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[REVIEW]

Recent Advances in the Understanding of Teleost Medaka Ovulation: The Roles of Proteases and Prostaglandins

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Ovulation is the process of liberating oocytes from the preovulatory follicles, and is observed in the ovaries of virtually all female vertebrate animals. Compared with mammalian species, there have been far fewer studies that address the ovulatory mechanisms of non-mammalian species. We have examined the molecular mechanism of follicle rupture during ovulation using the teleost model, medaka, or *Oryzias latipes*. Follicle rupture in medaka ovulation involves the cooperation of the tissue inhibitor of metalloproteinase-2b protein with at least three matrix metalloproteinases (MMP): membrane type-1 MMP (MT1-MMP), MT2-MMP, and gelatinase A. Our studies also indicate that the serine protease, i.e., plasmin, participates in the rupture for only a few hours prior to the activation of MMP-mediated hydrolysis at ovulation. The involvement of prostaglandin E_2 (PGE_2) in medaka ovulation was also demonstrated. Cyclooxygenase-2 and PGE_2 receptor subtype EP4b were respectively shown to be an enzyme responsible for PGE_2 synthesis and a receptor for the generated ligand in the preovulatory follicles. Based on the results obtained from our studies of fish, we discuss the similarities and differences in vertebrate ovulation compared with mammalian species.

Key words: ovulation, LH, prostaglandin E_2, progesterone receptor, medaka, ovary

INTRODUCTION

Ovulation is a complex process that eventually results in the liberation of the oocytes from the preovulatory follicles that grow in the cortex of the ovary. This process is induced by a gonadotropin, i.e., luteinizing hormone (LH), which is delivered from the pituitary gland of vertebrates. In response to an LH surge, various changes occur in the follicular compartments of the preovulatory follicles; meiosis resumes in the oocyte to complete a series of events called oocyte maturation, while follicular cells surrounding the oocyte are activated to produce a variety of biologically active factors and proteins that are required for successful ovulation (Richards et al., 1998; Nagahama and Yamashita, 2008). The term “ovulation” is generally used for the entire process of follicular responses to LH, and the rupture of the follicle wall upon ovulation is one of the follicular responses (Tsafriri and Dekel, 1994). In this article, we follow these generally accepted concepts of “ovulation” and “follicle rupture”.

Historically, follicle rupture during ovulation in mammalian species was thought to be accomplished by the physical breakdown of the follicle wall in the apical region of the follicle due to increased intrafollicular pressure. However, this hypothesis was rejected in the early 1960s as it became clear that the follicles nearing ovulation do not experience significantly increased pressure (Espey and Lipner, 1994). The hypothesis that the rupture is a result of restricted proteolysis occurring at the apical region of the follicles has since gained support. Interestingly, the involvement of proteolytic enzymes in follicle rupture during ovulation was first suggested in 1916 (Schochet, 1916). Using mammalian species, a number of investigations addressing the roles of proteolytic enzymes in follicle rupture have since been conducted (Ohnishi et al., 2005; Espey and Richards, 2006; Curry TE and Smith, 2006). The results of these studies apparently indicate that proteases, especially matrix metalloproteinases (MMPs), play a role in follicle rupture during ovulation in mammals (Curry TE and Smith, 2006; Espey and Richards, 2006). However, studies of mice using gene knockouts of candidate proteases failed to demonstrate essential roles for these proteases in follicle rupture. Thus, it remains to be established whether proteases play indispensable roles in follicle rupture during ovulation in mammals. Nevertheless, our overall knowledge of mammalian ovulation has greatly advanced over the last several decades, and has aided in exploring the mechanisms that govern ovulation in non-mammalian vertebrates.

Over the past ten years, our research group has been studying ovulation using the teleost medaka as a non-mammalian vertebrate model. The aims of our study are 1) to understand to what extent the molecular mechanisms of ovulation may be conserved throughout vertebrates and 2)

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to approach important unsolved problems that are difficult to clarify using mammalian experimental systems. In the present review, we highlight the progress towards understanding follicle rupture during ovulation in medaka. We propose a "two-step extracellular matrix hydrolysis model," in which both matrix metalloproteinases and serine proteinases play critical roles in follicle rupture. In addition, the involvement of prostaglandin E_2 (PGE_2) and its receptor in follicle rupture during ovulation in fish are discussed.

Oocyte maturation and ovulation in medaka

The medaka, *Oryzias latipes*, is a small freshwater teleost that offers advantages for use in genetics, developmental and reproductive biology, physiology, and toxicology (Iwamatsu et al., 1988; Nagahama, 1994; Ozato et al., 1992; Ishikawa, 2000; Wittbrodt et al., 2002; Kasahara et al., 2007). In particular, this non-mammalian vertebrate species has emerged as a powerful tool for the elucidation of reproductive processes, including the molecular mechanisms of ovulation. The fish usually spawn daily within 1 h of the onset of light for a number of consecutive days when maintained at ambient temperature (26°C) under a constant long photoperiod of 14 h light and 10 h dark. Using this method, the sequence of events leading to spawning, such as the completion of vitellogenesis, germinal vesicle breakdown and ovulation, can be timed accurately (Iwamatsu, 1978). Previous studies have elucidated the endocrinological background behind such reproductive events (Sakai et al., 1987; Sakai et al., 1988). In addition, the large follicles that are to ovulate on the next day are demonstrated to undergo a surge of gonadotropin at approximately 15–21 h before the expected time of ovulation (Iwamatsu, 1978). In this fish, germinal vesicle breakdown (GVBD), a critical process for oocyte maturation, occurs approximately 6 h before ovulation in the follicle that is destined to ovulate in vivo (Iwamatsu, 1978).

