Changes in Expression and Localization of Connexin 43 mRNA and Protein in Porcine Ovary Granulosa Cells during Follicular Atresia

Yuan CHENG1,2), Naoko INOUE3), Fuko MATSUDA-MINEHATA1), Yasufumi GOTO1,2), Akihisa MAEDA1) and Noboru MANABE1)

1) Research Unit for Animal Life Sciences, Animal Resource Science Center, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Ago 3145, Ibaraki-Iwama 319–0206, 2) Unit of Anatomy and Cell Biology, Department of Animal Sciences, Kyoto University, Kyoto 606–8502, and 3) Laboratory of Animal Morphology and Function, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464–8601, Japan

Abstract. Gap junctions contain channels that connect neighboring cells by allowing the movement of molecules smaller than 1,200 Da. They are formed by connexins and may play a crucial role in the regulation of apoptotic cell death. To determine the role of connexin 43 (Cx43), which is dominantly expressed in granulosa cells, in the regulation of granulosa cell apoptosis during follicular atresia, we examined the changes in the expression and localization of Cx43 mRNA and protein in granulosa cells during atresia using the quantitative real-time reverse transcription-polymerase chain reaction, in situ hybridization, Western blot, and immunohistochemistry. Stages of follicular atresia were assessed based on histochemical terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) and/or the ratio of progesterone and 17β-estradiol levels in follicular fluid measured by radioimmunoassay. Cx43 mRNA was detected in granulosa cells of secondary follicles and of healthy, early and progressed atretic tertiary follicles, but not in those of primordial or primary follicles. Both phosphorylated/activated and non-phosphorylated/native Cx43 proteins were detected in granulosa cells of secondary follicles and tertiar y follicles, but not in those of primordial or primary follicles. Moreover, in tertiary follicles, these Cx43 proteins were expressed most strongly in granulosa cells of healthy follicles, but only trace levels were noted in cells of early atretic and progressed atretic follicles, an indication that the expression levels of Cx43 protein decrease during follicular atresia. These findings indicate that Cx43 is involved in the apoptosis of granulosa cells during atresia in porcine ovaries.

Key words: Apoptosis, Connexin 43 (Cx43), Follicular atresia, Granulosa cell, Porcine ovary

has been seen in scattered granulosa cells located on the inner surface of the follicular wall, but not in cumulus cells, internal theca cells, external theca cells, or oocytes [7, 8, 11]. Electron microscope observation showed that pyknotic granulosa cells and apoptotic bodies increased in number in the granulosa layer as atresia progressed, that the apoptotic bodies were occasionally surrounded and phagocytosed by neighboring granulosa cells in the early stages of atresia, and that apoptotic bodies and cell debris were fed by macrophages in follicles in the progressed stages of atresia [9]. However, the apoptotic signal transducing system, which mediates apoptosis in granulosa cells of porcine ovaries, is still not known [12].

Communication among neighboring granulosa cells, between granulosa cells and cumulus cells, and between granulosa cells and oocytes is essential for successful follicular growth and development, oocyte maturation and ovulation, and direct cell-to-cell communication in follicular cells depends on gap junctions, which play crucial roles in regulating cell growth and development in various tissues, including the ovaries [13–23]. Gap junctions are aggregates of intercellular membrane channels which allow the direct exchange of nutrients, ions, metabolites, and other potential signaling molecules smaller than 1,200 Da between adjacent cells. Each channel is formed by two connexons (hemichannels), and each connexon is composed of six identical membrane proteins (hexamer) termed connexins, which constitute a large family of at least 20 related proteins that are distinguished by their molecular weights [24, 25]. Connexins are expressed in a variety of cells, and each cell type has its own characteristic pattern of expression of connexins. In ovarian follicles, intercellular communication between oocytes and somatic cells (granulosa cells and cumulus cells) through gap junctions is involved in folliculogenesis and oogenesis, and connexin 26, connexin 30.3, connexin 32, connexin 43 (Cx43) and connexin 60 are expressed in ovarian tissues [16, 17]. Only Cx43, a 43,000 Da protein sharing 97% amino acid identity among mammalian species, is expressed in granulosa cells. Gap junctional communication from granulosa cell to granulosa cell, granulosa cell to cumulus cell, and cumulus cell to oocyte depends on Cx43 [14], and Cx43 plays critical roles in early follicular growth and development. In porcine ovaries, Cx43 mRNA and protein have been identified and localized in growing follicles [16, 17, 21]. However, the importance of Cx43 to follicular selection, in other words, granulosa cell apoptosis, has not been examined yet.

