e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 70 (2022), No 4
https://doi.org/10.3409/fb_70-4.17

Ovarian mRNA Expression and Regulation of Matrix Metalloproteinase 16 in the Domestic Hen

Dominika WOLAK and Anna HRABIA

Accepted October 13, 2022 *Published online October 25, 2022* *Issue online December 06, 2022*

Original article

WOLAK D., HRABIA A. 2022. Ovarian mRNA expression and regulation of matrix metalloproteinase 16 in the domestic hen. Folia Biologica (Kraków) **70**: 133-140.

In mammals, membrane-bound matrix metalloproteinases (MT-MMPs) are thought to play an important role in ovarian remodeling. However, the role and regulation of these proteases in the ovary of birds remain largely unknown. One of MT-MMPs, i.e., MMP-16, has been found in the hen ovary; therefore, this study was undertaken to examine whether the transcript level of MMP-16 changes during follicle development and whether gonadotropins and estrogen are involved in the regulation of this enzyme expression. The relative expression of MMP-16 mRNA in the ovarian follicles (white, yellowish, small yellow, and the granulosa and theca layers of three of the largest yellow preovulatory [F3-F1]) was examined 22 h and 3 h before F1 follicle ovulation as well as following equine chorionic gonadotropin (eCG) or tamoxifen (estrogen receptor modulator, TMX) treatments by quantitative real-time polymerase chain reaction (qRT-PCR). MMP-16 transcripts were detected in all examined ovarian tissues of control and treated hens. The relative expression of MMP-16 depended on follicular size/maturation and the layer of the follicular wall. A relatively higher expression of MMP-16 mRNA in the granulosa layer at 3 h compared to 22 h before ovulation of F1 was found. The injections of eCG decreased transcript abundance of MMP-16 in white and small yellow follicles, as well as in the theca layer of F3-F2 and the granulosa layer of the F1 follicle. In turn, TMX caused an increase in mRNA expression of MMP-16 in the theca layer of the largest preovulatory follicles and a decrease in the granulosa layer of the F1 follicle. Our results provide the first mRNA expression analysis of MMP-16 in the hen ovary under different physiological states. In addition, results indicate a possible role of gonadotropins and estrogen in regulating the transcription of MMP-16 in the chicken ovary.

Key words: MMP-16, eCG, TMX, qRT-PCR, remodeling, ovary, chicken.

Dominika WOLAK, Anna HRABIA✉, Department of Animal Physiology and Endocrinology, University of Agriculture in Kraków, Al. Mickiewicza 24/28, 30-059 Kraków, Poland.
E-mail: anna.hrabia@urk.edu.pl; rzhrabia@cyf-kr.edu.pl

The ovary of the mature laying hen is composed of follicles at different developmental stages. The most numerous pool is represented by slow-growing prehierarchical follicles: primordial embedded in the ovarian stroma (<1 mm in diameter), white (1-4 mm; WFs), and yellowish (4-8 mm, YFs). The next group consists of the fast-growing follicles arranged into a distinct preovulatory hierarchy: small yellow follicles (8-12 mm; SYFs) and large yellow follicles (12-35 mm; F_n-F1), where the most mature follicle (F1) is the first in line to ovulate. The second largest follicle (F2) ovulates next day and so forth (BAHR & JOHNSON 1984). The structure remaining after oocyte release is a postovulatory follicle (POF) containing the theca (interna and externa) and granulosa layers. The POF gradually regresses and is completely resorbed within 7-10 days. Several prehierarchical atretic follicles are also present in the hen ovary (HRABIA 2022).

Growth, development, ovulation, and subsequent regression of ovarian follicles require intensive and extensive turnover of the extracellular matrix (ECM). The ECM components influence the proliferation, differentiation, apoptosis, and adhesion of cells; therefore, the ECM plays a significant role in proper follicle development, function, and atresia/regression (NY *et al*. 2002; SMITH *et al*. 2002). The structural changes in the ECM are regulated by various proteases. A growing body of evidence indicates that among them are matrix metalloproteinases (MMPs),

which are endopeptidases that are capable of degrading ECM components in different tissues, including reproductive tissues (HRABIA 2021).

MMPs are large group of zinc- and calcium-dependent enzymes also engaged in the activation and release of signaling proteins. Regarding their structural organization and substrate preference, five groups of MMPs are indicated: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), matrilysins (MMP-7 and MMP-26), membrane-bound MMPs (MT-MMPs: MMP-14, MMP-16, MMP-17, MMP-24, MMP-26), and other non-classified MMPs. MT-MMPs are unique because in contrast to other subgroups of MMPs, they are anchored onto the extracellular cell surface by a single-pass transmembrane domain (MMP-14, MMP-15, MMP-16, and MMP-24) or by a glycophosphatidylinositol (GPI) linkage (MMP-17 and MMP-25). Regulation of MMP transcription is under the influence of growth factors, cytokines, and hormones. Active forms of these endopeptidases are inhibited by non-specific inhibitors such as α2-macroglobulin, and specific tissue inhibitors of MMPs (TIMPs). For local activation of these enzymes, MT-MMPs are also responsible (KESSENBROCK *et al*. 2010; YAMAMOTO *et al.* 2015; HRABIA 2021).

