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Matrix metallopeptidases regulate granulosa cell remodeling through the hormone signaling pathway

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ABSTRACT

Objective: Granulosa cells (GCs) play a very important role in reproductive physiology due to their effect on developmental and functional changes. However, there are differing views regarding the mechanism by which hormones stimulate GCs. Therefore, our study aims to determine whether GCs, in the absence of initial stimulation (17β-estradiol), select specific types of MMPs that reconstitute cells by stimulation of major hormones [follicle-stimulating hormone (FSH) or/ and luteinizing hormone (LH)].

Materials and methods: Early GCs were extracted from immature follicles of the porcine ovary to analyze the MMPs levels. Using early GCs in pigs, the cell development rate was evaluated by adding 17β-estradiol, FSH, LH, or FSH + LH, respectively, to the DMEM containing 10% FBS. Real-time PCR, zymography, enzyme-linked immunosorbent assay, western blot, and immunofluorescence analysis were also performed to determine the MMPs activation in the GCs.

Results: Our results confirm that FSH or LH stimulation regulates cell development and intracellular MMPs. In particular, FSH activity kept the MMP-2 and MMP-9 expressions constant in GCs. Conversely, LH activity initially led to rapid increases in the MMP-9 expression, which 96 h later was similar to the MMP-2 expression. Simultaneous utilization of FSH + LH maintained a steady MMP-9 expression and the development of GCs increased. Additionally, when FSH and LH were processed simultaneously, the number of cells increased without changes in cell size, while the cell size changed when LH alone was used.

Conclusion: Therefore, the results of this study confirm that even without the initial stimulation of GCs, physiological changes occur according to hormonal changes in the environment, and there is variability in the expression of MMPs.

Introduction

Granulosa cells (GCs) play a very important role in the maturation and development of oocytes during embryogenesis as well as the functional maintenance of the ovary. The GCs undergo metabolic processes after stimulation by the follicle-stimulating hormone (FSH) in the early stages and dynamically differentiate cells. During estrus, the FSH and luteinizing hormone (LH), which are the representative hormones, cause functional changes in the ovaries. The follicles acquire the ability to synthesize estrogen in theca interna cells following stimulation by FSH and LH of a certain level, and subsequently, stimulation by estrogen increases the LH receptor

expression in the GC, expanding the cellular reorganization and follicle size [1,2]. At this stage of development, the GC with receptors for FSH and estradiol end cell growth by activating and binding a certain level of the LH receptor and play the role of expanding the follicle to the brink of final ovulation [3]. Due to the explosive expansion of the cumulus cells surrounding the oocyte after the sex hormone signaling process, the theca interna cells and GCs perform the role of luteal cells [4]. In addition, after ovulation, the GC increases the expression of the LH receptor and the production of progesterone [5]. As such, the role of a GC is to perform a rapid activation or inhibition of hormonal feedback to the environmental changes

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caused by hormones and form functional changes in cells [6]. The functional and physical changes of GCs cause successful physiological changes by secreting the miracle membrane-degrading enzymes matrix metallopeptidases-2 (MMP-2) and MMP-9 to decompose the type VI collagen component of the extracellular matrix [7,8]. Recent studies have shown that in early and mid-term follicles, the GC has not been able to activate the LH receptor, but has activated the LH receptor by estrogen constructed from theca cells [9]. In other words, estrogen activity must be actively reflected for smooth functional changes of the GCs. Comprehension of the key action of MMPs in physiological changes in GCs is also important. MMP-2 is understood to play an important role in the activation of extracellular membrane fragmentation, and MMP-9 is known to act upon cell nuclear fission or cytoplasmic changes [10,11]. Based on these findings, we have two questions. First, are GCs able to accept the role of FSH or LH without estrogen action? And does the action of major MMPs change with the action of FSH or LH? These two questions refer to important issues that could explain the autonomous physiological functions of GCs. Therefore, this study aims to obtain data on the independent functionalities of GCs for hormones by analyzing whether GCs, in which the initial stimulation of estrogen is removed, preferentially select MMPs according to the stimulation of major hormones.

Materials and Methods

Ethical approval

All animal procedures followed the protocol approved by the Animal Experimentation Ethics Committee at Hankyong National University (permission number: 2018-1).

