Expression of Relaxin Family Peptide Receptors 1 and 3 in the Ovarian Follicle of Japanese Quail

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In our previous studies, we demonstrated that the primary source of relaxin 3 (RLN3) in Japanese quail is ovarian granulosa cells. Although several relaxin family peptide (RXFP) receptors have been sequenced, the intricacies of these receptors in avian species remain insufficiently clarified. Therefore, we assessed the expression of RXFP receptors, RXFP1 and 3, in Japanese quail. Using RT-PCR, we found that both RXFP1 and 3 were ubiquitously expressed. The expression level of RXFP1 is significantly higher in the ovarian theca layer, indicating that it is the primary receptor for RLN3 in the ovary. During follicular development, there was an elevation in thecal RXFP1 expression, but it declined after the luteinizing hormone (LH) surge. We found that the protease activity of the 60 kDa band increased after the LH surge, suggesting the involvement of RLN3 signaling in ovulation. These results suggest a paracrine role of RLN3, involving its binding with RXFP1 in ovarian theca cells. This interaction may elicit biological actions, potentially initiating ovulation after the LH surge.

Key words: follicle, Japanese quail, ovulation, relaxin, relaxin family peptide receptor

Introduction

Relaxin (RLN) is a 6-kDa peptide that belongs to a superfamily of hormones structurally related to insulin[1] that includes RLN1-3 and insulin-like peptide (INSL3-6)[2]. Humans have three RLN isoforms (H1, H2, and H3) encoded by three different genes, whereas non-primate mammals have only two forms of RLN (RLN1 and RLN3)[1,2]. Receptors for this peptide family were discovered in 2002 and were characterized as leucine-rich repeats, and categorized as G protein-coupled receptors (LGR) [2–5]. Two LGRs have been identified, and the genes encoding LGR7 and 8 were cloned[2]. A subsequent study demonstrated that LGR7 is the receptor for RLN1 and 3, whereas LGR8 binds

INSL3[3].

RLN3 and INSL5 interact with two other G protein-coupled receptors (GPCRs) unrelated to LGRs[4,5]. GPCR135 is the receptor for RLN3, whereas GPCR142, which is structurally similar to GPCR135, is the receptor for INSL5. These receptors are now classified as RLN family peptide (RXFP) receptors, including RXFP1 (LGR7), RXFP2 (LGR8), RXFP3 (GPCR135), and RXFP4 (GPCR142)[5,6]. In addition, H1- and H2-RLN, and porcine RLN1 exhibit strong binding to and activation of both RXFP1 and RXFP2 with similar affinities. However, rat RLN1 exhibits only weak binding to RXFP2. H3-RLN binds to and activates both RXFP1 and RXFP3[3]. The receptors for INSL4 and INSL6 remain unknown.

In contrast to the extensive investigations in mammals, our understanding of RLN3 and its receptors in avian species remains poorly understood. Previously, we assessed the expression of RLN3 in Japanese quail; its mRNA expression increased in granulosa cells during follicular development, especially in the stigma region, before the LH surge[7]. Thus, RNL3 may be involved in follicular maturation and ovulation in Japanese quail. Recently, quantitative RT-PCR in chickens showed that *RXFP1* and *RXFP3* mRNA expression levels were highest in the ovarian theca layer and stroma, respectively[8]. Although these receptors

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

Gene	Name	Sequence (5' to 3')	GenBank accession No.
		Primers for semi-quantitative RT-PCR	
RXFP1	Forward primer	TGCGCAGCTGTAAGCCTAAT	XM_015860873.2
	Reverse primer	TGGCGTCTGTTTCCCTCTTC	
RXFP3	Forward primer	TCCCCCACTGAGAATGGGAT	XM_015848444.2
	Reverse primer	TTTCAACTGGCTCTTCGGCA	
S17	Forward primer	GGCGCGGGTGATCATCGAGAA	XM_015872709.2
	Reverse primer	GAGAGCGCCTCGTGGTGTTT	
		Primers for quantitative RT-PCR	
RXFP1	Forward primer	TGGTGGGATCGCTGGCTATT	XM_015860873.2
	Reverse primer	GTGTTTGAAGCCCAGAAAATGCA	
	Probe	[FAM] TGTCTCAGAGGTGTCAGTCTTACTGTTGA[TAMRA]	
RXFP3	Forward primer	CAAGCAAGGCTGGAGGAAGTC	XM_015848444.2
	Reverse primer	TCTGGGCTGTGGAGAATGCA	
	Probe	[FAM] TCCATCAACCTCTTTGTGACCAGCCTGGCT[TAMRA]	
S17	Forward primer	AACGAGAGCGCAGGGATAAC	XM_015872709.2
	Reverse primer	CGTCACCTGAAGGTTGGACA	
	Probe	[FAM] CGTACCCGAGGTCTCTGCTCTTGATCAGGA[TAMRA]	

