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The PGE₂/Ptger4b pathway regulates ovulation by inducing intracellular actin cytoskeleton rearrangement via the Rho/Rock pathway in the granulosa cells of periovulatory follicles in the teleost medaka

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Abstract

We have previously shown that the prostaglandin E₂/Ptger4b receptor system is involved in ovulation in teleost medaka and induces intracellular actin cytoskeleton rearrangement in the granulosa cells of preovulatory follicles. In this study, we investigated the signaling pathways through which prostaglandin E₂ induces a change in the actin cytoskeleton. Treating preovulatory follicles with GW627368X (Ptger4b antagonist), a Rho inhibitor, or Y-27632 [Rho-associated protein kinase (Rock) inhibitor] inhibited not only in vitro follicle ovulation but also intracellular actin cytoskeleton rearrangement. Active RhoA-c and Rock1 were detected in follicles immediately before ovulation. GW627368X also inhibited RhoA-c activation and cytoskeleton rearrangement. PGE₂-induced actin cytoskeleton rearrangement was not observed in the Ptger4b-, RhoA-c-, or Rock1-deficient OLHNI-2 cells. These results indicate that the PGE₂/Ptger4b pathway regulates intracellular actin cytoskeleton rearrangement via the Rho/Rock pathway in the granulosa cells of preovulatory follicles during medaka ovulation.

1. Introduction

Ovulation is a dynamic process of extrusion of mature oocytes from the interior of the ovary into the ovarian or peritoneal cavity, depending on the species (Espey and Richards, 2006; Lubzens et al., 2010), and is induced by the ovulatory surge of gonadotropin luteinizing hormone (LH). This process is closely coordinated by several intracellular and extracellular factors, proteins, and low-molecular-weight substances, including steroid hormones and prostaglandins (PGs). Previous studies have established that many genes/proteins are regulated and induced in the preovulatory follicles in response to LH stimulation, particularly in granulosa cells (GCs). These ovulation-associated genes/proteins have been studied intensively and the information from such studies aids in understanding the nature of LH-induced ovulation in vertebrates (Christenson et al., 2013; Espey and Richards, 2002; Espey and Richards, 2006; Gilbert et al., 2011; Lussier et al., 2017; Richards, 2007; Richards et al., 2015).

It is generally accepted that proteolytic degradation of the follicle wall extracellular matrix (ECM) components in the apical region of ovulating follicles is indispensable for successful vertebrate ovulation. However, the proteolytic enzymes responsible for follicle rupture have not yet been identified. In contrast, the mechanism underlying follicle wall degradation has been intensively studied in the teleost medaka. Sequential actions of two

distinct proteolytic enzyme systems, the urokinase-type plasminogen activator/plasmin system (Ogiwara et al., 2015) and the matrix metalloproteinase (MMP) system (Ogiwara et al., 2005), are required for the hydrolysis of ECM proteins present in the layers of ovulating follicles. Activation/regulation by intrinsic inhibitors and the roles of individual proteolytic enzymes involved in follicle rupture have been established (Takahashi et al., 2019). More recently, the signaling pathway of the LH-induced expression of MMP-15 (also known as MT2-MMP), one of the three MMPs required for follicle rupture during ovulation, has been elucidated (Ogiwara et al., 2021).

PGs are a group of biologically active lipid compounds that participate in various physiological processes in vertebrates. PGs have been implicated as important inducers of ovulation in many vertebrate species (Kim and Duffy, 2016; Richards, 1997; Sena and Liu, 2008; Shimada et al., 1986; Sorensen and Goetz, 1993; Takahashi et al., 2013). Mice deficient in cyclooxygenase-2, the rate-limiting enzyme in PG synthesis, exhibit an anovulatory phenotype (Davis et al., 1999). In addition, ovulation rate is reduced in mice lacking the PGE₂ receptor subtype EP2 (Hizaki et al., 1999, Kennedy et al., 1999, Tilley et al., 1999). These findings, together with those of ablate-and-replace experiments in nonhuman primates (Duffy et al., 2019), have established the role of PGE₂ and its receptor, EP2, in the ovulatory process in mammals. An interesting finding from experiments using