Granulosa cell culture

The ovaries of porcine (Landrace) from slaughterhouses were placed in saline solution at 35°C, transported to our laboratory within 2 h, and washed four times with saline solution. Liquor folliculi from the follicles (4–8 mm in diameter) was aspirated using an 18-gauge needle attached to a 5-ml syringe. After that, the supernatant containing mostly individual cells was discarded and transferred to Petri dishes. With the aid of a micropipette, pellets and debris were removed from the liquor folliculi and washed twice in the Dulbecco's modified Eagle's medium (DMEM). Aliquots of the resuspended cells were separated using a 1-ml syringe (23-gauge needle) and counted with a hemocytometer to measure cell consistency. The GC was plated in the DMEM containing 10% FBS (Life Technologies, cat no. 16000-044) at a density of $3.5 \times 10^7 \pm 0.3$ in a T-25 flask (Becton Dickinson and Co., Franklin Lakes, NJ) for 24 h, to facilitate cell attachment. After settling, by using each 3×10^3/ml GC in porcine, the medium was replaced with fresh DMEM containing 10% FBS supplemented with either 17β-estradiol (2 U/ml: Control), FSH (5 U/ml), LH (5 U/ml), or FSH+LH (5 U/ml). It was then cultured for 24, 48, or 96 h at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Quantification RT-PCR of MMPs and tissue inhibitors of metalloproteinases (TIMPs)

Total RNA was isolated and cDNA was synthesized in each hormone treatment group by applying the experimental method provided by TRIzol reagent (Invitrogen, Carlsbad, CA) and SuperScript II Reverse Transcriptase (Invitrogen). For qRT-PCR, it was analyzed in Line-gene K (Bioer Technology, Tokyo, Japan) using SYBR Green (TOYOBO, Osaka, Japan). The primers listed in Table 1 were used for the Quantification Real Time PCR (qRT-PCR).

The analysis of the results of the cycle threshold (Ct) for target gene was normalized to the porcine glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene) mRNA level to calculate the relative expression level of each gene using the 2-ΔΔCt method.

Table 1. Primer sequences for MMPs and TIMPs gene for real-time reverse transcription-PCR (RT-PCR).

Primer	Sequence	Product size
Porcine GAPDH for	5′-CCCGTTCGACAGACAGCCGTG-3′	NM_001206359.1
Porcine GAPDH rev	5′-CCGCCTTGACTGTGCCGTGG-3′	
Porcine MMP-2 for	5′-GGCAGCCCTCAGTTCTGTAA-3′	NM_214192.2
Porcine MMP-2 rev	5′-TACTTGAGCACAGGGCAGAG-3′	
Porcine MMP-9 for	5′-CTTGCCTTCTCATGCTGGGACT-3′	NM_001038004.1
Porcine MMP-9 rev	5′-GTGAGGATAGCACTTGGTCTGGCT-3′	
Porcine TIMP-2 for	5′-GGGTCTCGCTGGACATTG-3′	NM_003255.5
Porcine TIMP-2 rev	5′-AGCTGATCAGGATCCCCC-3′	
Porcine TIMP-3 for	5′-CCCTTCCCACTGAGCTTCCCTT-3′	NM_000362.5
Porcine TIMP-3 rev	5′-CTATCTGCTGGCTGCCCTTGAC-3′	

Analysis of MMP activity

To analyze the enzymatic reaction of MMPs, Kim et al.'s [11] methods were applied. *In vitro* maturation medium was centrifuged at 3,000 g; the supernatant was discarded, and the sediment was mixed with 20 μl of Fast of zymography (FOZ) loading buffer (5% Bromo phenol blue, 10% SDS and 2% Glycerol) and 4 μl of a zymography reaction solution. It was then reacted for 5 min on ice and then electrophoresed for 1.5 h at 150 V in sodium-dodecyl-sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of gelatin. After electrophoresis, the gel was induced to reform proteins twice for 20 min and washed with sterilized water. After the reformation, the enzymatic reaction was processed in an enzyme reaction buffer at 37°C for 18 h. Protein staining of the finished MMPs reactive gel was induced by Coomassie Brilliant Blue (Bio-Rad Laboratories, Hercules, CA) for 1 h, and the bleached regions were analyzed.

