—Original Article—

Changes in the Expression of Decoy Receptor 3 in Granulosa Cells During Follicular Atresia in Porcine Ovaries

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Abstract. During follicular development in mammalian ovaries, the majority of follicles undergo atresia. One of the characteristics of this process is apoptotic cell death in granulosa cells. Several death ligands and receptors, including Fas ligand (FasL) and Fas, have been detected in ovarian follicles and also demonstrated to be capable of inducing apoptosis in follicular cells. Decoy receptor 3 (DcR3) competes with Fas to bind FasL but lacks intracellular death domains, thus inhibiting the induction of apoptosis by the FasL/Fas system. In the present study, we examined the expression of putative porcine DcR3 (pDcR3) mRNA in porcine ovarian follicles. Total RNA was extracted from granulosa cells and thecal layer cells of tertiary follicles at healthy, early atretic and progressed atretic stages, and the expression of pDcR3 mRNA was demonstrated by reverse transcription-polymerase chain reaction (RT-PCR). The nucleic acid sequence in the coding region had 80% homology to that of human DcR3, and the deduced amino acid sequence was 73% identical to that of human DcR3. In an *in situ* hybridization experiment, pDcR3 mRNA expression was confirmed in granulosa and thecal layers, in both healthy and atretic follicles. Quantitative real time RT-PCR analysis showed that the expression of pDcR3 mRNA was weaker in granulosa cells of atretic follicles than those of healthy follicles. No notable changes were seen in the thecal layer cells. These results suggest that DcR3 plays a significant role in the regulation of apoptosis in granulosa cells, but not in thecal layer cells, during atresia.

Key words: Apoptosis, Decoy receptor-3 (DcR3), Follicular atresia, Granulosa cell, Porcine ovary

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In mammalian ovaries, the majority of follicles undergo a degenerative process known as atresia during follicular growth and maturation. Atretic follicles can be identified by their morphology, typically karyopyknosis in granulosa cells [1, 2]. Karyopyknosis is, however, also a typical morphological characteristic of apoptotic cells [3, 4]. Earlier studies [5, 6] have shown that degeneration of granulosa cells in atretic follicles is also associated with oligonucleosomal fragmentation of DNA, a biochemical hallmark of apoptosis, in agreement with the morphological observations. Our previous studies [7–11] demonstrated that porcine granulosa cells undergo apoptosis during follicular atresia through the histochemical and biochemical detection of DNA fragmentation and by electron microscopy. In antral follicles, apoptotic cells occurred at the inner surface of the granulosa layer at an early stage of follicular atresia and then diffused in the granulosa layer as atresia advanced. In thecal layer of atretic follicles, apoptotic cells were observed but at a low frequency compared with that in the granulosa layer.

Although it has been demonstrated that apoptotic cell death plays a significant role in ovarian follicular atresia, the mechanisms regulating apoptosis in granulosa cells are largely unclear. Apoptosis is classified into two types according to signal transduction

pathways; one is membrane ligand/receptor-dependent, and the other is ligand/receptor and mitochondrion-dependent [12]. Ligand/receptor-dependent apoptosis is triggered by the association of death ligands with death receptors that are members of the tumor necrosis factor (TNF) superfamily and TNF receptor (TNFR) superfamily, respectively. To elucidate the contribution of TNF and TNFR superfamily proteins to porcine follicular atresia, we searched for porcine homologues of human death receptors in the expressed sequence tagged (EST) database and found a series of sequences whose deduced amino acid sequences showed homology to human decoy receptor 3 (DcR3, also known as TR6). The decoy receptors, which are also members of the TNFR superfamily, compete with the cell death receptors to bind the endogenous ligands, but lack an intracellular death domain (DD), thus modulating apoptotic signal transduction through the cell death receptors. DcR3 is one of the decoy receptors found in human carcinoma cells, that binds with Fas ligand (FasL, also known as Apo-1 ligand and CD95 ligand) and can inhibit the induction of apoptosis by the Fas FasL/Fas system, a representative death ligand and a death receptor system [13–16]. A broad range of human tumor cells have been reported to express DcR3 at high levels, and it is supposed that certain tumors may escape FasL/Fas-dependent immune-cytotoxic attack by expressing DcR3 [13, 14, 17–21]. DcR3 is also shown to bind with LIGHT (homologous to lymphotoxins, exhibits inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes) and TNF ligand-related molecule 1A (TL1A) and modulates their sig-

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nals [16, 22–25]. Although DcR3 mRNA has been detected in the chicken [26] and DcR3 has been predicted to exist in the chimpanzee and bovine genomic sequences, there has been no report on other mammalian orthologs of human DcR3 to date. Its intrinsic function, therefore, has not been wholly clarified.