In the medaka, the ovary is a sac-like organ surrounded by an outermost thin layer that separates the ovary from the body cavity. The body of the ovary is surrounded by the surface germinal epithelium and contains growing follicles of various sizes (Fig. 1). A space between the outermost thin layer and the germinal epithelium of the ovary, called the ovarian cavity, is formed. Upon in vivo ovulation, oocytes are released from the body of the ovary into the ovarian cavity. In this review, we use the term "in vivo ovulation" to refer to the release of oocytes from the ovary body into the cavity. As we describe below, ovaries isolated from medaka or large preovulatory follicles dissected from the body of the fish ovary are employed for in vitro ovulation experiments. The term "in vitro ovulation" is used for oocytes' detaching from the ovarian follicle.

Medaka in vitro ovulation model

In vitro ovulation experiments can be performed using not only whole ovaries (Ogiwara et al., 2010) but also large preovulatory follicles dissected from the ovaries of the spawning fish (Schroeder and Pendergrass, 1976; Ogiwara et al., 2005). In vitro follicle ovulation experiments under various conditions have been used in our laboratory and are summarized in Fig. 2. For the preovulatory

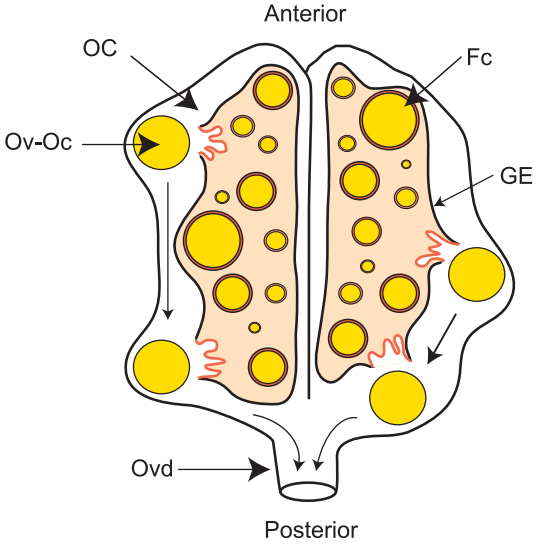

Fig. 1. Schematic representation of the medaka ovary. The medaka ovary is composed of apparently symmetric right and left parts that are connected in the center. The whole body of the ovary is wrapped by a thin layer that does not allow ovulated oocytes to escape out of the ovary. Ovulated oocytes move to the posterior cloaca and are eventually spawned. OC, ovarian cavity; Ov-Oc, ovulated oocyte, Fc, follicle; GE, germinal epithelium; and Ovd, oviduct.

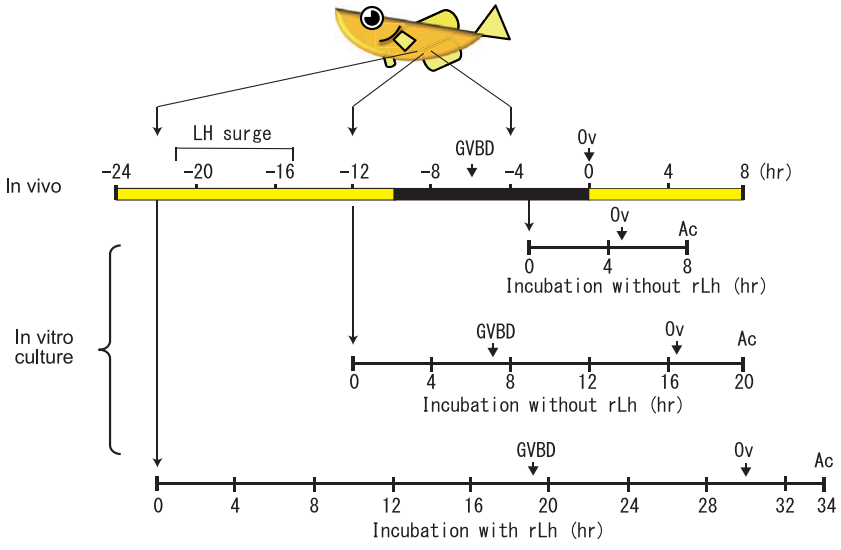

Fig. 2. In vitro culture methods established for medaka preovulatory follicles. Mature female medaka acclimated to artificial reproductive conditions (photoperiod, 10-h dark/14-h light; temperature, 27°C) ovulate in vivo on a 24-h cycle at the start of the light period. The timings of the LH surge and GVBD in vivo are also shown. In our in vitro follicle culture system, preovulatory follicles are isolated from the fish ovary 22, 12, or 3 h before the expected time of ovulation. Incubation of the follicles isolated 22 h before ovulation is conducted in the presence of recombinant medaka LH, while the follicles isolated 12 or 13 h before ovulation are incubated without recombinant medaka LH. For each in vitro incubation, the timings of GVBD and ovulation are indicated.

