**VARIATIONS IN THE EXPRESSION PATTERN OF *HSP27*
AND *MSK1* GENES DURING THE DEVELOPMENT
OF PREHIERARCHICAL FOLLICLES IN THE ZI GEESSE
(*ANSER CYGNOIDES*)***

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Abstract

The p38MAPKs (mitogen-activated protein kinases) signaling contributes a pivotal role in mammalian ovarian follicular development; however, the knowledge regarding their expression in geese remains unresolved. The objective of the current study was to determine the spatio-temporal expression of heat shock protein 27 (*HSP27*) and mitogen- and stress-activated protein kinase 1 (*MSK1*) genes in the prehierarchical follicles during geese ovarian development. The prehierarchical follicles samples were harvested from 35- to 37-week-old healthy laying geese. *HSP27* and *MSK1* relative expression in various sized prehierarchical follicles was detected by real-time quantitative polymerase chain reaction (RT-qPCR) and western blotting. Follicular wall localization of *HSP27* and *MSK1* was examined by using immunohistochemistry. Our results at mRNA level indicated that *HSP27* was highly expressed in middle white follicles whereas *MSK1* was predominantly expressed in small white follicles. The western blotting results for *HSP27* and *MSK1* were inconsistent with the RT-qPCR results in various stages of prehierarchical follicular development but noticeably, *HSP27* proteins were still expressed more in middle white follicles while *MSK1* proteins were more abundant in primary follicles. At different stages of prehierarchical development, immunodetections in the granulosa and theca cells revealed that *HSP27* was intensively localized in middle white follicles while strong detections of *MSK1* were observed in large white

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*Work financed from: Jilin Province Science and Technology Development Project, project no. 20180201034NY, 20150204025NY. Jilin Province Scientific Activities of Selected Returned Overseas Professionals, project no. 2018. Jilin Agricultural University youth talent support program, project no. 2018.

follicles. These results indicate *HSP27* and *MSK1* might be associated to the key regulators of folliculogenesis in geese.

Key words: ovarian development, gene, RT-qPCR, western blotting, immunohistochemistry

Geese are economically important agricultural poultry species with rapid growth and high quality meat production (Ding et al., 2015). However, strong broodiness, seasonal breeding and poor egg laying performance of geese are the key bottlenecks that hinder the development of the geese industry (Wang et al., 2014). Domestic fowl's egg laying production performance relies on the progressive stages of growth and the development of the ovarian follicles which are divided into two categories, namely non-hierarchical and hierarchical follicles (Wang et al., 2017). Folliculogenesis is a cyclic recruitment of follicles from a pool of resting primordial follicles into a well-established follicular hierarchy, which involves cell proliferation and differentiation before ovulation or follicle degeneration (Yu et al., 2016). Even though the anatomic-physiological functions during the follicle development are well demarcated, the molecular mechanisms underlying the regulation of follicle development in Zi geese are not fully understood.

The p38 MAPKs are subfamily members of MAPK superfamily ubiquitously distributed in various tissues and their activation can lead to many biological processes including cell proliferation, cell apoptosis and DNA repair (Cargnello and Roux, 2012). Previous studies have shown that p38 MAPKs have been implicated in promoting apoptosis in different cell types, including endothelial, neurons, fibroblasts, and cardiac muscle cells (Farley et al., 2006) and granulosa cells (Johnson, 2003). Recently studies have demonstrated that the p38 and other members of MAPK family play significant roles in promoting follicular development and growth in gilts and sows (Moreira et al., 2013) and rats (Hu et al., 2017) and are activated in the cytoplasm and nucleus of the oocyte by various stimuli including growth factors and protein cytokines.