In the present study, we identified the putative porcine DcR3 and examined changes in the expression and localization of DcR3 in porcine ovarian follicles during atresia by reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization, respectively.

Materials and Methods

Tissue collection

As previously reported [27], ovaries obtained from gilts at a local slaughterhouse were used. For the isolation of total RNA, tertiary follicles (3–4 mm in diameter) were dissected out using scalpels and classified into the healthy, early atretic and progressed atretic stages based on their morphology under a dissection microscope. The primary feature of atretic follicles is the presence of cellular debris in granulosa cells, since it correlates with neutral $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease activity [7] that catalyzes the internucleosomal cleavage of cellular DNA, which is characteristic of apoptosis. The granulosa layer and thecal layer were recovered from follicles at each stage by physical dissection, and total RNA was extracted from each sample using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). For preparation of histological sections, ovarian tissue pieces were frozen in 2-methyl butane, chilled with dry ice and kept at -80°C .

EST cloning, RT-PCR, 3'- and 5'-rapid amplification of cDNA ends (RACE), and sequencing

A partial sequence of putative porcine DcR3 (pDcR3) was initially identified during a homology search in the EST database at the National Center for Biotechnology Information (US National Library of Medicine) using the amino acid sequence for human death receptor 6 (DR6) as a query. The EST sequence (GenBank accession number: CF788807) was about 30% identical to human DR6. Another two EST sequences (CF361570 and CF362870) overlapping with the first sequence were found. These EST sequences were assembled, and an open reading frame (ORF) found in the assembled nucleotide sequence was transcribed into an amino acid sequence. There were six discrepant or undetermined nucleotides in the aligned EST sequences. Therefore, the deduced amino acid sequence contained two undetermined residues. A BLAST search revealed that this amino acid sequence had similarity to human DcR3.

The expression of pDcR3 in granulosa and thecal cells was examined by RT-PCR. The primers for RT-PCR, which were designed based on the sequence of pDcR3 using Primer3 [28], were as follows: 5'- CCA CTA CAC GCA GTT CTG GA-3', forward, and 5'- TGG AGG ACA AAT CCT GGA AA -3', reverse, with an expected PCR product size of 461 bp and representing the region from amino acid 72 to 224 of pDcR3. As templates, cDNAs were synthesized from total RNAs using a T-Primed First-Strand Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). PCR was

performed using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) with a PCRx Enhancer System (Invitrogen) in a thermal cycler (GeneAmp PCR Systems 9700; PE Applied Biosystems, Foster City, CA, USA). The hot-start PCR cycle was as follows: 2 min at 95°C , followed by 35 cycles of 30 sec at 95°C , 30 sec at 55°C and 60 sec at 68°C , and then a final extension period of 7 min at 68°C . To confirm the specific amplification of fragments of the expected size, each PCR product was electrophoresed in a 2% (w/v) agarose gel (Sigma Aldrich Chemicals, St. Louis, MO, USA). The Ready-Load 100-bp DNA Ladder Marker (Gibco BRL, Grand Island, NY, USA) was used for electrophoresis. A representative PCR product was sequenced using an automatic DNA sequencer (ABI PRISM 310; PE Applied Biosystems) according to the manufacturer's instructions.

