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Expression of Relaxin 3 in the Ovarian Follicle of Japanese Quail

Khoi X. Hoang[1], Mei Matsuzaki [2], Tetsuya Kohsaka[3] and Tomohiro Sasanami[1,4]

[1]United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[2]Program of Food and AgriLife Science, Graduate School of Integrated Sciences for Life, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima City, Hiroshima 739-8528, Japan
[3]Faculty of Health Science, Butsuryo College of Osaka, 3-33 Otorikita-machi, Sakai, Osaka 593-8328, Japan
[4]Department of Applied Life Sciences, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka, Shizuoka 422-8529, Japan

The relaxin (*RLN*) gene is expressed in the reproductive tracts, such as the ovary and uterus, of mammalian species. Although RLN expression is detected in the chicken ovary, detailed clarification of the physiological role of RLN has not yet been reported. To address this issue, in the present study we aimed to examine the spatiotemporal expression and hormonal control of RLN in Japanese quail. By performing semi-quantitative and quantitative reverse transcription-polymerase chain reaction analysis, we found that *RLN* mRNA was mainly expressed in the granulosa and theca layers of the ovary. The expression level in the granulosa layer increased with the stage of follicular development. Results from granulosa layer culture experiments revealed that *RLN* mRNA expression increased with the addition of estradiol-17β, whereas the addition of progesterone suppressed *RLN* transcription. More detailed analysis indicated that *RLN* expression was highest in the stigma region of the follicle but significantly decreased as the time of the expected luteinizing hormone (LH) surge approached. Together, our findings demonstrated that the granulosa cells in the mature preovulatory follicles constitute the main source of RLN in the Japanese quail. Because *RLN* expression was highest in the stigma region and the expression dramatically decreased following the LH surge, the results further suggest that RLN may be related to tissue remodeling for the ovulation process in birds.

Key words: follicle, granulosa layer, Japanese quail, relaxin, stigma

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Introduction

Relaxin (RLN), a peptide hormone approximately 6.5 kDa in size, was originally identified in the serum of pregnant guinea pigs as the factor causing the pubic ligament of guinea pigs to relax after estrus[1]. Similar relaxing activities were found in the extracts of pig corpus luteum and the rabbit placenta. Because the biological effects, such as inhibition of spontaneous myometrial contractions of the uterine and promotion of cervical softening, were subsequently confirmed using highly purified RLN preparations, RLN is considered to have physiological roles during pregnancy and at parturition in multiple mammalian species[2].

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Correspondence: Tomohiro Sasanami, Department of Applied Life Sciences, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka, Shizuoka 422-8529, Japan. (Email: atsasan@shizuoka.ac.jp)

The primary structure of the RLN peptide is similar to that of insulin, comprising an A chain and a B chain, which confer two inter-chains and one intra-chain. In addition, the middle of the B chain contains a consensus motif (Arg-X-X-X-Arg-X-X-Ile/Val, where X represents any amino acid), which is responsible for RLN receptor binding and biological activity[1–3]. Several isoforms of RLN have been identified; these peptides are classified as RLN family peptides, including RLN–3 and four insulin-like peptides (INSLs): INSL3, INSL4, INSL5, and INSL6[3].

Genomic library screening resulted in the cloning of human *RLN1* (encoding H1-RLN) and human *RLN2* (H2-RLN)[4]. The circulating form of RLN in humans is H2-RLN, which is considered to be the functional equivalent of all non-primate RLNs. Although no H1-RLN equivalent has been found, the homologous gene encoding H2-RLN in non-primates is referred to as *RLN1*[3,5]. RLN3, which has been identified in many species, acts as a neuropeptide in the brain and is expressed in peripheral tissues such as the heart, kidney, lung, liver, and pancreas. In the reproductive tissues in females, RLN1 has been found in the corpus luteum, placenta, and uterus in non-primates such as pigs, rats, mice, and guinea pigs[4]. In the ovary, locally produced

RLN may have stimulatory effects on follicular growth and corpus luteum function; moreover, RLN expressed in the uterus may be directly involved in the inhibition of uterine contractility and control of collagen remodeling in fetal membranes[1,2].

