Inhibition of medaka ovulation by gap junction blockers due to its disrupting effect on the transcriptional process of LH-induced Mmp15 expression

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Keywords: medaka ovulation, gap junction blocker, Mmp15, Pgr phosphorylation, Cdk9, cyclin I

Abstract

Using medaka, we found that *in vitro* follicle ovulation, but not germinal vesicle breakdown, was inhibited by three gap junction blockers, carbenoxolone, mefloquine, and flufenanic acid. The blockers specifically inhibited follicular expression of matrix metalloproteinase-15 mRNA and the protein (*mmp15*/Mmp15), a protease indispensable for medaka ovulation, indicating that gap junctional communication may be required for successful ovulation and *mmp15*/Mmp15 expression. Further experiments using carbenoxolone as the representative of the gap junction blockers showed that expression of nuclear progestin receptor (Pgr), a transcription factor required for *mmp15* expression, was not affected by carbenoxolone treatment, but the formation of phosphorylated Pgr was considerably suppressed. Carbenoxolone treatment caused a decrease in the Pgr binding to the promoter region of *mmp15*. mRNA expression of *cyclin-dependent protein kinase-9* (*cdk9*) and *cyclin I (ccni)*, whose translation products are demonstrated to be involved in Pgr phosphorylation in the medaka ovulating follicles, was suppressed by carbenoxolone treatment. Transcripts of *connexin 34.5 (cx34.5)* and *connexin 35.4 (cx35.4)* were dominantly expressed in the follicle cells of ovulating follicles. The results indicate that gap junctional communication plays an important role in medaka ovulation.

1. Introduction

The major function of the ovary is the release of the mature oocyte for fertilization and successful propagation of the species. In vertebrate ovaries, the individual follicles consist of an innermost oocyte and surrounding follicle cells. The fate of each follicle is controlled by various factors, among which gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) are the key regulators for the process. In addition, many other paracrine and autocrine factors are known to be involved in follicle development and ovulation (Rajkovic et al., 2006; Hsueh et al., 2015). Furthermore, the importance of gap junctional communication between two neighboring cells constituting the ovarian follicle has been recognized (Clarke, 2018).

Gap junctions, the clusters of intercellular channels that allow direct diffusion of ions and small molecules between adjacent cells, are composed of channel-forming membrane proteins termed connexins (Cxs) (Goodenough et al., 1996; Goodenough and Paul, 2009). In mammals, gap junctional communication is required for normal follicular development (Ackert et al., 2001). It is also established that gap junctions between the oocyte and cumulus cells play an important role in the arrest of meiosis and subsequent LH-induced resumption of meiosis in the Graafian follicles of mammalian species (Zhang et a1., 2010; Gilchrist et al., 2016). Similarly, the presence of gap junctions in ovarian follicles has been documented for various teleost species, including zebrafish (Kessel et a1., 1988), Atlantic croaker (York et a1., 1993; Yoshizaki et a1., 2001; Bolamba et a1., 2003), red seabream (Patiño and Kagawa, 1999), Ayu (Yamamoto et a1., 2007), striped bass (Weber and Sullivan, 2001), rainbow trout (Bobe et al., 2004; Rime et al., 2010), and coho salmon (Yamamoto et al., 2011). These studies indicate the possible involvement of gap junctions in oogenesis (Yamamoto et al., 2011; Marytyniuk et a1., 2013) and/or the acquisition of

oocyte maturational competence (Yoshizaki et al., 1994; Patiño and Kagawa, 1999; Yamamoto et a1., 2007; Yamamoto et al., 2008; Yamamoto and Yoshizaki, 2008; Rime et a1., 2010), implicating the importance of intercellular communication between the oocyte and follicle cells through gap junctions. However, the role of gap junctions in the process of follicle rupture during ovulation has not yet been investigated in any vertebrate species.

Ovarian follicle rupture is the culmination of ovulation and is triggered by LH in vertebrates; its mechanism has received much attention in mammalian (Ny et al. 2002, Smith et al. 2002, Curry and Smith 2006, Espey and Richards 2006, Liu et al. 2013) and piscine ovulation studies (Crespo et al., 2013; Takahashi et al., 2013; Crespo et al., 2015; Ogiwara and Takahashi, 2017). Although previous studies using both mammals and fishes have provided valuable information on the rupture process, at present our understanding of this issue is most advanced in the teleost medaka (*Oryzias latipes*), a good model for ovulation studies of non-mammalian vertebrates (Skai et al., 1988; Ogiwara et al., 2005; Takahashi et al., 2017; Takahashi et al., 2019). Therefore, medaka may be useful for investigating the gap junctions' role in follicle rupture during ovulation.

Previous studies using medaka have indicated that expression of some genes/proteins involved in the rupture process was enhanced by the ovulatory LH surge. Membrane type 2-matrix metalloproteinase (Mt2-mmp, official symbol Mmp15) is one of such enzymes; Mmp15 is markedly induced in the granulosa cells of ovulating follicles (Ogiwara et al., 2013). In our recent studies investigating how *mmp15*/Mmp15 expression would be regulated in the follicles during the hours preceding rupture, the LH-induced expression of *mmp15* was shown to be mediated by the two transcription factors, nuclear progestin receptor (Pgr) and CCAAT/enhancer-binding protein β (Cebpb) (Ogiwara and Takahashi,

2017). We have shown that Pgr is also LH inducible (Hagiwara et al., 2014; Ogiwara and Takahashi, 2017) and suggested that Pgr undergoes phosphorylation prior to binding to the promoter region of the *mmp15* gene (Ogiwara and Takahashi, 2019). Furthermore, we have reported that cyclin-dependent protein kinase 9 (Cdk9) and cyclin I (Ccni) are involved in Pgr phosphorylation in the granulosa cells of ovulating follicles (Ogiwara and Takahashi, 2019).

As a first step to determine whether gap junctions may contribute to medaka ovulation, the effect of the gap junction blockers on the ovulation rate was examined using an *in vitro* follicle ovulation system. We found that follicle ovulation was inhibited by the treatment with the blockers and that the inhibition was accompanied by the suppressed expression of *mmp15* mRNA in the ovulating follicle. Therefore, the current study was undertaken to investigate how gap junction blockers inhibit follicle ovulation. Our results showed that treatment of the preovulatory follicles with the gap junction blocker carbenoxolone (CBX) inhibits the generation of phosphorylated Pgr, which serves as a transcription factor for *mmp15* expression during medaka ovulation. We also provide data showing that the synthesis of both Cdk9 and its regulatory protein Ccni is affected by CBX treatment of the follicles. This is the first report that gap junctional communication is implicated as an important factor for the follicle rupture process in teleost ovulation.

2. **Materials and Methods**

2.1. Animals and Tissues

Adult orange-red variant medaka, *Oryzias latipe*s, was used. The fish were purchased from a local dealer, maintained under 14 h light/10 h dark condition at 26-28 °C. After acclimation to the artificial reproductive conditions, the fish ovulated every day at the

start of the light period in the 24-h spawning cycle, and we defined this time as ovulation hour 0. Ovarian follicles and follicular layers of the follicles were obtained as previously described (Hagiwara et al., 2014). Animal culture and the experimental procedures used in this study were conducted in accordance with the guidelines for animal experiments of Hokkaido University and were approved by the Committee of Experimental Plants and Animals, Hokkaido University.

2.2. In vitro follicle culture

Ovarian follicles were isolated from the ovaries of female medaka with an established 24 h spawning cycle at various time points prior to ovulation. Follicles obtained from two to three fish ovaries were pooled and then divided into control and test groups (approximately 20-25 follicles per group). The dissected follicles were used immediately or after incubation with gap junction blockers in 4 ml of 90% medium M199 solution (pH 7.4) containing 50 μM gentamicin, for the indicated time. The gap junction blockers that were used included CBX (Sigma-Aldrich, St, Louis, MO), mefloquine (Sigma-Aldrich), and flufenanic acid (LKT Laboratories Inc., St, Paul, MN). For follicle culture in the presence of gap junction blockers, preovulatory follicles isolated 14 h prior to ovulation (-14 h follicles) were incubated at 26-28 °C for either 14 h or 19 h depending on differences in target genes or the processes to be examined. CBX was dissolved in distilled water, whereas mefloquine and flufenanic acid were dissolved in dimethyl sulfoxide (DMSO). To determine the effective concentrations of the gap junction blockers in the *in vitro* follicle culture, dose-dependent inhibition tests were performed using the -14 h follicles. The concentrations tested were 12.5, 25, 50, and 100 μM for CBX; 20, 30, 40, and 100 μM for mefloquine; and 1, 10, 20, 50, and 100 μM for flufenanic acid. Based

on the results of the preliminary tests, the concentrations used for further experiments were set to 50 μM (CBX), 40 μM (mefloquine), and 50 μM (flufenanic acid). As a control, distilled water or DMSO (final concentration: 0.2%) was added to the medium. After incubation, the number of follicles that had ovulated and/or completed germinal vesicle breakdown (GVBD) was counted. Additionally, the expression levels of certain ovulation-related genes and/or the corresponding proteins were analyzed by quantitative real-time polymerase chain reaction (RT-qPCR) and western blotting, respectively. Experiments involving follicle culture were conducted at least four times to confirm the reproducibility of the results.

2.3. RNA isolation, reverse transcription (RT), and quantitative real-time polymerase chain reaction (qPCR)

Total RNA extraction and qPCR were performed as previously described (Fujimori et al., 2011). The primers used for PCR are listed in Supplemental Table S1. For qPCR, the KOD SYBR qPCR Mix (Toyobo, Osaka, Japan) (for *connexin* [*cx*] subtypes and *mmp15*) and the KAPA Fast qPCR Kit (Nippon Genetics Co., Ltd., Tokyo, Japan) (for other genes) were used to detect the expression of mRNA. The standards were processed in parallel with the cDNA samples, and were used to generate a standard curve. Dissociation curves were examined after each PCR run to ensure that a single PCR product had been amplified. PCR reactions were performed in triplicate on three separate RNA preparations from individual samples. The mRNA levels of the target genes were normalized to eukaryotic translation elongation factor 1 alpha 1 (*eef1a1*) or to cytoplasmic β-actin (*actb*), both of which were judged to be appropriate housekeeping genes for qPCR analysis of genes that were expressed in the medaka pre-ovulatory

follicles (Fujimori et al., 2011; Ogiwara and Takahashi, 2019).