are expressed in chicken ovaries, there is currently little understanding of RLN and its receptors. Therefore, we examine the expression of RXFP receptors in Japanese quail.

Materials and methods

Animals and tissue preparation

Female Japanese quail (*Coturnix japonica*), 15–30 weeks of age (Quail-Cosmos, Tahara, Japan), were individually caged under a photoperiod of 14 h light and 10 h darkness, with lights turned on at 5:00. We provided water and a commercial quail diet (Toyohashi-shiryo, Toyohashi, Japan) *ad libitum*. Almost all birds laid eggs regularly at the same time each day (15:00 to 18:00). Birds were monitored for oviposition, which was recorded manually every 1 h between 15:00 and 18:00, and ovulation was scored to occur 15–30 min after oviposition. For gene expression analyses, approximately 8–10 h before the expected time of ovulation, quail were euthanized by cervical dislocation. Tissues were harvested and stored at −80°C for RNA extraction. To isolate thecal layers, ovarian follicles were dissected 8 h before the expected time of ovulation (before the LH surge) and immediately after oviposition (after the LH surge), and subsequently placed in phosphate-buffered saline (pH 7.4). The granulosa and theca layers were separated using fine forceps and scissors as described by Gilbert *et al*[9].

All animal care and experimental procedures were approved by the Animal Care Committee of the Faculty of Agriculture at Shizuoka University (approval 2018A-5) and were conducted in accordance with the principles and specific guidelines presented in the Guide for the Care and Use of Agricultural Animals in Japan.

Semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was extracted using RNAiso Plus reagent (Takara Bio Inc., Shiga, Japan). RNA concentration and purity were determined by measuring absorption at 260 and 280 nm using a spectrophotometer (DS-11 Series Spectrophotometer, DeNovix Inc., Wilmington, DE, USA). Only RNA samples with A260/280 ratios of 1.8 or greater were used for reverse transcription. Aliquots (1 μg) of total RNA were used for first-strand cDNA synthesis in reaction mixtures (10 μl) using oligo(dT) primers with the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan).

Primer sets for quail *RXFP1*, *RXFP3,* and S17 ribosomal proteins have been designed from GenBank database entries and are listed in Table 1. Amplification of *RXFP1*, *RXFP3,* and S17 transcripts was performed using 1 μl of cDNA and separate reactions with varying numbers of cycles, in a final reaction volume of 50 μl with 5 mmol/ml MgCl$_2$, 0.4 mmol/ml dNTP, 2.5 U Taq DNA polymerase (Takara Bio Inc.), along with the relevant specific primers. After initial denaturation for 2 min at 94°C, a variable number of amplification cycles were performed at 94, 60, and 72°C (30 s each) for *RXFP1*; 94 and 58 °C (30 s each), followed by 72°C (45 s) for *RXFP3*; and 94 and 53.4°C (30 s each), followed by 72°C (20 s) for S17. Amplification of *RXFP1*, *RXFP3*, and S17 was performed using 35, 40, and 25 cycles, respectively. Amplification was followed by a final extension at 72°C for 2 min. PCR products were electrophoresed 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). Data are expressed as m RNA ratios of either *RXFP1* or *RXFP3* and S17 ribosomal protein.

Quantitative RT-PCR

Primers and probes (Table 1) were designed to align with the purified PCR products generated by the RT-PCR process outlined earlier. Quantification of *RXFP1* and *RXFP3* mRNA was performed in conjunction with standard curves for each gene obtained by amplifying 10-fold serial dilutions of purified PCR products. Amplification and detection were performed as previously described[7].

