulosa cell apoptosis. While the FASLG has been shown in atretic follicles, its treatment alone does not induce granulosa cell apoptosis *in vitro* (Porter *et al.* 2000). This indicates that other signalling pathways such as the STAT3 pathway may contribute to follicular atresia. In this context, another ligand, leukemia inhibitory factor (LIF), has been shown to play a central role in apoptosis through activating STAT3 by phosphorylation during mammary gland involution (Kritikou *et al.* 2003).

FSH-induced co-DF model in cattle is an excellent model for comparison of DFs, subordinate follicles (SFs) and co-DFs. Molecular differences present between the second-largest follicles – SF in control animals and co-DF2 in FSH-treated cows – will provide a mechanistic basis for follicular atresia during the decline of FSH concentrations. The results of this study could potentially contribute to developing novel therapeutic measures to enhance follicular development during assisted reproductive technologies (ARTs). Therefore, the aim of our study was to test if signalling pathways and their cytokine activators are differentially regulated in granulosa cells of SF and co-DF2.

Materials and methods

Co-DF induction and granulosa cell collection

All experimental procedures using cattle were approved by the Federal University of Santa Maria Animal Care and Use Committee (ACUC no. 23081.009594/2007-41). Cyclic adult beef cows ($n=20$; 4–10 years old) were synchronized as described previously (Gasperin *et al.* 2014). Eight cows with synchronous estrus were then used to carry out FSH or saline treatment during the first follicular wave. The ovaries of each cow were examined daily by transrectal ultrasonography and the day of ovulation was considered day 0. On the second day after ovulation (day 2), saline (control group, $n=4$) or a total dose of 100 mg FSH (FSH group, $n=4$, Folltropin-V; Bioniche Animal Health, Belleville, ON, Canada) was administered i.m. every 12 h for 48 h. The total dose of FSH was divided into two doses of 30 mg on day 2 and two doses of 20 mg on day 3. Ovaries were collected by colpotomy 12 h after the last FSH/saline treatment (morning of day 4). The two largest follicles were dissected out from the ovaries and granulosa cells were harvested. The largest and the second-largest follicles were respectively classified as DF and SF in control cows, and co-DF1 and co-DF2 in FSH cows. Relative variable (RV) was calculated for all molecular data to determine the effect of FSH

treatment, as detailed below in the 'Data processing and statistical analyses' section.

RNA extraction, RT and real-time PCR

Total RNA and protein from granulosa cells were extracted using AllPrep DNA/RNA/Protein Kit (Qiagen) as described previously (Gasperin *et al.* 2014). Quantity and RNA purity were measured using the NanoDrop (Thermo Scientific, Waltham, MA, USA). Absorbance 260/280 nm ratios above 1.8 were considered pure, and all samples used in the present study were above this threshold. To synthesize cDNA, 500 ng RNA was first treated with 0.1 U DNase (Invitrogen; 37 °C – 5 min). After DNase inactivation at 65 °C for 10 min, samples were incubated in a final volume of 20 µl with iScript cDNA Synthesis Kit (Bio-Rad Laboratory) following the manufacturer's protocols.

Designing and validation of primers (Table 1) and qPCR were performed as described previously (Sanchez *et al.* 2014). Samples were run in duplicates and transcript levels for each gene of interest were expressed relative to the average abundance of the reference genes, *CYCLOPHILIN* and *GAPDH*. To test cross-contamination with theca cells, PCR for detection of the mRNAs that encode *CYP17A1* in granulosa

Table 1 Primers used in the expression analysis of *Bos taurus taurus* genes.