2.4. Western blot analysis and immunoprecipitation/western blot analysis

Follicular layers were incubated in 50 mM Tris-HCl (pH 8.0), containing 1% sodium dodecyl sulfate (SDS) at 95 °C for 15 min, followed by centrifugation at 13,000*g* for 10 min. The resulting supernatants were used for western blot analysis. Protein concentration was measured using a Pierce™ BCA Protein Assay kit (ThermoFisher SCIENTIFIC, Waltham, MA). Western blot analysis was performed as previously described (Hagiwara et al., 2014). Polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA) on which separated proteins had been blotted were incubated with a primary antibody in phosphate-buffered saline containing 0.2% Tween-20 at 1:1000 dilution and subsequently with a secondary antibody at 1:5000 dilution. Detection of medaka Mmp15 (Ogiwara et al., 2005), Pgr (Hagiwara et al., 2014), and Cebpb (Ogiwara and Takahashi, 2017) proteins in follicle layer extracts was performed as previously described. For the detection of Pgr, Cdk9, and Ccni protein, immunoprecipitation/western blot analysis was conducted as described previously (Ogiwara and Takahashi, 2019). Ribosomal protein L7 (Rpl7) was used as loading control. All antibodies used in this study were characterized to be specific for their respective antigens in our previous reports. The signals were detected using an Immobilon Western kit (Millipore, Bedford, MA) according to the manufacturer's instructions.

2.5. Detection of phosphorylated Pgr

Phos-tag SDS-polyacrylamide gel electrophoresis (PAGE) using 7% polyacrylamide gel containing 50 μM Phos-tag®acrylamide (Wako Chemicals, Osaka, Japan) and 50 μM

$MnCl_2$ was performed as described previously (Ogiwara and Takahashi, 2019). Briefly, follicle layer extracts were immunoprecipitated with rat anti-medaka Pgr antibody or normal immunoglobulin G (IgG), and the resulting immunoprecipitated materials were separated by Phos-tag SDS-PAGE. The gel was then incubated twice in a buffer containing 0.2 M glycine, 20 mM Tris, 0.1% SDS, 20% methanol, and 10 mM EDTA, followed by the same buffer without EDTA. Pgr proteins blotted on Immobilon PVDF membranes (Merck Millipore) were detected using anti-mouse medaka antibody.

2.6. Pgr binding to the promoter region of mmp15 gene by chromatin immunoprecipitation (ChIP) assay

The -14 h-follicles were incubated for 14 h with or without 50 μM CBX. ChIP assay was conducted as previously described (Ogiwara and Takahashi, 2017; Ogiwara and Takahashi, 2019). A primer pair used in this study was the one designated primer pair-1 in our previous reports (Ogiwara and Takahashi, 2017; Ogiwara and Takahashi, 2019); this primer pair was designed to generate 101-bp nucleotides corresponding to the sequence between -101 and -1 upstream of the transcription start site of the *mmp15* gene. In parallel, the -14 h-follicles incubated for 14 h with 50 μM roscovitine (Merck Millipore), which is a well-known CDK inhibitor (Cicenas et al., 2015), were analyzed as a control.

2.7. Biotinylated DNA pull-down assay

Medaka genome was purified as previously described (Hagiwara et al., 2014). PCR was conducted using KOD-plus-Neo DNA polymerase (Toyobo) with the genome to amplify a 5' upstream region of a *mmp15* gene. The primer pairs used were 5'-Mmp15-

genome and 3'-Mmp15-genome (Supplemental Table S1). The amplified product was gel purified, phosphorylated, and ligated into a pBlueScript KS (+) plasmid (Agilent technologies, Santa Clara, CA). The nucleotide sequence of the resultant plasmid was confirmed by sequence analysis. PCR was carried out using a forward primer (5'-Mmp15-genome) biotinylated at its 5' site and a reverse primer (3'-Mmp15-genome) with the above plasmid. KOD-plus-Neo DNA polymerase was used for the reaction. The product was precipitated by ethanol and dissolved in 50 mM Tris-HCl (pH 7.5). The DNA solution (10 μg DNA per sample) was mixed with Streptavidin Sepharose High Performance beads (GE Healthcare, Boston, MA) (10 μl of bed volume) previously equilibrated in 50 mM Tris-HCl (pH 7.5) and incubated for 3 h at room temperature with gentle agitation. The beads were then washed with 50 mM Tris-HCl (pH 7.5) three times and incubated in 10 mM Tris-HCl (pH 7.5) containing 2 mM biotin, 1% bovine serum albumin, 0.15 M NaCl, and 0.1% Tween-20 for 3 h at room temperature for blocking. After incubation, the beads were washed with 50 mM Tris-HCl (pH 7.5) three times and used for a pull-down assay. Preovulatory follicles isolated 3 h before ovulation (-3 h-follicles) (30 follicles per sample) or cultured OLHNI-2 cells (1×10^6 cells per sample), which stably expresses Pgr but are deficient for Cdk9 gene (Ogiwara and Takahashi, 2019), were gently homogenized in a reaction buffer (50 mM Tris-HCl; pH 7.5) containing 1% Triton X-100, 1×Protease inhibitor (Wako, Osaka, Japan), and 1×Phosphatase inhibitor (Wako) and incubated for 3 h on ice. The samples were then centrifuged at 13,000*g* for 10 min at 4 °C. The resultant supernatants were used for a pull-down assay. The extracts and the beads were mixed in 1 ml of the reaction buffer and incubated for 16 h at 4 °C. The beads were washed with 50 mM Tris-HCl (pH 7.5) four times and then boiled in SDS-PAGE sample buffer for 10 min. The resultant samples

were analyzed by SDS-PAGE/western blotting. For a negative control, the extract and a PCR product (20 μg DNA per sample) amplified using a nonmodified primer (5'-Mmp15-genome) and a reverse primer (3'-Mmp15-genome) with the plasmid were mixed, incubated for 16 h at 4 °C, and used for a pull-down assay as a competitor.

2.8. Statistical analysis

Results are presented as the mean values ± SEM of 4-7 independent experiments. Statistical significance was verified using Student's *t*-test or a one-way analysis of variance (ANOVA) followed by the Dunnett's, Tukey, or Tukey-Kramer post hoc test or Kruskal-Wallis test followed by Steel or Scheffé post hoc test, as appropriate. Equal variation was confirmed by F-test or Levene's test, as appropriate. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of gap junction blockers on in vitro ovulation and GVBD of medaka preovulatory follicles

Preovulatory follicles were isolated from ovaries of the fish with a 24-h spawning cycle at 14 h before ovulation (-14 h-follicles) and used for *in vitro* follicle culture experiments. The -14 h-follicles, which had already undergone the surge of LH *in vivo* (Ogiwara et al., 2013), were incubated with or without CBX for 19 h (Fig. 1A, condition-1). After incubation, the rates of both GVBD, an important milestone for oocyte maturation, and ovulation were determined. Ovulation was inhibited when CBX was added into the medium at 12.5, 25, and 50 μM (Fig. 1B). In contrast, no significant inhibition was observed in the rate of GVBD under the condition (Fig. 1C).

We next examined the effects of two other gap junction blockers, mefloquine and flufenanic acid, on *in vitro* follicle ovulation and GVBD. Incubation of the -14 h-follicles with mefloquine at 40 μM caused 75% inhibition in the rates of ovulation (Fig. 2A). However, mefloquine treatment of the follicles was without effect on the rate of GVBD (Fig. 2B). Strong inhibitory activity was also observed for ovulation with flufenanic acid at 50 μM (Fig. 2C). The rates of GVBD showed a tendency to reduce with flufenanic acid, but the change was not significant (Fig. 2D).

We examined whether the -14 h-follicles incubated *in vitro* for 19 h with the blockers were viable using a trypan blue exclusion test. The oocyte and follicle cells of the follicles incubated with CBX (50 μM), mefloquine (40 μM), and flufenanic acid (50 μM) showed clear cytoplasm (data not shown), indicating that the inhibition of ovulation was not due to the toxic effect of the gap junction blockers.

3.2. Effects of CBX treatment on the expression of some ovulation-related genes

To gain insights into the mechanism by which gap junction blockers reduced the ovulation rates of preovulatory follicles that were destined to ovulate, the effect of CBX on the follicular expression of several genes/proteins, that were previously documented to be involved in the medaka ovulation, were examined. Ovulation-related genes/proteins tested were Pgr (Hagiwara et al., 2014; Ogiwara and Takahashi, 2017), Cebpb (Ogiwara and Takahashi, 2017), prostaglandin E_2 receptor subtype Ptger4b (Hagiwara et al., 2014), plasminogen activator inhibitor-1 (Pai1) (Ogiwara et al., 2015), and Mmp15 (Ogiwara et al., 2005; Ogiwara and Takahashi, 2017), all of which are LH-inducible in the ovulating follicles (Takahashi et al., 2017; Takahashi et al., 2019). Pgr protein levels did not change irrespective of whether the -14 h-follicles were previously incubated for 14 h with or

without CBX (Fig. 3A). Similarly, expression levels of Cebpb protein in the follicles were not affected by the addition of the inhibitor (Fig. 3B). Follicular expression levels of *ptger4b* and *pai1* mRNA were compared by qPCR using the -14 h-follicles that had been previously incubated with or without CBX; no significant difference in the expression of these genes was seen between CBX-treated and the control follicles (Fig. 3C and 3D). Unlike the genes/proteins described above, incubation of the -14 h-follicles with CBX for 19 h resulted in drastic reduction in the expression of *mm15* mRNA (Fig. 3E, left panel) and Mmp15 protein (Fig. 3E, right panel) in the follicles.

Experiments using two other gap junction blockers were also conducted. In the preovulatory follicles previously treated with these blockers, the expression of *mmp15* mRNA was reduced (Fig. 4A, left panel for mefloquine, and Fig. 4B, left panel for flufenanic acid). Consistent with the results of *mmp15* mRNA expression, Mmp15 protein levels in the follicles were lowered by the treatment with the blockers (Fig. 4A, right panel for mefloquine, and Fig. 4B, left panel for flufenanic acid).

The above results indicate that the suppression of follicle ovulation by the treatment with gap junction blockers seems to be associated with the reduced expression of *mmp15*/Mmp15.

