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Changes in the expression of FoxO1 and death ligand genes during follicular atresia in porcine ovary

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ABSTRACT. Follicular atresia, a key phenomenon in follicle development, eliminates most of the follicles in mammalian ovaries. To investigate the molecular mechanism of follicular atresia in porcine ovaries, we investigated the mRNA expression of three important cell death ligand-receptor systems and Fox O1 in follicles with a diameter of 3-5 mm. The phosphorylation and subcellular localization of Fox O1 during granulosa cell apoptosis was also determined. TRAIL and Fas L played an important role in follicular atresia at this stage. Fox O1 expression was upregulated during atresia, and was confined to the nucleus of granulosa cells; however, phosphorylated Fox O1 was localized to the cytoplasm. These results suggest Fox O1 involvement in the regulation of TRAIL and Fas L expression during follicular atresia in pigs.

Key words: Follicular atresia; Apoptosis; Fox O1

INTRODUCTION

In mammalian ovaries, more than 99% of follicles disappear during follicular growth and development in a phenomenon known as 'follicular atresia'; thus, only a limited number of follicles develop to ovulation (Takagi et al., 2007). Many researchers have investigated the molecular mechanism underlying atresia. Factors such as reproductive hormones, growth hormones, cell adherence molecules, and apoptosis-related genes have been reported to be involved in atresia (Kaipia and Hsueh, 1997; Hussein, 2005; Matsuda-Minehata et al., 2006). However, follicular atresia is thought to be a complex physiological process, and follicles at different stages of development may undergo atresia as a result of different mechanisms (Alonso-Pozos et al., 2003). The exact mechanism by which follicular atresia is regulated remains unknown.

Granulosa cell apoptosis has been suggested to be the trigger for follicular atresia (Grotowski et al., 1997; Rolaki et al., 2005), and investigations of the mechanism of follicular atresia regulation in porcine ovaries have been conducted. Some death ligand-receptor systems have been reported to be involved in the regulation of granulosa cell apoptosis during follicular atresia in porcine ovaries (Manabe et al., 2004, 2008). TNF, TRAIL, and Fas L, with their respective receptors, represent three important death ligand-receptor systems. Whether these work together or alone, and which plays the dominant role in the regulation of granulosa cell apoptosis during different phases of follicular atresia, remain to be elucidated.

Fox O1 is a transcription factor implicated in a number of physiological processes, including apoptosis, cell cycle progression, and signaling (Schmidt et al., 2002; Neufeld, 2003; Alikhani et al., 2005; McLoughlin et al., 2009). Three conserved residues (threonine 24, serine 256, and serine 319) are closely correlated with the phosphorylation and dephosphorylation of Fox O1. The PI3K/Akt signaling pathway is crucial for the regulation of Fox O1 phosphorylation. Akt directly phosphorylates Fox O1, which then binds to 14-3-3 proteins, which facilitate the translocation of Fox O1 from the nucleus to the cytoplasm (Biggs et al., 1999; Arden and Biggs, 2002; Birkenkamp and Coffer, 2003; Carter and Brunet, 2007). This nuclear exclusion and translocation to the cytoplasm inhibits Fox O1-dependent transcription. Fox O1 also regulates the expression of pro-apoptotic genes such as Fas L, TRAIL, and Bim (Brunet et al., 1999; Modur et al., 2002; Gilley et al., 2003).

In this study, we selected 3-5-mm (in diameter) follicles to determine which death ligand-receptor system(s) plays the dominant role in atresia at this stage. We also investigated the phosphorylation and distribution of Fox O1 in granulosa cells during apoptosis.

MATERIAL AND METHODS

Animals and follicle separation

Porcine ovaries were obtained from mature sows at a local slaughterhouse and transferred to the laboratory as soon as possible in PBS at 37°C. Next, individual pre-ovulatory antral follicles, 3-5 mm in diameter, were dissected from the ovaries under a surgical dissecting microscope (SZ40; Olympus, Tokyo, Japan) with small scissors and forceps. The follicles were placed in PBS in a plastic dish.