However, knowledge of RLN family peptides in nonmammalian species remains limited. In fishes, RLN-like peptides have been detected in the testis and ovaries of dogfish, sand tiger sharks, Japanese medaka, and zebrafish[6–10]. In avian species, RLN-like activity has been reported in the ovaries[11] and testis[12] of chickens. In 1997, Brackett *et al*.[13] demonstrated that purified RLN3 from ovarian follicles, which showed immunoreactivity to anti-porcine RLN antibodies, was bioactive as this material inhibited the spontaneous contractions of the estrogen-primed mouse uterus. More recently, RLN3 expression was reported in the granulosa and theca layers of chicken ovaries[14]. Quantitative polymerase chain reaction (PCR) analysis revealed that *RLN3* expression is highest in the granulosa layer and shows increased expression as follicles mature. However, no studies have elucidated the hormonal regulation and physiological role of RLN3 in birds.

To address this issue, in the present study we investigated whether the ovary serves as a source of RLN3 in Japanese quail and characterized *RLN3* expression in different-sized follicles. In addition, we cultured the granulosa layer with various steroid hormones to study the endocrine control of *RLN3* expression. Moreover, we considered the possible involvement of RLN3 in the process of ovulation.

Materials and methods

Animals

Female Japanese quail (*Coturnix japonica*), six weeks of age, were obtained from Quail-Cosmos (Tahara, Japan). They were individually caged under a photoperiod of 14 h light: 10 h dark (lights on at 5:00) and provided with water and a commercial quail diet (Toyohashi-shiryo, Toyohashi, Japan) *ad libitum*. Almost all birds laid eggs regularly, ranging from 15:00 to 18:00 every day. The birds were monitored for time of oviposition which was recorded manually every 1 h between 15:00 and 18:00; the ovulation was assumed to occur 15–30 min after the time of oviposition. For gene expression analysis, various tissues were harvested from egg-laying quail at 15–30 weeks of age following sacrifice by cervical dislocation approximately 8–10 h before the expected time of ovulation. The harvested tissues were frozen and stored at −80°C until RNA extraction. For isolation of the granulosa layer, the ovarian follicles were removed from the birds 8 h before the expected time of ovulation and placed in phosphate-buffered saline (pH 7.4). The granulosa layer (granulosa cells, inner layer of the vitelline membrane, and basal lamina) was isolated from the theca layer according to the procedure reported by Gilbert *et al.*[15]. Granulosa layers were obtained from the largest (F1), second largest (F2), and third largest (F3) follicles. To determine the detailed localization of *RLN3* in the follicles, the F1 follicle was collected and the theca and granulosa layers were separated as described above. The stigma (S), nonstigma (NS), and germinal disc (GD) regions of the granulosa layer were isolated as described by Jackson *et al*.[16].

All experimental procedures for the use and care of animals in the present study were approved by the Animal Care Committee of the Faculty of Agriculture at Shizuoka University (approval number: 2018A-5).

Cell culture

The granulosa layers isolated from the three largest follicles (F1, F2, and F3) were placed into a six-well culture plate (Corning Incorporated - Life Sciences, Durham, NC, USA) filled with 3 ml of Dulbecco's modified Eagle's medium (Sigma-Aldrich Inc., St. Louis, MO, USA). The medium was supplemented with various steroid hormones, such as estradiol-17β (E_2), progesterone (P_4), and testosterone (T), to final concentrations of 0, 10, 100, and 1000 nM. When steroid hormone was added to the medium, the ethanol concentration was never higher than 0.1%[17]. After incubation at 41°C in a humidified atmosphere of 5% CO_2 for 6 h, the granulosa layer was collected and stored at −80°C for RNA extraction.