Gene	Primer sequence (5'–3')	Conc. (nM)
<i>CYCLOPHILIN</i>	F: GGTCATCGGTCTCTTTGGAA	200
	R: TCCTTGATCACACGATGGAA	200
<i>GAPDH</i>	F: ACCCAGAAGACTGTGGATGG	200
	R: CAACAGACACGTTGGGAGTG	200
<i>CYP19A1</i>	F: GTGTCCGAAGTTGTGCCTATT	200
	R: GGAACTGCACTGGGAAATGA	200
<i>LHCGR</i>	F: GCACAGCAAGGAGACCAATAA	200
	R: TTGGGTAAGCAGAAACCATAGTCA	200
<i>FSHR</i>	F: AGCCCTTGTCACAACCTATGTC	200
	R: GTTCCTCACCGTAGGATAGATGT	200
<i>CYP17A1</i>	F: CCATCAGAGAAGTGTCCGAAT	200
	R: GCCAATGCTGGAGTCAATGA	200
<i>STAT3</i>	F: CTGCAGCAGAAGGTTAGCTACAA	200
	R: TTCTAAACAGCTCCACGATTCTCTC	200
<i>CCND2</i>	F: TGCCCCAGTGCTCCTACTTC	200
	R: CGGGTACATGGCAAACTTGA	200
<i>CASP3</i>	F: TGCAGAAGTCTGACTGGAAAACCCAAAC	200
	R: TCATCCTCAGCACCCTGTCTGTCTC	200
<i>AKT1</i>	F: GATTCTTCGGACAGCTGCTG	300
	R: GGCCGTGAACCTCTCATCAA	200
<i>AKT2</i>	F: GCCGAATAGGAGAAGTGGGG	200
	R: CACGTCTGAGGTGACACAA	200
<i>AKT3</i>	F: GTGGCGCACACTTAACTGA	200
	R: ACCCGCTCTCTCGACAAATG	200
<i>MAPK1</i>	F: TATTTCGAGCACCAGCATCG	200
	R: TGGAAGGTTTGAGGTACCGG	200
<i>MAPK3</i>	F: ACCCAAGGAACGACTGAAGG	200
	R: GCTGGGCACACAGTCCATT	200
<i>LIFR</i>	F: AAAGTCCGGCATCTAAGGT	200
	R: TCATGAGGTTGCTGGGACAG	200
<i>IL6ST</i>	F: AGAGTGGGACACCTTCTTA	200
	R: TCTGTGTAGGCTGCCATTCTG	200
<i>IL6R</i>	F: GACCAGAGGACAATGCCACA	200
	R: TCACTCTACTGAGGGGGCTC	200

F, forward primer; R, reverse primer; Conc., primer concentration used for gene amplification.

Table 2 Follicular diameter (mm) determined by transrectal ultrasonography.

Group	Follicle	Day 3	Day 4	Growth rate
Control	DF	6.3 ± 1.5	9.5 ± 1.3	3.2 ± 0.8
	SF	6.0 ± 1.1	6.1 ± 0.6*	0.1 ± 0.6*
FSH	co-DF1	5.3 ± 0.6	8.6 ± 0.9	3.4 ± 0.3
	co-DF2	4.7 ± 0.7	7.5 ± 0.7	2.8 ± 0.7

*Indicates significant difference from DF ($P < 0.05$).

cells was performed in each sample and all samples were free from contamination by theca cells (after 30 PCR cycles).

Immunoblot analyses

Granulosa cell protein samples obtained using AllPrep Kit (Qiagen) were boiled at 95 °C for 5 min, subjected to 7.5% SDS gel and transferred onto nitrocellulose membranes. After blocking for 2 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T), blots were incubated overnight at 4 °C with 1:1000 rabbit anti-human phosphorylated STAT3 (#9131; Tyr 705; Cell Signaling Technology, Danvers, MA, USA), phosphorylated MAPK3/1 (#4376; Erk1/2; Thr202/Tyr204; Cell Signaling Technology), phosphorylated AKT (#2965; Thr308; Cell Signaling Technology), total STAT3 (#9132; Cell Signaling Technology), total MAPK3/1 (#4695; Erk1/2; Cell Signaling Technology), cleaved CASPASE3 (Asp175; #9661, Cell Signaling Technology) or 1:5000 β -actin (bACTIN; ab8227; Abcam, Inc., Totonto, ON, Canada) with agitation, followed by three washes (10 min each) with TBS-T. The blots were then incubated with 1:7500 goat anti-rabbit IgG-HRP (ab6721; Abcam, Inc.) at room temperature for 2 h with agitation, followed by three washes (10 min each) with TBS-T. Immunoreactivity was detected with Immuno-Star WesternC Chemiluminescence Kit (Bio-Rad Laboratory) according to the manufacturer's instructions and visualized using Chemidoc System (Bio-Rad Laboratory). Quantification of bands of the western blots was performed using Image Lab Software (Bio-Rad Laboratory). ERK1 and ERK2 band densities were added together and were represented as MAPK3/1. The abundance of phosphorylated proteins (pMAPK3/1, pAKT and pSTAT3) was calculated relative to their respective total protein levels. CASPASE total protein abundance was calculated relative to bACTIN.