Morphological observation

Each follicle was classified as healthy (H), early atretic (EA), or progressively atretic (PA) according to previous studies. Briefly, the H follicles were round with a continuous membrane around the granulosa cells, a fixed and visible cumulus-oocyte complex (COC), and clear follicular fluid. The EA follicles could still have a visible COC but with gaps in the granulosa cell membrane, and turbid follicular fluid. The PA follicles lacked a COC or had a COC in the follicular fluid with a dark floccule.

Real-time PCR

Total RNA from each follicle was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA), and reverse-transcription reactions were performed with MLV according to the manufacturer protocol. Real-time PCR was performed using SYBR Premix Ex Taq in a reaction volume of 20 μL. Twenty follicles per group were used. The primer sequences are shown in Table 1.

Table 1. Primers for real-time PCR.

Gene	NCBI entry number	Primer sequence	Product (bp)	Annealing temperature (°C)
GAPDH	AF017079	F: GATGG TGAAG GTCGG AGTG R: CGAAG TTGTC ATGGA TGACC	500	58
TNF	X57321	F: ACG CTC TTC TGC CTA CTG C R: TCC CTC GGC TTT GAC ATT	162	58
TRAIL	AY639873	F: TAA TTG GCT AAA TGA TCT GC R: GCC TTA ACC TAT TGG CTC T	101	53
Fas L	AY033634	F: CTCAA GATCC ATCCC TCTGG R: TCATC TTTCC CTCCA TCAGC	227	58
Fox O1	NM_214014	F: TTCGGTCATGCCAGCCTAC R: TCGCTCAGCCTGACACCC	470	60

Immunocytochemistry

Granulosa cells were collected from follicles and subjected to immunofluorescence staining. Briefly, the cells were fixed, permeabilized, and blocked, followed by incubation with anti-Fox O1 antibodies (Epitomics) overnight. The cells were then rinsed and incubated with anti-rabbit IgG (Cy3-labeled). After counterstaining with DAPI, the cells were observed under a fluorescence microscope.

RESULTS

Morphological features of follicular atresia

The H follicles were red and round with abundant blood vessels: the membranes of the granulosa cells were continuous and tight. The EA follicles appeared pink with fewer blood vessels: the granulosa cells became apoptotic and fell into the follicular antrum, and the follicular fluid was slightly turbid. The PA follicles were white: the mass of granulosa cells were apoptotic and fell into the follicular cavity, the follicular fluid became very turbid, and floccules could be seen (Figure 1).

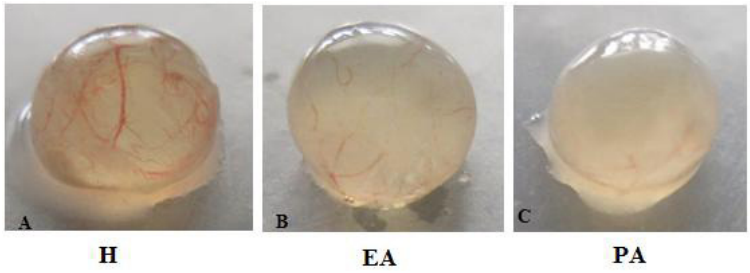

Figure 1. Features of follicular atresia in pigs. H = healthy; EA = early atretic; PA = progressively atretic.

Expression of cell death ligand genes during atresia

TRAIL, Fas L, TNF, and Fas gene expression was quantified by real-time PCR in both H and atretic follicles (Figure 2). TRAIL, Fas L, and Fas gene expression was significantly increased during the early stage of follicular atresia.