RNA extraction and semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was extracted from various tissues using the extraction reagent, RNAiso Plus (Takara Bio Inc., Shiga, Japan). After dissolving the resulting RNA pellet in RNase-free water, the concentration and purity were determined by absorption at 260 and 280 nm using a spectrophotometer (DS-11 Series Spectrophotometer, DeNovix Inc., Wilmington, DE, USA). Only RNA samples exhibiting an A260/280 ratio of 1.8 or greater were used for reverse transcription. Aliquots of 1 μg of total RNA were used for cDNA synthesis in reaction mixtures (10 μl) using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan).

Amplification of quail *RLN3* (GenBank accession no. XM_015848927) was carried out using the specific primer set: 5′-GAAGCGGCTCTCCCTACTG-3′ (forward) and 5′-GGAGCAGATGCAGAGAAGCA-3′ (reverse). The specific primer set: 5′-GGCGCGGGTGATCATCGAGAA-3′ (forward) and 5′-GAGAGCGCCTCGTGGTGTTT-3′ (reverse) for the gene encoding quail S17 ribosomal protein (GenBank accession no. XM_015872709) was employed for normalization of the data. All PCR reactions were carried out in reaction mixtures (50 μl) containing 5 mmol/ml $MgCl_2$, 0.4 mmol/ml dNTP, and 2.5 U Taq DNA polymerase (Takara Bio Inc.). The PCR protocol consisted of an initial denaturation for 2 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 20 s, then a final extension step at 72°C for 2 min. For the S17 primer pair, annealing at 53.4°C was performed, and 25 cycles were employed for amplification. The PCR product was separated on 1% agarose gels containing ethidium bromide (1 μg/ml) and visualized under UV transillumination. For quantitative evaluation, the intensity of the PCR product bands was measured using ImageJ software (https://imagej.nih.gov/ij/index.html). The results were expressed as the *RLN3* mRNA/S17 ribosomal protein mRNA ratio.

Table 1. **List of primers and probes for Japanese quail target genes**

Gene	Name	Sequence (5' to 3')	GenBank accession No.
RLN3	Forward primer	GAATACGAGCCTGTGGCAGA	XM_015848927.2
	Reverse primer	TAACTGCTGGGTCCTAGGGG	
	Probe	[FAM] AGTTCAAAGAACTTCTTCGCCAGGCAGAG [TAMRA]	
S17	Forward primer	AACGAGAGCGCAGGGATAAC	XM_015872709.2
	Reverse primer	CGTCACCTGAAGGTTGGACA	
	Probe	[FAM] CGTACCCGAGGTCTCTGCTCTTGATCAGGA[TAMRA]	

