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Involvement of ER–Calreticulin–Ca^{2+} Signaling in the Regulation of Porcine Oocyte Meiotic Maturation and Maternal Gene Expression

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SUMMARY

Calcium is one of the most ubiquitous signaling molecules, and controls a wide variety of cellular processes. It is mainly stored in the endoplasmic reticulum (ER), bound to lumenal proteins. Calreticulin is the major Ca^{2+}-binding chaperone in oocytes, and is integral to numerous cellular functions. To better understand the role of the ER–calreticulin–Ca^{2+} pathway in oocyte maturation and early embryogenesis, we characterized the porcine calreticulin gene and investigated its expression profile during oocyte maturation and early embryonic development. Calreticulin was widely expressed in pig tissues and its transcripts were downregulated during maturation, especially at 44 hr, and were undetectable at the blastocyst stage. We also investigated the effect of increased cytosolic Ca^{2+} induced by the Ca^{2+}-ATPase inhibitor, cyclopiazonic acid (CPA), on pig oocyte maturation and maternal gene expression. CPA at 10 μM did not inhibit germinal vesicle breakdown, but did result in the arrest of 38.6% oocytes at or before the MI stage. In addition, expression of the maternal genes C-mos, BMP15, GDF9, and Cyclin B1 was significantly increased in CPA-treated MII oocytes compared with control groups. These data were supported by the results of poly(A)-test PCR, which revealed that the cyclin B1 short isoform (CB-S), GDF9, and C-mos underwent more intensive polyadenylation modification in CPA-treated oocytes than control oocytes, suggesting that polyadenylation may influence Ca^{2+}-modulated changes in gene expression. Furthermore, CPA treatment decreased the percentage of four-cell parthenotes that developed into blastocysts, suggesting the need for functional SR/ER Ca^{2+}-ATPase pumps or Ca^{2+} signals during early embryo development after zygotic genome activation. Together, these data indicate that ER–calreticulin-associated Ca^{2+} homeostasis plays a role in oocyte and embryo development, and that alterations in maternal gene expression may contribute to the underlying molecular mechanism, at least partially, via polyadenylation.

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INTRODUCTION

Calcium (Ca^{2+}) is one of the most physiologically significant signaling molecules. Signaling cascades involving Ca^{2+} affect virtually every cellular function. The endoplasmic reticulum (ER) is a vital cellular organelle that functions in a range of diverse cellular processes. The lumen of the ER contains a high concentration of Ca^{2+}, and most of it is buffered by associating with chaperones that reside in

Additional Supporting Information may be found in the online version of this article.

the ER (reviewed in Michalak et al., 2002). Calreticulin and calsequestrin are two well-characterized Ca^{2+}-binding ER luminal proteins. Calreticulin is predominantly found in non-muscle cells, whereas calsequestrin is primarily restricted to the muscle sarcoplasmic reticulum (SR; Michalak et al., 1992; Coppolino and Dedhar, 1998). As the major Ca^{2+}-binding chaperone in the ER, calreticulin (CALR or CRT) is involved in numerous cellular processes (reviewed in Michalak et al., 1992; Coppolino and Dedhar, 1998; Johnson et al., 2001; Groenendyk et al., 2004; Gelebart et al., 2005). Park et al. (2001) demonstrated that calreticulin is important for proper development of *Caenorhabditis elegans* oocytes and showed that calreticulin mutants exhibited reduced fertility. Recently, calreticulin has been reported to participate in signal transduction events during or after mouse sperm–egg interactions at fertilization (Tutuncu et al., 2004).

In mammals, oocyte maturation proceeds after germinal vesicle breakdown (GVBD) through the stages of metaphase I (MI), anaphase I, and telophase I, up to the stage of metaphase II (MII), when meiosis is again arrested (Petr et al., 1999). After fertilization of a mature oocyte, meiosis is resumed and mitosis will be initiated only after that. The initial embryonic development is regulated by maternally inherited components stored within the oocyte. Accumulating evidence suggests that intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) play an important role in regulation of the meiotic maturation of oocytes and early embryonic development (Homa et al., 1993; Sousa et al., 1997; Whitaker, 2006). Variations in the $[Ca^{2+}]_i$ in the ER and cytoplasm play a key role in intraorganelle signaling pathways (Michalak et al., 2002). The ER-associated Ca^{2+}-signaling pathway is proposed to be a vital mediator of Ca^{2+} homeostasis. In response to intracellular stimulating signals, Ca^{2+} is released from internal ER stores via inositol (1,4,5)-trisphosphate ($InsP_3$) receptor channel, and is reloaded by SR/ER Ca^{2+}-ATPase (SERCA) after emptying the stored Ca^{2+}. It has been well established that $InsP_3$ receptor channel is dependently activated by sperm penetration. As the main Ca^{2+}-buffering protein, calreticulin modulates Ca^{2+} homeostasis and signaling by regulating the capacity of ER Ca^{2+} stores, and activity of both $InsP_3$ receptor and SERCA (Johnson et al., 2001). Cyclopiazonic acid (CPA) is a specific inhibitor of Ca^{2+}-ATPase, and thereby facilitates the release of Ca^{2+} from intracellular stores. CPA consequently elevates intracellular calcium levels through mobilization of intracellular deposits and through the influx of extracellular Ca^{2+} (store-operated Ca^{2+} entry; Demaurex et al., 1992).

The dynamic changes in Ca^{2+} oscillations during oocyte and early embryo development in mouse (Carroll et al., 1994; Stachecki and Armant, 1996) and human (Sousa et al., 1997) have been well characterized. However, in large domestic species such as bovine and porcine, the functional significance of changes in $[Ca^{2+}]_i$ levels during oocyte maturation and embryonic development is less well understood. In addition, previous studies of Ca^{2+} signaling pathways have focused primarily on the fertilization process, and a pivotal role for the $InsP_3$ receptor channel in fertilization of mammalian eggs has been firmly established (Miyazaki et al., 1993; Macháty et al., 1997; Malcuit et al., 2005).