HSP27 and *MSK1* are mitogen activated protein kinase activated by p38 MAPK pathway (Liu et al., 2016; Reyskens and Arthur, 2016). *MSK1* was primarily known as proteins that shared their homology with the N-terminal kinase domain (NTKD) associated to the AGC kinase family and a C-terminal kinase domain to the calmodulin kinase family (Ning et al., 2017). *HSP27* is a 27-kDa protein, a member of the ten mammalian small heat shock proteins family widely expressed in response to variety of stress deleterious stimuli such as apoptosis to enhance cell survival through cytoskeletal structural stability (Pavan et al., 2014). Although many studies have investigated the expression and localization of *MSK1* and *HSP27* in various animal tissues at different conditions, to the best of our knowledge the spatial temporal expression of *HSP27* and *MSK1* in different stages of prehierarchical follicular development has not been elucidated. The aim of this study was to determine the relative expression and localization of *MSK1* and *HSP27* during the prehierarchical follicular development of Zi geese using quantitative real time polymerase chain reaction (RT-qPCR), western blotting and immunohistochemistry (IHC) to further elucidate the molecular mechanisms of geese ovarian development. The results of this study may establish

the molecular foundation regarding the involvement of *HSP27* and *MSK1* genes at early stages of avian development and maturation.

Material and methods

Ethics statement

To ensure that animal welfare principal measures were observed, this experiment was authorized by Animal Health and Care Committee of the College of Animal Science and Technology, Jilin Agricultural University (Changchun, Jilin, China) (Per-mission number: GR(J)18-010. Date: 14 May 2018).

Experimental geese and tissue collection

Six healthy laying Zi white geese aged between 35 and 37 weeks with a regular laying sequence of 2–3 eggs were randomly selected from Jilin Agricultural University geese breeding farm. The geese were reared under the deep litter system of poultry management and fed locally formulated ration in the farm composed of 17.34% crude protein, energy, 12.36 ME (MJ/kg), 13.35 DE (MJ/kg), 2.57% calcium and 0.26% phosphorus at *ad libitum* with free access to water. All the geese were allowed free access to swimming pool and were exposed to natural sunlight and uncontrolled environmental climatic conditions. The geese were slaughtered by exsanguinations to obtain the prehierarchical follicles. Vernier caliper was used to measure the diameter of the prehierarchical follicles which were divided into five categories as primary follicles (Pe, <1 mm), small white follicles (SWF, 2–4 mm), middle white follicles (MWF, 4–6 mm), large white follicles (LWF, 6–10 mm), small yellow follicles (SYF, 10–15 mm). After classification, the follicles were ruptured to remove the follicular liquid and the oocyte, and thoroughly washed with phosphate buffered saline (PBS) buffer then immediately stored in a refrigerator at –80°C in preparation for gene expression analysis.

Isolation of RNA and cDNA synthesis

The total RNA of the prehierarchical follicles was isolated using TRIzol reagent (Invitrogen, USA), following the manufacturer's protocol. The total RNA for each sample was treated with DNase 1 (Ambion/Life Technologies) to avoid genomic DNA contamination. The RNA quality and concentration were assessed with Agilent 2100 Bio analyser (Agilent Technologies, USA). The purity and degradation were also assessed on 1.2% agarose gel. Reverse Transcription PCR (RT-PCR) was performed as instructed in manufacturer's manual of the first strand cDNA Synthesis Kit (TOYOBO, Japan). Briefly, the reverse transcription reaction consisted of 2 µg total RNA, 4 µl of 5XRT buffer, 1 µl of RT Enzyme Mix, 1 µl of Primer Mix and finally nuclease-free water was added to a total volume of 20 µl. The reaction conditions of the reverse transcription reaction were 37°C, 15 min and the enzyme deactivation was 98°C, 5 min. The final products were kept at 4°C for daily experimental usage and at –20°C for longer storage.

Primer design and quantitative real-time PCR

According to the gene sequences which were found from the GenBank for β -actin and coding sequence (CDS) from de novo transcriptome SRA (SRP071765) *HSP27* (ORTHOLOGY: K04455) and *MSK1* (ORTHOLOGY: K04445) from Kyoto Encyclopedia of Genes and Genomes (KEGG) database were used to design primers by using Primer Premier 5.0 software (Primer-E Ltd., Plymouth, UK) (Table 1). Quantitative real-time PCR (RT-qPCR) was performed to determine the expression levels of *HSP27* and *MSK1* in the prehierarchical follicles. The RT-qPCR reaction (20 μ l) consisted of 2 μ l of cDNA, 0.6 μ l of each of the forward and reverse primers (0.6 μ l), 0.4 μ l of ROX Reference Dye, 10 μ l of SYBR Green Master Mix, and 6.4 μ l of nuclease-free water. Amplification conditions were as follows: pre-denaturation at 95°C for 5 min, 45 cycles of amplification (95°C for 15 s and 59°C for 60 s). A melt curve analysis was performed from 60°C to 95°C by taking readings every 0.3°C. The relative quantification of gene expression was detected in triplicate per sample. Gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using β -actin as an internal control.