Zymography

Thecal layers were isolated from the F1 follicle 8 h before expected ovulation time (before LH surge) and 2 h before expected ovulation (after LH surge), and subsequently homogenized in ice-cold PBS supplemented with 0.5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor (SBTI), 1 mM phenyl-methyl-sulfonyl fluoride (PMSF) and 1 mM EDTA[10,11]. Homogenates were centrifuged at 20,000 x *g* for 10 min to remove cellular debris. Supernatants, referred to as thecal layer lysates, were divided into aliquots and stored at −80°C until use. Protein concentrations were determined using a Bradford Protein Assay kit (Bio-Rad), as per the manufacturer's instructions.

Thecal layer lysates (16 μg protein) were separated using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 0.1% gelatin as previously described[11]. After electrophoresis, gels were washed twice with 2.5% Triton X-100 for 30 min. Subsequently, gel strips were excised along the lane casting, and incubated with 0.1 M glycine (pH 8.0) buffer containing 0.5 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor (SBTI), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), and 10 μM carbobenzoxy-L-leucyl-L-leucyl-L-leucina (MG132) at 4°C for 1 h with gentle agitation[11]. The gel strips were then incubated overnight at 37°C to promote the enzyme reaction. After the reaction, the gel strips were stained with Coomassie Brilliant Blue and scored for halo formation.

Data analysis

All experiments were repeated three times. The normality of gene expression data was evaluated using the Shapiro-Wilk test, and homoscedasticity was evaluated using the F-test (comparisons between two groups) or Bartlett test (multiple comparisons). Expression data for RXFP1 and RXFP3 in each tissue and at each follicular stage were analyzed using the Steel-Dwass test as a nonparametric multiple comparison method. RXFP1 and RXFP3 levels before and after the LH surges were compared using Student's *t*-test because of their normality and homoscedasticity. P < 0.05 was considered statistically significant. Data are presented as means ± standard error of the mean (SEM).

Results

We first measured the expression of *RXFP1* and *RXFP3* mRNA using semi-quantitative RT-PCR of total RNA extracted from multiple tissues of mature female quails. As shown in Figure 1A, *RXFP1* mRNA was ubiquitously expressed, with the highest
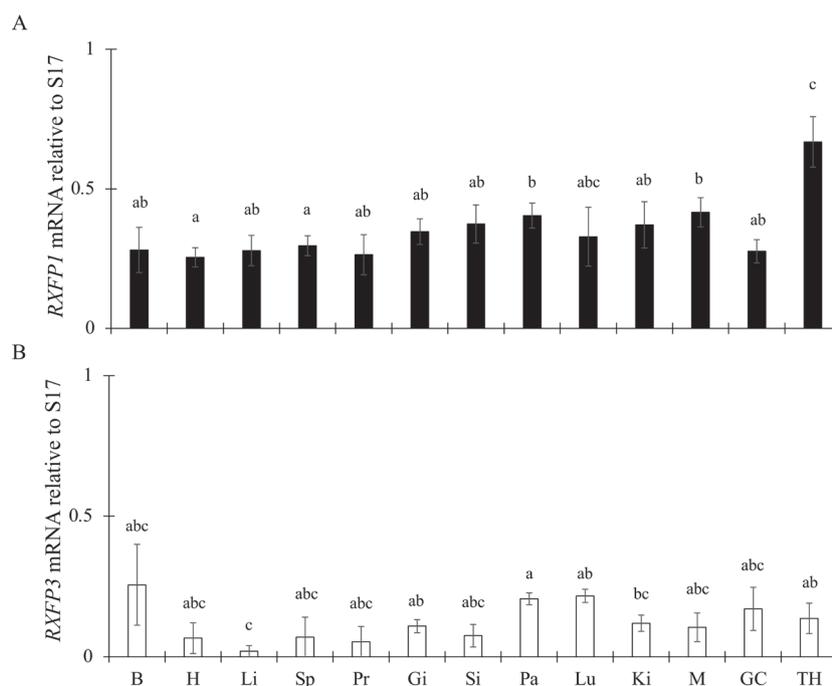

