Roles of poly (ADP-ribose) polymerase (PARP1) cleavage in the ovaries of fetal, neonatal, and adult pigs

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Abstract

Poly(ADP-ribosylation), which occurs rapidly in cells following DNA damage and is regulated by poly (ADP-ribose) polymerase 1 (PARP1), is a post-translational modification of proteins playing a crucial role in many processes, including DNA repair and cell death. Although PARP1 has recently been implicated in a variety of physiological and pathological processes, its role in the process of follicular development and atresia is not yet completely defined. This study was designed to investigate the cellular expression pattern and immunolocalization of PARP1, cleaved PARP1, caspase 3, and cleaved caspase 3 in fetal, neonatal, and adult porcine ovaries. Our results showed that in fetal and neonatal pigs, PARP1 cleavage is involved in the process of oocyte nest breakdown, primordial follicle formation, and transition to primary follicles. The results of immunohistochemistry indicated that PARP1 cleavage was involved in the process of follicular development and atresia, which was in accordance with our previous study; however, it was noted that cleaved caspase 3 was mainly localized in and around the nucleus of apoptotic granulosa cells (GCs), whereas cleaved PARP1 was mainly localized in the nucleus of the apoptotic GCs. RIA data showed increased serum progesterone and estradiol concentrations with age after birth. Collectively, our findings suggest that the PARP1 signaling pathway is involved in oocyte nest breakdown and primordial follicle formation in fetal and neonatal porcine ovaries, but is different from follicular atresia in adult porcine ovaries that involves cellular apoptosis.

Reproduction (2013) **146** 593–602

Introduction

Female fertility is affected by a variety of environmental, behavioral, and genetic factors that can alter folliculogenesis at various levels (Joffe 2003). These factors include a wide variety of endogenous and exogenous genotoxic agents that may lead to DNA damage and cell death. It was suggested that a germ cell can be lost to death processes before or after it is surrounded by somatic cells so as to form a follicle (Tingen *et al.* 2009a). The death of germ cells preceding follicle formation is termed attrition, whereas the degenerative process of follicle death once germ cells are surrounded by somatic cells or granulosa cells (GCs) is termed follicular atresia (Hsueh *et al.* 1994, Tingen *et al.* 2009b). In the mammalian ovary, each germ cell is either selected to grow and ovulate or be lost to an atretic pathway. In our previous work, we have reported that female reproductive capacity requires a balance between these two fates (Wei & Shi 2013).

Poly (ADP-ribose) polymerase 1 (PARP1) is a 116 kDa protein, the founding member of the PARP family which contains as many as 18 distinct proteins in humans, is

a nuclear, and NAD⁺-dependent enzyme present in eukaryotes (Virag & Szabo 2002, Kim *et al.* 2005). PARP1 can specifically bind and repair DNA-strand breaks generated by several genotoxic agents (Yelamos *et al.* 2011); apart from this, it is also implicated in the regulation of a wide range of important cellular processes including transcriptional regulation, chromatin modification, cellular homeostasis, and cell proliferation and death (Bernstein *et al.* 2002, Hassa 2009, Lu *et al.* 2011).

PARP1 protein has a modular structure composed of at least six domains, four of which have well-defined functions (Langelier *et al.* 2010, Megnin-Chanet *et al.* 2010). It is believed that these PARP1 domains may play roles in a variety of pathological processes leading to cell death, and which are mediated through PARP1 cleavage by suicide proteases including caspase 3. Cleavage of PARP1 then results in exposed structural domains that mediate specific forms of cell death (Kim *et al.* 2005, Luo & Kraus 2012). PARP1 cleavage produces an 89 kDa C-terminal fragment, which always contains the catalytic domain; and a 24 kDa N-terminal fragment (Nguewa *et al.* 2003, Agarwal *et al.* 2009). The 89 kDa C-terminus

fragment retains basal enzymatic activity due to the maintenance of the catalytic domain (Kim *et al.* 2005); however, PARP1 becomes inactive after cleavage, as it fails to recognize DNA damage. As a result, NAD^+ consumption is prevented.