Table 1. Primers used in RT-qPCR

Primers	Sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)
<i>MSK1</i>	F: TGGTGGAGTGTGGTGT	53.5	116
	R: CTTGTGGATAAGGTGGC		
<i>HSP27</i>	F: GAGCACGGCTTCATCTCCC	59.5	182
	R: TCTTGCCCTGGCTTTCG		
β -actin	F: GCGGCATGCCACACCGTGCCCATCTATGAG	59.0	205
	R: GCGAAGCTTGCCATCTCTGCTCGAAGT		

The candidate genes *HSP27* and *MSK1* encode hsp27 and msk1, respectively; β -actin. F denotes forward primers and R denotes reverse primers.

Western blot analysis

The prehierarchical follicles sample tissues were homogenized in radio-immunoprecipitation assay (RIPA) buffer (10 μ l of phosphatase inhibitor, 1 μ l of protease inhibitor and 5 μ l of 100 mM PMSF per 1 mL) followed by centrifugation at 14 000 rmp, at 4°C for 15 min and the supernatant was separated into a whole protein extract. The concentration of protein samples was determined by using the BCA protein content detection kit (Biotechnology Development, Kaiji, Nanjing, China). For every sample, 10 μ g total protein was pipetted onto a 12% SDS-PAGE Gel Electrophoresis Kit (Biotechnology Development, Kaiji, Nanjing, China) initially set at 60 V and the voltage was increased to 90 V for 90 min when the protein samples entered the separation gel. The electro blotting was carried out in a transfer liquid at 200 mA for 60–120 min. The membranes were incubated in 5% dry milk for 1.5–2 h at 25°C room temperature and afterwards the membranes were washed with TBST 3 times for 5 min then incubated overnight at 4°C immersed with the following primary antibody: mouse anti-HSP27 monoclonal antibody (clone G3.1,

1:2,000 dilution, LifeSpan BioSciences, Inc. Shanghai, China), rabbit anti-MSK1 polyclonal antibody (bs-2995R, 1:500–2,000 dilution, Bioss Antibodies, Shanghai, China). At the end, the membranes were rinsed with 1_× PBST (phosphate-buffered saline with 0.1% Tween-20), then incubated with HRP Affinipure conjugated goat anti-mouse IgG (H+L) secondary antibody (Wuhan, Hubei, China) for 1 h at room temperature. The membranes were washed three times with 1_× PBST at room temperature for a total of 15 min. The membranes were visualized with an ECL Test Kit (Millopore WB-KLS0100, Germany). The chemiluminescence of each protein band was quantified using the ImageJ software, and protein levels were normalized by β -actin as internal control.