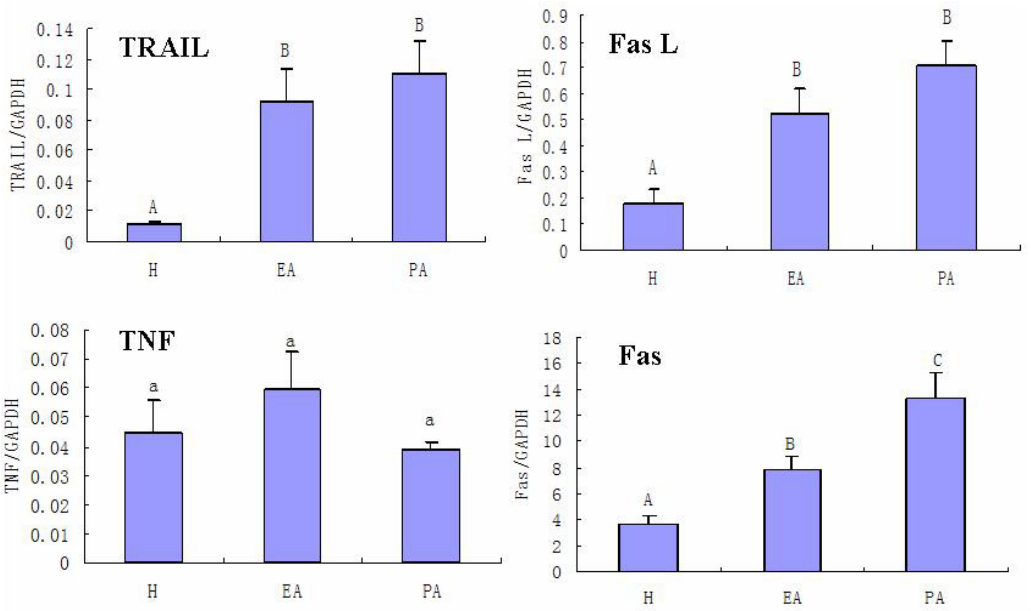

Figure 2. Gene expression during follicular atresia. Apoptotic pathway gene expression was detected by real-time PCR. Total RNA from 20 follicles per group was extracted. TRAIL, Fas L, and Fas expression was increased significantly during follicular atresia in porcine ovary. Different uppercase letters mean extremely significant difference ($P < 0.01$). The same lowercase letters indicate the difference was not significant ($P > 0.05$).

Variation in gene expression in H 3- to 5-mm follicles

Since the expression of TRAIL, Fas L, and Fas increased during porcine follicular atresia, we further analyzed their expression in H follicles. The expression of these genes in some H follicles reached or exceeded the mean in the early or late follicular atresia groups (Figure 3).

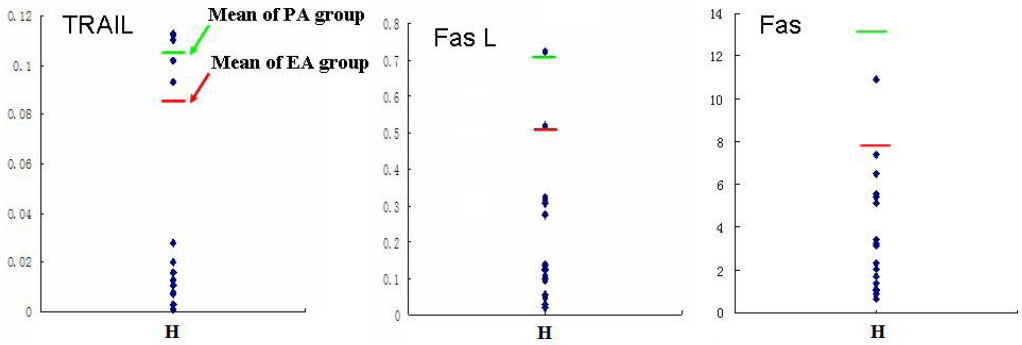

Figure 3. Gene expression variance in healthy (H) follicles. TRAIL, Fas L, and Fas gene expression was quantified by real-time PCR. The expression in each H follicle was compared with that in early atretic (EA) and progressively atretic (PA) follicles. The mean values of the EA and PA follicles are indicated by red and green lines, respectively.