Fig. 1.　**Tissue distribution of *RXFP1* and *RXFP3* mRNA expression measured using semi-quantitative PCR in multiple mature Japanese quail tissues.** (A) RLN receptor 1 (*RXFP1*). (B) RLN receptor 3 (*RXFP3*). Each data point represents the mean ± SEM from three animals for each tissue; values with different letters are significantly different (P < 0.05, Steel-Dwass test). B, Brain; H, heart; Li, liver; Sp, spleen; Pr, proventriculus; Gi, gizzard; Si, small intestine; Pa, pancreas; Lu, lung; K, kidney; M, muscle; GC, granulosa layer; TH, thecal layer.

levels observed in the thecal layer. Conversely, the expression of *RXFP3* mRNA was relatively low in all tissues, and increased expression was not detected in the ovary (Figure 1B). Next, we examined changes in *RXFP1* and *RXFP3* mRNA expression in the thecal layer during follicular development. As shown in Figure 2, a lower level of *RXFP1* mRNA was observed in F3. Gene transcription substantially increased in F2, and higher expression was maintained in the largest follicle (Figure 2A). However, the expression of *RXFP3* mRNA in the thecal layer remained low throughout the follicular development (Figure 2B). In subsequent experiments, we compared the expression levels of *RXFP1* and *RXFP3* in the F1 follicles of the thecal layer 8–10 h before the expected time of ovulation and immediately after oviposition, which were speculated to be before and after the LH surge, respectively. Figure 3 shows that *RXFP1* mRNA expression in the thecal layers significantly decreased after the LH surge compared to before the LH surge, whereas no such reduction in the expression of *RXFP3* was observed.

We next measured protease activity in thecal layer lysates using zymography to compare the banding pattern and protease activity before and after the LH surge. As shown in Figure 4A, a high-molecular-weight protease was detected in samples isolated before the LH surge. This band was also observed after the LH surge. In addition to the high-molecular-weight band, a clear protease band with a molecular weight of approximately 60 kDa was observed after the LH surge. The activity of the 60 kDa protease was inhibited by SBTI, PMSF, and leupeptin, whereas no such inhibitory effects were observed when the gel strip was incubated with EDTA and MG132. Finally, all the protease inhibitors tested failed to inhibit protease activity of the high-molecular-weight band.
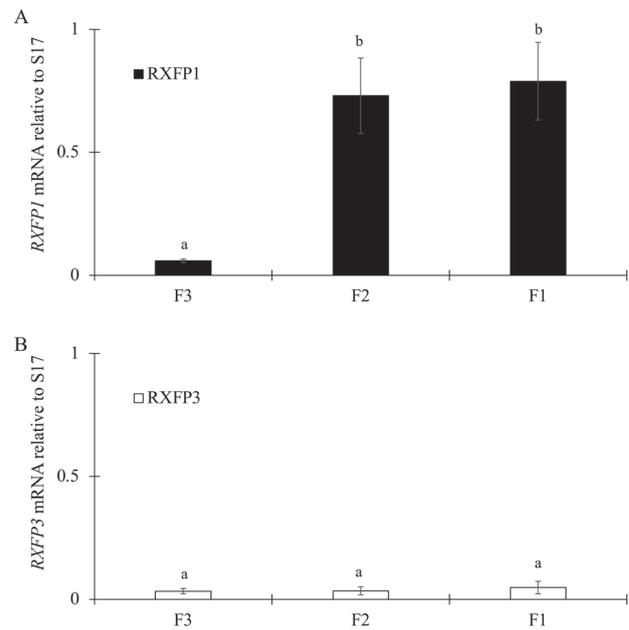

Fig. 2.   **Changes in *RXFP1* and *RXFP3* mRNA expression during follicular development**. Thecal layers obtained from the largest (F1), second largest (F2), and third largest (F3) follicles are isolated 8–10 h before the expected time of ovulation. RNA is isolated from the tissues and expression is measured by quantitative RT-PCR. Values represent means ± SEM of triplicate experiments; values with different letters are significantly different ($P < 0.05$, Steel-Dwass test).
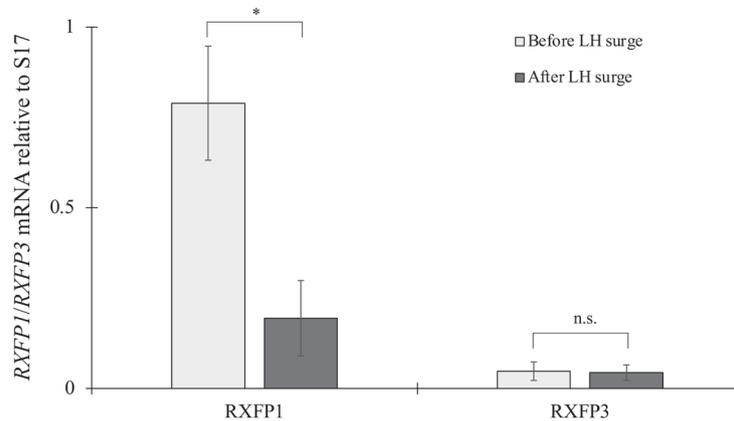