Enzyme-linked immunosorbent assay (ELISA) of MMP-9

For the ELISA, the MMP-2 and 9 levels were measured using a quantitative sandwich ELISA test (R&D Systems, Abingdon, UK) following the instructions of the manufacturer. All samples were measured three or more times (AVG ± SEM) and then determined according to the standard curve of each protein, and four parameters were considered based on the following equation: $y = (A-D)/(1 + (x/C)^B) + D$).

Western blot analysis

The GCs protein extract (30 μg) was separated by SDS-PAGE using 13% gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membrane was blocked overnight at 4°C with 5% skimmed milk and then washed for 10 min with the wash buffer (0.1% v/v Tween 20, 50 mM Tris-HCl and 200 mM NaCl; pH 7.6). The transferred membrane was incubated for 2 h with first antibodies (diluted 1:1,000 in blocking buffer) recognizing the active forms of TIMP-3 (sc-6836, Santa Cruz Biotechnology Inc., Dallas, TX) and β-actin (sc-47778, Santa Cruz Biotechnology Inc., Dallas, TX). After binding the antibodies, the membranes were washed three times for 15 min each with TBS-T (40 mM Tris-HCl pH 7.4, 25 mM NaCl, 0.1 % Tween-20) buffer and incubated for 2 h with horseradish peroxidase (HRP)-conjugated anti-goat (ab6721-1, Abcam, Cambridge, UK) or anti-mouse (ab6741, Abcam, Cambridge, UK) secondary antibodies (Abcam, Cambridge, UK; diluted 1:5,000 in blocking buffer). The transferred membranes were reacted with the enhanced chemiluminescence (ECL) detection reagent in the dark and then exposed to X-ray film for about 10 min; protein expression was normalized to that of β-actin protein, which acts as an internal control target, using the Alpha Innotech software (San Leandro, CA).

Immunofluorescence assay

Granulocyte cells were fixed with 4% paraformaldehyde, washed with PBS, and then blocked with 0.3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Dehydration and permeabilization were carried out by freezing the slides at −20°C in TBS (5 mM 0.1% Triton X-100 in PBS). After blocking with 3% BSA in PBS, the slides were incubated with a monoclonal antibody that specifically recognizes the active form of the MMP-2 (ab78796, Abcam, Cambridge, UK) and MMP-9 (Santa Cruz Biotechnology Inc., Dallas, TX) at a 1:200 dilution. After washing, all the slides were incubated for 2 h with anti-rabbit IgG conjugated to Alexa-488 or 594 phalloidin (Thermo Fisher Scientific, Waltham, MA). Nuclei staining was counterstained with Hoechst 33258, and all the slides were mounted using the fluorescent mount buffer (Dako, Carpinteria, CA). Phase difference and protein expression images were obtained using a fluorescence microscope (Olympus AX70).

Statistical data analysis of mRNA and protein ratios

Data were subjected to a t-test and the generalized linear model using the Statistical Analysis System software (SAS Institute, version 9.4, Cary, NC). Duncan's multirange test was used to determine the difference in mean values for each treatment group. Statistical significance was established at $p < 0.05$.

Results

Expression of MMPs-associated genes and cell development rate according to hormone added to the culture

The analysis of cell development according to the administration of each hormone confirmed that the cell development in the control group increased significantly in the initial 24 h, but increased in the FSH + LH group from 48 to 96 h. Comparatively, FSH and LH showed different patterns; FSH increased over 48 h and LH showed the greatest increase at 96 h. In other words, the FSH + LH group increased steadily from the initial 24 to 96 h, but LH increased at 96 h after hormone exposure (Table 2). Regarding the expression patterns of MMPs and TIMPs by culture time, MMP-2 significantly increased in the control group at 48 h, FSH at 48 h, and LH at 96 h. However, the FSH + LH group was confirmed of having the lowest expression. MMP-9 in the control group increased gradually but gradually decreased in the FSH and LH groups. In addition, the expression in the FSH + LH group decreased rapidly at 96 h. The expression patterns of TIMPs showed different results from MMPs. TIMP-2 expression was significantly higher in the FSH group than the other groups at all incubation times, and TIMP-3 did not show a particular pattern, but the LH and FSH + LH groups increased at 24 and 96 h. The results are shown in Figure 1.

