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Involvement of the nuclear progestin receptor in LH-induced expression of membrane type 2-matrix metalloproteinase required for follicle rupture during ovulation in the medaka, *Oryzias latipes*

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ABSTRACT

Hormonal regulation of the expression of Mmp15, a proteolytic enzyme indispensable for ovulation in the teleost medaka, was investigated. In an in vitro culture system using preovulatory follicles, Mmp15 expression and ovulation were induced in the presence of recombinant luteinizing hormone (rLh). Both rLh-induced Mmp15 expression and ovulation were 17α, 20β-dihydroxy-4-pregnen-3-one-dependent, suggesting the involvement of a nuclear progestin receptor (Pgr). In vitro follicle ovulation and Mmp15 expression were reduced by treatment with the Pgr antagonist RU-486. Like Pgr, the transcription factor CCAAT/enhancer-binding protein β (Cebpb) was induced by rLh. ChIP analyses indicated that Pgr and Cebpb bound to the *mmp15* promoter region. These results indicate that the rLh-induced expression of Mmp15 is mediated by Pgr and Cebpb. A differential timing of expression of Pgr and Cebpb in the preovulatory follicles appears to explain the considerably long time-lag from the *pgr* gene activation to *mmp15* gene expression.

1. Introduction

Ovulation denotes the shedding of one or more viable oocytes from fully grown ovarian follicles into the reproductive tract and is triggered by the binding of gonadotropin luteinizing hormone (LH) to the receptor in the follicle cells of the follicles destined to ovulate (Richards, 1994; Ma et al., 2004; Espey and Richards, 2006; Lubzens et al., 2010; Zhang et al., 2015; Takahashi et al., 2016). This process is accomplished through a highly regulated cascade of endocrine, morphological, and biochemical events (Espey and Richards, 2006). It is well known that follicle rupture in vertebrate ovulation involves proteolytic degradation at the apical region of ovulating follicles. In the teleost medaka, a variety of proteolytic enzymes are expressed in the follicles (Takahashi et al., 2013). Our previous studies demonstrated that the sequential actions of two distinct proteolytic enzyme systems, the plasminogen activator/plasmin (Plau/plasmin) system and the matrix metalloproteinase (Mmp) system, are required for the hydrolysis of extracellular matrix (ECM) proteins present in the follicle layers of ovulating follicles (Ogiwara et al., 2005, 2012, 2015). In the first step of ECM hydrolysis, active plasmin is produced by the proteolytic processing of liver-derived precursor plasminogen through the follicle-produced active plasminogen activator-1 (Plau1). Plasmin, thus activated, hydrolyzes laminin, a major ECM component constituting the basement membrane (Ogiwara et al., 2012, 2015). As a second step, another proteolytic system involving membrane type 1-Mmp (Mt1-mmp/Mmp14), membrane type 2-Mmp (Mt2-mmp/Mmp15), and gelatinase A (Mmp2) is activated. Mmp2, which is activated by Mmp14 and hydrolyzes type IV collagen, another principal component of the basement membrane, and Mmp15 degrade the type I collagen present in the theca cell layer (Ogiwara et al., 2005). Activation of the Plau/plasmin system and the Mmp system in the follicle is regulated by plasminogen activator inhibitor-1 (Ogiwara et al., 2015) and the tissue inhibitor of metalloproteinase-2b (Ogiwara et al., 2005), respectively. Interestingly, among the proteolytic enzymes involved in the process, Mmp15 alone was shown to be drastically induced with recombinant medaka Lh in the granulosa cells of ovulating follicles at the time of ovulation (Ogiwara et al., 2013). This suggests that Mmp15 may be an ovulation-related protein whose expression is under the control of LH in the fish. However, at present, it is not known how the *mmp15* gene expression would be controlled in the granulosa cells of the follicles that have undergone a surge of LH.

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The aim of the present study was to gain information on the mechanism underlying the LH-induced expression of *mmp15*/Mmp15 in the preovulatory follicles that are destined to ovulate. Our results indicate that the LH-induced expression of the *mmp15* gene is accomplished in two steps: Pgr is first induced by an LH surge, and the resulting Pgr subsequently may serve as a transcription factor for the expression of *mmp15* mRNA and protein. This study suggests that the transcription factor CCAAT/enhancer-binding protein β (Cebpb) is also involved in *mmp15* gene expression.