3.3. Inhibition of Pgr phosphorylation by CBX treatment of preovulatory follicles

The transcription factor Pgr, which is induced by the LH surge in the follicles destined to under ovulation in medaka, plays a critical role in the expression of *mmp15* gene (Ogiwara and Takahashi, 2017). We have recently reported that the LH-induced Pgr subsequently undergoes phosphorylation and that phosphorylated Pgr is detectable when transcription of the *mmp15* gene occurs in ovulating follicles (Ogiwara and Takahashi,

2019). Furthermore, a suppression of Pgr phosphorylation by CDK inhibitors resulted in a reduction of *mmp15* expression (Ogiwara and Takahashi, 2019). These findings suggest that the phosphorylated Pgr likely functions as a transcription factor for the *mmp15* gene expression. To validate this assumption, we first examined whether phosphorylated Pgr would indeed bind to the promoter region of the *mmp15* gene. For this purpose, the -3 h-follicles were used because they are expected to actively transcribe the *mmp15* gene in preparation for impeding ovulation (Takahashi et al., 2019). Extracts prepared from the follicles were subjected to DNA pull-down assay using Sepharose gel coupled with 683-bp DNA sequence corresponding to the *mmp15* promoter region. As shown in Fig. 5A, the band for Pgr was detected when the DNA pull-down assay was conducted using the extract without a competitor, but no band was detected when the DNA pull-down assay was conducted using the extract with the competitor, indicating that Pgr was specifically bound to the *mmp15* promoter sequence. Figure 5A also showed that Pgr bound to the *mmp15* promoter sequence was phosphorylated. We further confirmed the importance of Pgr phosphorylation for the binding of Pgr to the promoter region of *mmp15* gene by pull-down assay using OLHNI-2 cells stably expressing medaka Pgr (Ogiwara and Takahashi, 2017). When Cdk9, a protein kinase responsible for Pgr phosphorylation (Ogiwara and Takahashi, 2019), was knocked out, Pgr was no longer able to bind to the DNA sequence (Fig. 5B). These results indicate that the phosphorylated form of Pgr possibly functions as a transcription factor for *mmp15* expression.

Next, we examined whether CBX treatment of the follicles that are destined to ovulate would affect the status of Pgr phosphorylation. As shown in Fig. 5C, a difference in the extent of Pgr phosphorylation was obvious in the follicles previously incubated with or without CBX; CBX treatment noticeably suppressed the extent of Pgr phosphorylation.

We previously reported that Pgr binds to the promoter region of the *mmp15* gene in the granulosa cells of follicles during the hours preceding follicle rupture (Ogiwara and Takahashi, 2017). Therefore, we examined the effect of CBX treatment on Pgr binding to the *mmp15* promoter in the follicles undergoing ovulation using the ChIP assay (Fig. 5D). Because our previous studies established that Pgr most effectively binds to the sequence between -101 and -1 upstream of the transcription site of the *mmp15* gene (Ogiwara and Takahashi, 2017; Ogiwara and Takahashi, 2019), a primer pair was designed and used to generate 101-bp nucleotides corresponding to the above sequence. Preovulatory follicles isolated 14 h before ovulation were incubated for 14 h with or without CBX and then analyzed. Pgr recruitment to the promoter in the CBX-treated follicles was lower compared with the control. The extent of inhibition of Pgr recruitment by CBX was comparable to that by the CDK inhibitor roscovitine, which was recently documented to inhibit not only follicle ovulation but also follicular expression of *mmp15* mRNA in the medaka (Ogiwara and Takahashi, 2019).

The above results suggest that CBX treatment of ovulating follicles results in a reduced generation of phosphorylated Pgr and that the reduced levels of phosphorylated Pgr may be the obstacle to the expression of *mmp15* mRNA, which is supposed to be induced in the follicle under the normal condition.

3.4. Effects of CBX treatment on the expression of Cdk9 and Ccni

To examine the mechanism by which the extent of Pgr phosphorylation in ovulating follicles was reduced by CBX treatment, the effect of the gap junction blocker on the expression of Cdk9 and its regulatory protein Ccni were examined because they are possibly involved in Pgr phosphorylation in the follicles (Ogiwara and Takahashi, 2019).

Expression levels of both *cdk9* and *ccni* mRNA in the follicles were lowered by the treatment with CBX (Fig. 6A and 6B). When the Cdk9 protein in the follicles was analyzed, no apparent change was observed with CBX treatment (Fig. 6C). In contrast, the treatment of follicles with CBX strongly inhibited the expression of Ccni (Fig. 6D), the result being consistent with that of *ccni* mRNA expression.

3.5. Expression of connexins in the preovulatory follicles

The results described in the preceding sections suggest an involvement of gap junctional communication in Pgr phosphorylation by Ccni-activated Cdk9 for the successful ovulation of the follicles. We therefore examined the expression of connexins, the component of a gap junction. A computer search of *cx* genes using the medaka genome database (https://asia.ensembl.org/index.html) suggests that fish contain a total of 19 *cx* genes. Our recent analysis by next-generation sequencing identified five *cx* genes (*cx30.3*, *cx34.4*, *cx34.5*, *cx35.4*, and *cx43.4*) that are presumed to be transcribed in the preovulatory follicles. qPCR analysis was conducted to determine the follicular expression of the *cx* genes using isolated ovarian follicles. Expression profiles in the 24 h-spawning cycle of the fish varied in different *cx* genes (Fig. 7). We further analyzed *cx* mRNA expression using follicle layers of the follicles that were isolated 10 h and 7 h before ovulation (Fig. 8). Among the five *cx* genes, *cx34.5* mRNA was most abundantly expressed in the follicle layer at 10 h before ovulation (Fig. 8A), while *cx35.4* mRNA became dominant *cx* transcripts at 7 h before ovulation (Fig. 8B). Finally, we examined if CBX treatment affects *cx34.5* and *cx35.4* mRNA expression using pre-ovulatory follicles isolated 14 h prior to ovulation. The -14 h follicles were incubated for 7 h in the absence or presence of CBX (50 μM) and then analyzed for *cx* expression. No significant

difference was observed in *cx* expression levels between control and CBX-treated follicles for both *cx* genes (data not shown), indicating that *in vitro* treatment of preovulatory follicles with the gap junction blocker caused no inhibitory effect on *cx* expression.

The above results indicate that the two *cx* subtypes, *cx34.5* and *cx35.4*, are actively transcribed in the follicle layers of ovulating follicles around the time when Pgr undergoes phosphorylation by Cdk9.

4. **Discussion**

This study was conducted following our initial finding that medaka preovulatory follicles did not ovulate if they were incubated in the presence of CBX. Our attempts to define the nature of CBX's inhibitory effect on follicle ovulation have provided us further information on the process of LH-induced Mmp15 expression in normal ovulation of the medaka.

CBX acts as a modestly potent, reasonably effective, water-soluble blocker of gap junction (Willebrorods et al., 2017). This compound is also documented to have various gap junction-independent activities (Connors, 2012). CBX is also known to be an inhibitor of 11β-hydroxysteroid dehydrogenase (Armanini et al., 1982), an antagonist of GABAa receptor (Beaumont and Maccaferri, 2011; Ransom et al., 2017), and to have antiviral activities (Pu et al., 2017; Haga et al., 2018). Therefore, the outcome of experiments using CBX need to be treated with caution to determine whether CBX's effect would be due to its gap junction-blocking activity. In this study, we found that, in addition to CBX, two other gap junction blockers, mefloquine and flufenanic acid, showed an inhibitory effect on follicle ovulation. Furthermore, like CBX, the treatment

of preovulatory follicles with mefloquine and flufenanic acid caused the inhibition of *mmp15*/Mmp15 expression. Such a parallelism in the pattern of effects on the fish ovarian follicles between CBX and the other two blockers strongly argues for a gap junction-dependent role of CBX on preovulatory follicles. However, our results do not completely rule out the potential toxicity of these gap junction blockers at present.

Our previous studies indicate that *pgr*, *cebpb*, *ptger4b*, *pai1*, and *mmp15* are ovulation-related genes markedly induced in the granulosa cells of ovulating follicles by the ovulatory surge of LH and that decreased expression of these genes profoundly causes deterioration in the rate of ovulation *in vitro* (Takahashi et al., 2017; Takahashi et al., 2019). We found that among the five genes described, *mmp15* was only the gene whose follicular expression was significantly inhibited by CBX treatment. This finding indicates that the effect of CBX treatment on the expression of the *mmp15* gene is unique among the LH-inducible genes in the cells. The distinctive effect of CBX on the *mmp15* expression in the follicle was defined at the molecular level in this study.

The novel finding of the current study is that the expression of *cdk9* and *ccni* mRNA in the granulosa cells of ovulating follicles was inhibited by the treatment of the follicles with CBX. Consistent with the finding, Ccni protein was also not detected in the CBX-treated preovulatory follicles. However, we detected Cdk9 protein in the treated follicles at the levels comparable with untreated follicles. This observation could be explained as follows: the expression levels of *ccni*/Ccni in the follicles are very low before LH stimulation and are noticeably increased after the gonadotropin surge. Treatment of the follicles with CBX invalidates such induction of the gene expression, thereby keeping follicular *ccni*/Ccni expression levels low even after the LH surge in the treated follicles. On the other hand, *cdk9*/Cdk9 is constitutively expressed in the follicle, and its follicular

levels basically remain unchanged regardless of hormonal status under the physiological condition. In the follicles that have been exposed to CBX, *de novo* synthesis of *cdk9*/Cdk9 is probably inhibited. However, it is not surprising that a clear decrease in the follicular levels of Cdk9 protein, but not *cdk9* transcript, may not be seen if the preexisting Cdk9 protein is slowly degraded in the follicles. From these considerations, we tentatively conclude that the suppression of Pgr phosphorylation in the follicles by CBX treatment is primarily due to the absence of Ccni, which activates Cdk9.