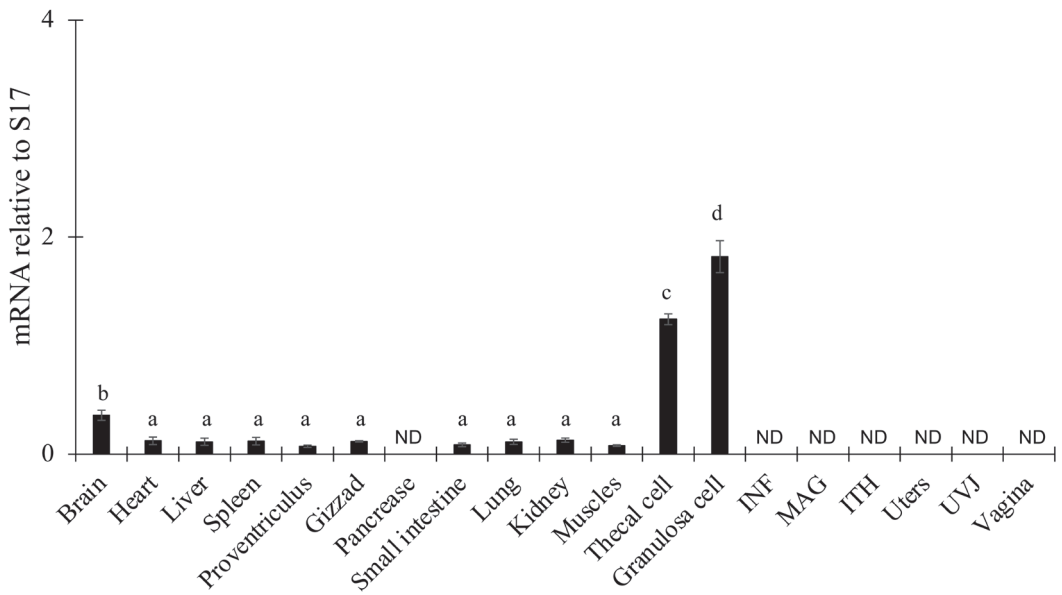

Fig. 1. **Tissue distribution and *RLN3* mRNA expression in Japanese quail**. Semi-quantitative RT-PCR analysis was performed using total RNA from various tissues of mature Japanese quail. The band intensities were normalized to those of the S17 ribosomal protein gene. Values represent the means ± SEM from five animals for each tissue; values with different letters are significantly different ($P < 0.05$). ND, not determined.

Quantitative RT-PCR

The primers and fluorescence probes (Table 1) for quail *RLN3* and the S17 ribosomal protein gene were constructed to be within the purified PCR product generated by RT-PCR, as described above. *RLN3* mRNA quantitation of test samples was performed in conjunction with a standard curve for each gene obtained by amplifying a 10-fold dilution series of purified PCR products. Quantification of gene expression by RT-PCR analysis was performed using a thermal cycler (DICE Real-Time System III; Takara Bio Inc.). PCR was carried out using reaction mixtures (50 µl) containing template DNA, 400 nM TaqMan probes, 1X Probe qPCR Mix (Takara Bio Inc.), and 200 nM of each specific primer. Amplification and detection of the samples and standards were performed using a thermal profile setup as follows: 5 min at 94°C followed by 45 cycles of 10 s at 94°C, 10 s at 57°C, and 10 s at 72°C. The expression level of *RLN3* was normalized to that of the S17 ribosomal protein gene.

Data analysis

All experiments were repeated 3–5 times. Normality and homoscedasticity of the data were confirmed using the Shapiro–Wilk test and Bartlett test, respectively. Differences between two groups were analyzed using the F-test followed by the Student's t-test. Differences between multiple groups were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered to reflect a statistically significant difference. Data are presented as the mean ± standard error of the mean (SEM).

Results

To investigate the tissue distribution of *RLN3* mRNA, we isolated various tissues from egg-laying birds and performed semi-quantitative RT-PCR analysis. As shown in Figure 1, *RLN3* was mainly expressed in the granulosa and theca layers, with relatively weak expression being detected in a wide range of tissues