To obtain the full-length mRNA/cDNA sequence, 3'- and 5'-RACE experiments were performed on RNA from granulosa cells using the 3'-RACE System for Rapid Amplification of cDNA Ends (Invitrogen) and 5'-RACE system, Version 2.0 (Invitrogen), respectively, according to the manufacturer's instructions. To overcome problems with in dC-tailing in 5'-RACE experiments, 10% (v/v) dimethyl sulfoxide (DMSO) was added to the reaction mixture. In PCR amplification, the PCRx Enhancer System was used at variable strength. Amplified fragments were analyzed by 2% (w/v) agarose gel electrophoresis and sequenced. The gene-specific primers used in the 3'- and 5'-RACE experiments were as follows: 5'- CCA CTA CAC GCA GTT CTG GA -3', 3'-RACE; 5'- TGG AGG ACA AAT CCT GGA AA -3', first round 5'-RACE RT: 5'- CTC GAG CTG CTG TCT GAA AA -3', first round 5'-RACE PCR: 5'- TCC AGA ACT GCG TGT AGT GG -3', second round 5'-RACE RT: 5'- CCA CAC ACC AGC CAC TCC -3', second round 5'-RACE PCR; and 5'- GGG TAG GTG GGT GCG TTC -3', second round 5'-RACE nested PCR. Sequences obtained in these experiments were analyzed using Genetyx-Mac, Genetyx-Mac/ATSQ and ClustalX (Software Development, Tokyo, Japan).

In situ hybridization

To reveal the localization of pDcR3 mRNA in the ovary, *in situ* hybridization was performed on frozen sections of porcine ovarian tissue using digoxigenin-labeled cRNA probes. As templates for cRNA probes, pDcR fragments and β -actin fragments were amplified by PCR using Platinum PCR supermix (Invitrogen). The primer pairs for partial cDNA sequences of pDcR3 were as follows: 5'- GGC ACC TTT TCA GAC AGC AG -3', forward, and 5'- TGG AGG ACA AAT CCT GGA AA -3', reverse, with an expected PCR product size of 223 bp. Those for β -actin were 5'- TCC CTG GAG AAG AGC TAC GA -3', forward, and 5'- ACA TCT GCT GGA AGG TGG AC -3', reverse, with an expected PCR product size of 368 bp. The conditions for amplification were as follows: denaturation at 94°C for 2 min and then 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. Amplified fragments were cloned into pDR-TOPO TA-cloning vector (Invitrogen) and reamplified using T7 promoter primer and each of the primers for amplification of pDcR and β -actin to prepare templates for sense and antisense probes. Digoxigenin (DIG)-labeled cRNA probes were synthesized by *in vitro* transcription using a DIG RNA Labelling Kit (Roche Diagnostics, Rotkreuz, Switzerland).

land). Tissue processing, hybridization and visualization were performed according to the manufacturer's directions. Frozen ovarian tissue pieces were cryosectioned at a thickness of 8 μ m and mounted on aminopropyltriethoxysilane-coated glass slides (Matsunami Glass, Osaka, Japan). Sections were soaked in xylene for 5 min to remove lipids and then air-dried. They were fixed with 4% (v/v) neutral buffered formalin (Wako, Osaka, Japan) for 7 min, washed with PBS and washed in 2 $\times$ standard saline citrate (SSC; Wako). The slides were prehybridized with 50% (v/v) formamide hybridization cocktail (Amresco, Solon, OH, USA) for 1 h at 37 C. Hybridization (probe concentration, 100 ng/ml) was performed at 37 C overnight. The sections were washed with 2 $\times$ SSC three times for 5 min each, 0.2 $\times$ SSC four times for 5 min each and 2 $\times$ SSC three times for 5 min each at 37 C and then equilibrated with 100 mM Tris-HCl, pH 7.5, containing 150 mM NaCl (THS). Sections were then treated with blocking solution (Roche) for 1 h at room temperature (22–25 C), incubated with alkaline phosphatase-conjugated sheep anti-DIG antibody (diluted 1:200 with blocking solution; Roche) for 2 h at room temperature, washed with THS, and then incubated with a 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT) solution (BCIP/NBT substrate system; Dako Cytomation, Glostrup, Denmark) containing 0.1 mM levamisole (Dako Cytomation). The reaction was allowed to develop for 6.5 h. The slides were washed in distilled water, lightly counterstained with methyl green, mounted in Ultramount (Dako Cytomation) and then examined under a light microscope.