In the present study, to reveal the role of Cx43 in granulosa cell apoptosis in porcine ovarian follicles, we examined the changes in the expression of Cx43 mRNA and protein in granulosa cells during follicular atresia by conducting quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses, respectively. Moreover, we demonstrated the changes in the localization of Cx43 mRNA and protein in follicular tissues during atresia using in situ hybridization and immunohistochemical staining, respectively.

Materials and Methods

Preparation of follicular granulosa cells

The ovaries were obtained from mature sows weighing more than 120 kg at a local slaughterhouse. Individual antral follicles, approximately 3 mm in diameter, were dissected from the ovaries under a surgical dissecting microscope (SZ40, Olympus, Tokyo, Japan). Each follicle was classified as morphologically healthy or atretic, and further subdivided into early atretic and progressed atretic follicles (approximately 10 follicles/ovary and more than 60 follicles/category were used). Follicular fluid from each follicle was collected using a 1-ml syringe (Terumo, Tokyo, Japan), separated by centrifugation at 3,000 g for 10 min at 4 C, frozen and kept at –80 C until radioimmunoassay (RIA). Each follicle was opened using fine watch-maker forceps, and granulosa layers and oocyte-cumulus complexes were removed. The granulosa cells were isolated with Pasteur’s pipettes, collected, washed 3 times in phosphate-buffered saline (PBS; pH 7.2) by centrifugation at 600 g for 5 min at room temperature (air-conditioned, 22–25 C), and immediately used for RT-PCR and Western blot analyses.

RIA for 17β-estradiol (E2) and progesterone (P4) levels in follicular fluid

After the molecular biological analyses were performed, E2 and P4 levels in follicular fluid were
measured using \(^{[125}\text{I}]-\text{RIA kits}\) (Bio-M rieux, Marcy-l’Etoille, France) to confirm the classification of the follicles. Follicles with a P4/E2 ratio of less than 15 were classified as healthy according to our previous findings \([24–26]\).

**Conventional and quantitative real-time RT-PCR analyses and DNA sequencing**

As we described previously \([24–27]\), for conventional and quantitative real-time RT-PCR analyses, total RNA was extracted from isolated granulosa cells using a RNeasy mini kit (Qiagen, Chatsworth, CA, USA), and treated with a RNase-free DNase kit (Qiagen) to eliminate residual genomic DNA. The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm, respectively. Then, isolated RNA was reverse-transcribed using a T-primed first-strand kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to synthesize cDNA. Briefly, the RNA sample was heated to 65 °C for 5 min, incubated at 37 °C for 5 min, mixed with first-strand reaction mixture \([\text{dATP, dCTP, dGTP, dTTP, murine reverse transcriptase, RNAguard, RNase and DNase-free bovine serum albumin (BSA), and Not I-d(T)18 primer]}\], preincubated for 5 min at 37 °C, and then incubated for 1 h at 37 °C. The products were used for PCR analysis.

Primers for the conventional PCR amplification of partial cDNA sequences of porcine Cx43 (GenBank accession number: AJ293888) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession number: AF017079, used as an intrinsic control) were as follows. Cx43 forward: 5’-TCGTA TTCCT GTGTG TCTC G-3’, and reverse: 5’-GGAGC AGCCA TTGAA ATAAG C-3’. GAPDH forward: 5’-GAGTC CACTG GTGTC TTCAC G-3’, and reverse: 5’-ATGAG TCCCT CCACG ATGC-3’. The expected PCR products for Cx43 and GAPDH were 173 and 236 bp long, respectively. Primers for quantitative real-time PCR amplification were as follows. Cx43 forward: 5’-GGTGG ACTGT TTCCT CTCTC G-3’, and reverse: 5’-GGAGC AGCCA TTGAA ATAAG C-3’. GAPDH forward: 5’-GGACT CATGA CCACG GTCCA T-3’, and reverse: 5’-TCAGA TCCAC AACCG ACACG T-3’. The expected PCR products for Cx43 and GAPDH were 232 and 220 bp long, respectively.