To achieve a better understanding of the role of calreticulin and Ca^{2+}-ATPase signaling during oocyte maturation and early embryogenesis, we characterized the porcine calreticulin gene and investigated its expression profile during oocyte maturation and early embryonic development. In this study, diploid parthenotes were employed to study early development in the pig because of the difficulty in obtaining homogenous pig embryos due to the relatively high incidence of polyspermy that occurs during in vitro fertilization (Cui et al., 2004). Next, we inhibited the activity of SERCA with CPA to investigate the effect of CPA-induced increases in oocyte $[Ca^{2+}]_i$ on the in vitro maturation (IVM) of pig oocytes and subsequent embryo development. To further explore the underlying molecular basis of these effects, the expression patterns and polyadenylation status of select maternal genes, including calreticulin, C-mos, cyclin B1, GDF9, and BMP15, were studied by real-time PCR and poly(A)-test (PAT) assays. As the markers of female germ cells, the latter four genes are well-studied maternal genes whose differential expression profile during pig oocyte maturation has been reported previously (Zhang et al., 2009).

RESULTS

Sequence Characterization of the Porcine Calreticulin Gene

The 1,919bp full-length porcine calreticulin cDNA (GenBank accession no. GQ984146) was obtained by assembling the sequences of expressed sequence tags (ESTs) and amplicons from porcine oocyte cDNA. Sequence analysis showed that the porcine calreticulin cDNA contained an open-reading frame (ORF) of 1,254 nucleotides (nt), flanked by a 63 bp 5′-UTR (untranslated region) and a 602 bp 3′-UTR. Comparison of human (NM_004343), mouse (NM_007591), and pig (GQ984146) calreticulin CDS revealed that the homology between human and pig is 88%, and that between mouse and pig is 85%. The pig calreticulin sequence is predicted to encode a protein of 417 amino acids with a calculated molecular mass of 48.29 kDa and an isoelectric point (pI) of 4.06. Comparison of cDNA and experimentally cloned DNA sequences established that the calreticulin gene spans 3.8 kb of genomic DNA and is made up of nine exons. All of the exon–intron splice junctions conformed to the GT/AG rule. After alignment of the DNA sequence to the existing pig genome database, the calreticulin gene was mapped to pig chromosome 2 (SSC2), which is syntenic with the human calreticulin chromosomal locus (19p13.3–p13.2) (https://www-lgc.toulouse.inra.fr/pig/compare/SSCHTML/SSC2B.HTM). A detailed gene structure is schematically depicted in Figure 1A. Sequence alignment analysis of the calreticulin protein of porcine and five other species indicated that it is evolutionarily conserved, with 96.2% identity to bovine and 69.8% to zebrafish (Fig. 1B).