Data processing and statistical analysis

Follicular dynamics data were analyzed by two-way ANOVA using SAS Software (SAS Institute, Inc., Cary, NC, USA). Differences between specific means were evaluated by the use of the Bonferroni's procedure on least squares means (Fricke *et al.* 1997). In order to determine the effect of FSH on molecular characteristics of the second largest follicles, we generated RVs for the second largest follicle in each cow. For this, variables of the second largest follicle (SF or co-DF2) in each cow were divided by the variables of the largest follicle (DF or co-DF1) from the same cow. RVs for mRNA data were compared between the two groups using the two-tailed Student's *t*-test. Based on the results of mRNA data analyses,

Figure 1 CYP19A1 (A), CCND2 (B) and CASP3 (C) mRNA expression and cleaved caspase 3 protein abundance (D) in granulosa cells of subordinate (SF) and co-dominant (co-DF2) follicles. In order to determine the effect of FSH treatment, data for SF and co-DF2 were normalized to the data of DF and co-DF1 respectively. *Indicates statistical significance ($P < 0.05$).

RVs for mRNA data were compared using one-tailed Student's *t*-test. Results are presented as mean $\pm$ S.E.M. A $P < 0.05$ was considered statistically significant.

Results

Dynamics of the two largest follicles in control and FSH-treated cows

Follicular dynamics measured by ultrasonography in control and FSH groups are shown in Table 2. As expected, the diameter of DF was larger than SF in control cows ($P < 0.05$) and there was no difference in diameters of co-DF1 and co-DF2 in FSH-treated cows ($P > 0.05$). The growth rate of DF, between days 3 and 4 after ovulation, was higher than that of SF in control cows ($P < 0.05$), whereas the growth rate of co-DF1 and co-DF2 during this period was similar in FSH-treated cows ($P > 0.05$). Furthermore, the diameter and growth rate of co-DFs of FSH-treated cows were similar to the DF of control cows ($P > 0.05$).

Figure 2 *LHCGR* (A) and *FSHR* (B) mRNA expression in granulosa cells of subordinate (SF) and co-dominant (co-DF2) follicles. In order to determine the effect of FSH treatment, data for SF and co-DF2 were normalized to the data of DF and co-DF1 respectively. *Indicates statistical significance ($P < 0.05$).

Figure 3 *MAPK1* (A) and *MAPK3* (B) mRNA expression and phosphorylated *MAPK3/1* abundance (C) in granulosa cells of subordinate (SF) and co-dominant (co-DF2) follicles. In order to determine the effect of FSH treatment, data for SF and co-DF2 were normalized to the data of DF and co-DF1 respectively. (D) Representative immunoblots showing protein abundance of phosphorylated and total MAPK along with β-Actin in granulosa cells of different follicle groups. *Indicates statistical significance ($P < 0.05$).