Conventional PCR amplification was performed as follows. Platinum Taq DNA polymerase (10,000 Units/ml; Gibco BRL, Grand Island, NY, USA) was added to the cDNA mixture and denatured. The mixture was subjected to PCR in a thermal cycler (GeneAmp PCR Systems 9700; PE Applied Biosystems, Foster City, CA, USA). The hot-start PCR cycles for Cx43 were as follows: after denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min-55 °C for 1 min-72 °C for 1 min, followed by final extension period at 72 °C for 7 min. PCR products were electrophoresed in 2% (w/v) agarose gels (Sigma Aldrich Chemicals, St. Louis, MO, USA) and stained with ethidium bromide (Wako Pure Chemicals, Osaka, Japan). A ready-load 100-bp DNA ladder marker (Gibco) was used as a molecular weight marker for electrophoresis. After electrophoresis, the PCR product for Cx43 was purified, and then used to make cRNA probes for *in situ* hybridization.

As we previously described \([27–29]\), mRNA levels of Cx43 and GAPDH were quantified using a Light-Cycler system (Roche Diagnostics, Rotkreuz, Switzerland), according to the manufacturer’s instructions. Briefly, after cDNA synthesis, PCRs were performed in 20 \(\mu\)l of reaction mixture (Light-Cycler-DNA master SYBR green I; Roche Diagnostics) containing 0.5 mM of each primer, nucleotides, Taq DNA polymerase and MgCl\(_2\). The optimized concentrations for each primer were 4 mM for Cx43 and 5 mM for GAPDH. The conditions for amplification were as follows: Cx43: after denaturation at 95 °C for 15 sec, 35 cycles of 65 °C for 5 sec, and 72 °C for 8 sec, followed by final extension at 72 °C for 1 min; and GAPDH: following denaturation at 95 °C for 15 sec, 35 cycles of 57 °C for 5 sec, and 72 °C for 8 sec, followed by final extension at 72 °C for 1 min. The quantification was performed using Light-Cycler analysis software (Roche Diagnostics) on an IBM-compatible computer.

To confirm the expression of porcine Cx43 mRNAs, the DNA sequence of each PCR product was determined using an automatic DNA sequencer (ABI Prism 310; PE Applied Biosystems) according to the manufacturer’s directions.

**Western blot**

As we previously reported \([25, 26]\), for Western blot analysis, the protein fraction (30 \(\mu\)g/lane) prepared from each isolated granulosa cell sample was separated by 10–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
PAGE; Wako), and then transferred onto nitrocellulose membranes (Hybond-C; Amersham Pharmacia, Piscataway, NJ, USA). The membranes were stained with a 0.2% (w/v) Ponceau S solution (Serva Electrophoresis, Heidelberg, Germany). They were immersed in blocking solution [100 mM Tris-HCl, pH 8.0, 5% (w/v) skim milk, 50 mM NaCl, and 0.1% (v/v) Tween 20; Sigma] for 30 min, and incubated with mouse anti-Cx43 monoclonal antibody (diluted 1:1,000 with blocking solution; Chemicon International, Temecula, CA, USA) for 18 h at 4 C. After a single wash, they were incubated with horseradish peroxidase (HR) conjugated anti-mouse IgG antibody (1:5,000; Jackson Immuno-Research Laboratories, West Grove, PA, USA) for 1 h at 20 C. Using the same Hybond-C membrane, the β-actin protein level was also examined as an internal control. Rabbit anti-human β-actin antibody (1:2,000; Abcam, Cambridge, UK) and HRP-conjugated goat anti-rabbit IgG conjugated with HRP were used as the primary and secondary antibodies, respectively. After incubation, chemiluminescence was visualized using an ECL system (Amersham Pharmacia) according to the manufacturer’s directions. The chemiluminescence was recorded with a digital fluorescence recorder (LAS-1000; Fuji Film, Tokyo, Japan), and the intensity of each protein band was quantified using ImageGauge software (Fuji Film) on a Macintosh computer. The relative abundance of specific Cx43 protein was normalized to the relative abundance of β-actin protein.

In situ hybridization

As we previously reported [25, 26], to visualize the distribution of Cx43 mRNA by in situ hybridization, digoxigenin (DIG)-labeled antisense and sense cRNA probes for Cx43 mRNA were synthesized using a Lig’n scribe kit (Ambion, Austin, TX, USA) and DIG-RNA-labeling kit (Roche Diagnostics) according to the manufacturer’s instructions. Briefly, the conventional PCR products described above were added to the T7 phage RNA polymerase promoter by ligation to generate antisense and sense RNA probe templates. This was followed by a PCR using promoter-specific primers and a target gene-specific 5’-primer and 3’-primer. The antisense and sense probe templates were transcribed, and then DIG was attached.