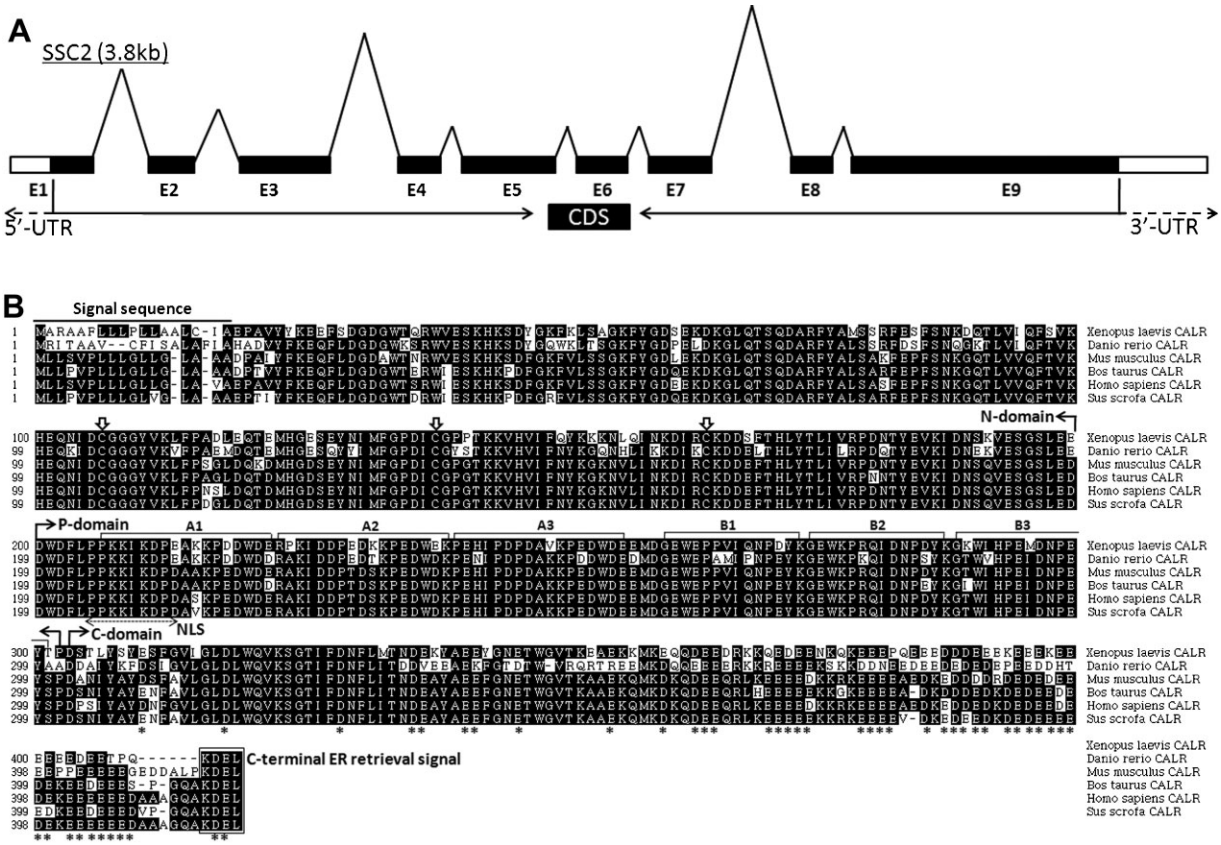

Figure 1. A: A schematic of the genomic structure of the porcine calreticulin gene. Nine exons separated by introns of different sizes are shown. The black regions represent the coding sequence (CDS) and the white region indicates the untranslated region (UTR). Detailed sequence information can be found in the Supplementary File. **B**: Multiple sequence alignment of calreticulin proteins of porcine and five other species (human, NP_004334; mouse, NP_031617; bovine, NP_776425; zebrafish, NP_571122; and frog, NP_001080765). The shaded areas indicate identical amino acids. The protein contains a typical N-terminal signal sequence and a C-terminal KDEL ER retrieval signal. The three functional domains, N, P, and C, are indicated by arrowheads. The highly conserved N-domain is globular in structure and contains a binding site for rubella virus RNA, a putative phosphorylation site, and a segment that binds to steroid hormone receptors and the cytoplasmic domains of integrin α-subunits (Coppolino and Dedhar, 1998). The three highly conserved cysteine residues within the N-domain are indicated by unfilled block arrows, whereas the last two cysteines form a disulfide bridge that facilitates the proper folding of calreticulin. The proline-rich P-domain, which binds Ca^{2+} with high affinity and low capacity, contains two characteristic triplets of conversed motifs, repeat A (consensus sequence PXXIXDPDAXKPEDWDE) and repeat B (consensus sequence GXWXPPXIXNPXYX). A putative nuclear localization signal (NLS; PPKKIKDPD) is also found in this domain. The C-domain is rich in acidic residues (D and E, marked by asterisks) and is highly negatively charged. This region binds Ca^{2+} with high capacity and low affinity. In short, the N and P domains are responsible for the chaperone function of this protein, whereas the C-domain is primarily involved in high-capacity Ca^{2+} storage.

Tissue Distribution of Calreticulin Determined by Porcine EST Database Search and RT-PCR

A bioinformatics method (pig EST database search) and experimental method (RT-PCR) were employed to investigate the tissue distribution of porcine calreticulin transcripts. To analyze the abundance of expression within the pig EST database, the full-length pig calreticulin cDNA (GQ984146) was used to search the 'EST_other' database, using the standard BLAST algorithm. ESTs sharing more than 80% sequence identity with the pig cDNA were collected, and a total of 34 homologous calreticulin ESTs were found. The source information from these ESTs yielded 33 unique sources for the pig calreticulin gene, and these data were used to determine its broad expression pattern (Supplementary File). To validate these results and to identify new expression patterns, semi-quantitative RT-PCR was used to analyze the expression of calreticulin mRNAs in various tissues (spleen, kidney, liver, fat, muscle, ovary, granulosa cells, and cumulus cells). Calreticulin was detected in all samples analyzed, suggesting broad-spectrum expression in multiple tissues, which is consistent with its role as a Ca^{2+}-binding chaperone (Supplementary File).

Expression and Polyadenylation of Calreticulin During IVM of Porcine Oocytes and Early Parthenote Development

The relative abundance of calreticulin transcripts during oogenesis was determined by real time RT-PCR. mRNA expression was initially increased at 12 hr of culture, followed by an aggressive decline during maturation, especially at

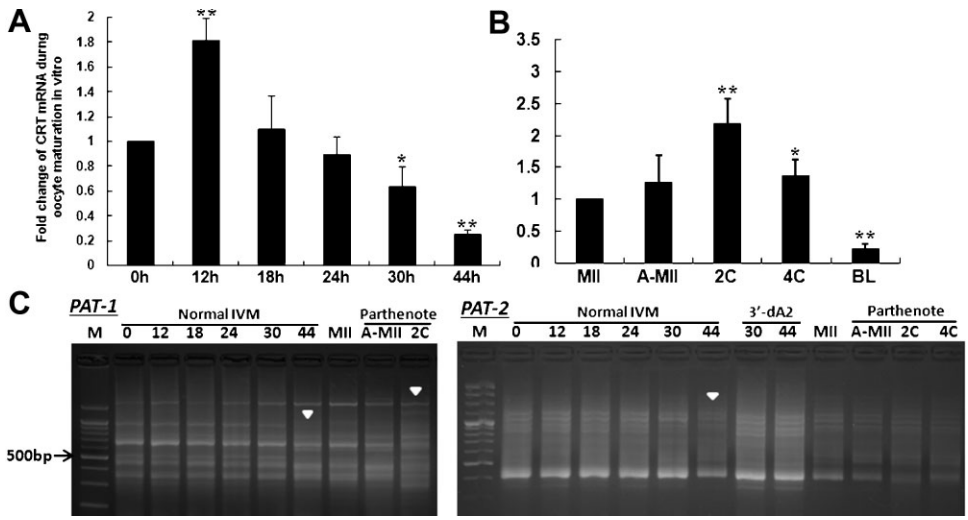

Figure 2. Real-time RT-PCR analysis of the expression patterns of calreticulin mRNAs during (**A**) maturation of pig oocytes and (**B**) parthenogenetically activated early embryo development in vitro. The relative abundance of mRNA was normalized to internal β-actin levels. The transcription levels in denuded oocytes at the GV-stage (0 hr) and MII-stage were arbitrarily set at one-fold. Statistically significant differences are indicated by $*P < 0.05$ and $**P < 0.01$. Data are presented as the means ± SEM of three to six separate experiments. A-MII, parthenogenetically activated MII-stage oocytes after 6 hr of *in vitro* culture; 2C, two-cell parthenotes; 4C, four-cell parthenotes; BL, blastocyst parthenotes. **C**: PAT assay to measure dynamic changes in the length of the poly(A) tails of maternal calreticulin transcripts during pig oocyte maturation and early embryonic cleavage. In vitro culture times, treatment, and embryonic stages are indicated at the top of the figure. 3′-dA was added to the culture medium at a concentration of 2 μg/ml, which suppressed cytoplasmic polyadenylation effectively in the oocytes.