The current data allow us to draw a picture representing the process of *mmp15* transcription occurring in the granulosa cells of the follicles *in vivo* (Fig. 9), where CBX's effects are also shown. When large postvitellogenic follicles in the medaka ovary undergo the ovulatory surge of LH, follicle cells expressing Lh receptors on the cell surface respond to LH stimulation. Although both theca and granulosa cells of postvitellogenic and preovulatory follicles are shown to express Lh receptors in medaka (Ogiwara et al., 2013), existing evidence indicates that granulosa cells play important roles in the ovulatory process of the fish (Takahashi et al., 2017; Takahashi et al., 2019). Therefore, the events occurring in the cells are shown in this model. Activated Lh receptor in the granulosa cells of the follicles induces the transcription factor *pgr* (Hagiwara et al., 2014), although the regulatory mechanism of *pgr* synthesis is unknown at present. At the time when Pgr protein levels are increased in the cells, the pathway synthesizing 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP), the physiological progestin ligand for medaka Pgr, is also activated (Nagahama and Yamashita, 2008). This activation facilitates the association of Pgr with 17,20βP to generate active Pgr. The resulting Pgr acts as a transcription factor for *ccni* mRNA expression. As soon as the translation product Ccni is synthesized, Cdk9, which is constitutively produced in the granulosa cells throughout

the 24-h spawning cycle of the fish, is activated and phosphorylates Pgr. Consistent with this hypothesis is our recent finding that the appearance of Ccni exactly times that of phosphorylated Pgr (Ogiwara and Takahashi, 2019). Phosphorylated Pgr now acts as active transcription factor for the expression of the *mmp15* gene. Another transcription factor, Cebpb, which is also induced in an LH-dependent, but not Pgr-dependent, manner (Ogiwara and Takahashi, 2017) by this time, participates in *mmp15* gene expression. Mmp15 thus synthesized is then activated and eventually expressed on the cell surface of the granulosa cells of ovulating follicles to degrade type I collagen, a major extracellular matrix protein present in the theca cell layer of the follicle (Takahashi et al., 2019). Our present data suggest that disruption of gap junctional communication by gap junction blockers, such as CBX, primarily causes inhibition of *ccni* and *cdk9* gene expression. It is not yet determined whether phosphorylated Pgr would be required for the expression of the *ccni* gene, as in the case of *mmp15* gene activation. Available data suggest that unphosphorylated Pgr may be involved in *ccni* transcription in the follicles, but future studies are necessary to validate this hypothesis.

Our results revealed that *cx34.5* and *cx35.4* mRNA were both dominantly expressed in the follicle layers of the follicles isolated at 10 h and 7 h prior to ovulation, respectively. The follicle layer-associated expression of these *cx* mRNAs suggests that the granulosa cells of the follicles may be the cells responsible for expressing the two connexins, leading us to speculate that the formation of gap junctions occurs between adjacent follicle cells (homologous gap junctions) surrounding the oocyte rather than those formed between follicle cells and the oocyte (heterologous gap junctions) during this time period. The above finding also indicates that a shift in the *cx* gene expression from *cx34.5* to *cx35.4* occurs at approximately the same time. Currently, the physiological meaning of this shift

in the expression of these two *cx* genes within the follicle cells is unclear. Assuming that expression profiles of these *cx* transcripts parallel the levels of translation products, Cx34.5 and Cx35.4 may be major connexins constituting functional gap junctions at these time periods within the follicle cells. We tentatively speculate that gap junctional communication via the junctions involving Cx34.5 and/or Cx35.4 may contribute to the expression of *ccni* mRNA in the granulosa cells of ovulating follicles and that CBX treatment of these follicles may cause a disturbance in gap junctional communication. Our current finding, that levels of *cx34.5* and *cx35.4* mRNA were not reduced within the follicles treated with CBX, appears to argue against the idea that CBX treatment itself may have resulted in the formation of gap junctions. At this point, our results do not completely rule out the possibility that Cx proteins other than Cx34.5 and Cx35.4 may be involved in this process.

Although our current data appear to point to the importance of gap junctional communication for *ccni* expression in the granulosa cells of the follicles, the molecular mechanism underlying the gap junction-involved *ccni* expression in the cells remains unknown. In this context, valuable future investigations would be as follows: i) The localization of Cx34.5, Cx35.4, and other Cx species in the follicles must be determined. Such information should be useful to judge whether heterologous gap junctions or homologous gap junctions. ii) The mechanism of transcription of the *ccni* gene occurring in the granulosa cells of the follicles in response to the LH surge, including identification of proteins/factors involved in the process, must be defined. Such information should help understand the inhibitory effect of CBX treatment on transcription of the *ccni* gene in the follicles. iii) The responsible ions or metabolites that exchange intercellularly through the gap junction in the ovulating follicles need to be identified.

We should note previous reports implicating possible functions of gap junctions during different stages of ovarian cellular differentiation (Yamamoto et al., 2011) and the acquisition of oocyte maturational competence (Yoshizaki et al., 1994; Chang et al., 1999; Chang et al., 2000; Bobe et al., 2004; Yamamoto et al., 2007; Yamamoto et al., 2008; Yamamoto and Yoshizaki, 2008; Rime et al., 2010) of the follicles in various teleost species. Maturational competence acquisition of the oocyte occurs in the postvitellogenic follicles during the preovulatory period and, like oocyte maturation and ovulation, is induced by the surge of LH. The LH-induced acquisition of the maturational competence precedes oocyte maturation and ovulation (Patiño and Thomas, 1990; Patiño et al., 2001). Using Atlantic croaker (Bolamba et al., 2003) and red seabream (Patiño and Kagawa, 1999), the increase of both homologous and heterologous gap junctions during the early phase of follicle maturation was demonstrated. It is also documented that heterologous gap junctions rather than homologous gap junctions contribute maturational competence acquisition of the oocyte (Yamamoto et al., 2007; Yamamoto and Yoshizaki, 2008). Several connexins have been implicated in the maturational competence acquisition in various teleost species: Cx32.2 in Atlantic croaker (Yoshizaki et al., 1994; Chang et al., 1999; Chang et al., 2000), Cx31.5 in red seabream (Choi and Takashima, 2000), Cx34.9 in Ayu (Yamamoto et al., 2007), and Cx43.2 in coho salmon (Yamamoto et al., 2011). However, no studies have been conducted to date exploring the involvement of gap junctions in the LH-induced ovulation in any teleost species. To the best of our knowledge, this is the first study implicating the role of gap junctional communication in teleost ovulation.

In the acquisition of oocyte maturational competence, gap junctional communication plays a role in the ovulatory process in teleost fish, and based on this, it is of interest to

consider the possible timing of the involvement of gap junctions in these two LH-induced events in spawning medaka. According to the two-stage model of LH-dependent follicle maturation proposed for teleosts (Pang and Ge, 2002; Patiño et al., 2001), the first stage is characterized as the period during which the post-vitellogenic follicles acquire the ability to produce maturation-inducing hormone (MIH or 17,20βP), and the oocyte becomes sensitive to MIH stimulation. The second stage represents the period during which the MIH-induced execution of meiosis resumes. Ovulating follicles must acquire ovulatory competence in parallel with the acquisition of oocyte maturational competence after the LH surge. It is well established that MIH also mediates the ovulatory action of maturational gonadotropin (Patiño et al., 2003), although little information is available regarding the mechanisms of LH-dependent ovulatory competence. In the medaka with an established 24 h spawning cycle, the post-vitellogenic follicles undergo this LH surge around approximately -18 h of ovulation *in vivo*, and this allows for the ability to rapidly synthesize MIH, which peaks within approximately -13 h before ovulation (Nagahama and Yamashita, 2008; Sakai et al., 1988). The follicles then undergo GVBD at approximately -6 h followed by sequential post-GVBD events such as chromosome condensation, assembly of the meiotic spindle, and formation of the first polar body (Nagahama and Yamashita, 2008). Therefore, it is conceivable that within the ovulating follicle in the medaka, the oocyte acquires maturational competence approximately 5 h after the surge of LH (-18 to -13 h of the fish 24 h spawning cycle; the first stage in the two-stage concept of LH-dependent follicle maturation) and then undergoes a series of events associated with meiosis resumption (-13 to 0 h; the second stage). In this study, we demonstrated a critical role of gap junctions in the expression of *ccni*/Ccni that occurs in the granulosa cells of medaka ovulating follicles. This is a genomic event, essential for

the generation of phosphorylated Pgr, which serves as active transcription factor for the expression of the ovulation-related gene *mmp15*. In the 24 h spawning cycle, *ccni*/Ccni expression in the ovulating follicle occurs between -9 to -3 h of ovulation concomitantly with the appearance of the phosphorylated form of Pgr (Ogiwara and Takahashi, 2019). This observation highlights the importance of gap junctional communication for the follicular expression *ccni*/Ccni in the latter half of the 24 h spawning cycle. If the acquisition of ovulatory competence is defined as the ability of ovarian follicles to ovulate under MIH stimulation (Patiño et al., 2003), the *ccni*/Ccni expression involving gap junctions that occurs within the granulosa cells of medaka ovulating follicles would then act as a component of the complex processes influencing ovulatory competence acquisition. Therefore, it would take longer to acquire ovulatory competence than it would to reach oocyte maturational competence. In this context, Patiño *et al*. (2003) reported interesting results, revealing that by using ovarian follicles from the Atlantic croaker, the acquisition of ovulatory competence lagged behind that of oocyte maturational competence. The precise mechanism of ovulatory competence acquisition in teleosts remains to be determined.

In summary, this study showed that, by analyzing the effect of the gap junction blocker CBX on medaka ovulating follicles, gap junctional communication is necessary for the process of LH-induced, Pgr-involved expression of the *mmp15* gene in the follicles. Our data indicate that gap junctional communication contributes not only to the *de novo* Ccni synthesis but also to ensuring a constitutive Cdk9 expression, thereby allowing Pgr to be phosphorylated at the appropriate time. This study has established the basis for further investigations to define the role of gap junctional communication in the ovulating follicle of the medaka.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research for the Ministry of Education, Culture, Science, and Technology of Japan (16H04810 to T.T.).

Footnotes

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Legends for Figures

Fig. 1. *In vitro* follicle culture with gap junction blockers. (A) An outline of the routinely used preovulatory follicle culture experiments is shown. The follicles isolated from ovaries 14 h before ovulation (-14-h follicles) were incubated with or without blockers for 19 h (Condition-1) or 14 h (Condition-2). The approximate timing of the LH surge (-18 h), GVBD (-6 h), and ovulation (0 h) *in vivo* are shown in the upper position. The rates of ovulation and germinal vesicle breakdown (GVBD) and the expression of *mmp15*/Mmp15 were determined using follicles incubated under Condition-1, whereas the expressions of Pgr, Cebpb, *ptger4b*, *pai1*, *cdk9*/Cdk9, and *ccni*/Ccni were determined using follicles incubated under Condition-2. (B) The -14-h follicles were cultured with distilled water (as control) or carbenoxolone (CBX). After a 19-h incubation, the rate of ovulation was determined. Asterisks indicate significance at $p < 0.05$ (*) or $p < 0.01$ (**) compared with the control (Kruskal-Wallis and Steel post hoc test, N = 7). (C) The -14-h

follicles were cultured with distilled water (as control) or CBX. After a 19-h incubation, the number of oocytes that had undergone GVBD was counted to determine the rate of GVBD.