The avian ovary is reported to be a site of synthesis and action of MMPs (HRABIA 2021). For the first time, NAKAJO *et al*. (1973) have suggested that proteolytic enzymes, such as collagenases, are engaged in a mechanism of follicle rupture during ovulation. In turn, ASEM *et al.* (2000) found the presence of six different MMPs in the basal lamina of chicken preovulatory follicles. Further experiments demonstrated changes in the expression of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 at the transcript and/or protein levels, which were dependent on the tissue and stage of follicle development, suggesting participation of these enzymes in folliculogenesis and ovulation (LEŚNIAK & HRABIA 2012; ZHU *et al*. 2014; YUAN *et al.* 2016; HRABIA *et al*. 2019; WOLAK & HRABIA 2021). The expression of MMP-3 mRNA was noted in cancerous chicken ovarian tissues (CHOI *et al*. 2011). Moreover, the involvement of gelatinases in the atresia of pre-hierarchical follicles and regression of POFs were also strongly suggested (HRABIA *et al.* 2018; 2019). We have recently found that gonadotropins (WOLAK *et al.* 2021), prolactin (HRABIA *et al.* 2021), and estrogen (WOLAK & HRABIA 2020) may play a significant role in the regulation of the expression and/or activity of chosen MMPs in the hen ovary. So far, there is no information concerning the synthesis and role of MT-MMPs in the ovary of birds. The mechanisms responsible for the regulation of the expression of these enzymes are also largely unknown. Follicle development and function are mostly under the control of pituitary gonadotropins (follicle stimulating hormone [FSH] and luteinizing hormone [LH]) and ovarian steroids. Estrogen is primarily responsible for the stimulation of cell proliferation and differentiation, as well as regulation of steroid synthesis, whereas progesterone induces a surge of LH just before ovulation. Since MMP-16 was recently predicted as a target gene for regulation by several microRNAs in hen ovarian compartments (OCŁOŃ & HRABIA 2021), the present studies were designed to examine whether transcript abundance of MMP-16 changes during follicle development and around the time of ovulation of the largest F1 follicle. We also tested the hypothesis that MMP-16 gene expression is affected by gonadotropins and estrogen. Accordingly, the relative abundances of MMP-16 mRNA in the wall of follicles were examined in the hen ovary following equine chorionic gonadotropin (eCG; originally known as pregnant mare serum gonadotropin [PMSG]) and tamoxifen (TMX; estrogen receptor blocker in birds) treatments.

Materials and Methods

Animals and experimental design

Three experiments were carried out and conducted in accordance with the research protocol approved by the Local Animal Ethics Committee in Kraków, Poland (approval no. 218/2015). Laying Hy-Line Brown hens (n = 42) between 25-32 weeks of age were purchased from a commercial farm. They were housed in individual cages of batteries under a photoperiod of 14 h light:10 h dark cycle. Animals had free access to water and commercial feed formulated for egg laying hens. Accurate timing of ovulation was determined by checking oviposition every 15 min between 7:00 a.m. and 3:00 p.m. Based on the recording of oviposition time, cloacal palpation, and autopsy (position of the ovum in the oviduct after sacrification), it was found that ovulation occurred within 5 min of oviposition of the previous egg in the series. Hens that weighed 1.68-1.82 kg were randomly assigned to control and experimental groups. In the first experiment, birds were euthanized by stunning, followed by bleeding at two physiologically different stages of the ovulatory cycle: 22 h (n = 6) and 3 h (n = 6) before the predicted ovulation of the largest preovulatory F1 follicle. In the second experiment, performed as described by WOLAK *et al*. (2021), the control birds (n = 6) received a subcutaneous injection in the abdominal area (just below the breastbone) of 0.9% NaCl with 0.05% bovine serum albumin (BSA), and experimental chickens (n = 6) were treated with eCG at a dose of 75 IU/0.2 ml 0.9% NaCl with 0.05% BSA/kg of body weight. The hens were treated daily (every morning between 9:00 and 11:00 a.m.). The eCG dose was chosen according to earlier *in vivo* studies (JOHNSON & LEONE 1985; NAKANISHI *et al*. 1991; GHANEM & JOHNSON 2019). eCG-treated chickens were eutha-

nized (by stunning, followed by bleeding) on the third or fourth day of cessation of egg laying by all eCG-treated hens, i.e., on day 7 of the experiment, within 2-5 h after eCG injection. In the last experiment, control birds (n = 8) were injected (as in the second experiment) with a vehicle (ethanol), and the experimental hens (n = 8) were treated with TMX at a dose of 6 mg/0.3 ml ethanol/kg of body weight, as recently described by WOLAK and HRABIA (2020). Hens were treated daily until a pause in egg laying occurred in all TMX-treated birds and euthanized on day 8 of the experiment. Laying hens (control) in the second and third experiment were euthanized on day 7 or 8 of the experiment, respectively, about 2 h after ovulation. The ovaries were rapidly harvested, weighed, placed on ice, and the ovarian follicles at different developmental stages were collected according to our previous studies (HRABIA *et al.* 2019; WOLAK & HRABIA 2020; WOLAK *et al*. 2021). Based on both diameter and color, follicles were categorized as white (WFs; 1-4 mm in diameter), yellowish (YFs; 4-8 mm, pre-recruitment follicles), small yellow (SYFs; 8-12 mm, the most recently recruited follicles), and three of the largest yellow preovulatory F3-F1 (F3<F2<F1; 26-35 mm). In eCG-treated hens, follicular hierarchy was disrupted, and thus the largest yellow follicles were divided into groups corresponding to the diameter of F3-F1 follicles of the control hens, and thereafter set as F3-F1 (WOLAK *et al.* 2021). From the preovulatory follicles, the theca and granulosa layers were separated as described by GILBERT *et al.* (1977). The follicle was cut with a razor blade and the granulosa layer was peeled, first from the yolk then from the theca layer. Subsequently, the layers were washed with phosphate-buffered saline (PBS). Tissues were immediately placed in RNAlater and stored until total RNA extraction.