Fig. 3.   **Changes in *RXFP1* and *RXFP3* mRNA expression before and after the LH surge**. Thecal layers obtained from the largest (F1) follicle are isolated approximately 8–10 h before the expected time of ovulation (before the LH surge) and immediately after oviposition (after the LH surge). Expression is measured by quantitative RT-PCR. Values represent means ± SEM of triplicate experiments; values labeled with (*) are significantly different ($P < 0.05$, Student's *t*-test); those labeled with n.s. are not significantly different ($P < 0.05$, Student's *t*-test). LH, luteinizing hormone.
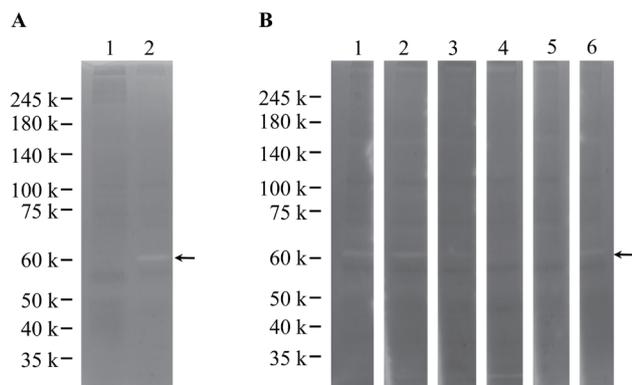
Fig.4.   **Protease activity in the thecal layer**. **A**) Zymography of thecal layer lysates before (lane 1) and after (lane 2) the LH surge. Proteins are resolved by SDS-PAGE with gelatin, and the gel is incubated with 0.1 M glycine buffer (pH 8.0). **B**) Zymography of thecal cell lysates after the LH surge. Proteins are separated by SDS-PAGE with gelatin, with gel strips cut along the lane castings. Each strip is incubated in glycine buffer with (lane 1) or without (lane 2) 1 mM EDTA, 50 μg/ml SBTI (lane 3), 1 mM PMSF (lane 4), 0.5 μg/ml leupeptin (lane 5), or 10 μM MG132 (lane 6). The position of the 60-kDa band is marked with an arrow. Representative results from repeated experiments are shown.

Discussion

In the present study, we investigated the expression of the relaxin receptors, RXFP1 and 3, in mature female quail. We found that *RXFP1* expression was higher in the ovarian thecal layer, but no ovary-specific expression was observed for *RXFP3*, indicating that RXFP1 mediates relaxin signaling in quail ovaries. This expression pattern is quite different from those of mammalian species such as mice, rats, macaques, and humans, which are expressed primarily in the brain[12–16]. Mammalian RLN3 binds with a high affinity to RXFP3 and has a lower affinity for RXFP1[2,3]. Although there are no reports regarding RLN3 receptor binding in birds, mammalian RXFP1 has been reported to couple with the Gs protein, suggesting that its stimulatory effects occur through a cAMP-mediated signaling pathway[17–19]. Contrastingly, RXFP3 is linked to the Gi/Go proteins, resulting in the inhibition of cAMP production[20]. Our previous study indicated that the primary source of RLN3 in Japanese quail is the granulosa layer of mature follicles[7]. Thus, we hypothesized that RLN3 derived from granulosa cells binds to RXFP1 in a paracrine manner and that this binding may stimulate cAMP-dependent signaling that leads to the activation of some biological responses in the thecal layers. Regarding the expression of RXFP3 in chicken, Ghanem and Johnson[8] reported that higher expression was detected in the chicken ovarian stroma. Although we did not examine the expression of the receptors in the ovarian stroma of Japanese quail, another ligand besides RLN3 may be responsible for binding to stromal RXFP3, because no expression of RLN3 was detected in the stroma[8]. During follicular development, receptor expression significantly increased in the largest and second-largest follicles, indicating that *RXFP1* mRNA expression fundamentally corresponds to the follicular developmental status. We also found that the expression of RXFP1 significantly decreased when ovulation was imminent and that this expression pattern was similar to that of RLN3 in granulosa cells. These findings indicate that RLN3 signaling that is mediated by RXFP1 is maintained during follicular development, and is attenuated substantially after the LH surge.