follicles that have undergone an LH surge in vivo between 15 and 21 h before ovulation (Iwamatsu, 1978), we often isolate the follicles 12 or 3 h before the expected time of ovulation. The follicles isolated in this manner spontaneously ovulate in vitro without requiring the addition of recombinant medaka LH to the culture medium. Compared with the in vivo situation, in vitro ovulation of the follicles takes a few more hours. Further, we have recently established an in vitro ovulation method for large preovulatory follicles isolated from the ovary prior to LH-priming in vivo (Ogiwara et al., 2013); the follicles successfully ovulate when cultured in the presence of recombinant medaka LH. In our in vitro follicle culture supplemented with recombinant medaka LH, we isolate the follicles from the ovary 22 h before ovulation, which is the time before the endogenous gonadotropin surge. These cultured follicles undergo GVBD and ovulation with a delay of approximately 3 and 8 h, compared with follicles that ovulate in vivo. Despite the delay in the timing of oocyte maturation and follicle ovulation in the LH-induced in vitro culture system, our in vitro follicle culture system has proven to be a useful experimental model for studying the ovulatory process in medaka.

To date, relevant in vitro methods using ovary fragments and ovarian follicles have been established for many teleost species. These species include zebrafish (Li et al., 1993; Liu and Ge, 2002; Lister and Van Der Kraak, 2010), Atlantic croaker (Patino and Thomas, 1990; Tubbs et al., 2010), rainbow trout (Bobe et al., 2004; Crespo et al., 2010), brook trout (Goetz et al., 1982), goldfish (Kagawa and Nagahama, 1981; Goetz, 1993), sea lamprey (Gazourian et al., 1997), Coho salmon (Luckenbach et al., 2010), European sea bass (Sorbera et al., 2001), and killifish (Raldua et al., 2005). These experimental models generally serve as good systems for studying oocyte maturation. However, in these teleost species, mature, healthy and intact oocytes cannot come off the follicle or ovarian fragments even if they have been primed by gonadotropins in vivo. To the best of our knowledge, the in vitro culture method using medaka preovulatory follicles is currently the only experimental system useful for both oocyte maturation and ovulation studies.

Follicle rupture by two-step ECM hydrolysis mechanism

There are clear differences in the tissue structures of ovarian follicles in mammalian and non-mammalian species. The large follicle in mammals consists of a round oocyte and two types of somatic cells: the granulosa cells and the theca cells. Some of the granulosa cells surround the oocyte and form the cumulus oocyte complex (COC), which protrudes toward the interior of an antrum filled with follicular fluid. The remaining granulosa cells are positioned just below the basement membrane in multiple cell layers known as the membrane granulosa. Theca cells, which are present on the outside of the basement membrane, also exist in multiple cell layers enriched with extracellular matrix (ECM) compo-

Table 1. Expression of proteolytic enzymes and the inhibitors in the medaka ovary.

Proteases and inhibitors	Expression	References
Matrix metalloproteinases		
MT1-MMP (MMP-14)	Oocytes of all growing follicles	Ogiwara et al., 2005
MT2-MMP (MMP-15)	Granulosa cells of peri- and postovulatory follicles	Ogiwara et al., 2005
MT5-MMP (MMP-24)	Oocytes of small growing follicles	Ogiwara et al., 2005; Kimura et al., 2001
Gelatinase A (MMP-2)	Oocytes of all growing follicles	Ogiwara et al., 2005; Matsui et al., 2000
Gelatinase B (MMP-9)	Granulosa cells of peri- and postovulatory follicles	Ogiwara et al., 2005; Matsui et al., 2000
Stromelysin-3 (MMP-11)	Oocytes of small growing follicles	Ogiwara et al., 2002
ADAMTSs		
ADAMTS-1	Oocytes of small growing follicles	Unpublished results
ADAMTS-20	Granulosa cells of peri- and postovulatory follicles	Unpublished results
Serine proteases		
Kallikrein-like enzyme	Granulosa cells of postovulatory follicles	Unpublished results
Trypsin	Not detectable	Ogiwara et al., 2007
Enteropeptidase	Oocytes of small growing follicles	Ogiwara et al., 2007
Urokinase-type plasminogen activator (uPA)	Oocytes of preovulatory follicles	Unpublished results
Tissue-type plasminogen activator (tPA)	Not detectable	
Plasminogen/plasmin	Precursor and activated enzyme, but not the transcripts, were detectable.	Ogiwara et al., 2012
Cysteine proteases		
Cathepsin L	Oocytes and granulosa cells of pre- and postovulatory follicles	Unpublished results
Cathepsin S	Oocytes of small growing follicles	Unpublished results
Protease inhibitors		
TIMP-2a	Granulosa cells of medium growing follicles	Ogiwara et al., 2005
TIMP-2b	Oocytes of preovulatory follicles	Ogiwara et al., 2005
TIMP-3	Granulosa cells of preovulatory follicles	Ogiwara et al., 2005
PAI-1	Granulosa cells of preovulatory follicles	Unpublished results
PAI-2	Localization not determined	Unpublished results