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The activity of MMPs and the expression pattern of TIMP-3 according to the treatment group

The analysis of MMPs' activity according to culture time identified that MMP-2 was very high in the FSH group at 24 h, while MMP-9 was relatively low. After 48 h, MMP-2 was relatively lower in the FSH group and MMP-9 was increased in the control and FSH groups. The expression of MMP-2 at 96 h was relatively low in the control and FSH + LH groups, and MMP-9 expression appeared similar, but slightly increased in the LH group. TIMP-3 mRNA showed a different expression pattern, it had a low expression in the FSH

Table 2. Cell development rate according to hormone addition.

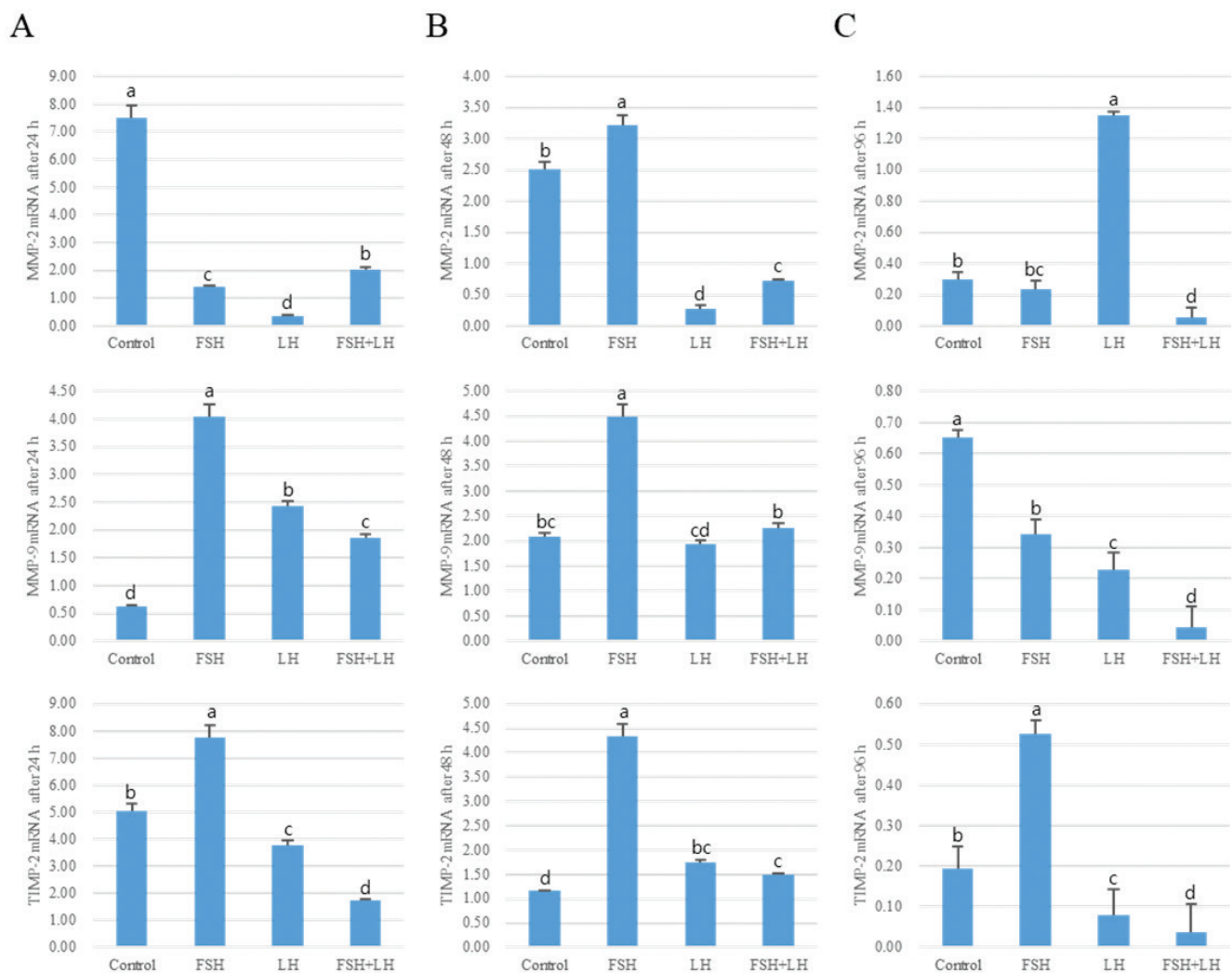| Group	Cell seeding	Culture times		
		24 h	48 h	96 h
17β-estradiol (control)		$4.5 \times 10^3 \pm 0.1^{**}$	$7.2 \times 10^4 \pm 0.1^{*}$	$4.3 \times 10^5 \pm 0.5$
FSH	$3.0 \times 10^3 \pm 0.2$	$3.0 \times 10^3 \pm 0.1$	$6.5 \times 10^4 \pm 0.2^{*}$	$5.0 \times 10^5 \pm 0.3$
LH		$3.5 \times 10^3 \pm 0.2$	$5.7 \times 10^4 \pm 0.3$	$7.0 \times 10^5 \pm 0.3^{*}$
FSH + LH		$4.0 \times 10^3 \pm 0.2^{*}$	$8.7 \times 10^4 \pm 0.3^{**}$	$6.3 \times 10^6 \pm 0.2^{**}$