In the porcine ovary, oocyte nest breakdown and primordial follicle formation take place ~ 90 days *post coitum* (90 dpc), and the major stage of transition from primordial follicles into primary follicles was between 90 dpc to 1 day *post partum* (1 dpp), according to our previous study (Ding *et al.* 2010). Ovarian follicle development is characterized by the processes of GC proliferation, differentiation, and apoptosis. Thereby, GCs are crucial for directing normal follicular development and atresia (Fan *et al.* 2008, Wei & Shi 2013); however, the exact signaling pathway is still unclear.

Our previous study demonstrated that cleavage of PARP1 by caspase 3 is strongly implicated in porcine follicular atresia (Wei & Shi 2013), and there is evidence that PARP1 may take part in folliculogenesis (Qian *et al.* 2010); however, the biological mechanisms responsible for follicular atresia are not as well studied as those responsible for earlier germ cell attrition. The aim of this study is to expand our knowledge of PARP1 cleavage signaling pathway during the process of follicular development and atresia in fetal, neonatal, and adult pigs.

Materials and methods

Ethics statement

Animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Nanjing Agricultural University. All pigs were killed in a state of unconsciousness, and then the ovaries were removed.

Reagents

Expression patterns and location of PARP1, PARP1 antibody (cleaved p25), caspase 3, and cleaved-caspase 3 (Asp175) were analyzed by immunohistochemistry (IHC) and immunoblotting. PARP1 antibody H-250 was purchased from Santa Cruz. PARP1 antibody (cleaved p25) is a reagent originally manufactured by Abgent (San Diego, CA, USA). The cleaved-caspase 3 (Asp175) antibody was purchased from Cell Signaling (Boston, MA, USA). Caspase 3 antibody and immunohistochemical kits (SABC method) were purchased from Boshide (Wuhan, China). 3,3-Diaminobenzidine tetra-chloride (DAB) was purchased from Sigma-Aldrich. Estradiol-17 β (E_2) and progesterone (P_4) RIA kits were purchased from Beijing North Biotechnology Institute, China. All other chemicals were purchased reagent grade.

Animals and conditions

Sexually mature gilts, all at 9–10 months of age and with body weights of 100–120 kg, were obtained from Jiangpu Farms of Nanjing Agricultural University (Nanjing, China). The animals

were kept indoors and provided with commercial foods three times per day and tap water *ad libitum*. After exhibiting at least two estrous cycles of normal duration, gilts were observed for estrous behavior twice daily and mated to fertile boars at the onset of estrus and 12 and 24 h later. The gestation day was estimated from the first mating day.

Collection of fetal, neonatal, and adult porcine ovaries

Fetal porcine ovaries from pregnant gilts were obtained by hysterotomy performed under anesthesia with lidocaine on day 90 dpc. Neonatal porcine ovaries from neonatal pups were obtained by ovariectomy performed under anesthesia with lidocaine on 1 dpp. Adult porcine ovaries were obtained from mature sows (150 dpp) at Nanjing Tianhuan Company (Jiangsu province, China), a local slaughterhouse. Before slaughter, pigs are first rendered unconscious using the following means: stunning using electric current applied with electrodes and then hoisted on a rail, after which they are exsanguinated via the carotid artery. After slaughter, the ovaries were removed and carried to the laboratory as soon as possible in physiological saline at 30–35 °C. One ovary was fixed in 4% paraformaldehyde at room temperature for 36 h and then kept in 70% alcohol for IHC; the contralateral ovary was frozen and kept refrigerated at -80 °C.

IHC of fetal, neonatal, and adult porcine ovaries

To examine the locations of PARP1, cleaved PARP 1, caspase 3, and cleaved caspase 3 in porcine ovaries, ovarian sections from fetal, neonatal, and adult pigs were prepared. Immunohistochemical staining was performed with the SABC method using MABs to PARP 1 (diluted 1:200), cleaved PARP 1 (1:300), caspase 3 (1:150), or cleaved caspase 3 (1:400). A few sections were picked randomly from the serial sections, and mounted on slides coated with 3-aminopropyl-triethoxysilane and dried for 24 h at 37 °C. The antibodies were diluted in PBS containing 1% (W/V) BSA, and the sections were incubated overnight at 4 °C with primary antibodies. The specific protein immunoreactivity was visualized with 0.05% DAB in 10 mM PBS containing 0.01% (V/V) H_2O_2 for 2 min, and counterstained with hematoxylin. The negative control was normal rabbit serum instead of primary antibody, and relative levels of immunostaining were evaluated according to our previous study (Ding *et al.* 2010). The images were captured under the microscope. To assign the intensities of staining for PARP 1, cleaved PARP 1, caspase 3, and cleaved caspase 3, three independent observers blinded to the experimental design were asked to examine the pictures, using a method previously described (Wei *et al.* 2012): –, no staining detected; +, weak; ++, moderate; and ++++, strong staining. Relative levels of immunostaining were evaluated and repeated at least four times.