Quantitative real time RT-PCR

Changes in pDcR3 mRNA/cDNA were examined by quantitative real-time PCR using a LightCycler system (Roche), according to the manufacturer's instructions. As an internal control, porcine β -actin mRNA/cDNA was also quantified. Reactions were performed in a 20 μ l reaction mixture (LightCycler Faststart DNA Master SYBR Green I; Roche) containing 0.5 mM of each primer, nucleotides, Taq DNA Polymerase and $MgCl_2$, whose optimized concentrations were 3 mM. DMSO 8% (v/v) was added to the reaction mixture for amplification of pDcR3. The primer pairs for partial cDNA sequences of pDcR3 and β -actin were the same as those used in the amplification of templates for cRNA probes for *in situ* hybridization. The conditions for amplification were as follows: denaturation at 95 C for 10 min and then 45 cycles of denaturation at 95 C for 15 sec, annealing at 61 C for 5 sec and extension at 72 C for 9 sec for DcR3 and 95 C for 10 min, then 40 cycles of 95 C for 15 sec, 55 C for 5 sec and 72 C for 9 sec for β -actin. Quantification was performed using the LightCycler analysis software (Roche) on an IBM computer.

Statistical analysis

Results of quantitative RT-PCR were analyzed by one-way ANOVA and Scheffé's multiple comparison using StatView J-4.5 (Abacus Concepts, Berkeley, CA, USA) on a Macintosh computer. Differences at $P < 0.05$ were considered significant.

Fig. 1. (a): RT-PCR analysis of pDcR3 mRNA expression in granulosa and theca layer cells. Fragments of the expected size (461 bp) were amplified in all groups. Lane 1: granulosa cells from healthy follicles. Lane 2: granulosa cells from early atretic follicles. Lane 3: granulosa cells from progressed atretic follicles. Lane 4: theca layer from healthy follicles. Lane 5: theca layer from early atretic follicles. Lane 6: theca layer from progressed atretic follicles. Lane 7: 100-bp ladder marker. (b): Quantitative analysis of pDcR3 mRNA expression in granulosa and theca layer cells by real-time RT-PCR. The expression level of pDcR3 was normalized to that of β -actin and presented as a percentage of that in the granulosa layer from healthy follicles. Results are expressed as the average $\pm$ SD of four pairs of measurements.

Results

Nucleic acid sequence and deduced amino acid sequence of pDcR3

RT-PCR amplification, fragments of the expected size in all groups (Fig. 1a), and the nucleic acid sequence of the fragments corresponded with that found in the EST database. The nucleic acid sequence of pDcR3 mRNA/cDNA obtained by the 3'- and 5'-RACE experiments spanned approximately 1.4 kb and contained an ORF for pDcR3 with a starting ATG at position 395 and a stop codon at nucleotide position 1,292 (Fig. 2). The similarity of pDcR3 to the human DcR3 variant M68C (GenBank accession number: NM 032945), which has a longer 5'-UTR than the variant M68E, was about 80% within the ORF, whereas that in the 5'- and 3'- untranslated region (UTR) was only 50%. The cDNA had a polyadenylation signal beginning at position 1,394.

The deduced amino acid sequence of pDcR3 (299 amino acid residues) was 73% identical (79% similar) to that of human DcR3 (GenBank accession number: NP 003814) and 47% identical (63% similar) to that of chicken DcR3 (GenBank accession number: XP 417434, Fig. 3). The predicted chimpanzee DcR3 protein (GenBank accession number: XP 514789) consisted of 220 amino acid residues and showed high similarity to human DcR3 in the C-terminal region, but cysteine-rich repeats were not conserved. High