30 hr (MI stage) and 44 hr (MII stage; Fig. 2A). The amount of calreticulin transcription was unchanged in MII-stage oocytes after parthenogenetic activation (A-MII). Transcription then increased in two- and four-cell parthenotes, and was followed by a a sharp decrease in the blastocyst stages (Fig. 2B). To further evaluate its protein expression, Western blotting was performed. The results indicated that calreticulin was expressed at relatively higher levels in GV-stage oocytes than MII-stage oocytes, whereas no signal was detected in BL-stage embryos (data not shown).

As a result of the long 3′-UTR of the calreticulin gene, two primers based on sequences in the beginning and middle regions of the 3′-UTR were designed for PAT PCR. PAT assay revealed that the polyadenylation status of pig calreticulin mRNAs was generally unchanged during oocyte maturation, with the exception of a slight deadenylation at 44 hr (MII; Fig. 2C). The addition of 2 μg/ml of the polyadenylation inhibitor 3′-deoxyadenosine (3′-dA) in the culture medium effectively suppresses the polyadenylation process. No difference in the poly(A) tails length of maternal calreticulin transcripts was detected by the PAT-2 primers in mature oocytes or during subsequent embryonic cleavage. However, a slight polyadenylation signal was detected by the PAT-1 primers in the 2C embryo (Fig. 2C).

Localization of Calreticulin in Pig Oocytes and Parthenotes

The subcellular distribution of calreticulin throughout the IVM of porcine oocytes and early embryonic development is shown in Figure 3. During the maturation of oocytes (immature GV and mature MII stages) and after parthenogenetic activation (A-MII, 6 hr), calreticulin accumulated primarily in the cortical/plasma membrane region. In pronuclear (PN, 18 hr) and early cleaved embryos (2C and 4C), the fluorescence signal could be detected weakly in the cytoplasm around the nucleus and more strongly in the region near the cortex. In embryos in the blastocyst stage, calreticulin staining was detected in all blastomeres (Fig. 3).

Effect of Calreticulin Antibody Injection on Porcine Oocytes Maturation and Maternal Genes Expression

To assess protein function, the effect of calreticulin antibody injection on porcine oocyte maturation and maternal gene expression was investigated. Calreticulin antibody injection into denuded GV stage oocyte (DO) had no effect on their nuclear maturation after 44-hr culture (Fig. 4A). However, as shown in Figure 4B, except GDF9, all other maternal genes showed statistically increased expression in calreticulin antibody-injected MII oocytes compared with control IgG-injected MII oocytes, suggesting that calreticulin may participate in oocyte cytoplasmic maturation. To further assess the role of calreticulin in early embryo development, antibody was injected into MII oocytes. Result indicated that depletion of calreticulin in MII oocytes, followed by parthenogenetic activation, had no obvious effect on developmental events up to the blastocyst stages (data not shown).

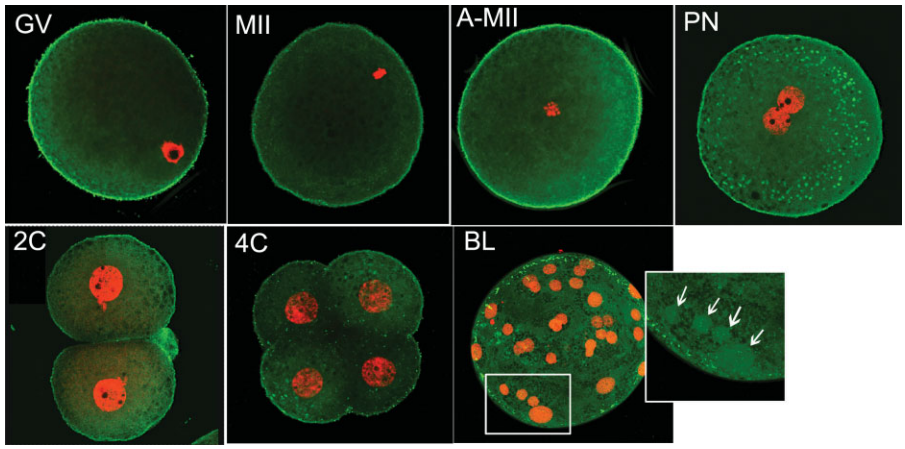

Figure 3. Representative laser scanning confocal microscopy images showing the immunolocalization of calreticulin in porcine immature GV-stage oocytes, mature MII-stage oocytes, parthenogenetically activated MII-stage oocytes (A-MII, 6 hr), pronuclear (PN, 18 hr), two-cell (2C), four-cell (4C), and blastocyst (BL) embryos developing in vitro. Green staining represents protein and red staining represents chromatin in merged images. At least 10 oocytes or embryos per group were examined in three independent experiments.

Effect of CPA on Cumulus Expansion and Porcine Oocyte Maturation In Vitro

The presence of CPA during the in vitro incubation period (44 hr) induced a concentration-dependent inhibitory effect on pig oocyte meiotic maturation (data not shown). At 10 μM CPA, 61.4% of oocytes arrested at MII compared with 87.29% in the control group (Fig. 5). During the normal maturation of pig oocytes, cumulus expansion was significantly increased at 44 hr. However, the addition of CPA to the culture medium at concentrations of 10 μM or above markedly suppressed cumulus expansion (Fig. 5).

To further assess the developmental competence of the oocytes, 10 μM CPA-treated MII-stage oocytes were parthenogenetically activated. The results showed that CPA-treated MII-stage oocytes had significantly lower developmental rates for two-cell cleavage (21%) and blastocyst formation (10.5%) compared with control MII oocytes (55.3% and 31.1%, respectively).