Fox O1 expression during follicular atresia

TRAIL, Fas L, and Fas expression is regulated by the transcription factor Fox O1; thus, the Fox O1 mRNA levels in H, EA, and PA follicles were quantified by real-time PCR. Fox O1 mRNA expression increased during follicular atresia ($P < 0.05$, Figure 4).

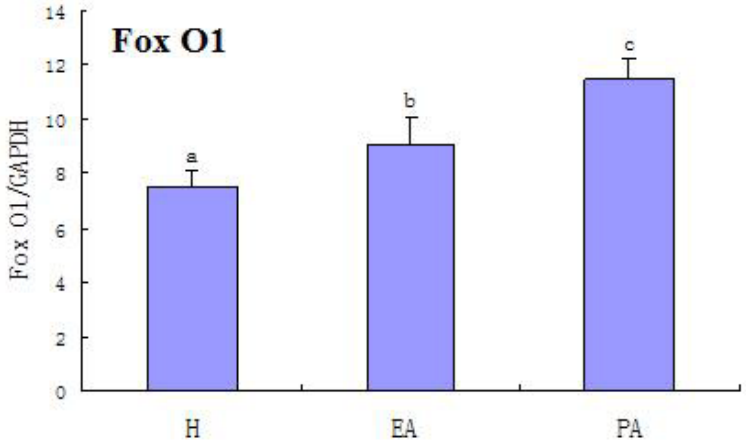

Figure 4. Fox O1 gene expression during follicular atresia. Fox O1 gene expression was detected by real-time PCR as mentioned above. For abbreviations, see legend to Figure 1.

Location of Fox O1 in porcine granulosa cells

Immunocytochemistry was performed to investigate the correlation between Fox O1 protein intracellular localization and its phosphorylation in pig granulosa cells. Unphosphorylated Fox O1 protein was located mainly in the nuclei of granulosa cells, and was translocated to the cytoplasm after phosphorylation (Figure 5).

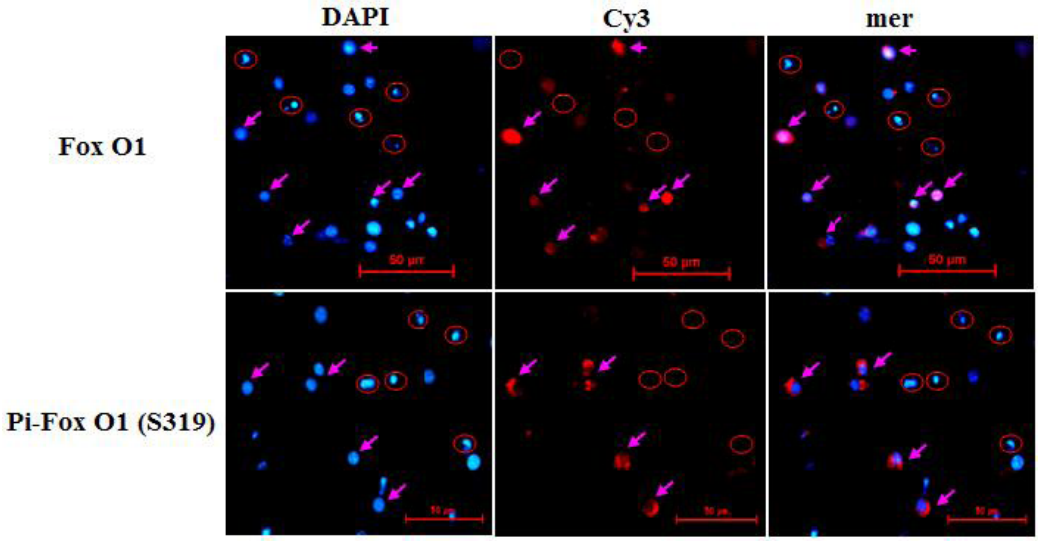

Figure 5. Intracellular location of Fox O1 in pig granulosa cells. Immunofluorescence cytochemistry was used to determine the subcellular localization of Fox O1. DAPI was used to stain the DNA. Fox O1 was expressed in the nuclei of some granulosa cells (top, arrows); however, it was not detected in apoptotic bodies (top, circled). Fox O1 phosphorylation occurs only in healthy granulosa cells, and phosphorylated Fox O1 is translocated to the cytoplasm (bottom, arrows). Fox O1 phosphorylation was not detected in apoptotic bodies (bottom, circled).