Total RNA isolation and qRT-PCR analysis

Total RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction (qRT-PCR) were performed as previously described (LEŚNIAK-WALENTYN & HRABIA 2016; HRABIA *et al.* 2019; WOLAK & HRABIA 2021). In brief, RNA was extracted from collected tissues using TRI-reagent according to the manufacturer's protocol (Sigma-Aldrich, St Louis, MO, USA). Two µg of RNA from each sample were reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA *via* Thermo Fisher Scientific, Waltham, MA, USA), including random primers as per the manufacturer's instruction. The complementary DNA (cDNA) was diluted 1:10 and used in qRT-PCR with the chicken-specific TaqMan Gene Expression Assay for the MMP-16 (assay ID: Gg03367051_m1; amplicon length 75 bp; RefSeq: NM_205197.2; Assay location 592; Applied Biosystems *via* Thermo Fisher Scientific) gene in a 96-well thermocycler (StepOne Plus; Applied Biosystems) according to the recommended cycling program: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95°C and 60 s at 60°C. The duplex PCR for the MMP-16 gene, and 18S rRNA as a reference gene, was performed in a 10 µl volume which contained 5 µl TaqMan Gene Expression Master Mix, 0.5 µl TaqMan Gene Expression Assay with specific TaqMan MGB-probe and one pair of primers, 0.5 µl of Eukaryotic 18S rRNA Endogenous Control (pair of primers and TaqMan probe-labeled VIC/TAMRA), 3 µl water, and 1 µl cDNA. Each sample was run in duplicate. The negative control (nuclease-free water) was included in all reactions. Relative quantification of the investigated gene was calculated after normalization with the 18S rRNA transcript, while the expression levels in the WFs, YFs, SYFs, theca of F1 22 h before ovulation or granulosa of F1 22 h before ovulation, or theca of F3-F1 or granulosa of F3-F1 follicles of control groups were considered as the calibrator using the $2^{-\Delta\Delta Ct}$ method (SCHMITTGEN & LIVAK 2008).

Statistical analysis

The variables were examined for normality and homogeneity of variance using the Shapiro-Wilk test and Brown-Forsythe test, respectively. Log transformations were performed as needed to meet homogeneity of variance and normality. Data were analyzed by one-way ANOVA (effect of ovarian tissue) followed by Duncan's multiple range test. The significance of differences between two means was analyzed using the Student's t-test or the nonparametric Mann-Whitney U test. Differences in values were considered to be significant at $p<0.05$. Calculations were performed with SigmaPlot (Systat Software Inc., San Jose, CA, USA). Results are presented as mean ± standard error of the mean (SEM).

Results

Expression of MMP-16 mRNA in the ovary of control, TMX-treated, and eCG-treated hens

RT-PCR analysis showed the presence of MMP-16 mRNA in all examined ovarian compartments (Fig. 1). The relative abundances (RQ) of MMP-16 transcript in the ovary of hens 22 h before ovulation differed among tissues. It was the highest in the WFs and the lowest in the granulosa layer of preovulatory follicles ($p = 0.025$ – $p<0.001$; Fig. 1). In the SYFs, the transcript level was lower by 63% than in the WFs and by 53% than in the YFs. The granulosa layer of F3, F2, and F1 follicles was characterized by lower

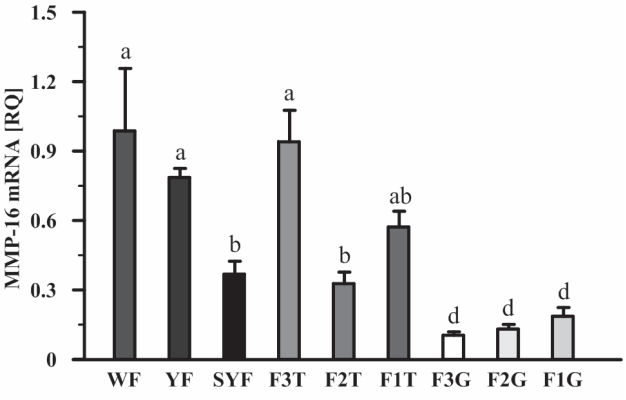

Fig. 1. Relative expression of MMP-16 mRNA in the chicken ovary. Data represents the mean relative quantity (RQ) ± SEM from five or six chickens normalized to the expression of 18S rRNA and standardized to the expression in the white follicles (WFs). Values marked with different letters differ significantly ($p<0.05$) among tissues (follicle types; one-way ANOVA followed by Duncan's multiple range test). WF – white follicles (1-4 mm in diameter); YF – yellowish follicles (4-8 mm); SYF – small yellow follicles (8-12 mm); F3-F1 – three of the largest yellow preovulatory F3-F1 follicles (26-35 m, F3<F2<F1); T – theca layer; G – granulosa layer.

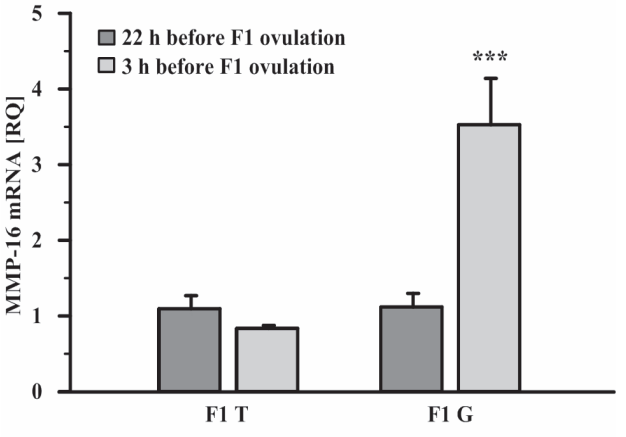

Fig. 2. Relative level of MMP-16 mRNA in the theca and granulosa layers of the largest yellow preovulatory follicle (F1) of hens, 22 h and 3 h before ovulation determined by RT-PCR. Each value represents the mean relative quantity (RQ) ± SEM from five or six chickens (biological replicates) normalized to the expression of 18S rRNA and standardized to the expression in the theca or granulosa layer of the F1 follicle 22 h before ovulation. ***$p<0.001$, compared with 22 h before ovulation (Mann-Witney U test). T – theca layer; G – granulosa layer.