Notably, our zymography data suggested that the 60-kDa protease in the thecal layer was strongly activated after the LH surge. The direct link between RLN3 signaling with the 60 kDa protease was not investigated, but it was speculated that RLN3 signaling through binding with RXFP1 may disturb the activity of the 60-kDa protease during follicular development. This substantial decline in RLN3 signaling may potentiate protease activity, and thus may be important for ovulation. Since ovulation occurs via rupture along the follicle stigma, it likely involves multiple biological activities, including proteolysis, movement of smooth muscle cells in the walls of follicles, and apoptosis[21]. In mammals, follicular atresia and luteal regression are accelerated in the ovaries of INSL3-deficient mice due to increased apoptosis, suggesting that relaxin family peptides induce apoptosis during ovulation[22]. In our previous study, we demonstrated that RLN3 expression was high in the stigma region, where follicular rupture occurs during ovulation, and the expression significantly declines when ovulation stops[7]. Therefore, protease activation in the stigma region may be more efficient. Because of its molecular mass, we hypothesize that the 60-kDa protease is plasmin, whose molecular weight is approximately 85 kDa in non-primate species and 75 kDa in humans[23–25]. This conclusion is also supported by the observation that the activity of this protease was inhibited by serine protease inhibitors PMSF and leupeptin, attenuating the proteolytic activity of serine and related proteolytic enzymes including plasmin[26]. Although the involvement of matrix metalloproteinases (MMPs) in ovulation has been reported in medaka fish[27,28], the 60-kDa protease is not likely to be a matrix metalloproteinase (MMP) because its activity is not inhibited by EDTA[29,30]. In addition, the proteasome inhibitor MG-132[31] did not affect the activity of the 60-kDa protease. Thus, it is unlikely that it is a cysteine protease such as a proteasome. Additionally, detailed studies are required to elucidate the direct relationship between RLN3 signaling and 60-kDa protease activation.

In summary, we found that RXFP1 may be a primary receptor for RLN3 in Japanese quail because of its abundant expression in the ovarian thecal layer. Our findings suggest the possible involvement of RLN3 signaling in ovulation because the significant decline in RLN3 and RXFP1 expression corresponds to the activation of the 60-kDa protease. These findings suggest ligand-receptor binding and the physiological role of RLN3 and its receptor in avian species.

Acknowledgments

Author contributions

The study was conceived and designed by Tomohiro Sasanami and Tetsuya Kohsaka. The manuscript was prepared by Khoi X. Hoang and Tomohiro Sasanami. Gene expression analysis and zymography were performed by Khoi X. Hoang, and statistical analysis was performed by Mei Matsuzaki. All the authors have approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

References

[1] Sherwood OD. Relaxin's physiological roles and other diverse actions. Endocr Rev, **25**: 205–234. 2004. https://doi.org/10.1210/er.2003-0013, PMID:15082520

[2] Bathgate RAD, Halls ML, van der Westhuizen ET, Callander GE, Kocan M and Summers RJ. Relaxin family peptides and their receptors. Physiol Rev, **93**: 405–480. 2013. https://doi.org/10.1152/physrev.00001.2012, PMID:23303914

[3] Ivell R, Kotula-Balak M, Glynn D, Heng K and Anand-Ivell R. Relaxin family peptides in the male reproductive system--a critical appraisal. Mol Hum Reprod, **17**: 71–84. 2011. https://doi.org/10.1093/molehr/gaq086, PMID:20952422

[4] Bathgate RA, Hsueh AJ and Sherwood OD. Physiology and molecular biology of the relaxin peptide family. In: Knobil and Neill's Physiology of Reproduction (Neill JD ed.), Academic Press, New York, 3rd ed, Vol. 1, pp. 679-768, 2006.