Figure 1. Gene expression analysis of MMPs and TIMPs in the GCs according to each hormone treatment group. (A) after 24 h, (B) after 48 h, (C) after 96 h, Real-time PCR data represent the mean ± SEM of five individual experiments and were normalized against β-actin (housekeeping gene) as an internal standard. *Different letters within the same column represent a significant difference ($p < 0.05$).
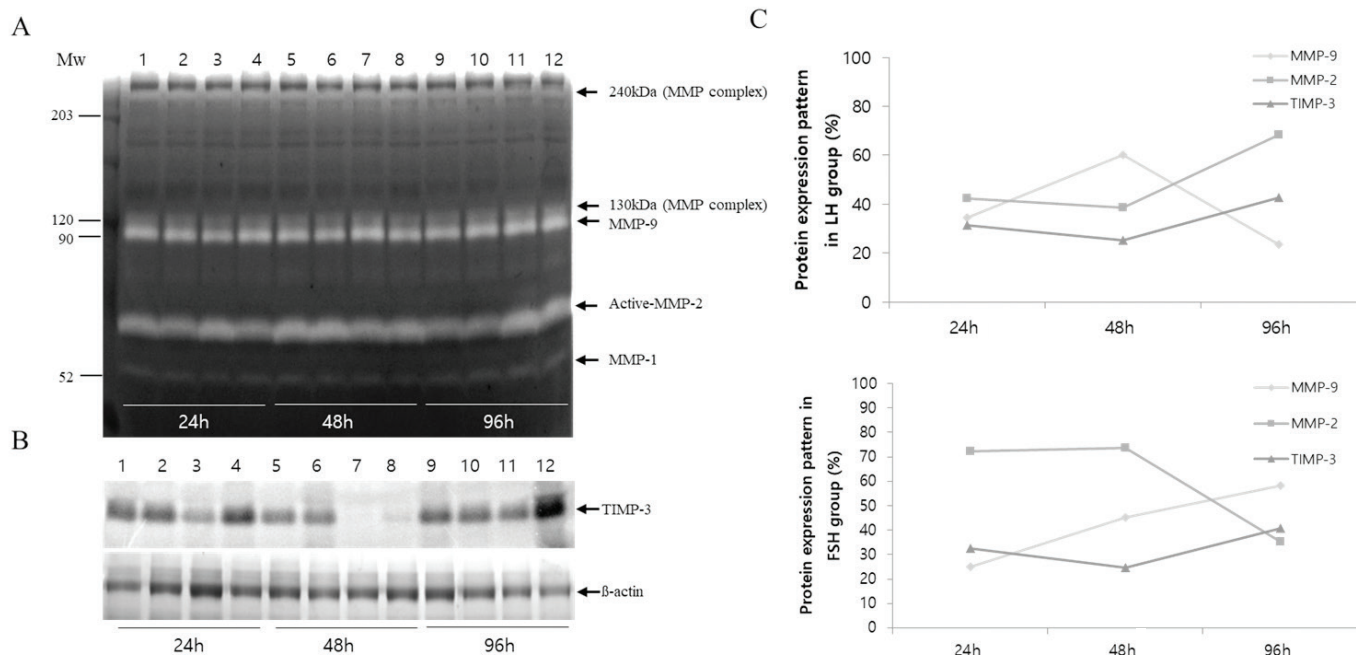