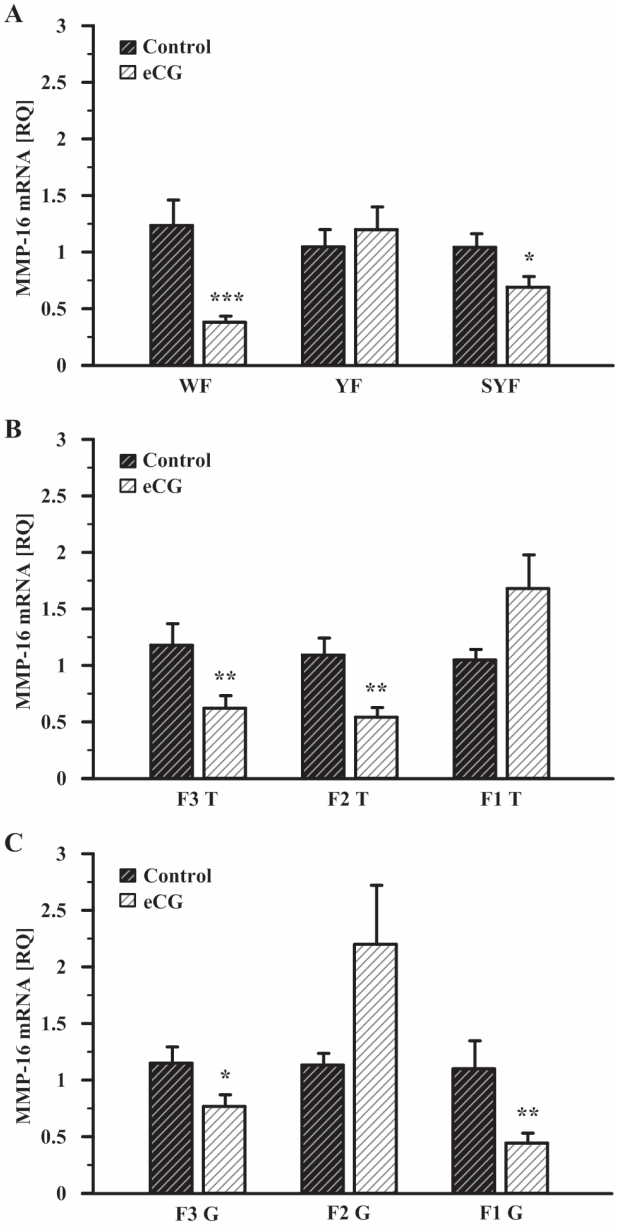

Fig. 3. Relative expression of MMP-16 mRNA in chicken ovarian follicles following equine chorionic gonadotropin (eCG) treatment (A-C). Each value represents the mean relative quantity (RQ) SEM from five or six chickens (biological replicates) normalized to the expression of 18S rRNA and standardized to the expression in the (A) white follicles (WF), yellowish follicles (YF), and small yellow follicles (SYF), (B) the theca layer of F3-F1, or (C) the granulosa layer of F3-F1 follicles of the control group. *$p<0.05$, **$p<0.01$, ***$p<0.001$, vs the control group (Student's t-test). T – theca layer; G – granulosa layer.

MMP-16 mRNA expression than the theca layer of the same follicles by 89%, 67%, and 61%, respectively. There were no significant differences in MMP-16 mRNA abundances in the granulosa layer between F3-F1 follicles, but there was a tendency to an increase along with follicle maturation to ovulation (Fig. 1).

The expression of MMP-16 mRNA was also evaluated in the wall of the F1 follicle at two stages of the ovulation cycle, i.e., at 22 h and 3 h before expected ovulation of F1. As shown in Figure 2, there was no difference in MMP-16 mRNA abundances in the theca layer between stages, but an increase (215%; $p<0.001$) in MMP-16 mRNA level was noted in the granulosa layer at the stage of 3 h before ovulation compared to 22 h before ovulation.