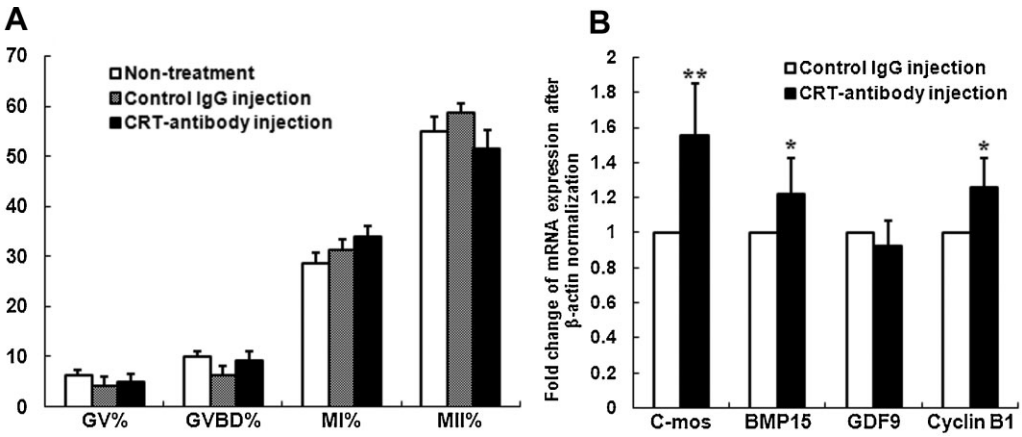

Figure 4. Effect of calerticulin antibody injection (200 μg/ml) on in vitro nuclear maturation of denuded porcine oocytes (**A**) and maternal genes expression (**B**). Cumulus-free GV-stage oocytes were injected with control rabbit IgG and calreticulin antibody or noninjected, and cultured in vitro for 44 hr in IVM medium. After maturation, the nuclear status of denuded oocytes was determined by Hoechst 33342 staining and the MII-stage oocytes were sampled for real-time RT-PCR. The relative mRNA level was normalized to internal β-actin levels. Statistically significant differences are indicated by $*P < 0.05$ and $**P < 0.01$.

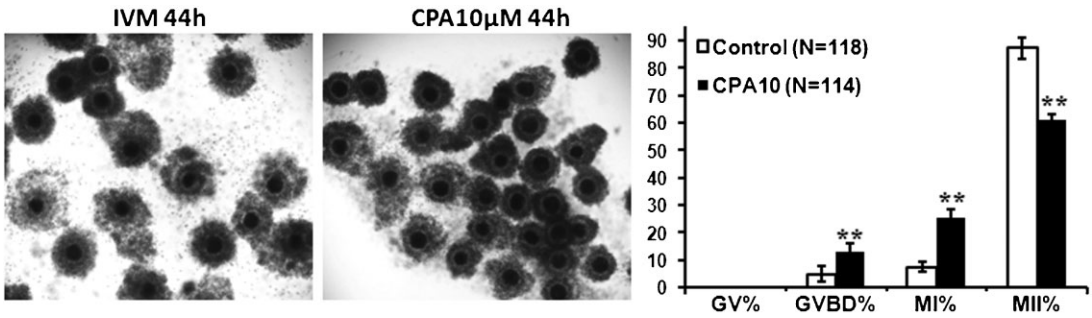

Figure 5. The effect of cyclopiazonic acid (CPA) on cumulus expansion and in vitro maturation of porcine oocytes. Only COCs of high quality were selected for culture in this study. After 44 hr of culture with or without 10 μM of CPA, the nuclear maturation status of denuded oocytes was determined by Hoechst 33342 staining. Statistically significant differences are indicated by $^{**}P < 0.01$.

Effect of CPA on Maternal mRNA Expression and Polyadenylation Patterns in Mature Porcine Oocytes

To investigate the mechanism responsible for the inhibitory effect of CPA on oocyte maturation at the molecular level, the mRNA expression patterns and polyadenylation status of important maternal genes were investigated by PCR analysis. In mature oocytes treated with CPA, the maternal genes C-mos, BMP15, GDF9, and cyclin B1 showed significantly increased expression levels compared with the control groups. The expression level of calreticulin showed no change (Fig. 6A). PAT assay analysis revealed more intensive polyadenylation signals for GDF9 (the shorter isoforms) and C-mos (isoform 2) in CPA-treated MII-stage oocytes than in control oocytes (Fig. 6B). Two isoforms of cyclin B1, both comprising the same ORF but differing in 3′-UTR length, were previously identified in pig oocytes by our group, and both the isoforms underwent polyadenylation during oocyte maturation (Zhang et al., 2010). No difference was observed in the polyadenylation patterns of the larger isoform of cyclin B1 (CB-L) or BMP15 transcripts between CPA-treated and control MII oocytes. However, the cyclin B1 short isoform (CB-S) in CPA-treated MII-stage oocytes appeared to have a higher level of polyadenylation compared with that of CB-S in control MII-stage oocytes (Fig. 6B).