Immunostaining analysis

The prehierarchical follicles were fixed for 48 h with 4% paraformaldehyde in PBS, pH 7.4, at 4°C. Then, the follicles were cut and the samples were immersed in different concentrations of ethanol (75%, 85%, 90% and 95%) and immersed in 100% xylene twice for 10 min each time. The treated samples were embedded in paraffin wax and the tissues were sliced at 5 μ m thickness then stretched and baked at 65°C for 3 h, and finally soaked twice in xylenes. The treated tissue sections were washed 3 times with PBS solution for 5 min each time. The slides were succumbed to antigen repair by heating in citrate buffer solution (pH 6) two times for 15 minutes. Tissue sections were washed 3 times with PBS solution for 5 min each time. Following deparaffinization, rehydration, and enzyme digestion, slides were blocked with 3% goat serum at room temperature for 20 min then incubated with 50 μ l of the target gene primary antibody (mouse anti-HSP27 monoclonal antibody, clone G3.1, 1:1,000 dilution, LifeSpan BioSciences, Inc. Shanghai, China), rabbit anti-MSK1 polyclonal antibody (bs-2995R, 1:400–800 dilution, Bioss Antibodies, Shanghai, China) at 4°C overnight and the sections subjected to the incubation were rewarmed for 45 min. The slides were washed 3 times in PBS for 2 min each time, and then incubated at room temperature for 60 min with 20 μ l secondary antibody. After the incubation with the secondary antibody the tissues were washed 3 times with PBS solution for 5 min each time followed by DAB staining (5–10 min). The stained sections were then rinsed with tap water for 10 min and then counterstained with hematoxylin for 2 min. Then, the sections were decolorized in 1% hydrochloric acid-ethanol for 10–15 sec, quickly taken out and placed in distilled water, and then placed in phosphate buffered saline with Tween-20 (PBST) with pH 7.4 for 5–10 min. The stained sections were rinsed with tap water for 15 min, then dehydrated and rendered translucent using different concentrations of ethanol and xylene. The film was then sealed with a neutral resin. The slides were examined with a BX53 electric fluorescent illuminator microscope (Olympus, Tokyo, Japan).

Statistical analysis

The relative expression levels of target genes in different grades of prehierarchical follicles were analysed by SPSS23.0 statistical software. Single factor analysis of variance (One-way ANOVA) was used among groups and least square significant difference (LSD) test was used to compare the means. The significant difference of the data was indicated as $P < 0.05$ and $P > 0.05$ denoted no significant difference.

Results

Expression pattern of *HSP27* and *MSK1* mRNA in prehierarchical follicles

The relative mRNA expression levels of the candidate genes were determined in five categories of prehierarchical follicles (< 1 mm to 15 mm) using the RT-qPCR. The expression level of each gene (*MSK1* and *HSP27*) at each stage of prehierarchical follicles was illustrated in Figure 1. Both genes were expressed during prehierarchical follicular development with varying expression patterns, the highest mRNA expression level of *MSK1* was observed in small white follicles ($P < 0.05$). However, there was no significance difference of *MSK1* expression between the large white follicles and the small yellow follicles ($P > 0.05$). Conversely, *HSP27* transcripts were dominantly expressed in the middle white follicles followed by the small white follicles ($P < 0.05$) and low amounts were quantified in large white follicles ($P < 0.05$) with no statistical significance difference between the primary follicles and small yellow follicles ($P > 0.05$).

Figure 1. Expression of genes encoding *HSP27* and *MSK1* in the prehierarchical follicles of Zi geese. The data are represented as means $\pm$ SEM; $n = 6$ geese and the different letters indicate the significant difference of mRNA expression between different sizes of prehierarchical follicles ($P < 0.05$) reported in arbitrary units (AU) normalised with β -actin. The vertical ordinate represents relative mRNA expression level of each gene while the horizontal ordinate indicates the sampled follicle sizes

Western blotting

The protein expression levels of *HSP27* and *MSK1* genes in the prehierarchical follicles of geese were detected by using western blot as illustrated in Figure 2. Both *HSP27* and *MSK1* proteins were expressed in all stages of prehierarchical follicular development. Similar to mRNA expression results *HSP27* was significantly high in middle white follicles ($P < 0.05$). There was no statistical difference in *HSP27* protein expression between small white follicles and small yellow follicles ($P > 0.05$), however, the *HSP27* protein expression between small white follicles and large white follicles had statistical difference ($P < 0.05$). Although *MSK1* protein was detected in all stages of prehierarchical development there existed different expression tendencies as compared to *MSK1* mRNA transcripts as there was abundant protein expression in primary follicles ($P < 0.05$) and significantly low in small white follicles ($P < 0.05$). There was significant difference between the middle white follicles and small yellow follicles ($P < 0.05$).