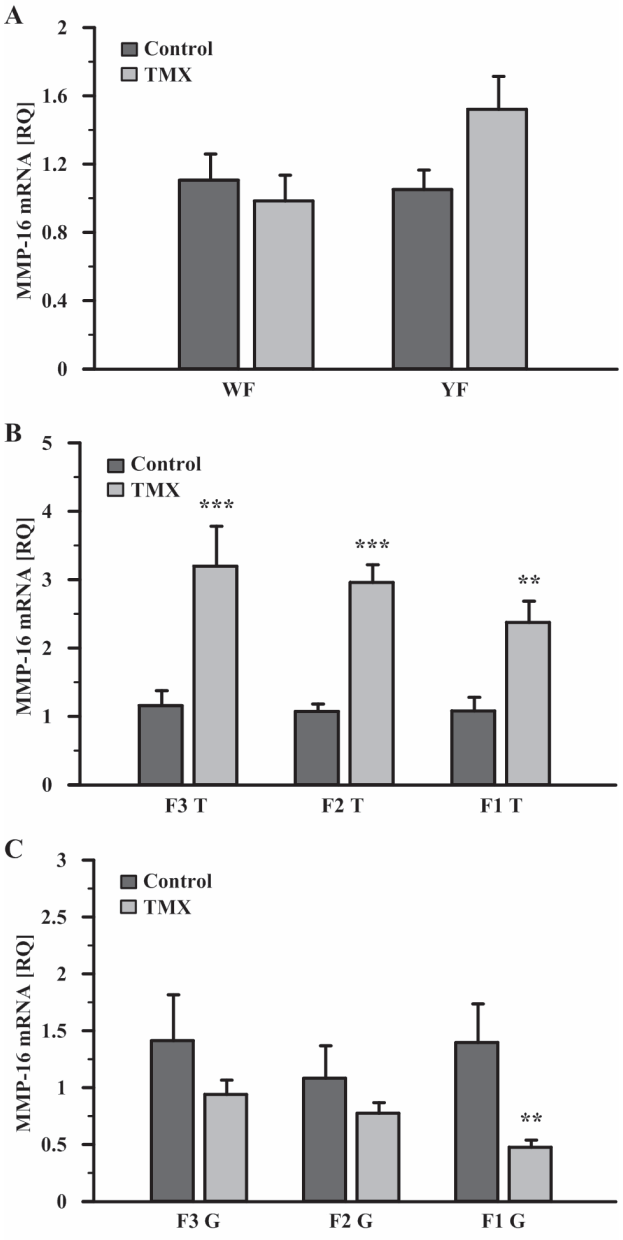

Fig. 4. Relative expression of MMP-16 mRNA in chicken ovarian follicles following tamoxifen (TMX) treatment (A-C). Each value represents the mean relative quantity (RQ)±SEM from five chickens (biological replicates) normalized to the expression of 18S rRNA and standardized to the expression in the (A) white follicles (WF) and yellowish follicles (YF), (B) the theca layer of F3-F1, or (C) the granulosa layer of F3-F1 follicles of the control group. **p<0.01, ***p<0.001, vs the control group (Student's t-test). T – theca layer; G – granulosa layer.

Compared with the control group, treating hens with eCG resulted in a lesser relative abundance of MMP-16 mRNA transcript in the WFs by 62% (p<0.001) and SYFs by 34% (p = 0.033). Moreover, within the hierarchical F3-F1 follicles, eCG caused a decrease in MMP-16 mRNA levels in the theca layer of F3 and F2 by 47% (p = 0.01) and by 51% (p = 0.005), respectively, and in the granulosa layer of F3 and F1 by 33% (p = 0.043) and 60% (p = 0.005), respectively. There was no influence of eCG treatment on transcript levels in other ovarian tissues (Fig. 3 A-C).

As depicted in Figure 4A, treatment of hens with TMX did not cause significant changes in MMP-16 mRNA levels in pre-hierarchical follicles, WFs (p = 0.557), or YFs (p = 0.051). Simultaneously, TMX increased MMP-16 mRNA expression in the theca layer of F3/F2 follicles by 176% (p = 0.004, p<0.001) and in the F1 follicle by 120% (p<0.003), while this transcript decreased in the granulosa layer of F1 by 66% (p =0.002) (Fig. 4B, C).

Discussion

Our experimental results revealed the mRNA expression of MMP-16 in different compartments of the hen ovary, i.e. WFs, YFs, SYFs, and three of the largest yellow preovulatory follicles (F3-F1), in relation to follicle maturation and after eCG or TMX treatments. Unfortunately, due to the lack of a commercially available antibody, there was no possibility to examine MMP-16 protein abundance or activity. The presented findings extend our previous results, demonstrating changes in transcript abundance of several MMPs and their TIMPs in chicken ovarian follicles at different physiological stages (HRABIA *et al*. 2018; HRABIA *et al.* 2019; HRABIA *et al*. 2021; WOLAK & HRABIA 2020; WOLAK & HRABIA 2021; WOLAK *et al.* 2021). The presence of the MMP-16 transcript was previously demonstrated in the rat, macaque, and human ovaries (PUTTABYATAPPAP *et al*. 2014). Our results provide additional information for a potentially possible role of MMP-16 in chicken ovarian follicles. The observed alterations in MMP-16 mRNA expression in follicular tissues along with follicle maturation and just before ovulation may indicate the involvement of this protease in follicle selection, maturation, and/or ovulation. MMP-16 is responsible for the processing of various components of the ECM, such as collagen type III, gelatins, or fibronectin, as well as for activation of the latent form of MMP-2, which in turn can activate pro-MMP-9. Both gelatinases are highly expressed in chicken ovarian follicles, and their participation in follicle development and ovulation was strongly proposed (LEŚNIAK & HRABIA 2012; HRABIA *et al.* 2019; HRABIA 2021). Thus, one of the roles of MMP-16 in the hen ovary may be the activation of MMP-2. Previous studies on mammals suggest an involvement of MMP-16 in promoting ovulation and remodeling of the ovulated follicle into the corpus luteum (PUTTABYATAPPAP *et al.* 2014), which may occur either directly or indirectly through activating other enzymes, modulating growth factor availability, and/or stimulating intracellular pathways (PAGE-MCCAW *et al*. 2007). It should be

noted that a negative correlation between MMP-16 and its related microRNAs was recently found in the theca and granulosa layers of the largest preovulatory follicles, which might also indicate an important role of MMP-16 in facilitation of follicle ovulation in the hen ovary (OCŁOŃ & HRABIA 2021). The relatively higher expression of MMP-16 mRNA noted herein in the WFs and YFs, and lower in SYFs, may be attributed to the participation of MMP-16 in the follicle destination, i.e., its atresia or selection into preovulatory hierarchy to complete development. It has been proven that WFs and YFs are highly susceptible for the process of atresia, and MMP-2 and MMP-9 were proposed as significant players in the atresia of prehierarchical follicles in the hen ovary (HRABIA *et al.* 2019). Within preovulatory follicles, the expression of MMP-16 mRNA was significantly higher in the theca layer than in the granulosa layer. This finding corresponds with previous results where mRNA levels of several MMPs were lowest in the granulosa layer and highest in the theca layers of the largest preovulatory follicles (HRABIA *et al.* 2019; WOLAK & HRABIA 2020; HRABIA *et al*. 2021; WOLAK & HRABIA 2021; WOLAK *et al.* 2021). The theca layer contains fibroblasts, macrophages, and mast cells (BARUA *et al.* 1998; PACZOSKA-ELIASIEWICZ 1999), and these cells are thought to be a source of MMPs (HRABIA 2021).