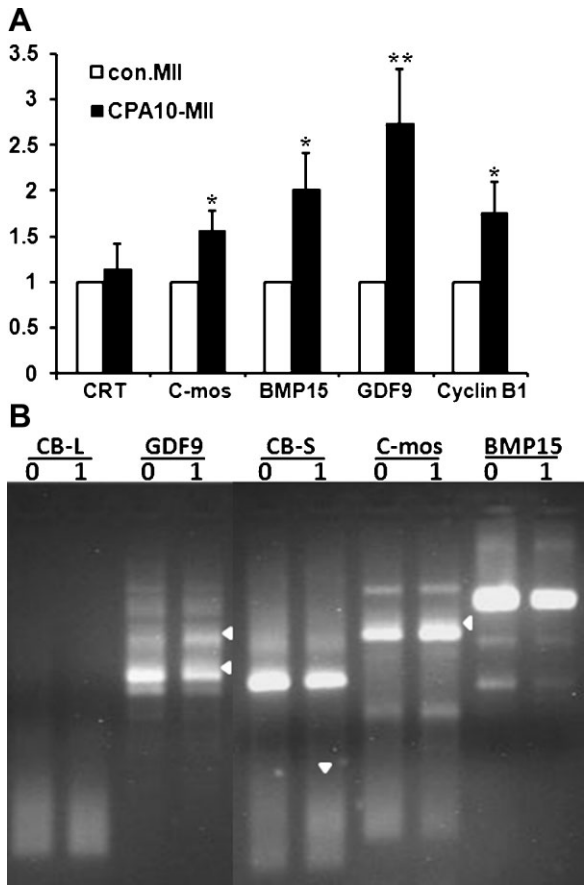

Figure 6. **A**: The effect of cyclopiazonic acid (CPA) on the expression of maternal genes. Pig MII-stage oocytes maturated with or without 10 μM of CPA were analyzed by real-time RT-PCR. The expression level of mRNAs in the control oocytes was taken as one-fold, and all expression levels were normalized to internal β-actin levels. Statistically significant differences are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$. Data are presented as the means ± SEM of three separate experiments. **B**: Effect of CPA on the polyadenylation status of maternal transcripts. '0' and '1' indicate the mRNA polyadenylation patterns of transcripts in MII oocytes treated with or without CPA, respectively.

Effect of CPA on the Development of Porcine Parthenotes

When CPA was present at 10 μM, fewer four-cell stage presumptive diploid parthenotes developed into blastocysts by day 7 (35.9%, n = 456) than when CPA was absent (64.8%, n = 229, $P < 0.01$). When the embryos were analyzed by Hoechst 33342 staining, the mean cell number of blastocysts in the CPA treatment group (36.23, n = 170) was significantly decreased compared with those in the control group (45.7, n = 101, $P < 0.05$).

DISCUSSION

Calreticulin has been characterized previously in various species, but not in pig. In this study, we reported the full-

length cDNA and genomic DNA sequences of porcine calreticulin. Analysis of pig and other mammalian calreticulin genes (such as human and bovine) indicates that they share a common gene structure comprised of 9 exons and a long 3′-UTR. RT-PCR analysis revealed that calreticulin was widely expressed in pig tissues, which is consistent with its role as a Ca^{2+}-binding chaperone. Interestingly, PCR with RT primers positioned within the CDS region of calreticulin amplified a single band when using pig oocyte cDNA as a template (data not shown), while two bands were produced from PCR using cDNA from somatic tissues as a template (Supplementary File). This suggests that calreticulin mRNAs may be alternatively spliced in porcine tissues.

In mammalian oocytes, gene expression is controlled at the post-transcriptional level, primarily by cytoplasmic polyadenylation, a process that plays an important role in protecting mRNAs from degradation and stimulating their translation (Bettegowda and Smith, 2007; Zhang et al., 2009). Sequence analysis indicated that a single weak polyadenylation signal (ATTAAA, consensus sequence AATAAA) and a weak cytoplasmic polyadenylation element (CPE, UUUUUAACU, consensus sequence $UUUUUA_{1-2}U$) were present in the 3′-UTR. The addition of 3′-dA, a polyadenylation inhibitor, did not dramatically change the polyadenylation patterns of maternal calreticulin mRNAs during pig oocyte maturation. This suggested that calreticulin transcripts were not subjected to significant polyadenylation modification, although a deadenylation signal at 44 hr of maturation (PAT-1 and 2) and a slight polyadenylation at the 2C-stage (PAT-1) were revealed by the PAT assay. This polyadenylation status may explain, at least in part, why the expression of calreticulin mRNA was downregulated during porcine oocyte maturation (especially at 44 hr) and upregulated in 2C and 4C stage parthenotes. Because the quantitative PCR conducted in this study utilized oligo-$(dT)_{12-18}$-primed cDNA, which uses a priming strategy that is influenced by both mRNA abundance and polyadenylation status (Thelie et al., 2007).

In this study, calreticulin strongly accumulated in the cortical region in pig oocytes (GV to MII stages). A similar pattern of localization has been reported in human (Balakier et al., 2002), mouse (Tutuncu et al., 2004), and bovine (Payne and Schatten, 2003) oocytes. Consistent with the observations published by Balakier et al. (2002) in human cleaved embryos, strong labeling in the cortex was observed in porcine 2C and 4C embryos. The cytoplasmic distribution of calreticulin around blastomere nuclei has also been reported in human 4C (Balakier et al., 2002) and bovine 2C (Payne and Schatten, 2003) embryos.

When the findings discussed above were considered in combination with the data obtained from analysis of mRNA expression levels, it was conceivable that the pig calreticulin gene is differentially expressed at various stages of oocyte and embryo development. To explore this possibility, calreticulin antibody was injected into denuded GV and MII stage oocytes to further assess its function during oocyte maturation and early embryogenesis. The results indicated that, although the antibody had no effect on oocyte maturation and embryo development up to the blastocyst stage, it changed the expression pattern of maternal genes but did not severely affect preimplantation embryonic events. This observation is directly supported by the findings published by Mesaeli et al. (1999), in which calreticulin-deficient embryos died at postimplantation day 14.5 as a result of insufficient development of the heart. Knock-down of calreticulin protein should result in an increase in intracellular free Ca^{2+} concentration due to a decreased availability of Ca^{2+} binding proteins. This raised the possibility that the aberrant gene expression in calreticulin-null oocytes or early embryos via the disturbance of cellular Ca^{2+} homeostasis contributed to defects in later fetal development.