[5] Kong RCK, Shilling PJ, Lobb DK, Gooley PR and Bathgate RAD. Membrane receptors: structure and function of the relaxin family peptide receptors. Mol Cell Endocrinol, **320**: 1–15. 2010. https://doi.org/10.1016/j.mce.2010.02.003, PMID:20138959

[6] Wilkinson TN and Bathgate RAD. The evolution of the relaxin peptide family and their receptors. In: Relaxin and Related Peptides. Advances in Experimental Medicine and Biology (Agoulnik AI ed.), Springer New York, New York, Vol. 612, pp. 1-13, 2007.

[7] Hoang KX, Matsuzaki M, Kohsaka T and Sasanami T. Expression of Relaxin 3 in the Ovarian Follicle of Japanese Quail. J Poult Sci, **60**: 2023025. 2023. https://doi.org/10.2141/jpsa.2023025, PMID:37795378

[8] Ghanem K and Johnson AL. Proteome profiling of chicken ovarian follicles immediately before and after cyclic recruitment. Mol Reprod Dev, **88**: 571–583. 2021. https://doi.org/10.1002/mrd.23522, PMID:34219321

[9] Gilbert AB, Evans AJ, Perry MM and Davidson MH. A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (*Gallus domesticus*). Reproduction, **50**: 179–181. 1977. https://doi.org/10.1530/jrf.0.0500179, PMID:864645

[10] Ito T, Yoshizaki N, Tokumoto T, Ono H, Yoshimura T, Tsukada A, Kansaku N and Sasanami T. Progesterone is a sperm-releasing factor from the sperm-storage tubules in birds. Endocrinology, **152**: 3952–3962. 2011. https://doi.org/10.1210/en.2011-0237, PMID:21810949

[11] Sasanami T, Yoshizaki N, Dohra H and Kubo H. Sperm acrosin is responsible for the sperm binding to the egg envelope during fertilization in Japanese quail (*Coturnix japonica*). Reproduction, **142**: 267–276. 2011. https://doi.org/10.1530/REP-11-0120, PMID:21642383

[12] Bathgate RAD, Samuel CS, Burazin TCD, Layfield S, Claasz AA, Reytomas IGT, Dawson NF, Zhao C, Bond C, Summers RJ, Parry LJ, Wade JD and Tregear GW. Human relaxin gene 3 (*H3*) and the equivalent mouse relaxin (*M3*) gene: Novel members of the relaxin peptide family. J Biol Chem, **277**: 1148–1157. 2002. https://doi.org/10.1074/jbc.M107882200, PMID:11689565

[13] Smith CM, Shen PJ, Banerjee A, Bonaventure P, Ma S, Bathgate RAD, Sutton SW and Gundlach AL. Distribution of relaxin-3 and RXFP3 within arousal, stress, affective, and cognitive circuits of mouse brain. J Comp Neurol, **518**: 4016–4045. 2010. https://doi.org/10.1002/cne.22442, PMID:20737598

[14] Ma S, Sang Q, Lanciego JL and Gundlach AL. Localization of relaxin-3 in brain of *Macaca fascicularis* : identification of a nucleus incertus in primate. J Comp Neurol, **517**: 856–872. 2009. https://doi.org/10.1002/cne.22197, PMID:19844992

[15] Bathgate RAD, Scott DJ, Chung SWL, Ellyard D, Garreffa AM and Tregear GW. Searching the human genome database for novel relaxin- and insulin-like peptides. Lett Pept Sci, **8**: 129–132. 2001. https://doi.org/10.1023/A:1016210004087

[16] Burazin TCD, Bathgate RAD, Macris M, Layfield S, Gundlach AL and Tregear GW. Restricted, but abundant, expression of the novel rat gene-3 (R3) relaxin in the dorsal tegmental region of brain. J Neurochem, **82**: 1553–1557. 2002. https://doi.org/10.1046/j.1471-4159.2002.01114.x, PMID:12354304

[17] Sudo S, Kumagai J, Nishi S, Layfield S, Ferraro T, Bathgate RAD and Hsueh AJW. H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. J Biol Chem, **278**: 7855–7862. 2003. https://doi.org/10.1074/jbc.M212457200, PMID:12506116

[18] Halls ML, Bathgate RAD and Summers RJ. Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. Mol Pharmacol, **70**: 214–226. 2006. https://doi.org/10.1124/mol.105.021691, PMID:16569707