Classification of porcine ovarian follicles

In order to study the histological changes as well as expression and localization of PARP1, cleaved PARP1, caspase 3, and cleaved caspase 3 by IHC during the process of porcine follicular development and atresia, each follicle was classified histologically as an unassembled oocyte (remaining in

Table 1 Classification criteria of porcine ovarian follicles^a.

Follicle	Morphologic characteristics of cells
Unassembled oocytes	Unassembled oocytes are surrounded by a single layer of squamous stromal tissue (remaining in oocyte nests)
Primordial follicles	Oocyte partially or completely encapsulated by squamous pregranulosa cells
Primary follicles	Granulosa cells (with single layer) show enlargement
Preantral follicles	Oocyte encapsulated by more than two layers of granulosa cells; no antrum formation
Antral follicles	Oocyte encapsulated by more than two layers of granulosa cells with antrum
Atretic follicles	Follicles with apoptotic and shedding granulosa cells in the follicular antrum, and apoptosis of granulosa cells accompanied by the presence of nuclei with condensed chromatin stained with hematoxylin

^aReferenced mainly from our laboratory's previous study (Ding *et al.* 2010, Wei *et al.* 2012, Wei & Shi 2013). The stages of follicle development (unassembled, primordial, primary, preantral, antral, and atretic follicles) were divided based on the classification criteria provided in this table.

oocyte nests), or as a primordial, primary, preantral, antral, or atretic follicle. According to our previous study (Ding *et al.* 2010, Wei *et al.* 2012, Wei & Shi 2013), porcine ovarian follicles were classified based on the classification criteria of Table 1.

RIA for E₂ and P₄ concentrations in the serum of neonatal, young, and adult pigs

Blood was collected from pigs of different ages and centrifuged at 5000 *g* for 10 min to separate the serum. The serum was used to quantify E₂ and P₄ using E₂ and P₄ RIA kits, respectively, in the General Hospital of the Nanjing Military Command.

SDS-PAGE and western blot analysis

Proteins from porcine ovaries were prepared for western blot analysis in RIPA lysis buffer (Beyotime, Nantong, China) containing 10 mM phenylmethylsulfonyl fluoride (Beyotime) using a Dounce homogenizer. After homogenization, samples were incubated for 30 min on ice, and centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was separated and the protein concentration was determined using a BCA Protein Assay Kit (Beyotime). In each experiment, equal amounts of sample lysate (15–40 µg) were separated by 10% (w/v) SDS-PAGE under standard reducing conditions with precision protein molecular weight markers (Fermentas, Shanghai, China), and then electrotransferred onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 2% BSA in TBST buffer (20 mM Tris-buffered saline, 0.05% Tween 20, pH 7.5) for 2 h at room temperature and incubated for 18 h at 4 °C with diluted antibodies specific to PARP1 (diluted 1:1000), cleaved PARP1 (1:2500), and α -tubulin (1:5000). Subsequently, the membranes were washed with TBST buffer and incubated with HRP-linked secondary goat anti-rabbit IgG antibody (diluted at 1:2000) for 3 h. Finally, the blots were washed four times and visualized with Super Signal West Pico Chemiluminescent Subs Kits (Pierce Biotechnology, Shanghai, China).

Statistical analysis

All results are expressed as mean $\pm$ S.E.M. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Students *t*-test was used to compare two groups, and a one-way ANOVA was used for more than two groups, with Tukey's range test applied for multiple comparisons. Statistical significant difference was considered to be $P < 0.05$.

Results

Neonatal, young, and adult pigs showed increased P₄ and E₂ concentrations with time after birth

During the process of oophorectomy, the serum of neonatal and young pigs (5, 35, 70, and 180 dpp) was also gathered.