The ovaries obtained from mature sows were cut (less than 1 × 1 × 1 cm), fixed with buffered 20% (v/v) formalin (Wako), dehydrated, and embedded in paraffin (Histosec; Merck, Darmstadt, Germany). Serial sections 3 µm thick were mounted on glass slides precoated with 3-aminopropyl-triethoxysilane (Silan; Sigma), deparaffined, rehydrated, and washed well in diethyl pyrocarbonate (DEPC; Sigma) treated water. They were treated with 0.2 N HCl for 10 min at room temperature, washed with phosphate-buffered saline (PBS; pH 7.2), and digested with protein-K (1 µg/ml; Sigma) in PBS for 25 min at 45 C. They were post-fixed with 4% (w/v) paraformaldehyde (PFA; Wako)-PBS for 5 min at 20 C, immersed in 2 mg/ml of glycine (Wako)-PBS for 10 min, washed well with PBS, and prehybridized with hybridization cocktails [10 mM Tris-HCl, pH 7.4, containing 600 mM NaCl, 1 x Denhardt’s solution, and 50% (v/v) deionized formamide; Amresco, Solon, OH, USA] for 1 h at 25 C. The slides were hybridized with the sense- or antisense-DIG-Cx43 cRNA probe for 18 h at 45 C. Each probe (1 µg/ml) was diluted with dilution solution [10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 600 mM NaCl, 1 x Denhardt’s solution, 10 mg/ml yeast tRNA, 10 mg/ml salmon testicular DNA, 5% (v/v) dextran sulfate and 50% (v/v) deionized formamide; Roche Diagnostics]. They were then washed well with 2 x SSC for 2 h, 0.5 x SSC for 2 h, and 0.2 x SSC for 1 h at 45 C, and then equilibrated with 100 mM Tris-HCl, pH 7.5, containing 150 mM NaCl (THS). They were treated with blocking solution for 1 h at room temperature, incubated with alkaline phosphatase (AP)-conjugated sheep anti-DIG antibody (diluted 1:500 with blocking solution) for 18 h at 4 C, washed with THS, and rinsed with 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 100 mM MgCl2 (THSM). Next, they were incubated with THSM containing 0.4 mM nitroblue tetrazolium chloride (Sigma), 0.4 mM 5-bromo-4-chloro-3-indolylphosphate-4-toluidine salt (Sigma) and 1 mM levamisole (Sigma) for 90 min at 25 C. The sections were then immersed in PBS, mounted with Histofine (Nichirei, Tokyo, Japan), and examined with a light microscope (BX51; Olympus). DEPC-treated water was used throughout the in situ hybridization staining process. The specificity of the hybridization signal obtained was confirmed by parallel incubation with antisense and sense cRNA probes. As further
negative controls, serial sections were hybridized without any probes, or they were incubated without anti-DIG antibody. All controls yielded completely negative results.

Detection of apoptotic cells and immunohistochemistry for Cx43

As we previously reported [25], apoptotic cells in porcine ovarian sections were histochemically detected. Briefly, serial 3-μm thick paraffin sections from each specimen were stained by the terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) method using an Apop-Tag kit (Intergen, New York, NY, USA) according to the manufacturer’s directions. The sections were counter-stained with methyl green, dehydrated, mounted with Entelan (Merck), and then examined under a light microscope. The following positive and negative controls were included in each experimental run. As negative controls, sections were incubated by omitting either terminal deoxynucleotidyl transferase or anti-DIG antibody. As a positive control, sections were treated with DNase I (1 mg/ml; Boehringer-Mannheim, Indianapolis, IN, USA), 140 mM sodium cacodylate, 4 mM MgCl₂, and 0.1 mM dithiothreitol in 30 mM Tris-HCl, pH 7.2, for 10 min at room temperature. Paraffin sections prepared from young adult rat testis were used as physiological positive controls.