The hormonal regulation of MMP-16 gene expression has not been investigated in the ovarian follicles of birds. Follicle development and function are, in large part, regulated by FSH and LH. These gonadotropin receptor abundances, as well as sensitivity to both gonadotropins, change with follicle development (HRABIA 2022). Namely, the greatest FSH receptor abundance is noted in the follicle around the time of recruitment (6-12 mm), and there is a progressive decrease in abundance with follicle maturation to ovulation (YOU *et al*. 1996; ZHANG *et al*. 1997). In turn, LH receptor abundance decreases in the theca layer and increases in the granulosa layer along with follicle maturation, reaching the highest level in the F1 follicle before ovulation (ZHANG *et al.* 1997). Taking into consideration that gonadotropins may potentially control MMP-16 gene expression in the chicken ovarian follicles, this hypothesis was evaluated in the study. For this purpose, the laying hens were treated with eCG for several days. It is well established that eCG has FSH-like properties (lesser LH-like activity) and a long half-life (JOHNSON & LEONE 1985; NAKANISHI *et al*. 1991; GHANEM & JOHNSON 2019). It was found that the relative transcript levels of MMP-16 were lower in most examined ovarian tissues of eCG-treated hens than in control hens. These findings suggest that the MMP-16 gene is a candidate target for direct and/or indirect eCG regulation at the transcriptional level. Our findings and hypothesis correspond with earlier results showing the effect of eCG treatment on MMP-2 and MMP-9 mRNA expression (WOLAK *et al.* 2021). It seems that the MMP-16 gene, in addition to genes of gelatinases, is negatively regulated by eCG, which primarily has FSH-like activities.

Gonadotropins, among others, regulate synthesis and secretion of steroids, and it is well known that estrogen plays a significant role in ovarian function. Moreover, it has been shown that alternations in the synthesis and secretion of steroid hormones and their receptor abundance in the follicular wall during ovarian follicle development, maturation, and ovulation in birds (YOSHIMURA & BAHR 1991; YOSHIMURA *et al.* 1993; HRABIA *et al.* 2008; HRABIA 2022; HANLON *et al*. 2022) are accompanied by changes in the expression and activity of several MMPs and their tissue inhibitors (OGAWA & GOTO 1984; ZHU *et al.* 2014; WOLAK & HRABIA 2020). Therefore, the question addressed in the next experiment presented in this study was whether estrogen's participation in the chicken ovarian function is associated with the regulation of mRNA expression of MMP-16. Accordingly, the hens were treated with TMX for several days. In avian species, TMX, a synthetic estrogen antagonist, binds to estrogen receptors with high affinity and blocks the transcription of estrogen-dependent genes in target tissues (SUTHERLAND 1981; ARAO *et al*. 1996). Transcript abundance of MMP-16 following TMX treatment was dependent on the ovarian tissue and stage of follicle development. TMX did not affect MMP-16 mRNA expression in pre-hierarchical follicles, WFs, and YFs, whereas it up-regulated MMP-16 gene expression in the theca layer of F3-F1 follicles and down-regulated this gene transcript abundance in the granulosa layer of the F1 follicle. These observations suggest that estrogen may be involved in the control of MMP-16 transcript levels mainly in preovulatory follicles, similarly as it was previously demonstrated in the case with several other MMPs and TIMPs in the hen ovary upon treatment with TMX (WOLAK & HRABIA 2020).

In conclusion, our results provide evidence that MMP-16 mRNA expression in the hen ovary depends on the tissue type and stage of follicle maturation, indicating the possibility that MMP-16 take part in ECM remodeling throughout follicle development. Changes in the transcript level of MMP-16 just before ovulation of the largest, yellow preovulatory follicle suggests that this enzyme may be potentially engaged in a complex signal system required to rapture the follicle during ovulation. Alternations in abundance of the MMP-16 transcript following eCG and TMX treatments indicate a possible role of gonadotropins, mainly FSH, and estrogen in regulating the transcription of MMP-16 in the chicken ovarian follicles. To better understand the role of MMP-16 in the hen ovary, further investigations evaluating MMP-16 protein levels and activity at different physiological states of this organ are warranted.

Funding

This research was financially supported by the Ministry of Sciences and Higher Education to the University of Agriculture in Kraków, Poland (subvention number: SUB-020002-D017).

Acknowledgements

The authors express their deepest gratitude to Mrs. Aleksandra FLORCZAK for technical assistance with RT-PCR.

Author Contributions

Research concept and design: A.H., D.W.; Collection and/or assembly of data: A.H., D.W.; Data analysis and interpretation: A.H., D.W.; Writing the article: D.W.; Critical revision of the article: A.H.; Final approval of article: A.H., D.W.

Conflict of Interest

The authors declare no conflict of interest.

References

ARAO Y., YAMAMOTO E., MIYATAKE N., NINOMIYA Y., UMEHARA T., KAWASHIMA H., MASUSHIGE S., HASEGAWA T., KATO S. 1996. A synthetic oestrogen antagonist, tamoxifen, inhibits oestrogen-induced transcriptional, but not post-transcriptional, regulation of gene expression. Biochem. J. **313**: 269-274. https://doi.org/10.1042/bj3130269.