The serum was used to quantify E₂ and P₄ levels using E₂ and P₄ RIA kits. The concentration of E₂ and P₄ in the serum obtained from pigs of different ages is shown in Fig. 1. The data showed that there were increased P₄

Figure 1 Serum concentrations of progesterone (P₄) and estradiol (E₂) in pigs of different ages (5, 35, 70, and 180 dpp). Data represent the mean $\pm$ S.E.M. from three duplicates. D5, D35, D70, and D180 stand for 5, 35, 70, and 180 dpp respectively. Significant differences by ANOVA and Tukey's *post-hoc* test, $n=3$.

and E₂ concentrations after birth with age (5, 35, 70, and 180 dpp).

The PARP1 cleavage signaling pathway exists in the unassembled oocytes (oocyte nests), but not in the primordial follicles of fetal and neonatal porcine ovaries

As described above, according to our previous study, two key time points (90 and 1 dpp) were selected as fetal and neonatal period to study the role of the PARP1 cleavage signaling pathway during the process of oocyte nest breakdown and primordial follicle formation and development in fetal and neonatal porcine ovaries. We investigated the cellular localization patterns of PARP1, cleaved PARP1, caspase 3, and cleaved caspase 3 in the ovary of pig by IHC. The porcine ovarian follicles were classified based on the classification criteria in Table 1. In the porcine ovary, positive reaction was observed as brown staining (marked with red arrows). No specific staining was observed in the negative control sections (Fig. 2E1 and E2, Fig. 3E1 and E2). The results showed that the immunostaining pattern of PARP1, cleaved PARP1, caspase 3, and cleaved caspase 3 was varied among different follicle stages during the process of oocyte

nest breakdown, primordial follicle formation, and transition to primary follicles. Relative expression levels of immunostaining of PARP1, cleaved PARP1, caspase 3, and cleaved caspase 3 in the ovaries are shown in Table 2.

Immunohistochemical results showed that PARP1 and caspase 3 were localized extensively to healthy oocytes of the oocyte nests and primordial follicles (Fig. 2A1, A2, C1 and C2, Fig. 3A1, A2, C1 and C2). The results also showed that oocyte nest breakdown was accompanied by caspase 3 activation (cleaved caspase 3) and PARP1 deactivation (cleaved PARP1) in the apoptotic oocytes (Fig. 2). In the nonapoptotic oocytes, there was almost no staining of cleaved caspase 3 and cleaved PARP1 (Fig. 2D1), while immunostaining of PARP1 and caspase 3 was clearly observed in the nucleus and cytoplasm of the healthy oocytes (Fig. 2D2). In pregranulosa cells, the staining of PARP1 and caspase 3 was weak, while that of the cleaved caspase 3 and cleaved PARP1 was almost invisible (Fig. 2E1, E2 and E3). Interestingly, Fig. 2C1, C2 and C3 clearly showed that cleaved PARP1 was mainly located in the nucleus; (however, the cleaved caspase 3 was located around the nucleus of the apoptotic oocytes; Table 2).

Figure 2 Immunostaining of PARP1 (A), cleaved PARP1 (B), caspase 3 (C), and cleaved caspase 3 (D) in the porcine ovary (90 dpc). The negative control (E) was normal rabbit serum (NRS) instead of primary antibody (photographs in differently colored individual square boxes marked above are enlarged below and marked A1, 2, B1, 2, C1, 2, D1, 2, E1, and 2). Staining of PARP1 (A), cleaved PARP1 (B), caspase 3 (C), and cleaved caspase 3 (D) was qualitatively different in oocytes at various stages of germ cell growth and development.

Figure 3 Immunostaining of PARP1 (A), cleaved PARP1 (B), caspase 3 (C), and cleaved caspase 3 (D) in the porcine ovary (1 dpp). The negative control (E) was normal rabbit serum (NRS) instead of primary antibody (photographs in differently colored individual square boxes marked above were enlarged below and marked A1, 2, B1, 2, C1, 2, D1, 2, E1, and 2). Staining of PARP1 (A), cleaved PARP1 (B), caspase 3 (C), and cleaved caspase 3 (D) was qualitatively different in the oocytes at various stages of germ cell growth and development.