ADAMTS, a disintegrin and MMP domain with thrombospondin-like motifs- ; TIMP, tissue inhibitors of metalloproteinase- ; PAI, plasminogen activator inhibitor.

nents. In contrast, a common tissue structure observed in the large follicles of non-mammalian species consists of a single layer of granulosa cells surrounding an oocyte, a single layer of theca cells, and a basement membrane between the two somatic cell layers. No COC is formed during folliculogenesis of non-mammalian vertebrate animals. Despite what appear to be differences in the follicular tissue structure in mammalian and non-mammalian species, the degradation of ECM components present in the extracellular space of the follicle layer of the preovulatory follicle in a regulated manner is a common requirement for successful ovulation. Previous studies have established that collagen type I is abundantly present in the tunica albuginea and theca externa of fully grown follicles in mammalian ovaries (Espey, 1967), while collagen type IV is present in the basement membrane separating the granulosa and theca cell layers (Berkholtz et al., 2006; Lind et al., 2006). Our recent work and that of another laboratory using the teleost medaka (Horiguchi et al., 2008; Kato et al., 2010) and *Prochilodus argenteus* (Santos et al., 2008) indicate that, as in mammalian species, collagens type I and IV are localized in the theca cell layer and basement membrane of the large preovulatory follicle, respectively. Thus, in terms of ECM degradation associated with follicle rupture during ovulation, a similar, if not identical, mechanism involving proteolytic enzymes capable of hydrolyzing ECM components is highly expected to operate across the vertebrates.

A variety of proteolytic enzymes are expressed in the medaka ovary (Table 1). In our attempts to identify the proteolytic enzymes responsible for follicle rupture during ovulation, various protease inhibitors were tested in preovulatory follicles using the in vitro ovulation system. Serine protease inhibitors, such as aprotinin, leupeptin, antipain and soybean trypsin inhibitor, and MMP inhibitors, such as EDTA, o-phenanthroline and GM6001, strongly inhibited follicle ovulation in vitro (Ogiwara et al., 2005; Ogiwara et al., 2012), suggesting the involvement of two different proteolytic enzyme systems. Our detailed study demonstrated that the serine protease plasmin plays an indispensable role in follicle rupture during medaka ovulation (Ogiwara et al., 2012). Interestingly, plasmin participates in the rupture for only a few hours prior to the activation of MMP-mediated hydrolysis at ovulation. We have proposed a sequential two-step ECM hydrolysis mechanism for follicle rupture in medaka ovulation (Fig. 3). In the first step of ECM hydrolysis, which occurs approximately 5–7 h before ovulation, active plasmin is produced by the proteolytic processing of liver-derived precursor plasminogen in the preovulatory follicle that is destined to ovulate. More recently, we have found that active plasmin is capable of hydrolyzing laminin, a major ECM component constituting the basement membrane, in vivo as well as in vitro (Our unpublished results). Only a few hours of detectable active plasmin in the follicle suggests the presence of well-regulated mechanisms for plasminogen activation. Participation of a plasminogen activator inhibitor(s) in this process is highly likely. As a second step, approximately 0–3 h before ovulation, another proteolytic system involving MT1-MMP, MT2-MMP and gelatinase A is activated for further ECM degradation events. Gelatinase A, which is activated by MT1-MMP, hydrolyzes type IV collagen, a principle component of the basement membrane, and MT2-MMP degrades the type I collagen present in the theca cell layer (Ogiwara et al., 2005). The activation of gelatinase A by MT1-MMP occurs in the plasma membrane of the ovulating oocyte, and this activation process is regulated by the tissue inhibitor of metalloproteinase-2b (TIMP-2b) (Ogiwara et al., 2005).

To what extent are the follicle layers of periovulatory follicles degraded for successful ovulation? This value was estimated on the basis of morphological observations of the follicles that ovulated in vitro. In vitro follicle ovulation starts

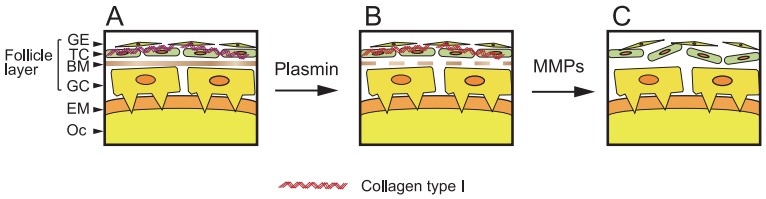

Fig. 3. A two-step ECM hydrolysis model of follicle rupture during ovulation in the medaka. In the preovulatory follicle, ECM proteins of the follicle layer are intact before proteolytic systems are activated **(A)**. At 5–7 h before ovulation, the urokinase-type plasminogen activator (uPA)/plasmin proteolytic system is activated to hydrolyze laminin, a major component of the basement membrane **(B)**. Subsequently, the MMP proteolytic system involving MT1-MMP, MT2-MMP and gelatinase A is activated. Gelatinase A activated by MT1-MMP hydrolyzes collagen type IV, another major ECM protein of the basement membrane, while MT2-MMP degrades collagen type I, the ECM protein residing in the GE/TC layer of the follicle (C). GE, germinal epithelium; TC, theca cell; BM, basement membrane; GC, granulosa cell; EM, egg membrane; and Oc, oocyte.