ASEM E.K., STINGLEY-SALAZAR S.R., ROBINSON J.P., TUREK J.J. 2000. Identification of some components of basal lamina of avian ovarian follicle. Poult. Sci. **79**: 589-601. doi: https://doi.org/10.1093/ps/79.4.589.

BAHR J.M., JOHNSON A.L. 1984. Regulation of the follicular hierarchy and ovulation. J. Exp. Zool. **232**: 495-500. https://doi.org/10.1002/jez.1402320316.

BARUA A., YOSHIMURA Y., TAMURA T. 1998. Localization of macrophages in the ovarian follicles during the follicular growth and postovulatory regression in chicken, *Gallus domesticus*. Poult. Sci. **7**: 1417-1421. https://doi.org/10.1093/ps/77.9.1417.

CHOI J.W., AHN S.E., RENGARAJ D., SEO H.W., LIM W, SONG D., HAN J.Y. 2011. Matrix metalloproteinase 3 is a stromal marker for chicken ovarian cancer. Oncol. Lett. **2**: 1047-1051. https://doi.org/10.3892/ol.2011.391.

GILBERT A.B., EVANS A.J., PERRY M.M., DAVIDSON M.H. 1977. A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (*Gallus domesticus*). J. Reprod. Fertil. **50**: 179-181. https://doi.org/10.1530/jrf.0.0500179.

GHANEM K., JOHNSON A.L. 2019. Response of hen pre-recruitment ovarian follicles to follicle stimulating hormone, *in vivo*. Gen. Comp. Endocrinol. **270**: 41-47. https://doi.org/10.1016/j.ygcen.2018.10.004.

HANLON C., ZIEZOLD C.J., BÉDÉCARRATS G.Y. 2022. The diverse roles of 17β-estradiol in non-gonadal tissues and its consequential impact on reproduction in laying and broiler breeder hens. Front. Physiol. **13**: 942790. https://doi.org/10.3389/fphys.2022.942790.

HRABIA A. 2021. Matrix metalloproteinases (MMPs) and inhibitors of MMPs in the avian reproductive system: an overview. Int. J. Mol. Sci. **22**: 8056. https://doi.org/10.3390/ijms22158056.

HRABIA A. 2022. Reproduction in the female. (In: Sturkie's Avian Physiology. Seventh Edition. SCANES C.G., DRIDI S. eds. Academic Press, New York): 941-986. https://doi.org/10.1016/B978-0-12-819770-7.00002-5.

HRABIA A., WILK M., RZĄSA J. 2008. Expression of alpha and beta estrogen receptors in the chicken ovary. Folia Biol. (Kraków). **56**: 187-191. https://doi.org/10.3409/fb.56_3-4.187-191.

HRABIA A., SOCHA J.K., SECHMAN A. 2018. Involvement of matrix metalloproteinases (MMP-2, -7, -9) and their tissue inhibitors (TIMP-2, -3) in the regression of chicken postovulatory follicles. Gen. Comp. Endocrinol. **260**: 32-40. https://doi.org/10.1016/j.ygcen.2018.02.008.

HRABIA A., WOLAK D., KWAŚNIEWSKA M., KIERONSKA A., SOCHA J.K., SECHMAN A. 2019. Expression of gelatinases (MMP-2 and MMP-9) and tissue inhibitors of metalloproteinases (TIMP-2 and TIMP-3) in the chicken ovary in relation to follicle development and atresia. Theriogenology. **125**: 268-76. https://doi.org/10.1016/j.theriogenology.2018.11.017.

HRABIA A., WOLAK D., SECHMAN A. 2021. Response of the matrix metalloproteinase system of the chicken ovary to prolactin treatment. Theriogenology **169**: 21-28. https://doi.org/10.1016/j.theriogenology.2021.04.002.

JOHNSON A.L., LEONE E.W. 1985. Ovine luteinizing hormone-induced steroid and luteinizing hormone secretion and ovulation in intact and pregnant mare serum gonadotropin-primed hens. Poult. Sci. **94**: 2171-2179. https://doi.org/10.3382/ps.0642171.

KESSENBROCK K., PLAKS V., WERB Z. 2010. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. **141**: 52-67. https://doi.org/10.1016/j.cell.2010.03.015.

LEŚNIAK A., HRABIA A. 2012. Expression of matrix metalloproteinase-2 mRNA in the chicken ovary in relation to follicle remodelling. Folia Biol (Kraków). **60**: 219-25. https://doi.org/10.3409/fb60_3-4.219-225.

LEŚNIAK-WALENTYN A., HRABIA A. 2016. Involvement of matrix metalloproteinases (MMP-2, -7, -9) and their tissue inhibitors (TIMP-2, -3) in the chicken oviduct regression and recrudescence. Cell Tissue Res. **366**: 443-454. https://doi.org/10.1007/s00441-016-2442-6.

NAKAJO S., ZAKARIA A.H., IMAI K. 1973. Effect of the local administration of proteolytic enzymes on the rupture of the ovarian follicle in the domestic fowl, *Gallus domesticus*. J. Reprod. Fertil. **34**: 235-40. https://doi.org/10.1530/jrf.0.0340235.

NAKANISHI A., MIYAKE M., UTSUMI K., IRITANI A. 1991. Fertilizing competency of multiple ovulated eggs in the domestic fowl (*Gallus domesticus*). Mol. Reprod. Develop. **28**: 131-135. https://doi.org/10.1002/mrd.1080280205.