Degeneration of the porcine ovarian follicle wall and GC apoptosis is accompanied by caspase 3 activation and PARP1 cleavage

Our previous study demonstrated that cleavage of PARP1 may serve a key role in controlling follicular atresia through degeneration of GC. In order to further study the characteristics of PARP1 cleavage and caspase 3 cleavage during the process of porcine ovarian follicle wall

degeneration, two kinds of atretic follicles (follicles with both healthy GCs and shedding, apoptotic GCs (Fig. 4A1, B1, C1, D1 and E1), and follicles with almost all the GCs as shedding and apoptotic (Fig. 4A2, B2, C2, D2 and E2)) were examined using PARP1, cleaved PARP1 caspase 3, and cleaved caspase 3 antibodies by IHC (Table 3).

The apoptosis of GCs was accompanied by the presence of nuclei with condensed chromatin stained with

Table 2 Relative levels of immunostaining of PARP1, cleaved PARP1, caspase 3, and cleaved caspase 3 in the nonapoptotic cells of fetal and neonatal porcine ovaries.

Protein	Age	Stages	Unassembled follicle	Primordial follicle	Primary follicle
PARP1	90 dpc	Oocyte	++	+++	NA
		Granulosa cells	NA	+	NA
	1 dpp	Oocyte	++	+++	+++
		Granulosa cells	NA	++	++
Cleaved PARP1	90 dpc	Oocyte	—	—	—
		Granulosa cells	NA	—	NA
	1 dpp	Oocyte	—	—	—
		Granulosa cells	NA	—	—
Caspase 3	90 dpc	Oocyte	+	++	NA
		Granulosa cells	NA	+	NA
	1 dpp	Oocyte	+++	+++	+++
		Granulosa cells	NA	++	++
Cleaved caspase 3	90 dpc	Oocyte	—	—	—
		Granulosa cells	NA	—	NA
	1 dpp	Oocyte	—	—	—
		Granulosa cells	NA	—	—

—, No staining detected; +, weak; ++, moderate; +++, strong staining; NA, not available.

Figure 4 Immunostaining of PARP1 (A), cleaved PARP1 (B), caspase 3 (C), and cleaved caspase 3 (D) in granulosa cells during the process of follicular atresia (150 dpp). The negative control (E) was normal rabbit serum (NRS) instead of primary antibody. From the enlarged pictures (A3–E3), it was more clearly demonstrated that PARP-1 and caspase-3 could not be detected in the apoptotic GCs, however, cleaved PARP-1 was mainly located in the nucleus of GCs, whereas cleaved caspase-3 was in and around the nucleus. Atretic follicles are characterized by shedding and apoptotic granulosa cells in the follicular antrum, and the apoptosis of granulosa cells was accompanied by the presence of nuclei with condensed chromatin stained with hematoxylin.

hematoxylin. The results showed that the cleavage of PARP1 by cleaved caspase 3 was in apoptotic GCs, which is consistent with our previous findings (Wei & Shi 2013). However, there was an interesting phenomenon – cleaved caspase 3 was mainly localized in and around the nucleus of apoptotic GCs, whereas cleaved PARP1 was mainly localized to the nucleus (Fig. 4A2, B2, C2, D2 and E2).

Expression patterns of PARP1 and cleaved PARP1 in fetal, neonatal, and adult porcine ovaries

To provide more evidence elucidating the role of PARP1 cleavage during the process of oocyte nest breakdown, primordial follicle formation, and development in fetal and neonatal porcine ovaries, the expression patterns of PARP1 and cleaved PARP1 in porcine ovarian extracts from fetal neonatal and adult porcine ovaries were analyzed by western blotting.

The data showed that PARP1 cleavage produces an 89 kDa C-terminal fragment, and a 24 kDa N-terminal fragment in fetal (90 dpc), neonatal (1 dpp), and adult (150 dpp) porcine ovaries (Fig. 5). Thus, the results of western blotting indicated that porcine ovarian development was accompanied by PARP1 cleavage.