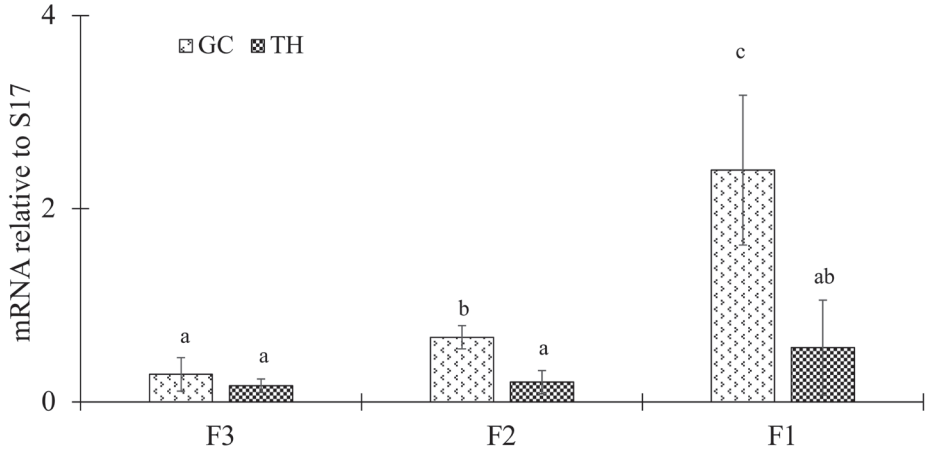

Fig. 2. **Changes in *RLN3* mRNA expression during follicular development**. Granulosa layers obtained from the largest (F1), second largest (F2), and third largest (F3) follicles were isolated 8–10 h before the expected time of ovulation. The tissues were extracted for RNA and expression was measured by quantitative RT-PCR. Values represent the means ± SEM of triplicate experiments; values with different letters are significantly different ($P < 0.05$). GC,–; TH,–

except for the pancreas, any parts of the oviduct, or the vagina. Next, we investigated the change in *RLN3* mRNA expression during follicular development. As shown in Figure 2, higher expression was observed in the granulosa layer than in the theca layer in all follicles; in addition, the expression in the granulosa layer increased with increased follicle size.

To elucidate the hormonal regulation of RLN3 expression, granulosa layers obtained from F1, F2, and F3 were incubated for 6 h with 0, 10, 100, and 1000 nM of E_2, P_4, or T, and the expression of *RLN3* was measured by quantitative RT-PCR analysis. As shown in Figure 3, the addition of 1000 nM E_2 increased the *RLN3* mRNA expression in all follicles, whereas the addition of more than 10 nM P_4 suppressed *RLN3* transcription. The addition of T into the medium did not affect *RLN3* expression in F2 and F3, but tended to decrease *RLN3* expression at lower concentrations in F1. The expression of *RLN* in F1 was restored at higher T concentrations.

To investigate the spatiotemporal expression of *RLN3* mRNA, samples were separately isolated from the granulosa and theca layers 8 h before the expected time of ovulation and immediately after oviposition, which were assumed to be before and after the LH surge, respectively. Furthermore, the expression in the granulosa layer around the S, NS, and GD regions of the granulosa layer were also quantitated. We found that the expression of *RLN3* mRNA in the granulosa and theca layers significantly decreased after the LH surge compared with that before the LH surge (Fig. 4). In addition, the highest expression was observed in the S region of the follicle, with this expression significantly decreasing after the LH surge. Relatively lower expression levels were observed in the NS and GD regions; these did not exhibit a similar reduction in expression (Fig. 5).

Discussion

In this study, we demonstrated the profiles of *RLN3* expression in mature female quail. We found that 1) *RLN3* expression was detected in the granulosa layer of the ovarian follicle and gene expression increased during follicular development; 2) *RLN3* expression was enhanced by E_2, whereas it was suppressed by P_4; and 3) *RLN3* expression was significantly higher in the S region than that in other regions of the granulosa layer of the F1 follicle, with this expression decreasing following the LH surge.

Ghanem and Johnson[14] reported that the expression of RLN3 in hen ovaries was highest in the granulosa layer and significantly increased during follicular development. Although these authors did not measure the expression levels in mature follicles (i.e., F1, F2, or F3), our data suggesting that the main source of RLN3 in Japanese quail is the granulosa layer of mature follicles are consistent with the results from chicken. In pig, it was reported that the non-pregnant or immature animals expressed a large amount of RLN in the largest preovulatory follicles[18], which may be linked to the fact that the changes in RLN3 expression correspond fundamentally with the developmental stage of the follicles.

In granulosa layer cultures, we found that E_2 stimulated *RLN3* expression and that the effect of E_2 was greater in the larger follicles than in smaller ones (F3<F2<F1). In female birds, E_2 plays an important role in reproduction by stimulating follicular development [19]. Thus, the elevated effects of E_2 on *RLN3* expression during follicular development may reflect the role of E_2-stimulated RLN3 expression on the process of follicular growth. In contrast, we found that the addition of T in the culture medium showed no obvious effects on *RLN3* expression in F3 and F2.