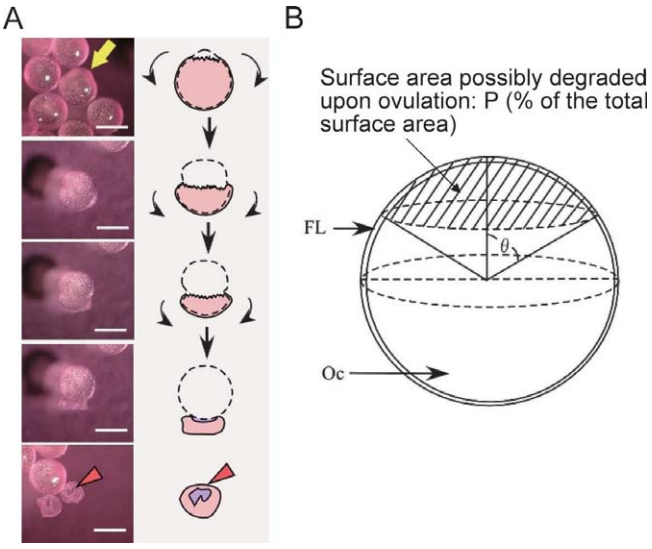

Fig. 4. Calculation of degraded surface area in follicles relative to the total surface area upon ovulation. **(A)** The process by which the oocyte of the ovulating follicle ruptures is shown. A yellow arrow indicates the site of follicle layer degradation. A red arrowhead indicates a hole remained in the follicle that had lost the oocyte by ovulation. Bars indicate 1 mm. A schematic representation of the process is also shown at the right. **(B)** The surface area possibly degraded upon in vitro follicle ovulation is represented by hatched lines. To calculate the hatched area size relative to the total surface area of the follicle, the equation $P = 50(1-\cos\theta)$ was used. The values of θ were experimentally determined. FL, follicle layer; Oc, oocyte.

with the appearance of a small hole on the surface of spherical follicles around the vegetal pole (Fig. 4A). The hole then enlarges with a concomitant appearance on the surface of the ovulating oocyte that is covered up with a thin layer of follicle cells before the start of ovulation. When the hole reaches a certain size, the oocyte frees itself from the follicle layer. The oocyte, which is just about to come off the follicle layer, becomes dumbbell-shaped, indicating that an extensive degradation of the follicle layer may not be necessary for in vitro medaka follicle ovulation. We determined the extent to which the follicle layer of ovulating follicles could be spatially deteriorated upon ovulation using the equation $P = 50(1-\cos\theta)$, where P is the percent of the degrading surface area relative to the total surface area (Fig. 4B). Values of θ could be determined by morphological observations of in vitro ovulating follicles. The θ values were found to be 54.6 ± 3.4 (the mean $\pm$ SEM of six independent determinations, $n = 6$), given that $P = 20.9 \pm 1.8$ ($n = 6$). These results indicate that follicle ovulation could occur by the dissolution of as little as 1/5 of the total ECM components in the layer of a fully-grown spherical follicle.

We have recently found that the treatment of preovulatory follicles prior to LH surge with recombinant medaka LH in the in vitro experimental system drastically induces the expression of MT2-MMP, but not gelatinase A, MT1-MMP, or TIMP-2b (our unpublished results). Our data also indicate that induction of MT2-MMP may be mediated by nuclear progesterone receptor (nPR) (Ogiwara et al., 2013).

INVOLVEMENT OF PROSTAGLANDINS IN OVULATION

Prostaglandins, prostaglandin synthesis, and the receptors in teleosts

Prostaglandins (PGs) play roles in a wide range of physiological processes (Simmons et al., 2004; Sugimoto and Narumiya, 2007). PGs are produced from arachidonic acid through the sequential actions of cyclooxygenase (COX) and specific PG synthases. Previous studies have established that COX plays a key regulatory role in PG synthesis. In mammals, two COX paralogs, a constitutive (COX-1) and inducible enzyme (COX-2), have been identified. In contrast, teleosts have additional copies of COX-1 and/or COX-2. For example, the medaka genome contains two COX-1 genes (*ptgs1a* and *ptgs1b*) and one COX-2 gene (*ptgs2*). This fact is thought to be the result of a teleost-specific genome duplication and subsequent genome loss event (Jarving et al., 2004; Ishikawa and Herschman, 2007; Ishikawa et al., 2007; Havird et al., 2008). It is generally accepted that PGs have a fundamental role in the mechanism of ovulation (Espey and Richards, 2006). Recent studies using mice lacking the gene encoding COX-2 or the PGE_2 receptor EP2 have elucidated the role of PGs in the process of cumulus oocyte complex (COC) expansion during ovulation (Hizaki et al., 1999; Richards et al., 2002).