Figure 2. Protein quantitative analysis of *HSP27* and *MSK1* in different stages of prehierarchal follicles development using Western blot. The upper two panels show the measurable quantities of *HSP27* and *MSK1* in prehierarchal follicles at different growth stages. The lower panel indicates the β -actin as an internal control in all samples. Each sample is shown as mean $\pm$ SEM of the ratio of the relative density of *HSP27* or *MSK1* to β -actin. The different superscripts indicate statistically significant differences ($P < 0.05$)

Figure 3. Immunohistochemical localization of *HSP27* and *MSK1* in geese prehierarchal follicles. Positive staining (brown colour) was observed in the granulosa cell (GC) and theca cells (TC). Illustrative micrographs of sections through five different stages of ovarian prehierarchal follicles development at five stages and incubated with *HSP27* and *MSK1* antibody (A–E). Panel A represents primary follicle (PE), Panel B represents small white follicles (SWF), Panel C represents middle white follicles (MWF), Panel D represents large white follicles (LWF) and Panel E represents small yellow follicles (SYF). Representative negative control incubated with PBS instead of anti-*HSP27* and anti-*MSK1* antibody (F). Scale bars: *HSP27* = 400 μ m (A), 200 μ m (B, D and F), and 100 μ m (C and E) and *MSK1* = 200 μ m (A, B, C, E, F), and 400 μ m (D)

***HSP27* and *MSK1* proteins immunolocalization in geese prehierarchical follicles**

In order to determine the localization and distribution of p38 pathway related genes, this study localized *HSP27* and *MSK1* proteins for the first time in the granulosa and theca cells of the prehierarchical follicles of geese by immunohistochemistry. The immunolocalization results for *HSP27* and *MSK1* are shown in Figure 3. *HSP 27* and *MSK1* were both observed in the granulosa cells and theca cells as anticipated, with *HSP27* weak staining observed in the large white follicles and *MSK1* weak staining was detected in small white follicles. The expression intensities of *MSK1* protein were comparative even in the granulosa and theca cells for both the primary and small yellow follicle during the different follicular stages of development. Strong immunoreactive signals of *MSK1* were observed in the large white follicles, whereas *HSP27* was more intense in the middle white follicles. The negative controls did not show any positive signal.

Discussion

The optimal follicular development in poultry thrives when the growing follicles in the ovary progress gradually from a cohort of the primordial follicles into large preovulatory follicles through a well-defined follicular hierarchy (Johnson, 2012). Contrary to litter-bearing species, fowls are monovulatory species in which the healthy dominant follicle is normally selected for maturation and ovulation while other follicles at various stages of development are subjected to follicular cell degeneration (atresia) and basically regulating the clutch size (Johnson, 2014). The p38 mitogen-activated protein kinase belongs to MAPKs signal transduction pathways commonly found in all eukaryotic cells to regulate cell physiological responses such as gene transcription and protein synthesis by responding to various types of stimuli (Koul et al., 2013). This study has illustrated that *HSP27* and *MSK1* are implicated during the geese follicular development. By using the RT-qPCR our results demonstrated the spatiotemporal expression patterns of *HSP27* and *MSK1* at various stages of prehierarchical development. This research finding is in agreement with previous study that reported the inconsistency in bovine follicles *HSP27* transcripts expression patterns (Velázquez et al., 2011). Miyagaki et al. (2011) showed the different expression levels of *MSK1* in various mouse tissues germinal vesicle (GV)- and metaphase II (MII)-stage oocytes, ovary, and brain. These results suggest that *HSP27* and *MSK1* genes may actively participate in regulating the geese ovarian follicular development and growth in a context dependent manner.

HSP27 is a member of small heat shock protein (HSPs), which is temporarily prompted in response to a wide range of cellular stimuli such as heat stress to stabilize the cytoskeletal structure and exert the anti-aggregation features to sequester the damaged proteins, therefore enhancing cell motility and survival (Velázquez et al., 2010). At the protein level, the *HSP27* expression was predominantly high in middle white follicles and this result was consistent to its expression at gene level. This result indicates that *HSP27* may play critical protective functions at this stage.