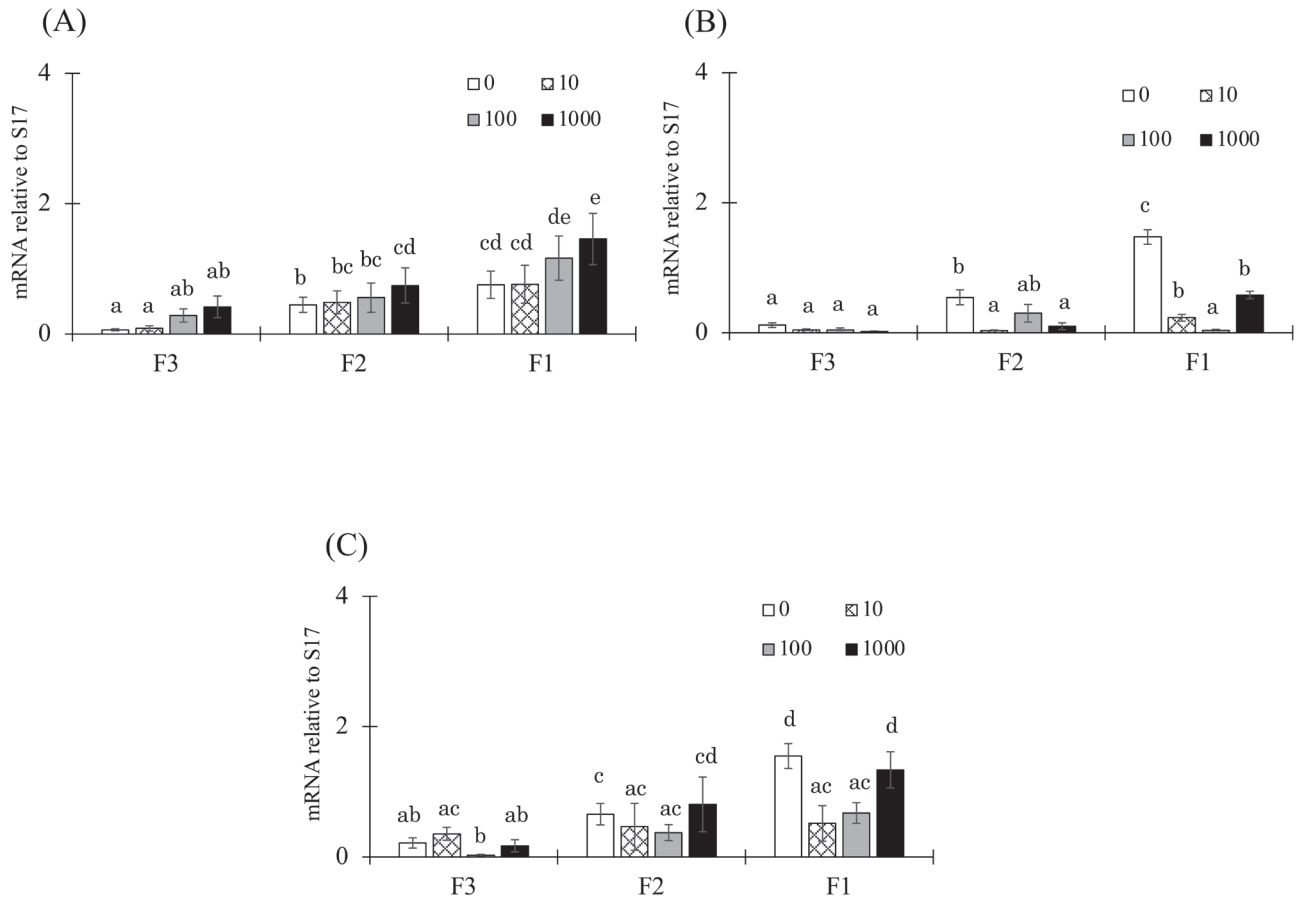

Fig. 3. **Effect of various steroid hormones on *RLN3* mRNA expression**. The Granulosa layers of the largest (F1), second largest (F2), and third largest (F3) follicles were cultured with 0, 10, 100, and 1000 nM of estradiol-17β (A), progesterone (B), or testosterone (C) for 6 h. After incubation, granulosa layers were collected to extract RNA, and expression was measured by quantitative RT-PCR. Values represent the means ± SEM of triplicate experiments; values with different letters are significantly different ($P < 0.05$).