Fig. 2. Effects of mefloquine (MEF) and flufenanic acid (FLU) on *in vitro* ovulation and GVBD of preovulatory follicles. (A) The -14-h follicles were incubated with DMSO (as control) or 40 μM MEF. After 19 h of incubation, the rate of ovulation was determined. Asterisks indicate significance at $p < 0.01$ compared with the control (*t*-test, N = 7). (B) The -14-h follicles were incubated as in (A). After a 19-h incubation, the rate of GVBD was determined (N = 7). (C) The -14-h follicles were incubated with DMSO (as control) or 50 μM FLU. After a 19-h incubation, the rate of ovulation was determined. Asterisks indicate significance at $p < 0.01$ compared with the control (*t*-test, N = 7). (D) The -14-h preovulatory follicles were incubated as in (C). After a 19-h incubation, the rate of GVBD was determined (N = 7).

Fig. 3. Effect of CBX on the follicular expression of several genes involved in medaka ovulation. (A) The -14-h follicles were incubated for 14 h with CBX. After incubation, the follicle layer extract was prepared and analyzed for Pgr by western blot analysis. An arrow indicates the position of Pgr. Rpl7 protein was also detected as a control. (B) Follicle incubation and the preparation of a follicle layer extract were conducted as in (A). The extract was subjected to western blot analysis for Cebpb. An arrow indicates the position of Cebpb. Rpl7 protein was used as control. (C) Follicle incubation was conducted as in (A). Total RNA extracted from the incubated follicles was used for qPCR using specific primers of *ptger4b*. The mRNA expression levels were normalized to the

expression of *eef1a1* (ANOVA and Tukey post hoc test, N = 4). (D) Follicle incubation and total RNA preparation were conducted as in (C). The RNA was used for qPCR using specific primers of *pai1*. The mRNA expression levels were normalized to the expression of *eef1a1* (Kruskal-Wallis and Scheffé post hoc test, N = 5). (E) The -14-h follicles were incubated for 19 h with CBX. After incubation, total RNA extract and the follicle layer extract were prepared from the incubated follicles and used for qPCR using specific primers of *mmp15* (left panel) and western blot analysis (right panel), respectively. The mRNA expression levels of *mmp15* were normalized to the expression of *eef1a1*. A significant difference at $p < 0.01$ is indicated by asterisks (Kruskal-Wallis and Scheffé post hoc test, N = 5). An arrow indicates the position of Mmp15. Rpl7 protein was used as a control in western blot analysis.

Fig. 4. Effects of mefloquine (MEF) and flufenanic acid (FLU) on the follicular expression of *mmp15*/Mmp15. (A) The -14-h follicles were incubated for 19 h with MEF. After incubation, total RNA extract and the follicle layer extract were prepared from the follicles and used for qPCR analysis of *mmp15* mRNA expression (left panel) and for western blot analysis of Mmp15 protein expression (right panel), respectively. The mRNA expression levels of *mmp15* were normalized to the expression of *eef1a1*. A significant difference at $p < 0.05$ is indicated by an asterisk (ANOVA and Tukey-Kramer post hoc test, N = 6). An arrow indicates the position of Mmp15. Rpl7 protein was a control in western blot analysis. (B) The -14-h follicles, which had been incubated for 19 h with FLU, were processed as in (A). Follicular expression of *mmp15* mRNA and Mmp15 protein was examined by qPCR (left panel) and western blot analysis (right panel), respectively. The mRNA expression levels of *mmp15* were normalized to the

expression of *eef1a1*. A significant difference at $p < 0.01$ is indicated by asterisks (Kruskal-Wallis and Scheffé post hoc test, N = 4). An arrow indicates the position of Mmp15. Rpl7 protein was used as a control in western blot analysis.

Fig. 5. CBX inhibition of Pgr phosphorylation in preovulatory follicles. (A) The -3-h follicles were isolated, and the follicle extracts were prepared. The extracts were directly used (-Competitor) or used after incubating with the DNA sequence corresponding to the *mmp15* promoter region (+Competitor). The extracts were analyzed by DNA pull-down assay (upper panel). Aliquots of the extracts were immunoprecipitated with rat anti-medaka Pgr antibody followed by western blot analysis using mouse anti-medaka Pgr antibody (middle panel). The materials precipitated by DNA pull-down assay were analyzed by Phos-tag SDS-PAGE/western blotting (lower panel). An arrow and arrowhead indicate phosphorylated Pgr and unphosphorylated Pgr, respectively. An asterisk indicates nonspecific bands recognized by the antibody. (B) The extracts of OLHNI-2 cells stably expressing Pgr (-CDK9 KO) or OLHNI-2 cells stably expressing Pgr but deficient for Cdk9 (+CDK KO) were used. The cell extracts were analyzed by DNA pull-down assay (upper panel). Aliquots of the extracts were analyzed by western blotting using anti-medaka Pgr antibody (lower panel). (C) The -14-h follicles were incubated with or without CBX for 14 h, and the extracts of the incubated follicles were immunoprecipitated with anti-medaka antibody (Anti-Pgr) or control IgG (IgG). Immunoprecipitated materials were analyzed by Phos-tag SDS-PAGE/western blotting using anti-medaka Pgr antibody. An arrow and arrowhead indicate phosphorylated Pgr and unphosphorylated Pgr, respectively. An asterisk indicates the bands corresponding to the antibody used for immunoprecipitation. (D) The -14-h follicles were incubated with

CBX or roscovtine (Rosc) or with no additives (Cont) for 14 h, and the follicles were used for ChIP assay using primer pair-1 (Supplementary Table S1). Asterisks indicate a significant difference at $p < 0.01$ (ANOVA and the Dunnett post hoc test, N = 4).

Fig. 6. Effect of CBX on the expression of Cdk9 and Ccni in the preovulatory follicle. (A) The -14-h follicles were incubated for 14 h with CBX. Total RNA extracted from the incubated follicles was used for qPCR using specific primers of *cdk9*. The mRNA expression levels were normalized to the expression of *eef1a1*. A significant difference at $p < 0.01$ is indicated by asterisks (ANOVA and Tukey-Kramer post hoc test, N = 4-7). (B) Follicle incubation and total RNA preparation were conducted as in (A). The RNA was used for qPCR using specific primers of *ccni*. The mRNA expression levels were normalized to the expression of *eef1a1*. A significant difference at $p < 0.05$ (*) or $p < 0.05$ (**) is indicated by asterisks (Kruskal-Wallis and Scheffé post hoc test, N = 4-7). (C) The -14-h follicles were incubated for 14 h with CBX. After incubation, the follicle layer extract was prepared and immunoprecipitated using anti-medaka Cdk9 antibody or normal IgG. The resulting precipitates were analyzed by western blotting using the Cdk9 antibody. An arrow indicates the position of Cdk9. Bands corresponding to the antibody used for immunoprecipitation are indicated by asterisks. (D) Follicle incubation and preparation of the follicle layer extracts were conducted as in (C). The extracts were immunoprecipitated using anti-medaka Ccni antibody or normal IgG, and the resulting precipitates were analyzed by western blotting using the Ccni antibody. Arrows indicate the position of Ccni. Bands corresponding to the antibody used for immunoprecipitation are indicated by asterisks.

Fig. 7. Expression of connexin mRNA in the preovulatory of the medaka. (A) Total RNAs were extracted from preovulatory follicles isolated from ovaries at the indicated time points. qPCR was conducted using specific primers for *cx30.3* (A), *cx34.4* (B), *cx34.5* (C), *cx35.4* (D), or *cx43.4* (E). The mRNA expression levels were normalized to the expression of *actb* (N = 5).

Fig. 8. Expression of connexin mRNA in the follicle layers of the -10-h and -7-h follicles. (A) The follicle layers isolated from the -10-h follicles were used. Total RNAs were extracted from the resulting layers, and qPCR was conducted for five connexin expression. The mRNA expression levels were normalized to the expression of *actb* (N = 3). (B) Total RNAs were extracted from the follicle layers of the -7-h follicles. qPCR was conducted for five connexin expression. The mRNA expression levels were normalized to the expression of *actb* (N = 4).

Fig. 9. A proposed model for the signal pathway of LH-induced expression of *mmp15*/Mmp15 in the granulosa cells of ovulating follicles. Gap junctional communication may be required for the LH-induced, Pgr-dependent *ccni* expression as well as the constitutive *cdk9* expression in the cells under the normal condition, and disturbance of gap junction communication by the blockers causes the inhibition of transcription of these genes. Transcription of *pgr*, *cebpb*, *ccni*, and *cdk9* occurs in the nucleus of the granulosa cells. See the text for details.