Involvement of the PARP1 cleavage signaling pathway during the process of follicular development and atresia in adult porcine ovaries

To study the role of PARP1 cleavage during the process of follicular development and atresia, we investigated the characteristics of localization of PARP1, cleaved PARP1 caspase 3, and cleaved caspase 3 proteins in the adult porcine ovary by IHC. In the ovary, a positive reaction was observed as brown staining. No specific staining was observed in the negative control sections. The porcine ovarian follicles were classified based on the classification criteria in Table 1. The results showed that the immunostaining patterns of PARP1, cleaved

Table 3 Relative levels of immunostaining of PARP1, cleaved PARP1, and caspase 3 in the granulosa cells (GCs) from porcine ovaries.

Status	Healthy	Apoptotic
PARP1	+++	–
Cleaved PARP1	–	+++
Caspase 3	+++	–
Cleaved caspase 3	–	+++

–, No staining detected; +, weak; ++, moderate; +++, strong staining; NA, not available.

Figure 5 (A) Analysis of PARP1, cleaved PARP1, and tubulin expression in fetal (90 dpc), neonatal (1 dpp), and adult (150 dpp) porcine ovaries. dpc, days post coitum; dpp, day post partum. *T* Significant differences by ANOVA and Tukey's *post-hoc* test, $n=3$. (B) Bars with different letters are significantly different from one another ($P<0.05$).

PARP1 caspase 3, and cleaved caspase 3 were varied between healthy and atretic follicles. Relative levels of immunostaining of PARP1, and cleaved PARP1 and caspase 3 in the GCs of porcine ovaries are shown in Table 4.

Immunohistochemical results showed that PARP1 and caspase 3 were extensively localized to healthy GCs and oocytes of primary follicles, secondary follicles, early antral follicles, and antral follicles in the porcine ovary (Fig. 6). In the atretic follicles, there was almost no staining of PARP1 (Fig. 6A5) or caspase 3 (Fig. 6C5) in the apoptotic GCs, while immunostaining of cleaved caspase 3 (Fig. 6D5) and cleaved PARP1 (Fig. 6B5) was clearly observed.

Discussion

In this study, we make a case for cleavage of PARP1 being strongly implicated in porcine follicular development and atresia of fetal, neonatal, and adult porcine ovaries. We demonstrated that PARP1 cleavage might play an important role during the process of follicular development and atresia of fetal, neonatal, and adult porcine ovaries using IHC and western blotting. The RIA data showed increased P_4 and E_2 concentrations with age after birth (5, 35, 70, and 180 dpp).

We provided evidences that the PARP1 cleavage signaling pathway exists in the unassembled oocytes (oocyte nests) during the process of oocyte nest breakdown and primordial follicle formation. However, according to our previous findings, the mechanisms involving PARP1 cleavage and controlling follicle atresia were in GCs but not in oocytes. Therefore, we concluded that PARP1 cleavage in oocytes was involved in the process of oocyte nest breakdown of porcine ovaries. Thus, the mechanisms underlying oocyte nest breakdown may be different from those of follicular atresia. The IHC results showed that cleaved caspase 3 was mainly localized in and around the nucleus of apoptotic GCs, whereas cleaved PARP1 was mainly localized in the nucleus; this indicated that the cleavage of PARP1 occurred in the nucleus after activation of caspase 3 in the cytoplasm during the process of follicular atresia. However, these findings need to be corroborated by more evidence.

DNA strand breaks can lead to PARP1 activation, and the activation of PARP1 may be an attempt to repair the DNA damage caused by nuclease activation in the dying cell (Spina-Purrello *et al.* 2008, Tanori *et al.* 2008). However, this attempt to repair damage proves futile as PARP1 is cleaved by cleaved caspase 3 into a catalytic fragment of an 89 kDa and DNA binding unit of 24 kDa (D'Amours *et al.* 1999, Agarwal *et al.* 2009). Therefore, this cleaved PARP1 could serve as a biochemical marker of caspase-dependent apoptosis.