To visualize the localization of Cx43 protein, the ovaries obtained from mature sows were cut (less than 1 × 1 × 1 cm) and rapidly frozen in liquid nitrogen. Frozen serial sections (8 μm thick) of ovaries cut on a cryostat (CM1500; Leica, Heidelberger, Germany) were mounted on glass slides precoated with Silan, and then fixed in precooled acetone (−80 C) for 10 min. After being washed wish PBS, the slides were preincubated with nonphosphorylated/activated Cx43 protein, or with mouse anti-Cx43 monoclonal antibody (diluted 1:250 with PBS; Chemicon International), which can detect non-phosphorylated/native Cx43 protein, for 18 h at 4 C. After being washed with PBS containing 0.1% (v/v) Tween 20 (PBS-T) for 5 min, they were incubated with Cyan-3-conjugated goat anti-rabbit IgG antibody (diluted 1:800 with PBS-BSA; Jackson Immunoresearch) or with Alexa 488-conjugated goat anti-mouse IgG antibody (diluted 1:800 with PBS-BSA; Molecular Probes, Poort Gebouw, NZ, USA) for 2 h at room temperature. After a single wash with PBS-T, they were mounted with glycerol, and examined with a confocal laser-scanning microscope (Fluoview FV3000, Olympus).

Statistical analysis

All experiments including the isolation of follicles and preparation of granulosa cells were repeated three times with separate groups (nine sows/group) for independent observation. Before ANOVA, the homogeneity of variance was assessed using the StatView-4.5 program (Abacus Concepts, Berkely, CA) on a Macintosh computer. ANOVAs with Fisher’s least significant differences test for biochemical data and Wilcoxon’s signed-rank tests for histological estimation were carried out using the StatView-4.5 program on a Macintosh computer. Differences at \( P<0.05 \) were considered significant.

Results

Changes in the expression of Cx43 mRNA during follicular atresia

The RT-PCR products detected in the present study were confirmed to be porcine Cx43 by sequencing the cDNA as follows: The cDNA sequence of Cx43 amplified by conventional RT-PCR was 5’-TCGTA TCCTT GGTGT CTCTC GCCCT GAACA TACAC CAGGC ACTTT AAGGG TGGAT CTCCA-3’ (173 bp), while that amplified by quantitative real-time RT-PCR was 5’-GGTGG ACTGT TTCCT CTCTC GTCCC ACGGA GAAAA CACAT TTCAT TACAT TCAAG TGGAT CTCCA-3’ (173 bp), while that amplified by quantitative real-time RT-PCR was 5’- GGTTG ACTGT TTCAT TACAT TCAAG TGGAT CTCCA-3’ (173 bp), while that amplified by quantitative real-time RT-PCR was 5’-GGTTG ACTGT TTCAT TACAT TCAAG TGGAT CTCCA-3’ (173 bp).
AAATA CGCTT ATTTC AATGG CTGCT CC-3’ (232 bp). That of GAPDH amplified by conventional RT-PCR was 5’-GAGTC CACTG GTGTC TTCAC GACCA TGAGG AAGGC CGGGA CTCAC TTGAA GGTTG GGGCC AAGAG GATCA TCAAC TCGCC GATGC CCCCA TGTTT GTGAT GGCGG TGAAC CATGA GAAGT ATGAC AACTC CCTCA AGATC GTCA GAAAT CCTCC TGCCG CACCA ACTGC TTGGC ACCCC TGGCC AAGGT CATCC ATGAC CACTT CGGCA TCGTG GAGGG ACTCA T-3’ (236 bp), and that amplified by quantitative real-time RT-PCR was 5’-GGACT CATGA CCACG GTCCA TGCCA TCACT GCCAC CCAGA AGACT GTGGA TGGCC CTGCT GGGAA GCTGT GGGGT ATGAG CCGAG GGCT GACCA AACAA TCAAC CTGCT TTCTA CCGGC GCTGC CAAAG GTGTC GGCAC GGTCA TCCCT GAGCT CAACG GGAGG CTCAC TGGCC TGGCC TTCCG TGTCC CCACC CCCAA CGTGT CGGTT GGGGA TCTGA-3’ (220 bp).

Cx43 and GAPDH mRNAs were detected in isolated granulosa cells of healthy, early atretic and progressed atretic follicles by quantitative real-time RT-PCR (Fig. 1A). Cx43 mRNA was constantly expressed in granulosa cells. Lower levels of Cx43 mRNA were seen in granulosa cells of early atretic and progressed atretic follicles than in granulosa cells of healthy follicles. The quantitative real-time RT-PCR data indicated that Cx43 mRNA expression slightly decreased during follicular atresia (P<0.01; Fig. 1B).