Proper Ca^{2+} signaling is crucial from the earliest stages of development. During pig oogenesis and oocyte maturation in vitro, oocyte development is accompanied by dynamic changes in Ca^{2+} deposits in the ooplasm (Macháty et al., 1997; Petr et al., 2001; Rozinek et al., 2006). In addition to the role of calreticulin in cellular Ca^{2+} homeostasis, SERCA is also an important regulator of cytosolic free Ca^{2+} concentrations. To better understand this process, CPA, a specific inhibitor of SERCA, was used to mobilize intracellular Ca^{2+} deposits and increase Ca^{2+} concentration in pig oocytes. Petr et al. (2000) suggested that CPA artificially activates mature pig oocytes by elevating $[Ca^{2+}]_i$ levels, even the parthenogenetic development can not proceed beyond 8C stage. Recently, Koh et al. (2009) showed that thapsigargin, another inhibitor of SERCA, exhausted intracellular Ca^{2+} stores and consequently stimulated extracellular Ca^{2+} influx through store-operated channels. In this study, CPA inhibited both pig oocyte maturation and cumulus expansion in a dose-dependent manner. It has been reported that Ca^{2+} deposits gradually increase during in vitro culture of pig oocytes (Petr et al., 2001). CPA-induced elevation in $[Ca^{2+}]_i$ at the onset of maturation may be detrimental to the maturation of COCs, suggesting that the proper Ca^{2+} signaling and the concentration of intracellular Ca^{2+} stores are crucial for normal meiotic maturation. This notion was supported by the finding that brief treatment with CPA (2 hr) can help overcome the MI block in growing oocytes that had not attained full meiotic competence (Petr et al., 1999). While these findings appear to be in contrast to our report, our data showed that changes in Ca^{2+} signaling caused by inhibiting SERCA pumps for 44 hours had no effect on GVBD, but significantly blocked the development of oocytes in the MI stage. This may have been due to the long duration of CPA treatment (44 hr) that was used, which may have induced a prolonged elevation in $[Ca^{2+}]_i$. However, the results in both cases indicate that the Ca^{2+} dynamics around the MI stage is important for the MI to MII transition.

Interestingly, previous studies of pig oocytes suggested that the intracellular Ca^{2+} levels are indicative of oocyte competence (Petr et al., 1999; Rozinek et al., 2006). Our results consistently indicated that CPA-treated oocytes exhibited lower developmental competence than control oocytes. To further explore the molecular basis of CPA-mediated up-regulation of maternal transcripts, PAT assay was used to determine their polyadenylation status. The resulting data partially confirmed our real-time PCR results by revealing increased polyadenylation of GDF9, CB-L and C-mos in CPA-treated MII-stage oocytes. During normal

TABLE 1. List of Porcine Primers Used in This Study

Gene	Primer name	Sequence (5′–3′)	Full-length cDNA	Genomic DNA
Calreticulin	P1-L	CGGCTGCTGGAGGAGTGAC	Upper part	Introns 1, 2, 3
	P1-R	AACTTTCTTGGTGCCAGGGC		
	RT-F	GCCCTGGCACCAAGAAAG	Middle part	Introns 4, 5
	RT-R	ACTGGCGGTTCCCACTCT		
	Intron 6F	GACGAGCGAGCCAAAATTGA	Middle part	Introns 5, 6
	Intron 6R	TTCCCCTCCAAAAGCACTAC		
	P2-L	AAAACTTTGCTGTGCTGGGC	Lower part	Introns 7, 8
	P2-R	AGTTCTCCAGTCTCATAGAGACATTA		
	PAT-1[b]	CAGGCCAAGGATGAGCTGTAG	End of CDS	
	PAT-2	CTTTGATTCTTCTTCAGCCCTCA	Middle 3′-UTR	
β-actin[a]	RT-F	CTTCCCTTGGTCTCTGTTTAATAC		
	RT-R	AGACCGGCAAGACAGAAATGA		

[a]The pig sequence (GenBank no. AK237086) was used for real-time RT-PCR primer design.
[b]Primer for PAT assay was located in the end of CDS. The stop codon (TAG) is underlined.

IVM, C-mos, BMP15 and GDF9 showed higher levels of expression in oocytes in the MI stage than those in the MII stage (Zhang et al., 2009). Based on this, we hypothesized that CPA-treatment inhibited cytoplasmic maturation by maintaining the ooplasmic status of MII-stage oocytes at an MI-like stage, and that the distorted expression pattern of maternal transcripts may be involved in the underlying mechanism. This was further confirmed by the finding that CPA-treated oocytes exhibited an aberrant pattern of maternal gene expression and possessed low embryonic developmental competence. Furthermore, when CPA was added to parthenote culture medium to confirm the association of CPA treatment with embryo viability, the results suggested that fewer 4C embryos progressed to the BL stage in the presence of CPA, and that the CPA-derived BL embryos were of lower quality than control embryos. This suggests that the proper Ca^{2+} signaling also play a role during early embryo development. In particular, the zygotic genome is activated at the 4C stage in pigs. Therefore, CPA treatment would have an impact on transcription, which may affect subsequent embryonic development. Additionally, Ca^{2+} stores were not equally distributed in oocyte or embryo organelles (nucleus, mitochondria, vacuoles, cytoplasm) and the reorganization of organelles was observed during oocyte maturation (Petr et al., 2001; Rozinek et al., 2006). Therefore, the effect of CPA may result, at least partially, in the redistribution of organelles within oocytes. Furthermore, dysfunction of the ER caused by depletion of Ca^{2+} store via blocking SERCA pumps may also be affected by CPA action since proper ER function is necessary for protein folding and trafficking.

In summary, we have isolated and characterized the porcine calreticulin gene. Its differential expression patterns at both the mRNA and protein level indicated the involvement of calreticulin-Ca^{2+}-mediated pathways in the regulation of oocyte maturation. Furthermore, our experiments focused on the effect of CPA treatment on meiotic maturation, maternal gene expression and embryo development. The results showed that the proper timing of Ca^{2+} release from deposits and the concentration of cytoplasmic Ca^{2+} are crucial for proper oocyte and early embryo development. The data presented here will contribute to a better understanding of the molecular mechanism of Ca^{2+}-dependent events in the development of mammalian oocytes and early embryos.