[19] Halls ML, Bathgate RAD and Summers RJ. Comparison of signaling pathways activated by the relaxin family peptide receptors, RXFP1 and RXFP2, using reporter genes. J Pharmacol Exp Ther, **320**: 281–290. 2007. https://doi.org/10.1124/jpet.106.113225, PMID:17065365

[20] van der Westhuizen ET, Werry TD, Sexton PM and Summers RJ. The relaxin family peptide receptor 3 activates extracellular signal-regulated kinase 1/2 through a protein kinase C-dependent mechanism. Mol Pharmacol, **71**: 1618–1629. 2007. https://doi.org/10.1124/mol.106.032763, PMID:17351017

[21] Johnson AL. Reproduction in female. In: Sturkie's Avian Physiology (Scanes CG ed.), Academic press, New York, 6th ed, pp. 635-665, 2015.

[22] Spanel-Borowski K, Schäfer I, Zimmermann S, Engel W and

Adham IM. Increase in final stages of follicular atresia and premature decay of corpora lutea in Insl3-deficient mice. Mol Reprod Dev, **58**: 281–286. 2001. https://doi.org/10.1002/1098-2795(200103)58:3<281::AID-MRD6>3.0.CO;2-0, PMID:11170269

[23] Austen DEG. Clinical Biochemistry of Blood Coagulation. In: Scientific Foundations of Biochemistry in Clinical Practice (Williams DL and Marks V eds.). Butterworth-Heinemann, Oxford, 2nd ed, pp. 495-513, 1994.

[24] Novokhatny V. Structure and activity of plasmin and other direct thrombolytic agents. Thromb Res, **122**: S3–S8. 2008. https://doi.org/10.1016/j.thromres.2008.06.018, PMID:18718639

[25] Barlow GH, Summaria L and Robbins KC. Molecular weight studies on human plasminogen and plasmin at the microgram level. J Biol Chem, **244**: 1138–1141. 1969. https://doi.org/10.1016/S0021-9258(18)91819-3, PMID:4237416

[26] Ogiwara K, Minagawa K, Takano N, Kageyama T and Takahashi T. Apparent involvement of plasmin in early-stage follicle rupture during ovulation in medaka. Biol Reprod, **86**: 113. 2012. https://doi.org/10.1095/biolreprod.111.093880, PMID:22278979

[27] Matsui H, Ogiwara K, Ohkura R, Yamashita M and Takahashi T. Expression of gelatinases A and B in the ovary of the medaka fish *Oryzias latipes*. Eur J Biochem, **267**: 4658–4668. 2000. https://doi.org/10.1046/j.1432-1327.2000.01513.x, PMID:10903498

[28] Ogiwara K, Takano N, Shinohara M, Murakami M and Takahashi T. Gelatinase A and membrane-type matrix metalloproteinases 1 and 2 are responsible for follicle rupture during ovulation in the medaka. Proc Natl Acad Sci USA, **102**: 8442–8447. 2005. https://doi.org/10.1073/pnas.0502423102, PMID:15941829

[29] Hahn-Dantona EA, Aimes RT and Quigley JP. The isolation, characterization, and molecular cloning of a 75-kDa gelatinase B-like enzyme, a member of the matrix metalloproteinase (MMP) family. An avian enzyme that is MMP-9-like in its cell expression pattern but diverges from mammalian gelatinase B in sequence and biochemical properties. J Biol Chem, **275**: 40827–40838. 2000. https://doi.org/10.1074/jbc.M006234200, PMID:11010969

[30] Simsa S, Hasdai A, Dan H and Ornan EM. Differential regulation of MMPs and matrix assembly in chicken and turkey growth-plate chondrocytes. Am J Physiol Regul Integr Comp Physiol, **292**: R2216–R2224. 2007. https://doi.org/10.1152/ajpregu.00864.2006, PMID:17332158

[31] Prasad R, Atul, Kolla VK, Legac J, Singhal N, Navale R, Rosenthal PJ and Sijwali PS. Blocking Plasmodium falciparum development via dual inhibition of hemoglobin degradation and the ubiquitin proteasome system by MG132. PLoS One, **8**: e73530. 2013. https://doi.org/10.1371/journal.pone.0073530, PMID:24023882