Figure 1

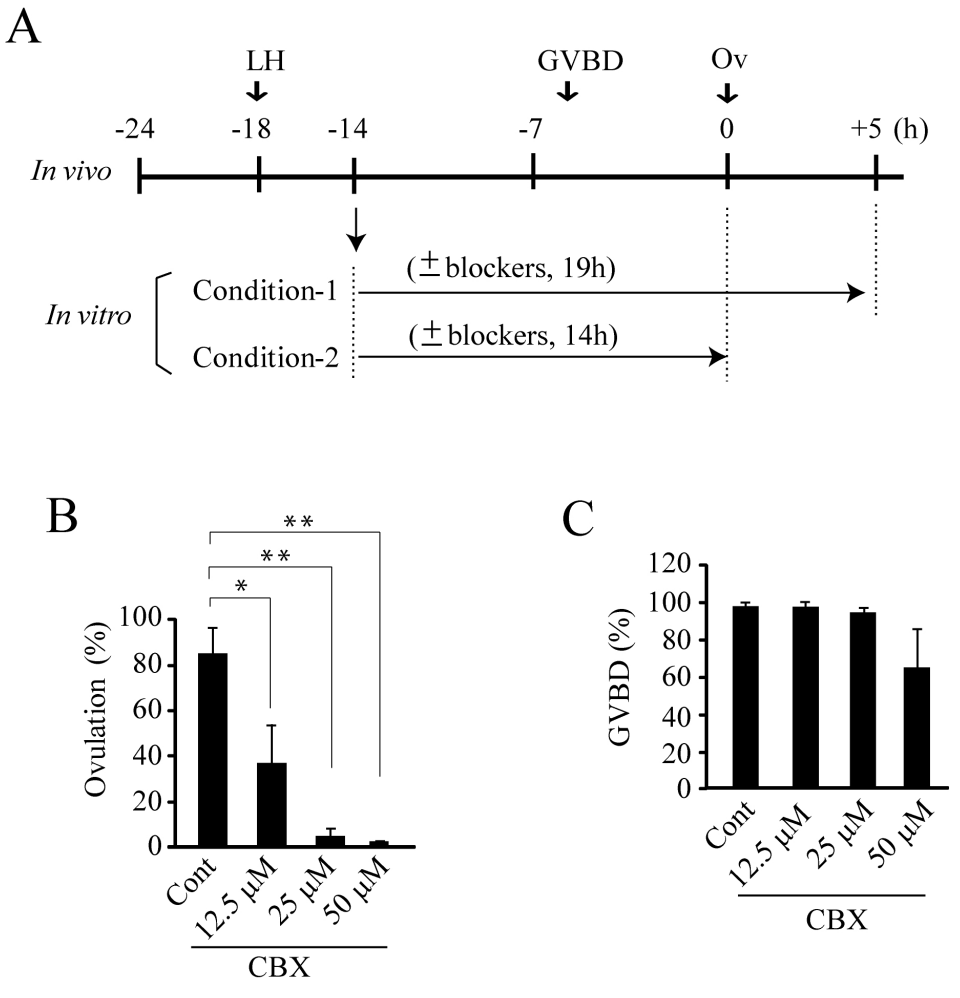

A
LH
GVBD
Ov
-24
-18
-14
-7
0
+5 (h)
In vivo
In vitro
Condition-1
Condition-2
(±blockers, 19h)
(±blockers, 14h)
B
Ovulation (%)
100
80
60
40
20
0
**
**
*
Cont
12.5 μM
25 μM
50 μM
CBX
C
GVBD (%)
120
100
80
60
40
20
0
Cont
12.5 μM
25 μM
50 μM
CBX

Figure 2

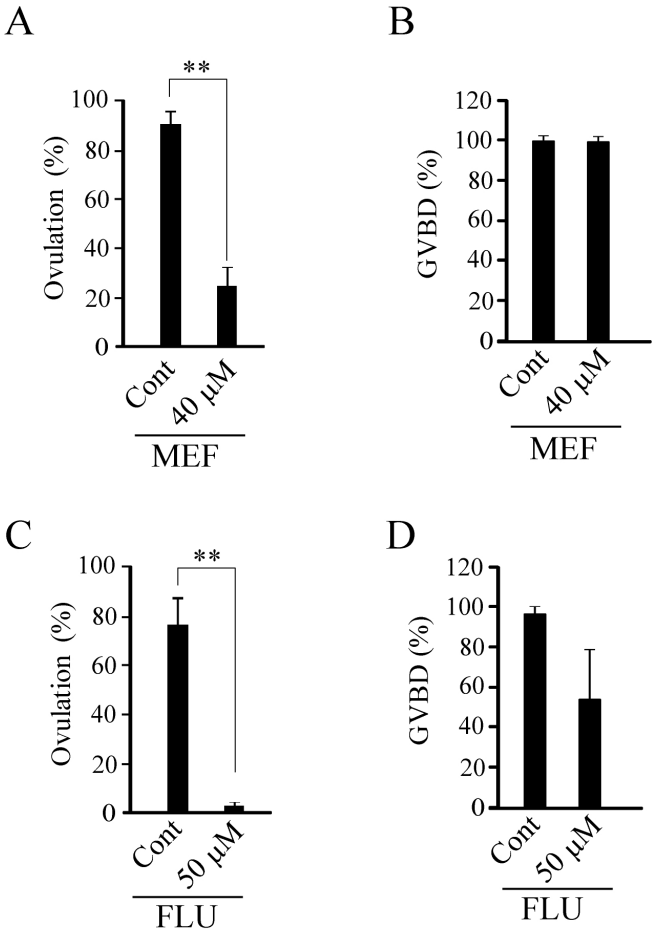

A
B
C
D
**
**
Ovulation (%)
GVBD (%)
Ovulation (%)
GVBD (%)
100
80
60
40
20
0
120
100
80
60
40
20
0
Cont
40 μM
MEF
Cont
40 μM
MEF
Cont
50 μM
FLU
Cont
50 μM
FLU

Figure 3

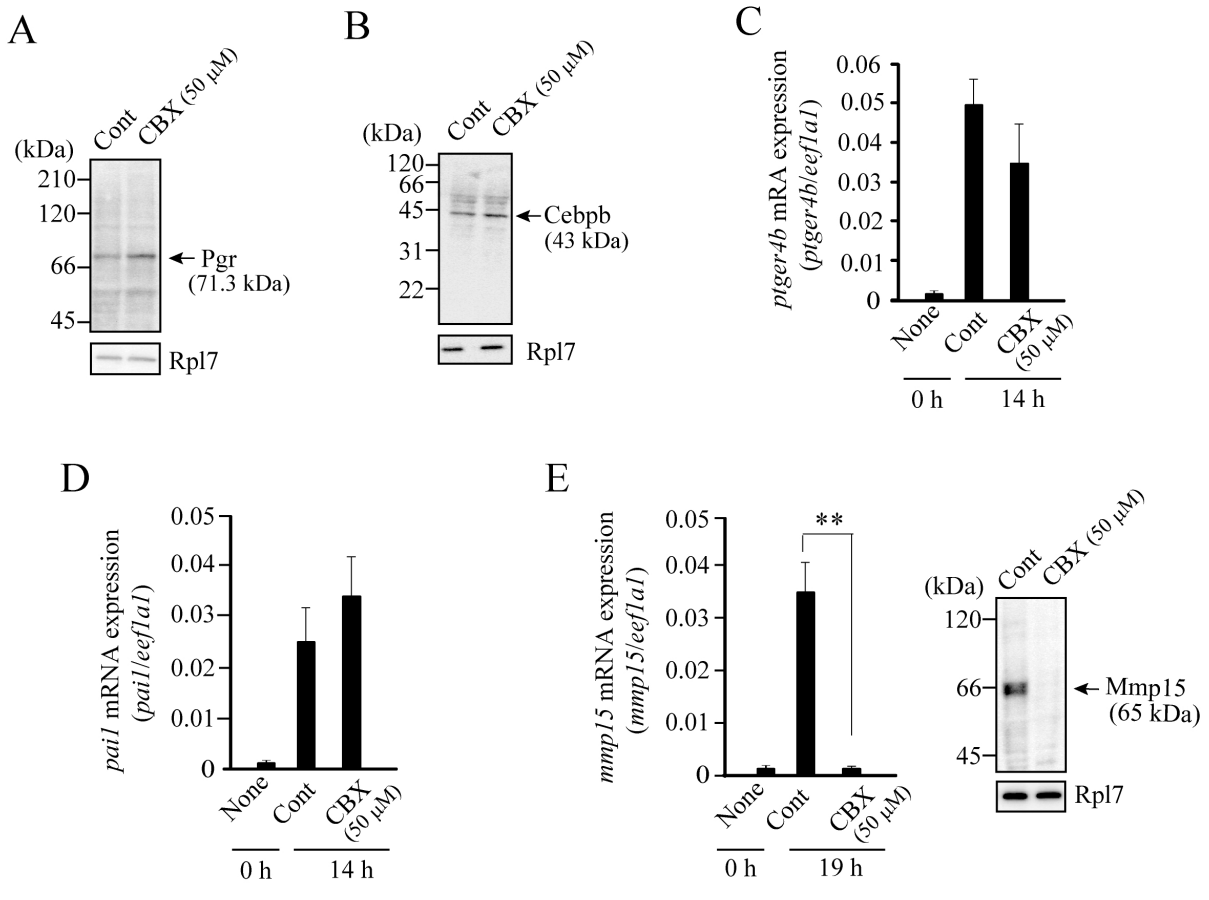

A
Cont
CBX (50 μM)
(kDa)
210
120
66
45
Pgr
(71.3 kDa)
Rpl7
B
Cont
CBX (50 μM)
(kDa)
120
66
45
31
22
Cebpb
(43 kDa)
Rpl7
C
ptger4b mRNA expression
(ptger4b/eef1a1)
0.06
0.05
0.04
0.03
0.02
0.01
0
None
Cont
CBX (50 μM)
0 h
14 h
D
pai1 mRNA expression
(pai1/eef1a1)
0.05
0.04
0.03
0.02
0.01
0
None
Cont
CBX (50 μM)
0 h
14 h
E
mmp15 mRNA expression
(mmp15/eef1a1)
**
0.05
0.04
0.03
0.02
0.01
0
None
Cont
CBX (50 μM)
0 h
19 h
Cont
CBX (50 μM)
(kDa)
120
66
45
Mmp15
(65 kDa)
Rpl7

Figure 4

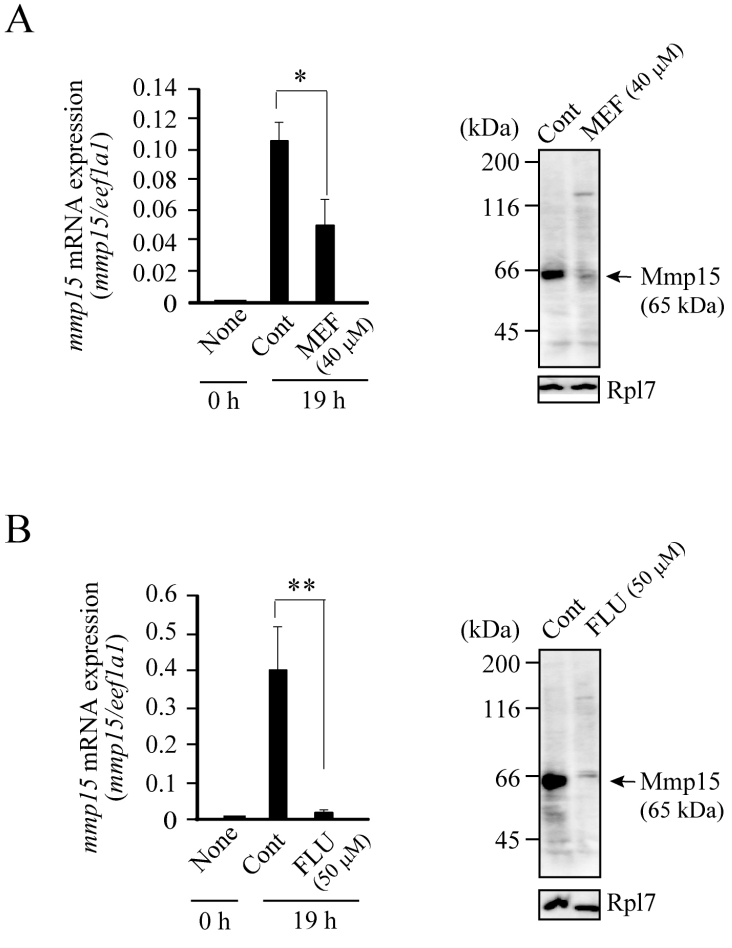

A
mmp15 mRNA expression
(mmp15/eef1a1)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
*
None
Cont
MEF
(40 μM)
0 h
19 h
(kDa)
Cont
MEF (40 μM)
200
116
66
45
Mmp15
(65 kDa)
Rpl7
B
mmp15 mRNA expression
(mmp15/eef1a1)
0.6
0.5
0.4
0.3
0.2
0.1
0
**
None
Cont
FLU
(50 μM)
0 h
19 h
(kDa)
Cont
FLU (50 μM)
200
116
66
45
Mmp15
(65 kDa)
Rpl7