1	cccg tgttactcaggggac agggctttaataaaag ttgctggcactacag	49
50	gggtggacggggtct gtgtggcctgggcct ggtggtggggttga gggcgccagagagga	109
110	tggggccttttgggca gtctgtctccgacct ggtttcaggggaggg gactccagcgctct	169
170	gtcccaactttgtcca gaagaggggggggcg ccggggccccccag gaccgctctggggag	229
230	aagggggcatccctt gggacaaagcccgt ctccccgccccgc cccccaggcaggga	289
290	gttgctgccccgggc ggggaggggaggggc tccgcggggcggtg ccagcccggccctta	349
350	taggacgcgcggtcc ggccgcgcgcgcgc cgcagcagccagacc atgagggccctccg	409
	M R A P P	
410	tggcgtggtggcgtg ccgctgcccgcctg ctgctggcggttcaca gcgcgcgggagggcg	469
	W P W P W P L P A L L L A F T A R G E A	
470	gcgaacgcacccacc tacccgtggcgggac gcggagaccggggag tggctggtgtgtggc	529
	A N A P T Y P W R D A E T G E W L V C G	
530	cagtgcgcgcgggc accttcgtgcggcgg ccttggtggcccgac agccccacgacgtgc	589
	Q C P P G T F V R R P C G P D S P T T C	
590	gacgcgtgcccgcgc gcgcactacacgcag ttctggaactacctg gagcgtgcccgtac	649
	D A C P P R H Y T Q F W N Y L E R C R Y	
650	tgcaacgtcttctgc ggggagcgcgaggag gaggcgcggccgtgc ggggccaccacaaac	709
	C N V F C G E R E E E A R P C G A T H N	
710	cgcgtctgcccgtgt gcggcggtcttcttc gcgcgtgcggccttc tgctggagcacgcg	769
	R V C R C R P G F F A H A G F C L E H A	
770	ccctgcccgcctggc gccggcgtggcgccc ctgggcacccccagc cagaacacgcagtgc	829
	P C P P G A G V A A L G T P S Q N T Q C	
830	caggcgtgccccccg ggcaccttttcagac agcagctcgagttcg gagcagtgcggccc	889
	Q A C P P G T F S D S S S S S E Q C R P	
890	caccgcaactgcacg gccgtgggcctggcc gtcaacgtgcccggc tcctccttccacgac	949
	H R N C T A V G L A V N V P G S S F H D	
950	gccctgtgcaccaac tgcacgggcttccca ctggctcgtctggag cggggggcgccgggg	1009
	A L C T N C T G F P L G S L E R G A P G	
1010	accgaggagtgcgag cgcgctgtcgtcgac ttctgtagctttccag gatttgcctccaag	1069
	T E E C E R A V V D F V A F Q D L S S K	
1070	aggctcctgcggctg cagcaggccctcgcc ggcccaggggcgctg agtccgcgcgcgcg	1129
	R L L R L Q Q A L A G P G P L S P P P P	
1130	agggaggaccgcgcg gccctgcagctcagg ctgctgcgacagctc acggagctccgtgag	1189
	R E D R A A L Q L R L L R Q L T E L R E	
1190	gcgcggccccggggcg ctgggtggcgcggtg ctgccggcgctgcgc acggccaggctgcgc	1249
	A R P G A L V A R L L P A L R T A R L P	
1250	gggctggagcgcgga gtccgcgcgcgcttc ctccgggcgcgctga gcgcctccccgtta	1309
	G L E R G V R A R F L R A R *	
1310	tttatttcgcgccccg tcccagcgcgccctg tgccgcacgaagga ggcttctgccccggc	1369
1370	tcgggcggcgctaac tttatttttataaag ccgattcctaagga aaaaaaaaaa	1427

Fig. 2. Nucleic acid and deduced amino acid sequences of pDeR3. The asterisk at the end of the deduced amino acid sequence indicates the stop codon. A putative polyadenylation signal is underlined.

Fig. 3. Deduced amino acid sequence of pDcR3 aligned with the human and chicken DcR3 sequences. Dark boxes indicate identical amino acid residues; lightly shaded boxes represent similar amino acid residues. Cysteine residues (asterisks) within four TNFR cysteine-rich repeats (TNFR-Cys 1 to 4) reported in human DcR3 (Swiss-Prot: O95407) were conserved. The signal peptide region in each amino acid sequence is indicated with a dotted underline. The putative signal peptide cleavage site in pDcR3 was predicted using the GENETYX-MAC software.

similarity (>90%) was noted between the porcine and human sequences at amino acid residues corresponding to the four cysteine-rich repeats in human DcR3, and cysteine residues in these domains were conserved in pDcR3. Another ORF with a starting ATG at position 109 and a stop codon at nucleotide position 1,183 was found in the pDcR3 mRNA/cDNA. Part of the deduced amino acid sequence in the frame of the latter ORF showed moderate (43% identical, 47% positive) homology to human regulator of telomere elongation helicase 1 (RTEL1) isoform 2 (GenBank accession number: NP 116575).