As in mammalian species, ovarian PG synthesis in non-mammalian vertebrates is known to occur during spontaneous or artificially-induced ovulation. In some teleosts, indomethacin, which is a non-selective inhibitor of COX, has been reported to effectively block ovulation (Cetta and Goetz, 1982; Patino et al., 2003; Lister and Van Der Kraak, 2008). Other investigations have reported that PGs induce in vivo and in vitro ovulation (Jalabert and Szollosi, 1975; Stacey and Pandey, 1975; Goetz and Theofan, 1979; Kagawa and Nagahama, 1981; Goetz and Nagahama, 1985; Pankhurst, 1985; Kagawa et al., 2003; Lister and Van Der Kraak, 2008). The possible involvement of PGs in ovulation has also been documented for amphibians (Schuetz, 1986; Chang et al., 1995; Chang et al., 1997; Ramos et al., 2008; Sena and Liu, 2008). These previous studies strongly suggest that PGs have a conserved role in ovulation in vertebrates, including teleost fish. Generally, the COC is formed only for the grown ovarian follicles of mammalian vertebrates; the role of PGs in the expansion of the COC in preovulatory follicles is not applicable to non-mammalian vertebrate species.

In teleosts, the particular molecular species of PGs involved in ovulation appears to differ by species. $PGF_{2\alpha}$ and PGE_2 are the two major PGs that are thought to control fish ovulation (Stacey and Pandey, 1975; Goetz and Theofan, 1979). $PGF_{2\alpha}$ and its metabolite 15-keto-$PGF_{2\alpha}$ are well known to be postovulatory prostaglandin pheromones (Sorensen et al., 1988; Sorensen and Goetz, 1993; Stacey and Sorensen, 2002; Munakata and Kobayashi, 2010) that trigger female sexual behavior in a variety of externally fertilizing species. A close association between ovulation and $PGF_{2\alpha}$ was reported for rainbow trout (Jalabert and Szollosi, 1975), carp (Epler et al., 1985), brook trout (Goetz et al., 1982), and goldfish (Stacey and Pandey, 1975; Sorensen et al., 1988), while PGE_2 was found to play a dominant role in ovulation for yellow perch (Goetz and Theofan, 1979) and medaka (Fujimori et al., 2011; Fujimori et al., 2012).

The presence of $PGF_{2\alpha}$ and/or PGE_2 was demonstrated by direct measurement using the ovaries of zebrafish (Lister and Van Der Kraak, 2008; Lister and Van Der Kraak, 2009), yellow perch (Berndtson et al., 1989; Goetz, 1997), goldfish (Goetz, 1991), European sea bass (Sorbera et al., 2001), brook trout (Cetta and Goetz, 1982; Goetz, 1991), and medaka (Fujimori et al., 2011). Recently, the PGE_2 receptor subtypes EPs, EP1, EP2, EP3, and EP4, as well as a $PGF_{2\alpha}$ receptor (FP) from zebrafish (Villablanca et al., 2007; Kwok et al., 2012) were characterized. In addition, the expression of EP1, EP2, EP3, and EP4 transcripts in the medaka ovary was examined (Fujimori et al., 2011). Teleost fishes generally contain both EP and FP receptors, while medaka appears to contain only EP receptors. Indeed, our attempt to isolate the FP receptor using medaka tissues was not successful (our unpublished results). FP receptor sequences for zebrafish, fugu, tilapia, cod, coelacanth, and stickleback are available from the Ensembl Genome Database, whereas that of the medaka FP receptor is lacking in the database. Further, no sequence information for medaka PGF synthase, which is responsible for converting PGH_2 to $PGF_{2\alpha}$, is available from the Ensembl Database. This result may indicate that the medaka fish is incapable of producing $PGF_{2\alpha}$, thus lacking the $PGF_{2\alpha}$/FP signaling system. Further thorough investigations are required for determining whether the medaka possesses a $PGF_{2\alpha}$ receptor and/or PGF synthase, however.

Roles of prostaglandins in medaka ovulation

The indispensable role of PGs in medaka ovulation was demonstrated by the inhibition of in vitro follicle ovulation using culture medium containing indomethacin, a COX inhibitor, and GW627338X, an EP4 antagonist (Fujimori et