DISCUSSION

Most porcine ovarian follicles undergo atresia before reaching 6 mm in diameter (Manabe et al., 2004), so the mechanism of atresia within follicles 3-5 mm in diameter is representative. There are about five million primordial follicles in pigs ten days after birth, of which at most 1600 develop to ovulation during the breeding period (Manabe et al., 2004). The vast majority of follicles undergo atresia at various stages of development. Follicular atresia in pigs is regulated by many factors (Kimura et al., 1999; Wada et al., 2002; Matsuda-Minehata et al., 2005; Maeda et al., 2007; Cheng et al., 2005, 2008; Matsuda et al., 2008; Sugimoto et al., 2010). Manabe et al. (2004) summarized five apoptosis signaling pathways and emphasized the importance of three cell death ligand-receptor systems, TNF-TNFR, Fas L-Fas, and TRAIL-TRAILR. Different stages of follicular atresia may have different regulatory mechanisms (Alonso-Pozos et al., 2003), and which system plays the most important role in the middle atresia of follicles with a diameter of 3-5 mm is not known. Therefore, we investigated the expression of the three aforementioned death ligand genes during atresia. Our results suggest that the TRAIL and Fas L, but not TNF, pathways play an important role at this stage. TRAIL, Fas L, and Fas gene expression in EA follicles was significantly increased and remained high during late-stage atresia. Therefore, these two pathways play an important regulatory role not only in the initiation of follicular atresia, but also in its further promotion.

Analysis of variance of gene expression in the healthy group indicated that Fas and TRAIL expression in some H follicles met or exceeded that in the EA or PA follicles. This implied that abnormal gene expression may be an early event in follicle atresia and that gene

expression abnormalities may precede the appearance of features of follicular atresia, because healthy follicles were confirmed by morphology in this research. Moreover, this suggests that follicles with increased expression of apoptosis pathway genes may undergo atresia in the future.

The transcription factor Fox O1 remains in the nucleus and stimulates the expression of genes such as Fas L, TRAIL, and Bim, so as to inhibit cell proliferation and promote apoptosis (Brunet et al., 1999; Modur et al., 2002; Carter and Brunet, 2007). Our data showed that the atresia of porcine follicles 3-5 mm in diameter is regulated mainly by the Fas L and TRAIL pathways. Thus, we conjectured that Fox O1 could be involved in follicular atresia by the regulation of Fas L and TRAIL expression. Indeed, Fox O1 expression was significantly increased during follicular atresia. Furthermore, we investigated the phosphorylation and intracellular distribution of Fox O1 in granulosa cells. Fox O1 protein was detected in the nucleus of granulosa cells, but phosphorylated Fox O1 was localized to the cytoplasm. Fox O1, acting as a transcription factor, may contribute directly to the increased Fas L and TRAIL expression during follicular atresia. Analysis of the 5'-regulatory sequence of the porcine TRAIL gene revealed a Fox O1 binding site (Furuyama et al., 2000), with the sequence 'TTGTTTAC', at -77 bp. This further confirms our hypothesis. However, final confirmation of the direct regulation of TRAIL expression by Fox O1 requires further direct experimental evidence, such as by CHIP analysis.

CONCLUSIONS

Follicles 3-5 mm in diameter undergo atresia mainly through the TRAIL and Fas L pathways in pigs. The patterns of Fox O1 gene expression and intracellular localization observed in this study indicate the involvement of this transcription factor in the regulation of follicular atresia.

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