In situ hybridization experiments revealed the expression of pDcR3 mRNA in the granulosa and thecal layers of tertiary follicles (g and t in Fig. 4a, respectively). Expression was observed in granulosa layers of both healthy and atretic follicles (H and A in Fig. 4a, respectively), and no difference in expression level between healthy and atretic follicles was evident by *in situ* hybridization, which is not suitable for quantitative analysis.

As summarized in Fig. 1b, the normalized expression levels of pDcR3 mRNA were lower in granulosa cells prepared from early atretic and progressed atretic follicles than in those prepared from

healthy follicles ($P<0.05$), whereas there was no notable difference in levels in the thecal layer cells between healthy and atretic follicles. Expression levels in thecal layer cells were lower than those in granulosa cells from healthy follicles ($P<0.05$) and similar to those in granulosa cells from atretic follicles.

Discussion

In the present study, pDcR3 mRNA expression in ovarian follicles was demonstrated. Analysis of the mRNA/cDNA sequence of pDcR3 revealed an alternative out-of-frame ORF starting upstream of the start codon for pDcR3. Several factors affecting the selection of a translation initiation site have been reported. The presence of a Kozak context is one of them. The nucleic acid sequence around the start codon of pDcR3 is a sub-optimal Kozak context (ACCATGA) identical to that in human DcR3, while the sequence around the start codon of the alternative ORF is an optimal Kozak context (AGGATGG). The variant M68C also has a start codon with a Kozak context (GGCATGG) at position 124, 311 bases upstream of the translation initiation site. It is supposed that there is some bias to select a downstream translation initiation site in human DcR3, and a similar phenomenon possibly occurs in pDcR3. In pDcR3, a stem-loop structure is expected around the start codon of the alternative ORF, which is a cause of the leaky

Fig. 4. Localization of pDcR3 mRNA expression demonstrated by *in situ* hybridization. Ovarian sections were hybridized with pDcR3 antisense probe (a), β -actin antisense probe (c) for a positive control, or pDcR3 sense (b) or β -actin sense (d) probe for a negative control. pDcR3 mRNA was expressed in granulosa layers of both healthy (H) and atretic (A) follicles. g, granulosa layer; t, theca interna. Bars=100 μ m.

scan.

In the human genome, DcR3 overlaps partially with the C-terminal coding region and 3'-UTR of RTEL1 isoform 2. The putative pDcR3 nucleic acid and deduced amino acid sequences showed similarities with those of human RTEL1 isoform 2 in the region overlapping with DcR3, in which the porcine and human DcR3 sequences have high similarity. It is doubtful that the pDcR3 sequence obtained in the present study is a partial sequence of the porcine RTEL1 homologue for two reasons; the stop codon in human RTEL1 isoform 2 was not conserved in the nucleic acid sequence of pDcR3, and the deduced amino acid sequence translated from the pDcR3 nucleic acid sequence in the reading frame corresponding to RTEL1 did not show significant similarity to human RTEL1 in the 5'-upstream region of pDcR3 mRNA/cDNA. It can not be denied that there are some alternatively spliced variants that produce the porcine RTEL1 homologue, which were not detected in the 5'-RACE experiments, and they have affected the quantitative analysis in the present study. For confirmation and further characterization of pDcR, its protein expression should be

proven.

Several death ligands and receptors have been detected in ovarian follicles and have been demonstrated to be capable of inducing apoptosis in follicular cells [10–12]. Our previous studies demonstrated that TNF superfamily and TNFR superfamily proteins are also expressed in porcine ovarian follicles and that they are involved in regulation of survival and apoptosis of granulosa cells [27, 29–32]. Not only death receptors, but also decoy receptors were expressed in porcine ovarian follicles. TNF-related apoptosis-inducing ligand (TRAIL) and its receptors, death receptor-4 (DR4), death receptor-5 (DR5) and decoy receptor-1 (DcR1), were expressed in porcine granulosa cells [29–31], and expression of DcR1 is reduced in granulosa cells in atretic follicles [31]. In addition, removal of DcR1 from the cellular surface sensitizes granulosa cells to TRAIL induced-apoptosis *in vitro* [30].