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Figure 2. Activation and expression analysis of MMPs and TIMPs proteins in the GCs according to each hormone treatment group. (A) Gelatin zymography, (B) Western blot, (C) ELISA: *1–4* After 24 h, *5–8* After 48 h, *9–12* After 96 h, *1; 5; 9* Control, *2; 6; 10* FSH + LH, *3; 7; 11* FSH, *4; 8; 12* LH. Normalized against β-actin (housekeeping gene) as an internal standard. *Different letters within the same column represent a significant difference ($p < 0.05$).
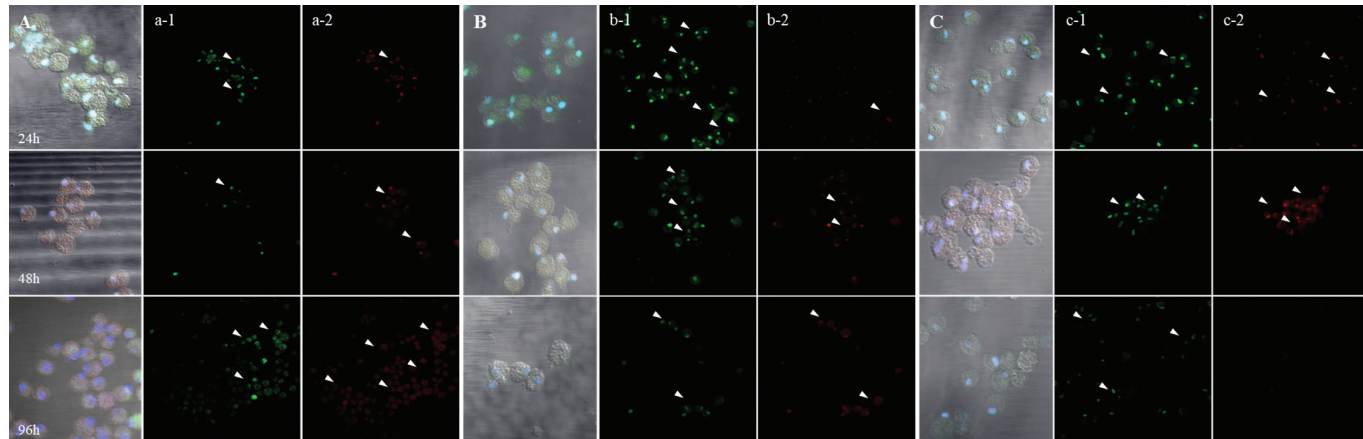