Changes in the expression of Connexin 43 (Cx43) mRNA in granulosa cells

By Western blot, a strong immunoreaction for Cx43 protein (43 kD) was demonstrated in the granulosa cells prepared from healthy follicles (Fig. 2A). Decreased expression of Cx43 protein was seen in granulosa cells of early atretic and progressed atretic follicles, and only, trace levels of Cx43 protein were noted in granulosa cells from progressed atretic follicles. Changes in chemiluminescence intensity quantified using an automatic image analyzer showed that Cx43 protein expression decreased during follicular atresia (P<0.001; Fig. 2B).

Localization of Cx43 mRNA in follicles

A constant expression of Cx43 mRNA was detected in the granulosa cells including cumulus cells of secondary and tertiary follicles, but not in granulosa cells of primordial or primary follicles. Signals of Cx43 mRNA observed in granulosa cells of secondary follicles were weaker than those seen in granulosa cells of tertiary follicles. In healthy, early atretic, and progressed atretic tertiary follicles, Cx43 mRNA was detected in granulosa cells, and its expression decreased during follicular atresia (Figs. 3A, B and C, respectively). When the sections were incubated with sense cRNA probes, no positive staining was seen.

Localization of phosphorylated/activated and native Cx43 proteins and apoptotic cells in follicles

Positive stainings for both phosphorylated/activated and native Cx43 proteins were detected in granulosa cells including cumulus cells of
secondary and tertiary follicles, but not in granulosa cells of primordial or primary follicles. Weak staining for these Cx43 proteins was seen in internal thecal or external thecal layers of primordial, primary, secondary, or tertiary follicles. Stronger staining for phosphorylated and native Cx43 proteins was observed in granulosa cells of healthy tertiary follicles (Figs. 3D and G, respectively). During follicular atresia, decreased levels of staining for these phosphorylated and native Cx43 proteins were seen (Figs. 3E and F, and H and I; early atretic and progressed atretic follicles, respectively). Interestingly, distinct immunostaining was observed in cumulus cells of healthy and early atretic follicles.

No TUNEL-positive/apoptotic cell was observed in primordial, primary, or secondary follicles. As with our previous studies [11, 25], in tertiary follicles, no TUNEL-positive cell was seen in the granulosa, internal thecal or external thecal layers of healthy follicles (Fig. 3J). Apoptosis occurred in granulosa cells located on the inner surface of the follicular wall of follicles in the early stages of atresia (Fig. 3K). Finally a TUNEL-positive reaction was seen in most granulosa cells of progressed atretic follicles (Fig. 3L).

Discussion

It has not been clearly shown which molecule(s) acts as an initial trigger for granulosa cell apoptosis during follicular atresia in mammalian ovaries [1–6]. The balance between survival and death factors is crucial in determining the fate, proliferation or death, of granulosa cells [6]. Gap junctions are essential for maintaining interactions between cells within a tissue and their homeostasis, and regulate growth/proliferation and death [24, 25]. In ovarian follicles, gap junctions are considered to play important roles in follicular growth and development and in the prevention of follicular atresia in developing preantral secondary follicles [13–23]. Although the role of gap junctions in mammalian follicles has been investigated, there is insufficient information on whether or not Cx43, which is a dominant gap junction protein in porcine follicles [16, 17] and has multiple phosphorylated/activated and non phosphorylated/native forms [30], is involved in granulosa cell apoptosis during atresia in porcine ovaries. In the present study, we examined the changes in the expression and localization of Cx43 mRNA and its protein in granulosa cells of porcine ovaries during follicular atresia.

In primordial or primary follicles, in which follicular epithelial cells, the precursors of granulosa cells, form a monolayer surrounding the oocyte, no mRNA or protein of Cx43 was detected. In secondary follicles, in which follicular epithelial cells begin to rapidly proliferate and configure the stratified epithelium, production of Cx43 was detected. In secondary follicles, in which follicular epithelial cells begin to rapidly proliferate and configure the stratified epithelium, production of Cx43 was detected. Both phosphorylated and native Cx43 proteins were detected in granulosa cells of these follicles. Phosphorylation modulates the conformation of Cx43 protein, and thus phosphorylated Cx43 opens the gap junction hole [15, 30]. In tertiary follicles, in which the follicular
The localization of connexin 43 (Cx43) mRNA was examined by in situ hybridization. Porcine ovarian sections from healthy, early atretic, and progressed atretic follicles were hybridized with Cx43 antisense cRNA probe (A, B, and C, respectively). Positive staining for Cx43 mRNA was detected in granulosa cells including cumulus cells (inlets) of healthy, early atretic, and progressed atretic tertiary follicles. Decreases in Cx43 mRNA expression were seen in granulosa cells during follicular atresia. Porcine ovarian sections were stained immunohistochemically with antibodies that recognize phosphorylated/activated (D, E, and F) and native (G, H, and I) Cx43 proteins. During follicular atresia, decreases in the staining for these phosphorylated and native Cx43 proteins were seen. Apoptotic cells were detected using the TUNEL method (J, K, and L). Magnification: × 200.