During the process of cellular apoptosis, PARP1 activation and cleavage play active but complex roles (Boulares *et al.* 1999, Chaitanya *et al.* 2010). Although PARP1 cleavage is considered to be a prominent marker of apoptosis, it is worth recalling that apoptosis cannot be assessed exclusively by evaluating PARP1 proteolysis. PARP cleavage is an important step in the apoptotic process, but is only one of many. Although cleavage of PARP1 by cleaved caspase 3 at the DEVD site is a universal phenomenon during the process of cell apoptosis, the significance of this cleavage *in vivo* is largely unknown (Petrilli *et al.* 2004). Some studies showed that PARP1 cleavage might be an attempt to inactivate its capacity to repair DNA so as to preserve energy pools (Herceg & Wang 2001). There are findings that suggest that the 24 kDa N-terminal PARP1 fragment irreversibly binds to DNA in order to prevent DNA repair

Table 4 Relative levels of immunostaining of PARP1, and cleaved PARP1, and caspase 3 in the granulosa cells (GCs) from porcine ovaries.

Status	Healthy	Apoptotic
PARP1	+++	—
Cleaved PARP1	—	+++
Caspase 3	+++	—
Cleaved caspase 3	—	+++

—, No staining detected; +, weak; ++, moderate; +++, strong staining; NA, not available.

Figure 6 Immunostaining of PARP1 (A), cleaved PARP1 (B), caspase 3 (C), and cleaved caspase 3 (D) in the adult porcine ovary during follicular development and atresia (150 dpp). The negative control (E) was normal rabbit serum (NRS) instead of primary antibody (photographs in differently colored individual square boxes marked above were enlarged below and marked A1, 2, B1, 2, C1, 2, D1, 2, E1, and 2). Staining of PARP1 (A), cleaved PARP1 (B), caspase 3 (C), and cleaved caspase 3 (D) was qualitatively different in the oocytes at various stages of germ cell growth and development. Porcine ovarian follicles were classified based on the classification criteria in Table 1.

(Smulson *et al.* 1998, Petrilli *et al.* 2004). Thus, these findings indicate that the cleavage of PARP1 is an important regulatory event in cellular functions, but the mechanisms involved require clarification. In addition to apoptosis, necrosis and autophagy may play significant roles in cell death (Edinger & Thompson 2004). What's more, evidences indicated that there was complex interplay between apoptosis, necrosis, and autophagy (Walsh & Edinger 2010, Chaabane *et al.* 2013). It was reported that mTOR signaling, which may play key roles in autophagy, can be modulated by PARP1 (Munoz-Gamez *et al.* 2009, Ethier *et al.* 2012). Thus,

a better understanding of the signaling pathway involved in cell death needs future studies to dissect the interplay among death, apoptosis, and necrosis in cell death and survival more thoroughly.

Our previous study demonstrated that oocyte nest breakdown and primordial follicle formation take place at ~90 dpc, and the transition from primordial follicles to primary follicles was primarily between 90 dpc and 1 dpp in the porcine ovary (Ding *et al.* 2010); however, the mechanisms controlling these processes remain unclear. Follicular development and atresia are complex processes that are governed by a series of endocrine,

paracrine, and autocrine signals (Matsuda *et al.* 2012). E_2 and P_4 are two of the main steroid hormones that play essential roles during follicular development. The fate of follicles is then determined by the balance between/among these factors, and it is believed that E_2 and P_4 play vital roles during the process of folliculogenesis (Kezele & Skinner 2003, Wen *et al.* 2010). Our findings may provide further insight into understanding these processes.

Conclusions

In conclusion, our data suggest that the cleavage of PARP1 by activated (cleaved) caspase 3 plays an important role during the process of oocyte nest breakdown and primordial follicle formation, as well as in follicular development and atresia. We believe that these findings will prove to be helpful in investigating the mechanisms controlling follicular development and atresia.

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.

Funding

This study was supported in part by the National Nature Science Foundation of China (no. 31172206), Nature Science Foundation of Jiangsu Province, China (no. BK 2011499), and a Grant-in-Aid for Innovative Training of Doctoral Students in Jiangsu Province, China (no. CXZZ12-0294).

Acknowledgements

We thank Jiangpu Farms of Nanjing in China for supplying experimental animals. We also express our gratitude to Dr Reinhold J Hutz of the Department of Biological Sciences, University of Wisconsin-Milwaukee, USA, for reading the original manuscript and offering valuable suggestions.

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Received 23 April 2013

First decision 23 July 2013

Revised manuscript received 28 July 2013

Accepted 23 September 2013