Figure 3. *In situ* detection of MMP-2 and MMP-9 proteins in each cultured porcine GC. Cells were counterstained with Hoechst 33258. White arrows indicate cells expressing specific proteins. (A) FSH + LH, (B) FSH, and (C) LH: MMP-2, *a-1*; *b-1*; *c-1*; MMP-9, *a-2*; *b-2*; *c-2*.

group at 24 h, and the expression was only confirmed in the control and FSH + LH groups at 48 h, and the LH group showed a very high expression at 96 h. The analysis of the MMPs and TIMP-3 expression patterns in the concentrated FSH and LH culture medium showed the same pattern as seen in the zymography and western blot results. Particularly in the LH group, MMP-2 and TIMP-3 increased gradually, but MMP-9 gradually decreased. Comparatively, MMP-2 expression in the FSH group remained steady from

24 to 48 h and decreased at 96 h, while MMP-9 gradually increased. However, TIMP-3 showed lower expression than MMPs but gradually increased from 24 to 96 h (Fig. 2).

Analysis of expression patterns of MMPs in the cytoplasm of cells according to hormone treatment

It is confirmed that the MMP expression increased in the cytoplasm of all the treatment groups. In the FSH + LH group, MMP-2 increased at 24 h and MMP-9 increased from

48 to 96 h. Also, the density between cells increased. In the FSH group, MMP-2 expression appeared to remain steady from 24 to 96 h, and MMP-9 increased from 48 to 96 h, showing a similar expression pattern to MMP-2. However, the density between cells decreased. In the LH group, the MMP-2 expression in the cytoplasm showed a similar pattern to that of the FSH group, but the expression of MMP-9 increased from 24 to 48 h and was hardly observable at 96 h. In particular, the cell sizes of the FSH and LH groups were relatively larger than that of the FSH + LH group (Fig. 3).

Discussion

A feature of GCs is the functional role changes with the development of follicles. In particular, the LH receptors are expressed by the stimulation of FSH and estradiol. Thereafter, as the LH concentration in the blood increases, the LH receptor in the GC increases [12,13]. We developed two questions stemming from our previous research. First, is there an absence of estradiol stimulation in early GCs, and can cell reconstitution be activated only by the FSH or LH stimulation? Second, if cells begin to reconstitute, are there differences in the types and roles of MMPs that are primarily used for reconstitution? Through these two questions, our study attempts to prove whether changes in the physiological functions of GC are actively developed. The physiological changes of GCs are known to play a very important role from estrus to gestation, as they are secretory cells that secrete progesterone by expanding the follicle, maturing the egg, and maintaining the corpus luteum after ovulation. The role of GCs becomes more closely related to hormones during the development of oocytes when they are stimulated by the signal transduction of hormones built from theca cells and carry out the central function of metabolism [14,15].

The results of our study confirm that the development of cells in porcine GCs is different in the group with only estradiol or FSH, LH, and FSH + LH. In particular, compared to the group treated with only estradiol or FSH, the generation of cells was significantly increased in the group treated with FSH + LH. There was also a difference in the cell development pattern and MMPs gene expression according to the hormone added. GCs cause apoptosis in the process of performing functional and physiological changes and reorganize tissue by forming new cells [5,16]. This series of processes is thought to determine the major expression of MMP-9 or MMP-2 as indicated in Kim et al.'s [10] study, which confirmed that the selection of MMPs varies depending on the stimulation of hormones. Regarding FSH, it is confirmed that MMP-2 and MMP-9 act simultaneously to stimulate GC growth and increase metabolic processes. In addition, in the case of LH, MMP-9 is expressed at an extremely high level in the cytoplasm at 48 h and appears to influence cell size and reconstitution. FSH + LH also maintained the development of cells

while maintaining a steady MMP-9 expression up to 96 h. In other words, unlike our findings from Xu et al.'s [16] study, the GC is thought to increase the metabolism of cells through hormone receptors according to various environments, even if there is no initial stimulation of estradiol. Specifically, estradiol is thought to be a stimulation tool that increases the function of GCs [9]. GCs start many physiological actions after ovulation until corpus luteum is formed, and after corpus luteum is formed, it increases the apoptosis of aging cells, and new cells reconstitute and attain MMP mechanisms [11,12].

In particular, our research findings, similar to Dominguez et al. [17], indicate that GCs are likely to increase the supplementary role of apoptosis in cell remodeling and increase MMP-9 expression when cell generation is maintained, thereby facilitating cell differentiation. Our research findings answer our two questions. It is confirmed that in an environment without stimulation of estradiol, GCs can release LH or FSH receptors only by the stimulation of key hormones, and promote cell differentiation through selective MMP cell reconstitution. However, questions remain about how different stimuli of hormones determine the type of MMPs. Our findings may provide new information about GC remodeling, and provide important clues regarding infertility due to abnormal hormonal mechanisms. In addition, future research on the association of hormones acting on the GCs and mechanisms of MMPs may lead to the identification of new GC functionalities.

Conclusion

With regard to oocytes maturation in pig polycystic follicles, the choice of the hormonal environment is very important, of which is the regulation of ovulation by proper stimulation of GC. In the meantime, we have little understanding of ovarian management to improve piglet production. Therefore, our study finds that GC itself can determine its development according to the hormonal environment, and such a study can be important data for the increase in the litter size in the future.

Acknowledgment

Conflict of interests

The authors declare that they have no conflict of interest.

Authors' contribution

SH Kim participated in developing the protocol, the sample granulosa cell, and in drafting the manuscript. SH Kim also

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