al., 2011). The medaka fish contains three COX genes, *ptgs1a*, *ptgs1b*, and *ptgs2*. Of these, *ptgs2* is expressed most abundantly in the ovary (Fujimori et al., 2011). During a 24-h spawning cycle, the *ptgs2* mRNA levels in the ovary are fairly constant. Consistent with this finding, ovarian PGE_2 levels do not fluctuate in the spawning cycle. This finding was rather surprising because, as established by previous studies using mammalian species (Espey and Richards, 2006) and teleost species (Grosser et al., 2002; Ishikawa et al., 2007; Lister and Van Der Kraak, 2009; Zou et al., 1999; Ishikawa and Herschman, 2007), the expression of COX-2 enzyme was reported to be inducible. As revealed by immunohistochemical analysis using a specific antibody against the medaka COX-2 protein, the follicle layer and oocyte cytoplasm of the large preovulatory follicle contain the protein (Fig. 5). The strongest signal was observed in the theca cells of the follicle, suggesting that the thecal cells predominantly produce PGE_2 in the follicles that are destined to ovulate. The EP4b receptor, a subtype of six medaka PGE_2 receptors, was expressed dominantly in the fish ovary, and transcripts of the PG receptor were expressed in the follicle cells of large preovulatory follicles. Further, EP4b receptor mRNA expression was drastically induced in the preovulatory follicles as ovulation approached (Fujimori et al., 2012). The expression of EP4b mRNA was inducible in vitro not only by pregnant mare serum gonadotropin (PMSG) (Fujimori et al., 2012) but also by recombinant medaka LH (our unpublished result). We have recently shown that the EP4b antagonist GW627368X completely abolishes the in vitro ovulation of large follicles even when added only 1 h before the time of ovulation (Fujimori et al., 2012). This result suggests that PGE_2 functions to induce the ovulation of large preovulatory follicles by binding to the EP4 receptor just before the time of ovulation. Further, this result suggests that PGE_2/EP4b signaling is required for fish ovulation at the time that follicle rupture occurs. More recently, we found that nuclear progesterone receptor (nPR) but not membrane progesterone receptor (mPR) is involved in the induction of EP4b expression (Hagiwara et al., unpublished results). Figure 6 shows a model for EP4b expression induced by LH in which the transcription factor nPR is implicated. In this model, we assume that the theca cells of the preovulatory follicle are mainly responsible for the production of PGE_2 and that granulosa cells are the cells expressing EP4b receptor at the time of ovulation.

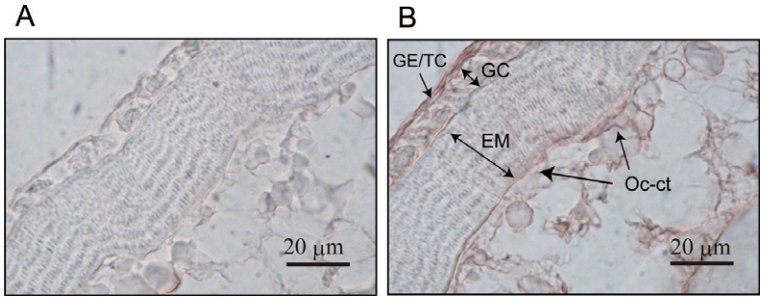

Fig. 5. Immunohistochemical localization of COX-2 in the preovulatory follicle of the medaka ovary. Paraffin sections (10 μm) of the mature female medaka ovary were incubated with normal mouse serum **(A)** or anti-medaka COX-2 serum **(B)**. Signals were detected using an AEC kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions. GE/TC, germinal epithelium/theca cell layer; GC, granulosa cell layer; EM, egg membrane of the oocyte; Oc-cy, oocyte cytoplasm.

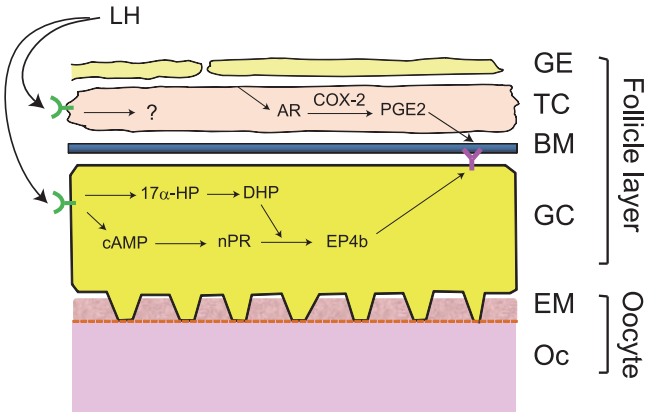

Fig. 6. Endocrine regulation of EP4b expression in the preovulatory follicle of the medaka. The LH surge results in the increased production of steroid hormone 17α-hydroxyprogesterone (17α-HP) via the activation of LH receptors on the granulosa cell (GC). 17α-HP is then converted to 17α, 20β-dihydroxyprogesterone (DHP). The gonadotropin surge simultaneously induces the expression of nuclear progesterone receptor (nPR) in the GC. nPR binds to DHP and acts as a critical transcription factor for EP4b gene expression. Translated EP4b receptor protein is expressed on the surface of GC in the follicle that is about to ovulate. The interaction of EP4b with PGE_2, which may be generated from arachidonic acid (AR) in the theca cell (TC), evokes an intracellular signal transduction reaction(s) in GC that eventually leads to ovulation. BM, basement membrane; GE, germinal epithelium; EM, egg membrane; Oc, oocyte.

Similarities and differences in ovulation between mammals and medaka

Previous studies on mammalian ovulation have revealed that proteases, prostaglandins, and progesterone are critically involved in the process (Espey and Richards, 2006). Compared with the sheer number of references arguing for their roles in mammalian ovulation, information on the roles of the compounds in ovulation of the teleost medaka is very limited. Nevertheless, existing evidence indicates that they are indispensable for medaka ovulation as well, although clear differences exist in the precise roles and mechanisms in ovulation of the compounds between mammals and medaka.