Rather, T suppressed *RLN3* expression at lower doses (10 and 100 nM), whereas 1000 nM T resorted *RLN3* expression in F1 follicles. Although the reason for this discrepancy in F1 remains unclear, we consider that T may act as a negative regulator of RLN3 expression in avian species. Further experiments will be required to better understand the effects of T on RLN3 expression

In female birds, T is synthesized and secreted from the theca interna of the medium sized follicles, and is converted to E_2 by aromatase in the theca externa [20, 21]. The increase in circulating T that occurs approximately 6 h before ovulation is the result of secretion from the four largest follicles [19]; the follicular venous plasma of the fourth to second largest follicles contains a significantly higher concentration of T than that in peripheral plasma [22, 23]. Similarly, preovulatory follicle secretion of E_2 is greatest from the third and fourth largest follicles 3–6 h prior to ovulation [19], with the concentration of E_2 in follicular venous plasma also being higher than that in peripheral plasma [22, 23]. Although the effective concentrations of E_2 and T in granulosa cell culture were much higher than those of the circulating hormones, it is possible that the local concentrations of these hormones around the granulosa cells may also be much higher than the blood hormone concentrations.

In contrast to the stimulatory effects of E_2, *RLN3* expression was suppressed by P_4. In particular, *RLN3* expression in the granulosa layers of all tested quail follicles was decreased by the addition of P_4. In the domestic hen, 4–6 h before ovulation, the amount of P_4 produced by granulosa cells in the F1 follicle increased in response to LH, suggesting that LH stimulated P_4 production by the granulosa layer, concurrent with the decrease in RLN expression in an autocrine manner. This assumption is also supported by the observation that the granulosa layer isolated from large follicles is more sensitive to LH because the number of receptors for LH increases as the follicles mature [24].

In this study, we found that *RLN3* mRNA expression significantly decreased after the LH surge compared with that before the LH surge. This result is consistent with culture experiments

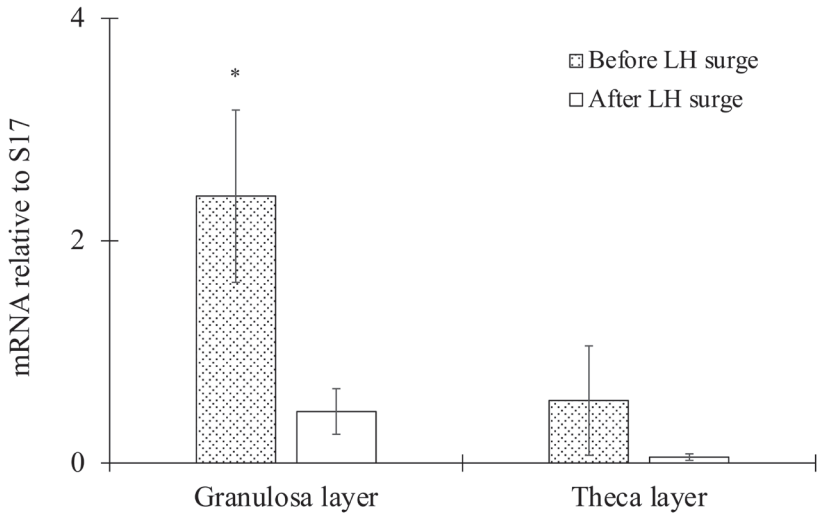

Fig. 4. **Changes in *RLN3* mRNA expression before and after the LH surge**. Granulosa and theca layers obtained from the largest (F1) follicle were isolated approximately 8–10 h before the expected time of ovulation (before the LH surge) and immediately after ovulation (after the LH surge). The tissues were extracted for RNA and expression was measured by quantitative RT-PCR. Values represent the means ± SEM of triplicate experiments; values with (*) are significantly different ($P < 0.05$). LH, luteinizing hormone.