MATERIALS AND METHODS

In Vitro Porcine Oocyte Maturation and Parthenogenetic Activation

Ovaries were collected from prepubertal gilts at a local slaughterhouse, and cumulus–oocyte complexes (COCs) were aspirated from the antral follicles (3–6 mm in diameter). Only COCs with multiple layers of intact cumulus cells and uniform ooplasm were selected for IVM. Approximately 50 COCs were matured in 500 μl of IVM medium under mineral oil at 38.5°C for 44 hr in a humidified atmosphere of 5% CO_2. In the drug treatment groups, 10, 50, or 100 μM of CPA was added to the culture medium. After maturation, the cumulus cells were removed by pipetting in the presence of 0.1% hyaluronidase for 2 min. For parthenogenetic activation, cumulus-free MII oocytes with the first polar body were exposed to 5 mg/l ionomycin diluted in North Carolina State University (NCSU)-23 medium for 5 min. Following 3 hr of culture in NCSU-23 medium supplemented with 7.5 μg/ml of cytochalasin B (CB; Sigma, St. Louis, MO), porcine embryos were washed thrice and transferred into NCSU-23 medium containing 0.4% BSA (Sigma) under mineral oil for culturing.

Full-Length cDNA Cloning and Sequence Analysis of the Porcine Calreticulin Gene

No sequences were available in GenBank for the pig calreticulin gene. Therefore, human calreticulin cDNAs (GenBank accession no.: NM_004343) were used to search the 'EST_other' database with the standard BLAST (http://www.ncbi.nlm.nih.gov/blast/) algorithm for homologous pig ESTs. ESTs sharing more than 80% sequence identity with human sequences were assembled to produce a pig EST-contig. Porcine gene-specific primers were designed based on these contigs using Primer Premier 5.0 software (http://www.premierbiosoft.com; Table 1). According to the comparison of experimentally cloned pig cDNA and the corresponding human sequence, primers predicted to span the introns were designed to amplify pig muscle genomic DNA (Table 1). All the PCR products were subcloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced commercially. Finally, full-length cDNA and genomic DNA sequences of the pig calreticulin gene were obtained by re-alignment of all the PCR fragments and EST sequences. For sequence analysis, the ORFs of the cloned pig cDNA were determined based on the homologous

human mRNA sequences, and the predicted protein sequences were obtained using the EXPASY Translate Tool (http://us.expasy.org/tools/dna.html). Alignment analysis of calreticulin proteins of different species was performed using the Megalign program of the DNASTAR software package.

Real-Time RT-PCR and PAT Assays

mRNAs from groups of 20 porcine oocytes or embryos derived from in vitro culture were isolated with Dynabeads mRNA Direct Kit(Dynal Asa, Oslo, Norway). First-strand cDNA synthesis was achieved by reverse transcription of mRNA using an Oligo(dT)$_{12–18}$ primer (for RT-PCR) or Oligo(dT)-Anchor (5′-GCGA-GCTCCGCGGCCGCGT$_{12}$-3′, for PAT assay) and SuperScript™ II Reverse Transcriptase (Invitrogen). Real-time PCR was performed in a final reaction volume of 20 μl with SYBR Green, a fluorophore that binds to all double-stranded DNA (qPCR kit from FINNZYMES, Finland). The primers used for PCR are shown in Table 1. PCR conditions were as follows: 10 min at 94°C followed by 39 cycles of 30 sec at 94°C, 34 sec at 59°C, 22 sec at 72°C, and a final extension of 5 min at 72°C. Finally, the relative quantification of gene expression was performed using the 2-ddCt method (Livak and Schmittgen, 2001) by normalization to internal β-actin mRNA levels (Seli et al., 2005; Zhang et al., 2010). For determination of the poly(A) tail length of maternal transcripts, PAT PCR was carried out as described previously (Zhang et al., 2009). The PCR protocol was initiated with 8 min at 94°C, followed by 33 cycles of 30 sec at 93°C, 1 min at 59°C, and 50 sec at 72°C, and was completed by a final extension of 5 min at 72°C. PCR products were electrophoresed on 2.5% agarose gel stained with ethidium bromide. Differences in poly(A) tail length were observed as smears of different lengths on the gel. As one gene may express multiple mRNA isoforms with varying 3′-UTR length, the PAT product may appear as several main bands and smears. The band represents a length intermediate between that of the gene-specific primer (GSP) and the poly(A) tail.

Immunocytochemistry

After removing the zona pellucida in acidic Tyrode medium (pH 2.5), porcine oocytes and embryos were fixed in 3.7% paraformaldehyde in PBS for 1 hr at room temperature. Cells were permeabilized for 2 hr in 1% TritonX-100. To determine the cellular distribution of calreticulin, the cells were incubated overnight at 4°C with primary anti-calreticulin rabbit polyclonal antibody (Abcam Cat.# Ab4) diluted 1:100 in 1% BSA-PBS. FITC-conjugated anti-rabbit IgG was used as the secondary antibody. After staining the nuclei with DAPI for 15 min at 37°C, the oocytes and embryos were washed thrice and mounted on slides. Each group that was subjected to immunocytochemistry contained a negative control consisting of oocytes and embryos that were incubated only with the secondary antibody and DAPI, respectively. Slides were viewed with a Leica confocal laser scanning microscope.

Antibody Microinjection Into Pig Denuded Oocytes

The fresh aspirated pig COCs were collected and released of cumulus cells. Denuded GV stage oocytes (DO) were injected with control rabbit IgG and calreticulin antibody (Cat. Ab4, Abcam, 200 μg/ml) or noninjected in a 20-μl drop of Hepes-buffered Tyrodes medium containing dbcAMP, under paraffin oil. After microinjection, the oocytes were transferred into inhibitor-free IVM medium and cultured in vitro for 44 hr.

Statistical Analysis

The general linear models (GLM) procedure in the SAS program (SAS Institute, 1985) was used to analyze the data from all experiments. Significant differences were determined using Tukey's multiple range test (Steel and Torrie, 1980). $P < 0.05$ was considered significant.

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