Figure 5

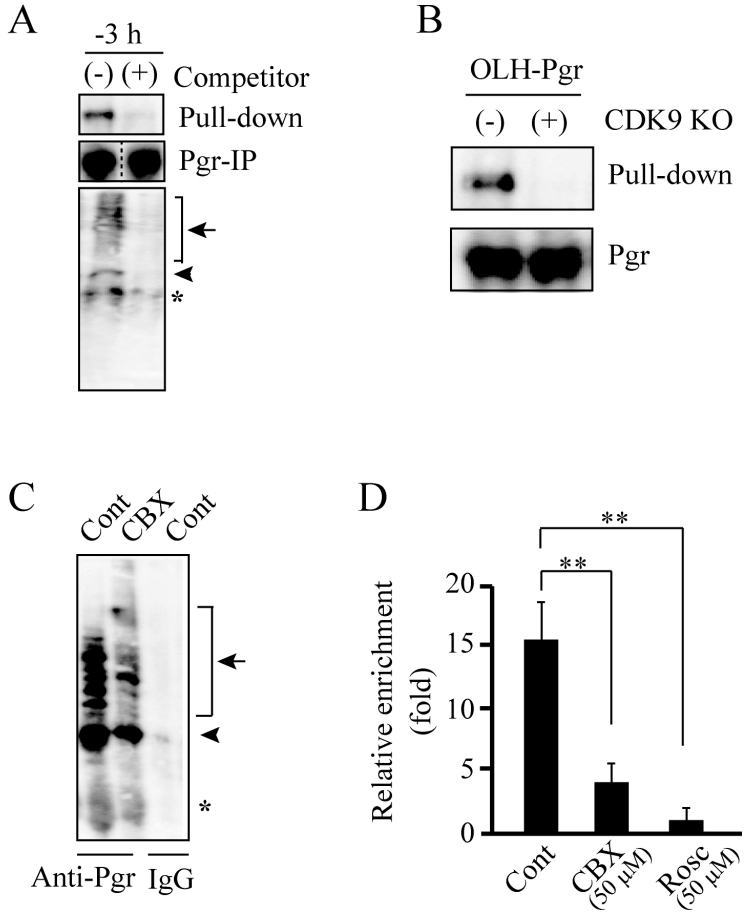

A
-3 h
(-) (+) Competitor
Pull-down
Pgr-IP
*
B
OLH-Pgr
(-) (+) CDK9 KO
Pull-down
Pgr
C
Cont
CBX
Cont
*
Anti-Pgr IgG
D
**
**
Relative enrichment (fold)
20
15
10
5
0
Cont
CBX (50 μM)
Rosc (50 μM)

Figure 6

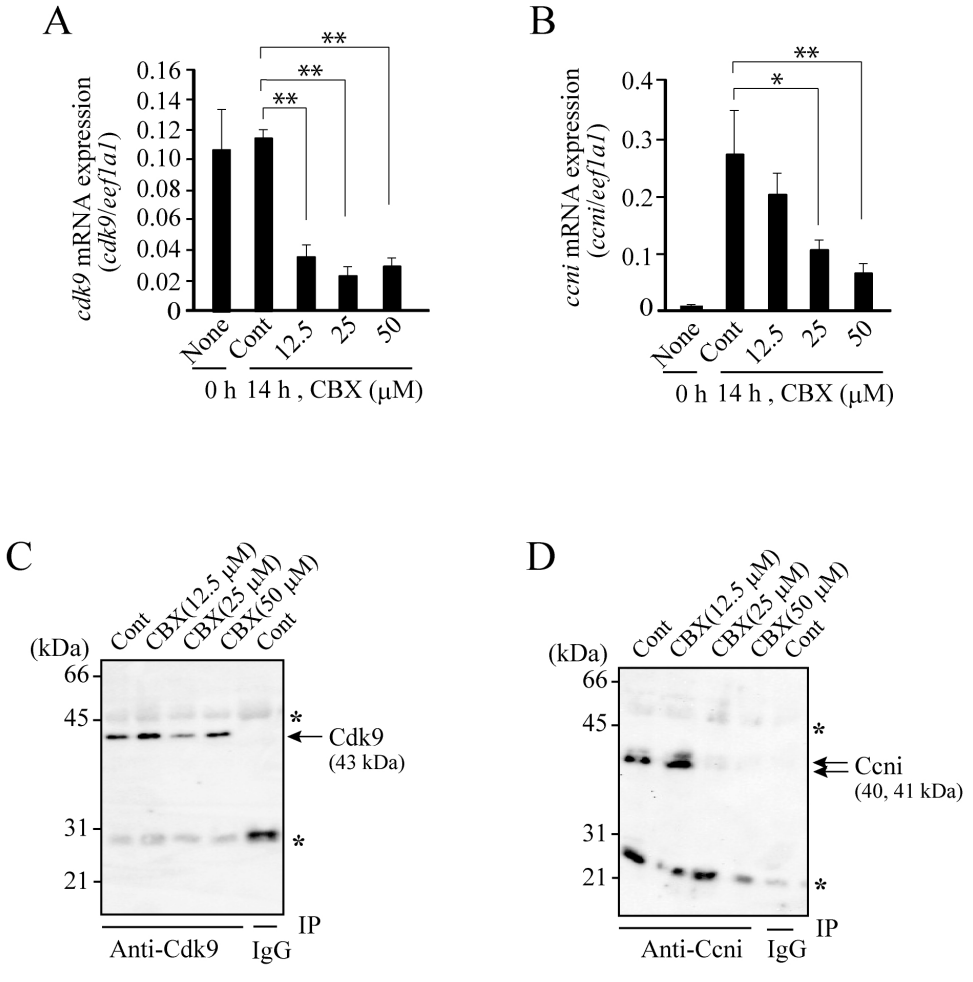

A
cdk9 mRNA expression
(cdk9/eef1a1)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
**
**
**
None
Cont
12.5
25
50
0 h
14 h , CBX (μM)
B
ccni mRNA expression
(ccni/eef1a1)
0.4
0.3
0.2
0.1
0
**
*
None
Cont
12.5
25
50
0 h
14 h , CBX (μM)
C
(kDa)
Cont
CBX(12.5 μM)
CBX(25 μM)
CBX(50 μM)
Cont
66
45
31
21
*
Cdk9
(43 kDa)
*
IP
Anti-Cdk9
IgG
D
(kDa)
Cont
CBX(12.5 μM)
CBX(25 μM)
CBX(50 μM)
Cont
66
45
31
21
*
Ccni
(40, 41 kDa)
*
IP
Anti-Ccni
IgG

Figure 7

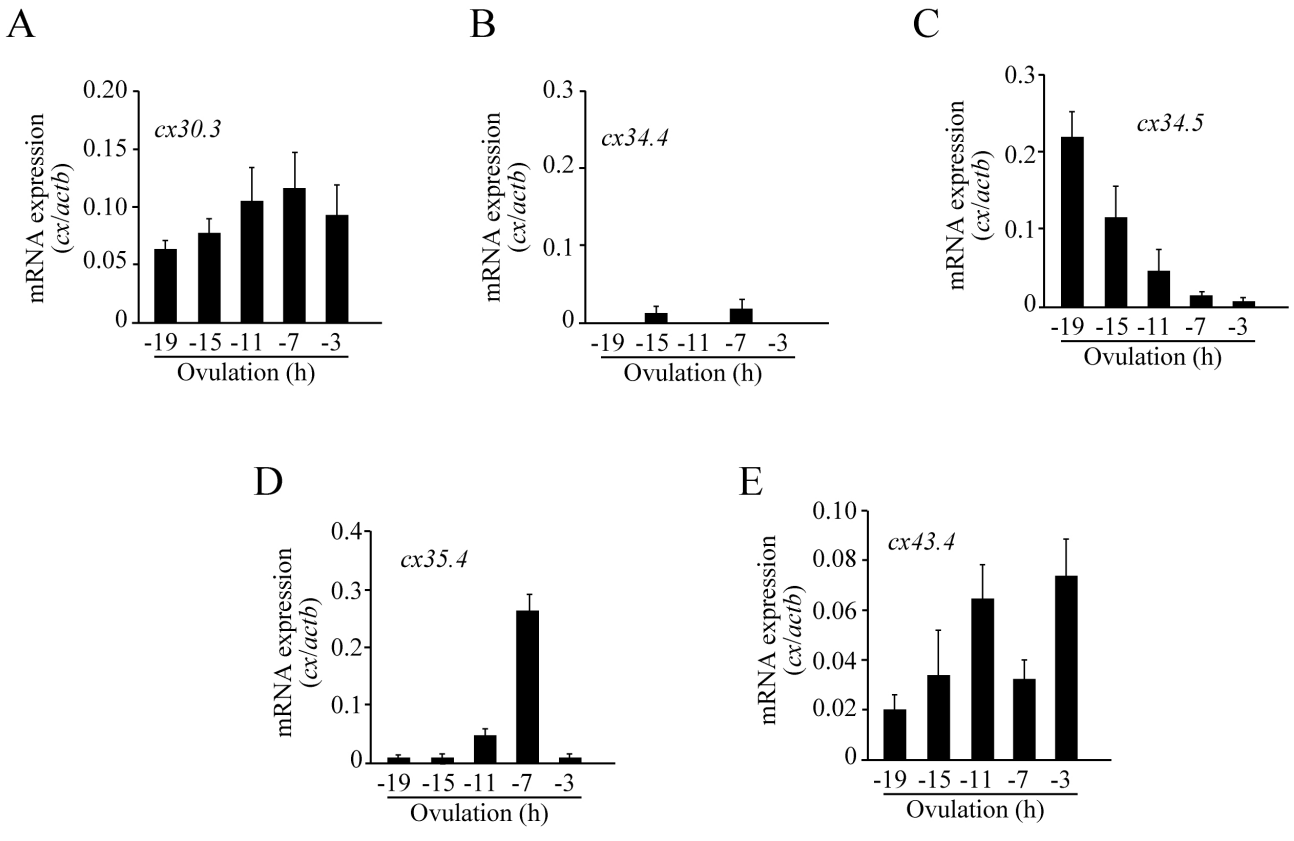

A
B
C
D
E
cx30.3
cx34.4
cx34.5
cx35.4
cx43.4
mRNA expression (cx/actb)
Ovulation (h)
-19 -15 -11 -7 -3
0.20
0.15
0.10
0.05
0
0.3
0.2
0.1
0.4
0.08
0.06
0.04
0.02