2. Materials and methods

2.1. Animals and tissues

Adult orange-red variant medaka, *Oryzias latipes*, were purchased from a local dealer and used for experiments. Maintenance and acclimation of the fish to the artificial reproductive conditions (14 h light and 10 h dark, 26–27 °C) were conducted as previously described (Hagiwara et al., 2014). Under these conditions, the fish ovulated every day around the transition time from the dark to the light period. In the present study, ovulation hour 0 was set to the start of the light period. Ovaries, ovarian follicles, and follicle layers of the follicles were isolated from the spawning female fish as previously described (Ogiwara et al., 2013). Animal cultures and experimentation 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 culture of dissected follicles

The culture was performed as previously described (Ogiwara et al., 2013). The outline of in vitro follicle culture is shown in Fig. 1. Follicles, which had not yet been exposed to the in vivo surge of LH, were isolated at 22 h before ovulation from ovaries of the fish with established 24-h spawning cycle and were cultured in 4 ml of 90% M199 medium containing 50 μM gentamycin (pH 7.4). The follicles were obtained from two to three fish ovaries, pooled, and then divided into control and test groups. The isolated follicles (approximately 20–25 follicles per group) were cultured with 100 μg/ml medaka recombinant LH (rLh), 25 μM trilostane (TLS, Sigma-Aldrich, St. Louis, MO), 1 μM 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20βP, Sigma-Aldrich), or 0.1 mM RU-486 (also known as mifepristone, Sigma-Aldrich). The duration of incubation and the time points when the follicles and/or follicle layers were collected for analysis of the expression levels of target genes are shown in Fig. 1. The rate of follicle ovulation was determined after the follicles were cultured for 30 h. Medaka rLh was produced in Chinese hamster ovary k-1 cells as previously described (Hagiwara et al., 2014).

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

Total RNA isolation, RT, and real-time PCR were conducted as previously described (Fujimori et al., 2011), except that a Thermal Cycler Dice® Real-Time *II* MRQ (Takara Bio. Osaka, Japan) was used for analyses. *Cytoplasmic actin* (*actb*) was used as a reference gene to normalize the expression of the target genes examined. The primers used in this study are listed in Supplemental Table S1.

2.4. Preparation of antigens and antibodies

A 858-bp cDNA coding for the complete Cebpb protein (286 residues) was amplified by RT-PCR using KOD Neo DNA polymerase (Toyobo, Tokyo, Japan) with ovary cDNA. The amplified product was phosphorylated and ligated into the vector pET30a (Novagen, Madison, WI), which had been previously digested by *Eco*RV. The protein antigen was produced in the bacterial expression system. Expression, purification, and dialysis of the protein were performed as previously described (Ogiwara and Takahashi, 2007). Anti-medaka Cebpb antibody was generated using mice according to the method previously described (Ogiwara et al., 2013). Recombinant Pgr was produced as described previously (Hagiwara et al., 2014) and used to immunize rats to obtain anti-medaka Pgr antibody. Mouse anti-medaka Pgr antibody (Hagiwara et al., 2014), rabbit anti-medaka Mmp15 antibody (Ogiwara and Takahashi, 2007), and rabbit anti-medaka Actb antibody (Ogiwara et al., 2012) were prepared as described previously. Antibodies were purified using an Immobilon polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA) membrane as described (Ogiwara et al., 2012), and the resulting purified antibodies were used for the experiments.

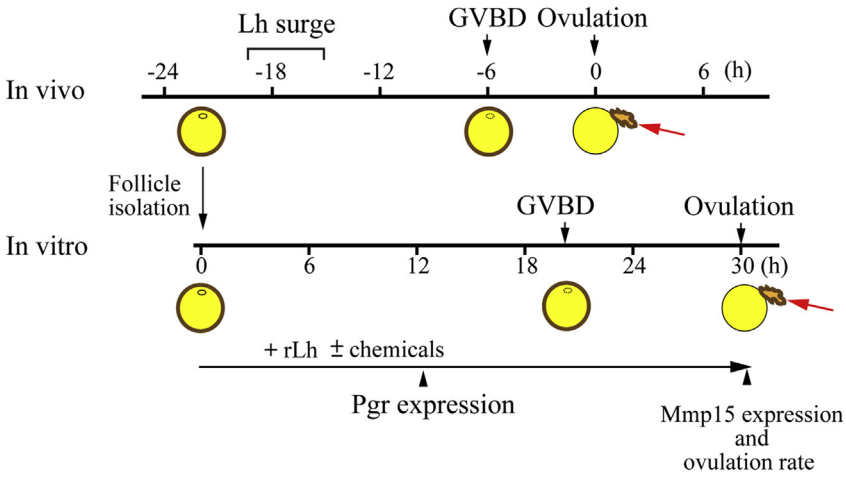

Fig. 1. An outline of the preovulatory follicle culture experiments carried out in this study. The follicles isolated from ovaries 22 h before ovulation were incubated in the presence of medaka recombinant LH (rLh) (100 μg/ml) with or without chemicals. The expected timing of the LH surge, GVBD, and ovulation in vivo and in vitro are shown. Red arrows indicate the follicle layers of ovulated follicles. Arrowheads indicate the time points of analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)