Granulosa cell health in the two largest follicles from control and FSH-treated cows

Granulosa cells were harvested from the two largest follicles of each of the saline and FSH-treated cows. To test if FSH treatment enhanced the health of granulosa cells of co-DF2 compared to SF, we analyzed *CYP19A1* mRNA abundance in granulosa cells. As expected, mRNA abundance of *CYP19A1* in granulosa cells of the SF was dramatically lower in control cows compared to co-DF2 of FSH-treated cows ($P < 0.05$; Fig. 1A). Likewise, transcript levels of cyclin D2 (*CCND2*) were higher in co-DF2 than SF ($P < 0.05$; Fig. 1B). On the other hand, *CASP3* mRNA was more abundant in granulosa cells of SF in control cows than co-DF2 in FSH-treated cows ($P < 0.05$; Fig. 1C). Also, immunoblot assays revealed that the cleaved *CASP3* protein was detected only in granulosa cells of SF but not in those of DF and co-DFs (Fig. 1D). Selection of the DF is associated with increased expression of gonadotropin receptors, especially LH receptor (*LHCGR*) in granulosa cells (Ginther *et al.* 1999). Thus, we evaluated the transcript abundance of gonadotropins receptors in granulosa cells. Relative mRNA abundance of *LHCGR* was higher in co-DF2 than SF ($P < 0.05$; Fig. 2A). Relative mRNA abundance of *FSHR* in granulosa cells did not differ between follicles in control and FSH-treated cows (Fig. 2B).

Phosphorylation status of MAPK3/1, AKT and STAT3 in granulosa cells of the two largest follicles in control and FSH-treated cows

We first measured mRNA and protein levels of MAPK3/1, AKT and STAT3 in granulosa cells of the second largest follicles relative to the largest follicles from control and FSH-treated cows. Levels of *MAPK1* and *MAPK3* mRNA were higher in granulosa cells of SF of control cows than co-DF2 of FSH-treated cows ($P < 0.05$; Fig. 3A and B). In contrast to the mRNA profile, the abundance of phosphorylated MAPK3/1 proteins was lower in granulosa cells of SF in control cows than co-DF2 in FSH-treated cows ($P < 0.05$; Fig. 3C and D). Relative mRNA levels of *AKT1*, *AKT2* and *AKT3* were higher in control SF than FSH-treated co-DF2 ($P < 0.05$; Fig. 4A, B and C respectively). However, the abundance of phosphorylated AKT did not differ between the second-largest follicles of both groups ($P > 0.05$; Fig. 4D and E), albeit FSH-treated co-DF2 had numerically higher pAKT. Relative abundance of *STAT3* mRNA was markedly higher in granulosa cells of SF than in co-DF2 ($P < 0.05$; Fig. 5A). In line with the mRNA pattern, the relative abundance of both total and phosphorylated isoform of *STAT3* protein was higher in granulosa cells of SF compared to co-DF2 ($P < 0.05$; Fig. 5B, C and D).

Figure 4 *AKT1* (A), *AKT2* (B) and *AKT3* (C) mRNA levels and phosphorylated AKT abundance (D) in granulosa cells of subordinate (SF) and co-dominant (co-DF2) follicles. In order to determine the effect of FSH treatment, data for SF and co-DF2 were normalized to the data of DF and co-DF1 respectively. (E) Representative immunoblots showing protein abundance of phosphorylated and total AKT along with β -Actin in granulosa cells of different follicle groups. *Indicates statistical significance ($P < 0.05$).

Figure 5 STAT3 mRNA abundance (A) and levels of total (B) and phosphorylated (C) STAT3 in granulosa cells of subordinate (SF in Control group) and co-dominant (co-DF2 in FSH group) follicles. In order to determine the effect of FSH treatment, data for SF and co-DF2 were normalized to the data of DF and co-DF1, respectively. (D) Representative immunoblots showing protein abundance of phosphorylated and total STAT3 with β -Actin in granulosa cells of different follicle groups. *Indicates statistical significance ($P < 0.05$).