However, as the prehierarchical follicles develop towards attaining the hierarchical stage (preovulatory follicles), the *HSP27* protein decreased exponentially to the lowest level at small yellow follicles, suggesting that *HSP27* might have little physiological protective role during the follicle selection into the preovulatory stage and this might also predict high occurrence of follicular degeneration. Liu et al. (2010) found that the down regulation of *HSP27* in mouse oocyte significantly increased the ratio of early stage apoptosis while the primary apoptotic factors such as caspase 3 and caspase 8 expression also drastically increased. *MSK1* is a nuclear serine/threonine kinase that resides downstream of the mitogen-activated protein kinases (MAPK) signaling pathway phosphorylated by extracellular signal-regulated kinases (ERK1/2) through G protein-couple receptors and tyrosine kinases receptors and p38 signaling pathway is activated by cellular response to stress such as cytokine signaling, chemical stress, UV irradiation, and osmotic fluctuations (Carpenter and Wu, 2014). Deak et al. (1998) found that *MSK1* is widely expressed across a variety of tissues, including heart, brain, placenta, lung, liver, kidney and pancreatic tissue, with predominant expression observed in brain, placenta and skeletal muscle. In this pilot study, we found that *MSK1* protein was highly expressed in primary follicles (PE) and with no significant difference between the MWF and SYF. This indicates that the *MSK1* protein contributes significantly during the initial stage of prehierarchical follicular development.

Folliculogenesis in mammals and birds is the process accompanied by the functional morphological changes and proliferation of the follicular walls (granulosa cell layer and the theca cell layer) to support and protect the growing and developing oocyte to reach ovulation stage (Richani and Gilchrist, 2017). The essential growth promoting factors including insulin like growth factor (IGF) and estradiol are secreted by granulosa cells, and the fade of these major life promoting factors causes loss of important functions by the granulosa cells resulting in cell death (Matsuda et al., 2012). The theca cells produce androgens (testosterone) in response to luteinizing hormone (LH), which are eventually converted into estradiol (estrogen) by follicle stimulating hormone (FSH)-induced aromatase located in the granulosa cells of growing follicles (Young and McNeilly, 2010; Zhang et al., 2013). For the first time, this study localized the *HSP27* and *MSK1* proteins in both granulosa and theca cells of geese prehierarchical follicles. *HSP27* and *MSK1* proteins were localized in all sampled prehierarchical follicles with varying color intensities. The weak *HSP27* staining was observed in SYF and the strong immunolabeling was detected in MWF. These results may speculate that *HSP27* exerts its biological activities in a stage dependent manner during prehierarchical follicle growth/development through intra ovarian follicular autocrine and paracrine interaction. Similar to our results, Velázquez et al. (2013) found that *HSP27* was expressed in both granulosa and theca cells of bovine follicles with significant varying staining intensities in follicles sample classifications following follicular development. The greatest *MSK1* immunodetections were found in LWF followed by MWF whereas the weak staining was observed in SWF. These findings suggest that *MSK1* may have different roles in molecular mechanism regulating various-sized prehierarchical follicular recruitment to dominant follicle selection. In the same way, other authors have found discrepancy

in localization expression patterns of *MSK1* in various tissues in rats (Brami-Cherrier et al., 2009; Li et al., 2013; Ning et al., 2013). It is worth noting that the comparative expression of *HSP27* and *MSK1* at gene level (RT-qPCR), protein level (western blot) and tissue level (immunohistochemistry) did not completely follow the identical expression patterns possibly due to complex processes such as translation rate modulation and protein synthesis delay beyond transcript concentration that contribute to establishing the expression level of proteins (Liu et al., 2016).

Conclusion

In summary, the present study revealed the *HSP27* and *MSK1* are associated with follicular development in geese. Their expressions in a stage specific manner in prehierarchical follicles could provide additional basic information to already existing knowledge of the biological functions of *HSP27* and *MSK1* in the reproductive physiology of geese and other animals. However further investigations are required to elucidate a direct interaction and the role played by *HSP27* and *MSK1* in geese prehierarchical follicular cell degeneration and selection of healthy mature dominant follicle.

Acknowledgements

We appreciate the Jilin Agricultural University Goose Farm for providing the experimental animals.

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Received: 16 II 2019

Accepted: 8 VII 2019