NY T., WAHLBERG P., BRANDSTROM J.M. 2002. Matrix remodeling in the ovary: regulation and functional role of the plasminogen activator and matrix metalloproteinase systems. Mol. Cell. Endocrinol. **187**: 29-38. https://doi.org/10.1016/S0303-7207(01)00711-0

OCŁOŃ E., HRABIA A. 2021. miRNA expression profile in chicken ovarian follicles throughout development and miRNA-mediated MMP expression. Theriogenology **160**: 116-127. https://doi.org/10.1016/j.theriogenology.2020.11.004.

OGAWA K., GOTO K. 1984. Effects of gonadotrophins, sex steroids and adenohypophysectomy on the activities of proteolytic enzymes in the ovarian follicle wall of the domestic fowl (*Gallus domesticus*). J. Repr. Fertil. **71**: 545-549. https://doi.org/10.1530/jrf.0.0710545.

PACZOSKA-ELIASIEWICZ H.E. 1999. Udzial histaminy w czynnosci jajnika kury domowej (*Gallus domesticus*). Zesz. Naukowe Akad. Rol. Krakowie Rozpr. **248**: 1-84.

PAGE-MCCAW A., EWALD A.J., WERB Z. 2007. Matrix metalloproteinases and the regulation of tissue remodelling. Nat. Rev.

Mol. Cell Biol. **8**: 221-233. doi: 10.1038/nrm2125. https://doi.org/10.1038/nrm2125.

PUTTABYATAPPAP M., TERRY A.J., LINAH F.A., *et al*. 2014. Ovarian membrane-type matrix metalloproteinases. Induction of MMP-14 and MMP-16 during the periovulatory period in the Rat, Macaque, and Human. Biol. Reprod. **91**: 1-12. https://doi.org/10.1095/biolreprod.113.115717.

SCHMITTGEN T.D., LIVAK K.J. 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Prot. **3**: 1101-1108. https://doi.org/10.1038/nprot.2008.73.

SMITH M.F., RICKE W.A., BAKKE L.J., DOW M.P., SMITH G.W. 2002. Ovarian tissue remodeling: role of matrix metalloproteinases and their inhibitors. Mol. Cell Endocrinol. **191**: 45-56.

SUTHERLAND R.L. 1981. Estrogen antagonist in chick oviduct: antagonist activity of eight synthetic triphenylethylene derivates and their interactions with cytoplasmic and nuclear estrogen receptors. Endocrinology **109**: 2061-2068. https://doi.org/10.1210/endo-109-6-2061.

WOLAK D., HRABIA A. 2020. Tamoxifen-induced alternations in the expression of selected matrix metalloproteinases (MMP-2,-9-10 and-13) and their tissue inhibitors (TIMP-2 and TIMP-3) in the chicken ovary. Theriogenology **148**: 208-215. https://doi.org/10.1016/j.theriogenology.2019.11.008.

WOLAK D., HRABIA A. 2021. Alternations in the expression of selected matrix metalloproteinases (MMP-2, -9, -10, and -13) and their tissue inhibitors (TIMP-2 and -3) and MMP-2 and -9 activity in the chicken ovary during pause in laying induced by fasting. Theriogenology **161**: 176-186. https://doi.org/10.1016/j.theriogenology.2020.12.005.

WOLAK D., SECHMAN A., HRABIA A. 2021. Effect of eCG treatment on gene expression of selected matrix metalloproteinases (MMP-2, MMP-7, MMP-9, MMP-10, and MMP-13) and their tissue inhibitors (TIMP-2 and TIMP-3) in the chicken ovary. Anim. Reprod. Sci. **224**: 106666. https://doi.org/10.1016/j.anireprosci.2020.106666.

YAMAMOTO K., MURPHY G., TROEBERG L. 2015. Extracellular regulation of metalloproteinases. Matrix Biol. **44-46**: 255-263. https://doi.org/10.1016/j.matbio.2015.02.007.

YOSHIMURA Y., BAHR J.M. 1991. Localization of progesterone receptors in pre- and postovulatory follicles of the domestic hen. Endocrinology **128**: 323-330. https://doi.org/10.1210/endo-128-1-323.

YOSHIMURA Y., CHANG C., OKAMOTO T., TAMURA T. 1993. Immunolocalization of androgen receptor in the small, preovulatory, and postovulatory follicles of laying hens. Gen. Comp. Endocrinol. **91**: 81-89. https://doi.org/10.1006/gcen.1993.1107.

YOU S., BRIDGHAM J.T., FOSTER D.M., JOHNSON A.L. 1996. Characterization of the chicken follicle-stimulating hormone receptor (cFSH-R) complementary deoxyribonucleic acid, and expression of cFSH-R messenger ribonucleic acid in the ovary. Biol. Reprod. **55**: 1055-1062. https://doi.org/10.1095/biolreprod55.5.1055.

YUAN Z., CHEN Y., CHEN Q., GUO M., KANG L., ZHU G. *et al*. 2016. Characterization of chicken MMP13 expression and genetic effect on egg production traits of its promoter polymorphisms. G3 (Bethesda). **6**: 1305-1312. https://doi.org/10.1534/g3.116.027755.

ZHANG C., SHIMADA K., SAITO N., KANSAKU N. 1997. Expression of messenger ribonucleic acids of luteinizing hormone and follicle-stimulating hormone receptors in granulosa and theca layers of chicken preovulatory follicles. Gen. Comp. Endocrinol. **105**: 402-409. https://doi.org/10.1006/gcen.1996.6843.

ZHU G., KANG L., WEI Q., CUI X., WANG S., CHEN Y. *et al*. 2014. Expression and regulation of MMP1, MMP3, and MMP9 in the chicken ovary in response to gonadotropins, sex hormones, and TGFB1. Biol. Reprod. **90**: 1-11. https://doi.org/10.1095/biolreprod.113.114249.