Transcript abundance of the receptors of known STAT3-activating ligands in granulosa cells of the two largest follicles in control and FSH-treated cows

It is well established that leukaemia inhibitory factor (LIF) acting through its receptor (LIFR) stimulates STAT3 by phosphorylation (Heinrich *et al.* 1998). Also, LIFR forms heterodimers with the glycoprotein IL6ST to form a high affinity receptor through which LIF signaling is triggered (Heinrich *et al.* 1998). In line with the activation of STAT3, *LIFR* and *IL6ST* mRNA levels were higher in granulosa cells of control SF than FSH-treated co-DF2 ($P < 0.05$; Fig. 6A and B respectively). On the other hand, mRNA levels of interleukin 6 receptor (*IL6R*), which competes with LIFR to form heterodimers with IL6ST for STAT3 activation, were lower in SF than co-DF2 ($P < 0.05$; Fig. 6C).

Discussion

Understanding the molecular mechanisms involved in atresia of follicles in response to declining FSH concentrations is important for developing novel methods to enhance follicular development during ARTs. SFs undergo atresia due to declining FSH (Rivera & Fortune 2001, Lopez *et al.* 2005). Our recent study demonstrated that the STAT3 signalling pathway is activated in granulosa cells of atretic SFs (Gasperin *et al.* 2014). These temporal data did not establish that lack of FSH concentrations resulted in activation of STAT3. In the present study, we used a FSH-induced co-DF model to investigate signalling pathways in granulosa cells of SFs and examined if altered pathways of SFs are rescued in the second-largest follicle that would have undergone atresia in FSH-treated cows. Administration of small doses of FSH at the expected time of follicular deviation prevents the decline in FSH levels and thus one or more SFs escape atresia to become co-DFs (Mihm *et al.* 1997, Rivera & Fortune 2001). Indeed, in FSH-treated cows of the present study, the two largest follicles continued growing at a similar growth rate and did not differ in size. There were no significant differences in *FSHR* mRNA abundance in granulosa cells from the second largest follicles in both groups. Taken together with the previous observations (Luo *et al.* 2011, Gasperin *et al.* 2014), these data indicate that the presence *FSHR* alone does not rescue the SF from atresia. The *LHCGR* mRNA levels were lower in SFs relative to DFs compared to co-DF2 relative to co-DF1. These data are similar to previous reports (Beg *et al.* 2001, Luo *et al.* 2011) and together indicate that FSH-induced *LHCGR* prevented the potential SF from undergoing atresia in response to declining FSH concentrations. The lower *LHCGR* expression in SFs was associated with a remarkable increase in the mRNA abundance of *CASP3* and cleaved *CASP3* protein. FSH treatment inhibited *CASP3* expression and cleavage in granulosa

cells of co-DF2. These results suggest that follicular atresia in low FSH environment involves induction of CASP3 expression and cleavage, leading to granulosa cell apoptosis.

Figure 6 *LIFR* (A), *IL6ST* (B) and *IL6R* (C) mRNA expression in granulosa cells of subordinate (SF) and co-dominant (co-DF) follicles. In order to determine the effect of FSH treatment, data for SF and co-DF2 were normalized to the data of DF and co-DF1 respectively. *Indicates statistical significance ($P < 0.05$).

STATs are a family of transcription factors that mediate cytokine signalling and stimulate cell proliferation, migration and apoptosis (Levy & Darnell 2002, Rawlings *et al.* 2004). The receptors of cytokines such as LIF and IL6 share a common transmembrane protein receptor, IL6ST (a.k.a. gp130) to activate STATs (Heinrich *et al.* 1998). After ligand binding of the receptor, STATs become activated through tyrosine phosphorylation and regulate transcription (Bromberg & Darnell 2000). In the present study, phospho-STAT3 protein and mRNA levels were high in granulosa cells from SF, confirming our previous observations (Gasperin *et al.* 2014). We further found that in high FSH environment of FSH-treated cows, there was no such activation of STAT3 in granulosa cells of co-DF2, suggesting that FSH treatment inhibits activation of STAT3. The increased phospho-STAT3 abundance in granulosa cells of SF suggests that this pathway is potentially activated by factors involved in the inhibition of granulosa cell estradiol synthesis, proliferation and/or differentiation.