FasL and Fas has also been considered to participate in apoptosis during follicular atresia in the mammalian ovary, based on their expression in ovarian follicles [27, 33–42] and pro-apoptotic activities of FasL and antibodies to Fas in follicular cells. Increases in

the expression of either or both of Fas and FasL have been observed during follicular atresia in mice [36], rats [37, 42], cattle [43] and pigs [27]. It has been reported that FasL was expressed in porcine granulosa cells and that its expression level and the percentage of apoptotic cells were increased by the effect of haem oxygenase, whose expression was increased in atretic follicles [44]. Fas and FasL are also expressed in normal follicles, although at lower levels compared with atretic follicles [27]. In addition, Fas-mediated apoptosis is potentiated by cycloheximide in murine granulosa cells [45]. These findings suggest that follicular cells have certain inhibitors of Fas-mediated apoptosis. There are several reports on anti-apoptotic factors in ovarian follicles, including Fas-associated phosphatase (FAP-1) [45], members of the inhibitor of apoptosis protein (IAP) family [46–49] and cellular FLICE-like inhibitory protein (c-FLIP) [50–58]. Their participation in the apoptosis of granulosa cells and follicular atresia has been suggested, although their significance in the regulation of follicular atresia *in vivo* remains to be clarified.

In the present study, the expression level of pDcR3 mRNA in granulosa and thecal layers in both healthy and atretic follicles was demonstrated by RT-PCR and *in situ* hybridization experiments. Quantitative RT-PCR analysis showed the expression level to be lower in granulosa cells prepared from atretic follicles than in those prepared from healthy follicles, but no notable changes were seen in the thecal layer cells. These results suggest that DcR3 plays a significant role in the regulation of apoptosis in granulosa cells, but not in thecal layer cells, during follicular atresia. The expression of the DcR3 homologue in the ovary has been reported in chickens and Northern blotting showed increased expression of DcR3 in atretic follicles [59]. The granulosa layer and thecal layer were not separated in that study, and so, the results cannot be directly compared to our results. DcR3 can inhibit Fas-mediated apoptosis, therefore, pDcR3 would play a significant role in the regulation of apoptosis relevant to follicular atresia in porcine granulosa cells. Although the expression of pDcR3 mRNA in the granulosa layer decreased in atretic follicles, the levels were still detectable. It is possible that some death ligand/receptor systems other than the FasL/Fas system [27], presumably the TRAIL/DR4 and DR5 systems [29–31], but not the TNF α /TNFR2 system [12, 32], are the main inducers of the receptor-mediated apoptosis of granulosa cells during follicular atresia in porcine ovaries.

In another set of *in situ* hybridization experiments, positive signals were observed in corpora lutea, but non-specific signals were also noted in this region. The FasL/Fas system plays a central role in the apoptosis of luteal cells during luteolysis [60]. Our previous study showed that the expression of soluble-Fas (FasB), which antagonizes FasL signaling, regulates luteolysis in murine ovaries [61]. In the ovaries, pDcR3 might inhibit FasL before starting luteolysis in the same manner cFLIP [62]. The expression and function of the FasL/Fas system in luteolysis in porcine ovaries, as well as pDcR3, need to be elucidated.

It has been reported that human DcR3 over-expressed in mice functions as a decoy for endogenous FasL [63], but its intrinsic function has not been clarified since knockout animal experiments have not been carried out. We have not yet revealed the properties of pDcR3 protein; therefore, it is still unclear whether or not pDcR3

has similar properties to human DcR3. If pDcR3 is the functional homologue of human DcR3, clarification its physiological function will be possible by generation and analysis of DcR3 knockout pigs.

In conclusion, DcR3 mRNA was localized in the granulosa and thecal layers of both healthy and atretic follicles in porcine ovarian follicles. Lower levels of DcR3 mRNA were noted in granulosa cells prepared from atretic follicles than in those prepared from healthy follicles, but no notable changes in DcR3 mRNA were seen in the thecal layer cells during atresia. These present results suggest that DcR3 plays a significant role in the regulation of apoptosis in granulosa cells, but not in thecal layer cells, during atresia.

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