Fig. 3. The localization of connexin 43 (Cx43) mRNA was examined by in situ hybridization. Porcine ovarian sections from healthy, early atretic, and progressed atretic follicles were hybridized with Cx43 antisense cRNA probe (A, B, and C, respectively). Positive staining for Cx43 mRNA was detected in granulosa cells including cumulus cells (inlets) of healthy, early atretic, and progressed atretic tertiary follicles. Decreases in Cx43 mRNA expression were seen in granulosa cells during follicular atresia. Porcine ovarian sections were stained immunohistochemically with antibodies that recognize phosphorylated/activated (D, E, and F) and native (G, H, and I) Cx43 proteins. During follicular atresia, decreases in the staining for these phosphorylated and native Cx43 proteins were seen. Apoptotic cells were detected using the TUNEL method (J, K, and L). Magnification: × 200.

antrum is formed, and a proportion of the stratified follicular epithelial cells/granulosa cells, which surround the oocyte, differentiate into cumulus cells, and accelerated production of Cx43 was noted. The histochemical localization of phosphorylated and native Cx43 proteins in
healthy follicles of porcine ovaries is similar to that in ovine and bovine follicles but not in rodent follicles [18–20]. In atretic tertiary follicles, less Cx43 mRNA was noted, and a rapid disappearance of phosphorylated and native Cx43 proteins was observed. Granulosa cells readily proliferate in healthy tertiary follicles [11], but pyknotic granulosa cells and apoptotic bodies in the granulosa layer were surrounded and phagocytosed by neighboring granulosa cells, which had normal morphology, of the follicles in the early stages of atresia [9]. A specific sugar residue, Siaα2,6Gal/GalNAc, which binds with the terminal position of the sugar chain in complex-type N-glycans, of cell-membranous glycoproteins, appeared only in granulosa cells of atretic follicles [24, 31]. We confirmed the up-regulation of the mRNA expression of ST6Gal I, which is a α2,6 sialyltransferase and catalyzes the transfer of α2,6-sialic acid in cell-membranous glycoproteins, in granulosa cells during follicular atresia [24]. Moreover, our previous findings on extracellular matrix (ECM) components [32, 33] and cell adhesion factors, cadherins and β-catenin [34] in porcine follicles showed that the cell adhesion factors act as a survival factor, and intracellular signaling through these factors may depend on the protein tyrosine-kinase cascade [35]. It is considered that the expression of cell death receptors [36–39] and decoy receptors [40, 41], and apoptosis inhibiting factors [42] also depends on cell to cell contact through the cell adhesion factors.

We presume that gap junction components may also affect these cell death regulatory factors similar to cell adhesion factors.

Based on our previous and present findings [7–12, 24–26, 31–43], we hypothesize that the apoptotic process in granulosa cells is as follows. Granulosa cells in healthy tertiary follicles maintain interaction with neighboring granulosa cells through gap junction components, dominantly Cx43, and cell adhesion factors, and vigorously proliferate. However, early atretic follicles lose their cell to cell connections, become detached from neighboring granulosa cells, and progress to apoptosis. The loss of gap junctions induces an up-regulation in the expression of ST6Gal I, and then the cell membrane glycoconjugates are altered by ST6Gal I. Such changes in the cell-membranous signal act as a trigger for phagocytosis, and the neighboring granulosa cells begin to phagocytose the crumbling apoptotic granulosa cells. Based on the results of the present study, we conclude that Cx43 acts as a survival factor and is involved in the apoptosis of granulosa cells in porcine ovaries.

Acknowledgements

This study was supported by a Grant-in-aid for Creative Scientific Research (13GS0008) and a Grant-in-aid for Scientific Research (S) (16108003) to N. M. from the Japan Society for The Promotion of Science.

References