A switch from survival to death signalling induces physiological apoptosis. Deletion of STAT3 resulted in reduced levels of apoptosis and delayed mammary gland involution (Chapman *et al.* 1999, Kritikou *et al.* 2003). Therefore, our current data support the hypothesis that STAT3 signalling pathway is involved in granulosa cell apoptosis during atresia and that its suppression is one of the mechanisms by which FSH supports follicular development in cows.

Inhibition of AKT and MAPK pathways abrogates the stimulatory actions of FSH and insulin-like growth factor (IGF) on cultured bovine granulosa cells (Ryan *et al.* 2008). We found that despite high expression of MAPK1/3 and AKT1/2/3, the phosphorylated isoforms of these proteins were lower in granulosa cells of control SF than FSH-treated co-DF2. Higher levels of pAKT and pMAPK proteins were shown in the follicle wall of DFs in cattle (Evans & Fortune 1997) and sheep (Evans & Martin 2000). Our observations that the co-DF2 in FSH-treated cows had higher amounts of pMAPK and pAKT proteins suggest that FSH maintains AKT and MAPK protein activity in growing co-DFs.

The deprivation of key survival factors or stimulation by death ligands are the main causes of apoptosis and both contribute to granulosa cell apoptosis (Matsuda-Minehata *et al.* 2006). Estradiol, IGF1, FSH, epidermal growth factor and IL6 have been characterized as anti-apoptotic factors (Hsu & Hammond 1987, Chun *et al.* 1995, Hsu & Hsueh 1997, Heinrich *et al.* 1998, Rosenfeld *et al.* 2001, Mao *et al.* 2004). These factors act by activating the AKT signalling pathway (Matsuda *et al.* 2012). On the other hand, the death ligand-receptor systems such as FASLG, Fas (CD95), tumour necrosis factor alpha (TNF α and its receptor TNFR) and BCL2 family proteins have been implicated in follicular atresia (Baud & Karin 2001, Matsuda-Minehata *et al.* 2006, Jaaskelainen *et al.* 2009, Matsuda *et al.* 2012).

These signals induce different intracellular pro-apoptotic pathways, which result in cleavage of CASP3 leading to apoptosis (Matsuda *et al.* 2012).

LIF is a STAT3 activating ligand that has been shown to play a role in the apoptotic process during the mammary gland involution (Kritikou *et al.* 2003). LIF/STAT3 signalling coincides with induction of apoptosis in mammary epithelial cells (Abell *et al.* 2005). Our results demonstrated a positive association of higher mRNA levels of *LIFR*, *IL6ST* and *STAT3* with increased STAT3 activity in SF granulosa cells. On the other hand, *IL6R* mRNA abundance was lower in SF granulosa cells. As both LIFR and IL6R require dimerization with IL6ST for their signalling, the expression pattern of these three receptors suggests that there is an overall increase in LIFR signalling while IL6R signalling is reduced. These results suggest that LIF may be the primary ligand activating the STAT3 pathway in granulosa cells undergoing apoptosis. Our study is the first to suggest that LIF/STAT3 signaling may play a role in granulosa cell apoptosis. Further mechanistic studies are required to identify the relationship between the three signaling pathways, MAPK, AKT and STAT3, in ovarian granulosa cells, as well as to identify the origin of LIF in atretic follicles.

In summary, atresia of SF was associated with increased STAT3 phosphorylation, which could be attributed to an increased expression of *LIFR* and *IL6ST* in granulosa cells. Such increased STAT3 signalling was inhibited by the FSH treatment in granulosa cells of co-DF2. Temporal relationship between phosphorylated STAT3 and cleaved CASP3 in granulosa cells of SFs indicates that an increase in STAT3 signalling may be involved in apoptosis during follicular atresia. We conclude that FSH treatment rescued granulosa cells of co-DF2 through inhibition of STAT3 pathways. Further studies are required to delineate the mechanisms by which declining FSH concentrations result in increased LIF–STAT3 signalling in granulosa cells of SFs. It would also be interesting to study the potential contribution of STAT3 in ovarian dysfunction in lactating dairy cows.

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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