Figure 8

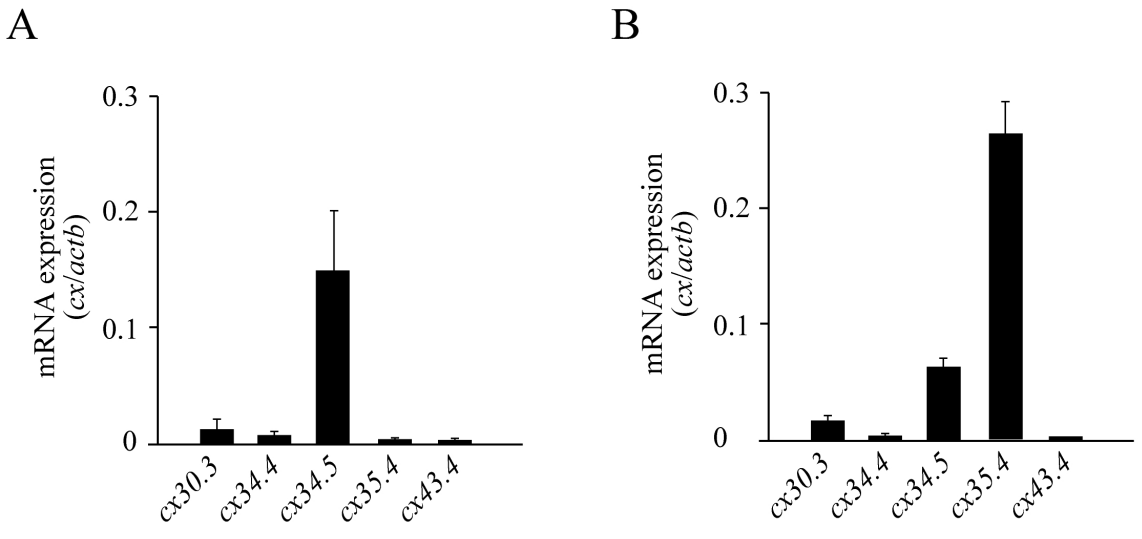
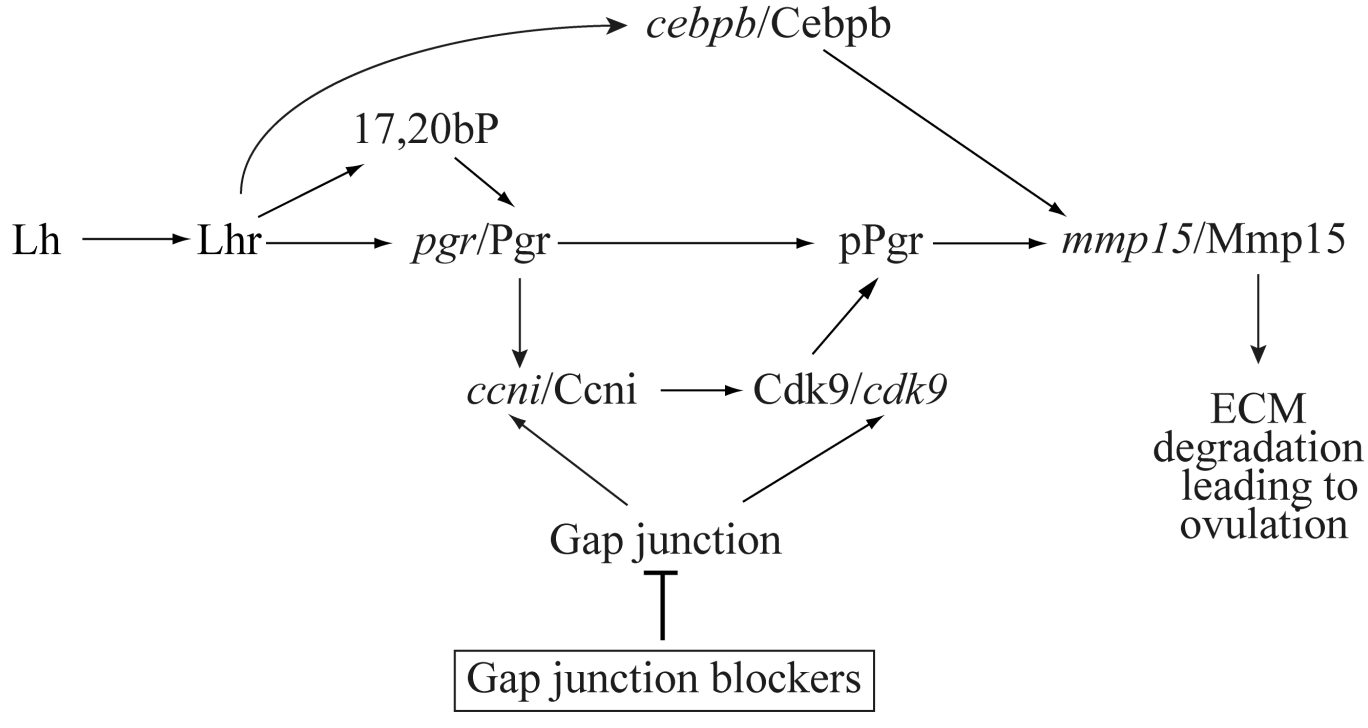

A
B
mRNA expression
(cx/actb)
0.3
0.2
0.1
0
cx30.3
cx34.4
cx34.5
cx35.4
cx43.4

Figure 9


cebpb/Cebpb
17,20bP
Lh
Lhr
pgr/Pgr
pPgr
mmp15/Mmp15
ccni/Ccni
Cdk9/*cdk9*
ECM degradation leading to ovulation
Gap junction
Gap junction blockers

Supplemental Table S1. Primers used in this study.

Primer name	gene	Sequence	Accession No.
Real-time PCR			
mmp15 ss	*mmp15*	5′-GTACACTTTTTTCTACAAGGGT-3′	AB072928
mmp15 as	*mmp15*	5′-CAAAGTCCAGTTCAAGTTCAGA -3′	
pai-1 ss	*pai1*	5′-CCCTTCAACCCCAAACTGACA-3′	AB571478
pai-1 as	*pai1*	5′-TCACATCGTAGTCCACCCCATC-3′	
EP4b ss	*Ptger4b*	5′-CAGATGGTGATCCTGCTCAT-3′	AB563504
EP4b as	*Ptger4b*	5′-GCCAGGAGGTCTTCATTGAT-3′	
Cx 30.3 ss	*cx30.3*	5′-ATGAGTTGGGATAAGCTCTACACC-3′	XM_004081622
Cx 30.3 as	*cx30.3*	5′-CCACTGTTCACACTTCACAA-3′	
Cx 34.4ss	*cx34.4*	5′-ACAAGCACTCCACAGCCTTC-3′	XM_004082640
Cx 34.4 as	*cx34.4*	5′-GGCGATGAAGCAGTCCACTG-3′	
Cx 34.5 ss	*cx34.5*	5′-CAAAGCCACTCCACAGTCATG-3′	XM_004083781
Cx 34.5 as	*cx34.5*	5′-ACATAGCGAGCTTCCAGAGTG-3′	
Cx 35.4 ss	*cx35.4*	5′-TTCCAAGCTCTCCTCAGTGG-3′	XM_004082639
Cx 35.4 as	*cx35.4*	5′-GGTGAAACCTCGCACTTGAC-3′	
Cx 43.4 ss	*cx43.4*	5′-AGGTGCCAACAGGGACTATG-3′	XM_011489951
Cx 43.4 as	*cx43.4*	5′-GCGGGAGAAGGAGGATCTAT-3′	
CDK9 ss	*cdk9*	5′-ATGCAACGAGACAAAACAAGCA-3′	XM_004086584
CDK9 as	*cdk9*	5′-TCGTACTTGGAGAACTCGTCGC-3′	
Cyclin I ss	*ccni*	5′-ATGAAGAGCCCAGGAGCCGCAG-3′	LC435346
Cyclin I as	*ccni*	5′-GATGTTGGGAAAAGGAGATGTC-3′	
ef1a ss	*eef1a1*	5’-CACCGGTCACCTGATCTACA-3’	AB013606
ef1a as	*eef1a1*	5’-GCTCAGCCTTGAGTTTGTCC-3’	
β-actin ss	*actb*	5′-TGACGGAGCGTGGCTACTC-3′	D89627
β-actin as	*actb*	5′-TCCTTGATGTCACGGACAATTT-3′	
ChIP			
Primer pair-1 SS for mmp15	*mmp15*	5′-GAAAGTCATGACGTCACTGG-3′	
Primer pair-1 AS for mmp15	*mmp15*	5′-GGCTCCGCTCCTCCAGAGCC-3′	
Primer pair-8 SS for mmp15	*mmp15*	5′- AGATATTGCAGTTGCTATACGG-3′	
Primer pair-8 AS for mmp15	*mmp15*	5′- TCATCTGTGTTCCTCTTTCAC-3′	
DNA pull-down			
5’-Mmp15-genome	*mmp15*	5′-AACAGACTTTAAGCAAAG-3′	
3’-Mmp15-genome	*mmp15*	5′-GGCTCCGCTCCTCCA-3′	