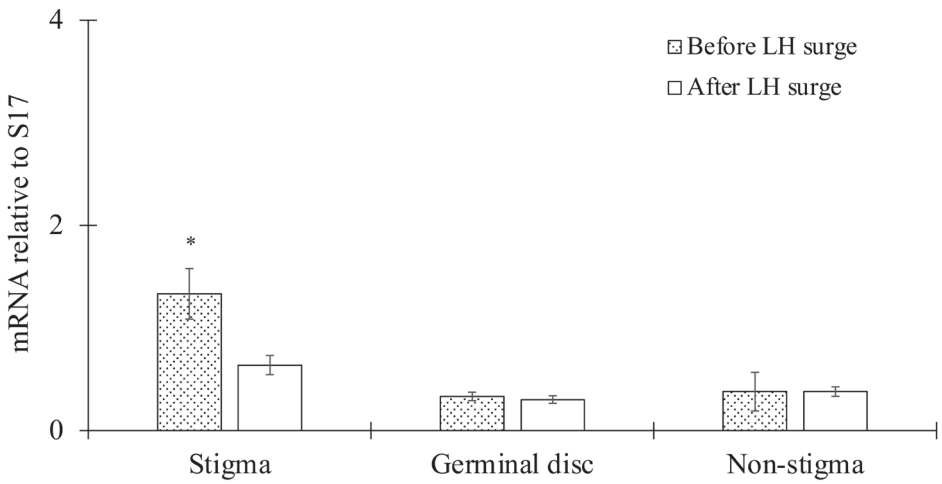

Fig. 5. ***RLN3* mRNA expression in the different regions of the granulosa layer before and after the LH surge**. The largest (F1) follicle was collected approximately 8–10 h before the expected time of ovulation (before the LH surge) and immediately after oviposition (after the LH surge). The stigma (S), nonstigma (NS), and germinal disc (GD) regions of the granulosa layer were separated. RNA was extracted from the tissues and the expression was measured by quantitative RT-PCR. Values represent the means ± SEM of triplicate experiments; values with (*) are significantly different ($P < 0.05$). LH, luteinizing hormone.

showing that the addition of P_4 suppressed *RLN3* expression. Moreover, we found that *RLN3* expression in the stigma region of the granulosa layer of the F1 follicle significantly decreased after the LH surge. Jackson *et al*.[16] reported that plasminogen activator, a proteolytic enzyme, plays an essential role in the extracellular matrix remodeling required for follicular development and ovulation. Although we did not assess either plasminogen activator or protease in this study, our data showing the dramatic decline of *RLN3* expression in the stigma region after the LH surge may relate to the process of ovulation. Notably, the expression of RLN family peptide receptor 1 (RXFP1), which is an authentic receptor for RLN3, was detected in the theca layer but not the granulosa layer in hen ovaries[14]. Therefore, we hypothesize that the RLN3 expressed by the granulosa layer may stimulate the theca layer by binding with RXFP1, resulting in the alteration of protease activity and expression to promote ovulation. Further studies are required to support this hypothesis regarding RLN functions in birds.

In conclusion, our findings shed light on the novel functions of RLN gene family peptides in avian species. Our results indicated that the granulosa layers in mature follicles express *RLN* genes, with the expression being highly localized in the stigma region. Although we did not examine the roles of RLN on protease expression or activity, we hypothesize that RLN may be involved in ovarian tissue remodeling and ovulation because *RLN* expression dramatically declined after the LH surge.

Acknowledgments

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Author contributions

Tomohiro Sasanami and Tetsuya Kohsaka conceived and designed the present study. Khoi X. Hoang and Tomohiro Sasanami prepared the manuscript. Khoi X. Hoang performed gene expression analysis and cell culture; Mei Matsuzaki performed the statistical analysis. All authors approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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