The potential roles in ovulation of many proteases have been studied using mammalian species. Three proteolytic enzyme systems, namely, plasminogen activator (PA)/plasmin, MMPs, and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) enzymes, have been targets of intensive studies. The results of these studies indicate that MMPs have a significant function in the degradation of the follicle wall (Espey and Richards, 2006; Curry TE and Osteen, 2003). The idea that the PA/plasmin system may be important for ovulation does not appear to be firmly supported (Ny et al., 1999; Leonardsson et al., 1995; Ny et al., 1997; Curry TE and Smith, 2006). Further, the implication of ADAMTS enzymes in ovulation remains to be investigated (Espey and Richards, 2006; Curry TE and Smith, 2006). In medaka ovulation, a sequential action of PA/plasmin and

MMPs is required for successful ovulation (Ogiwara et al., 2005; Ogiwara et al., 2012). A significant role of the PA/plasmin system is unique in fish ovulation because, as described above, recent evidence has argued against an essential role of the proteolytic enzyme system for follicle rupture during ovulation in mammals.

As in mammalian species, COX-2 is responsible for the generation of PGE_2 in the preovulatory follicles that are destined to ovulate in the medaka. However, a notable difference in the expression of COX-2 between mammalian species and the teleost medaka is their responsiveness to gonadotropins. In mammals, an LH surge or human chorionic gonadotropin treatment drastically induces the expression of COX-2 in ovarian granulosa cells and cumulus cells (Wong et al., 1989; Joyce et al., 2001; Sirois et al., 2004). In contrast, the medaka counterpart is constitutively expressed (Fujimori et al., 2011), and the expression levels are not affected by gonadotropins such as PMSG and recombinant medaka LH (our unpublished observation). Instead, PGE_2 receptor subtype EP4b is readily induced by the treatment of recombinant medaka LH. Thus, it has been concluded that the effect of PGE_2 on ovulation is regulated through the expression of EP4b receptor in the preovulatory follicles in the fish. Another clear difference in the role of PGE_2 in ovulation between mammalian species and the medaka is that this bioactive compound is involved in the process of COC expansion during mammalian ovulation, while it has a direct role in follicle rupture during fish ovulation.

Mammalian ovaries begin producing a significant amount of progesterone in response to an LH surge (Bahr, 1978; Goff and Henderson, 1979; Hubbard and Greenwald, 1982). An LH surge also induces the expression of nuclear progesterone receptors (nPRs) (Li and O'Malley, 2003). The synthesis of progesterone and nPR both take place in the follicle cells of the preovulatory follicles, and their association results in the formation of an active transcription factor that directly regulates the expression of a variety of ovulation-related genes (Li and O'Malley, 2003; Ellman et al., 2009; Robker et al., 2009; Sriraman et al., 2010). In the medaka, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) is the naturally occurring steroid hormone (Sakai et al., 1987; Fukuda et al., 1994) that functions as a maturation-inducing hormone (MIH). The levels of DHP in the fish ovary rapidly increase after an LH surge (Sakai et al., 1987). Emerging evidence suggests that DHP has a dual role in the preovulatory follicle; the steroid hormone is essential not only for oocyte maturation, but also for ovulation in the medaka. Drastically induced expression of nPR was observed in the follicle cells of the fish ovarian follicle after treatment with PMSG (Nagahama and Yamashita, 2008) or recombinant medaka LH (our unpublished results). Because the expression of the two ovulation-related proteins, MT2-MMP and EP4b, in the medaka preovulatory follicle appears to be closely related to nPR, the activation of nPR is likely a prerequisite for the transcription of ovulation-related genes in the fish. Our recent morphological observation that the LH receptor, but not the FSH receptor, is localized to the follicle cells of the large preovulatory follicles (Ogiwara et al., 2013) is consistent with the idea that, as for mammalian species, nPR-mediated gene expression of ovulation-related proteins occurs in the granulosa cells of the follicles nearing ovulation.

CONCLUSIONS

Significant progress has been achieved in improving our understanding of the control of teleost oocyte maturation in recent years (Nagahama and Yamashita, 2008; Lessman, 2009; Thomas, 2012). In contrast, information on the mechanism of ovulation for non-mammalian vertebrate species has been very limited. However, results from our recent studies using the teleost medaka have highlighted the mechanism of follicle rupture during ovulation. Using the fish model, we have determined the proteases and the inhibitor that are involved in the rupture and have elucidated their respective roles in the process. We have also clarified the involvement of PGE_2 in follicle rupture during fish ovulation. The accumulation of our knowledge of medaka ovulation has enabled us to consider the differences and similarities between the ovulatory process in mammalian and non-mammalian vertebrates at the molecular level. Although much has been learned about ovulation of the fish, much more remains to be solved. Areas of future study include the following: 1) defining the regulatory mechanisms of ovulation, particularly the LH-dependent induction mechanism of MT2-MMP and EP4b via the action of the transcription factor nPR and 2) determining the nature of the effect of PGE_2 on the follicle cells of ovulating follicles at the time of ovulation. Another exciting challenge is to unravel the mystery of how these two important biological processes, i.e., oocyte maturation and ovulation, are properly timed in the follicle that is destined to ovulate.

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