EP2-knockout mice is that the cumulus-oocyte complex (COC) is abortive in multiple ovulated complexes, indicating that the PGE₂/EP2 receptor system activated after LH stimulation also plays a role in COC expansion in periovulatory follicles (Hizaki et al., 1999). However, the mechanisms underlying PGE₂-induced ovulation are not yet fully understood. Accumulating evidence indicate a critical role of PGs in teleost ovulation. PGF_{2α} and/or PGE₂ play important roles in the ovulatory processes in fish (Takahashi et al., 2018). In vitro ovulation has been shown to be inhibited by indomethacin using yellow perch (Bradley et al., 1994), Japanese eel (Kagawa et al., 2003), zebrafish (Lister et al., 2008) and medaka (Fujimori et al., 2011). In medaka, in which PGE₂ regulates ovulation (Fujimori et al., 2011; Fujimori et al., 2012), the PGE₂ receptor subtype Ptger4b is drastically induced in an LH-dependent manner in large-sized follicles as ovulation approaches. Similar to medaka, studies have demonstrated that Pgr and Ptger4b have pivotal roles in zebrafish ovulation (Baker et al 2019; Baker and Van Der Kraak, 2021; Tang et al., 2016), favoring the possibility of a functional role of PGE₂ in ovulation across teleost species. Despite evidences supporting the contribution of PGE₂ to teleost ovulation, the mechanism by which PGE₂ promotes ovulation remains largely unknown. However, in this context, we previously reported that Ptger4b receptor activation by PGE₂ in the GCs of ovulating follicles of medaka progressively leads to actin depolymerization within

the cells as the time of ovulation approaches (Ogiwara and Takahashi, 2016), suggesting a role for the PGE₂/Ptger4b system in actin cytoskeleton remodeling in the GCs of follicles at follicle rupture during ovulation.

Dynamic remodeling of the actin cytoskeleton involves the physiological processes, such as cell migration, invasion, and metastasis (Yamaguchi and Condeelis, 2007). The remodeling is regulated by actin polymerization and depolymerization. Rho GTPases, such as Rac, Rho, and Cdc42, are the main regulators of remodeling and play pivotal roles in controlling actin cytoskeleton dynamics (Ridley, 2001). Regulators, such as cofilin, cortactin, LASP-1, Mena, AFAP-110, and profilin are also reported to be involved in controlling the actin cytoskeleton (Najm and El-Sibai, 2014; Yamaguchi and Condeelis, 2007). Some studies suggest that the reorganization of the actin cytoskeleton is regulated via the PGE₂/EP pathway in many cellular systems. Adhesion dynamics in dendritic cells are mediated via the PGE₂/EP pathway (van Helden et al., 2008). It is suggested that PGE₂/EP4 signaling mediates actin filament depolymerization in podocytes, resulting in the suppression of filtration barrier function in the cells (Martineau et al., 2004). PGE₂/EP signaling has been demonstrated to modulate the migration of dendritic cells by reorganizing the F-actin cytoskeleton (Diao et al., 2021). The above studies have also shown that cAMP acts as a signaling molecule downstream of the PGE₂ receptor.

To extend our hypothesis that the activated PGE₂/Ptger4b system in medaka follicles around the time of follicle rupture induces intracellular actin cytoskeleton rearrangement in follicle cells (Ogiwara and Takahashi, 2016; Takahashi et al., 2018), we examined the signaling pathway involved in the PGE₂/Ptger4b system that leads to cytoskeleton rearrangement during ovulation in medaka. Herein, we report a possible role for the Rho/Rho-associated protein kinase (Rock) pathway in actin rearrangement in the GCs of periovulatory follicles. Our results strengthen the notion that PGE₂ plays a pivotal role in intracellular actin filament rearrangement in the GCs of periovulatory follicles during ovulation in the fish.

2. Materials and methods

2.1. Medaka culture and tissue preparation

Orange-colored medaka fish (*Oryzias latipes*) variants purchased from commercial vendors were used in this study. The fish were kept in a 60-liter tank for at least 14 days to acclimate to 26 °C under a 14/10 h light/dark cycle (Ogiwara et al., 2005). Under these conditions, they spawn daily and ovulate immediately before the light period begins. In this study, the timing, called ovulation time, was set at ovulation hour 0. Preovulatory follicles destined to ovulate (≥ 1.0 mm, post-vitellogenic phase, stage IX-X) were isolated

(Ogiwara et al., 2013). The follicle layers were separated as previously described (Ogiwara et al., 2005). The experimental procedures used in this study were approved by the Committee of the Experimental Plants and Animals, Hokkaido University.

2.2. In vitro follicle culture and ovulation

In vitro follicle culture was performed as described previously (Ogiwara and Takahashi, 2019). Preovulatory follicles already exposed to the endogenous LH surge were isolated from the ovary 3 h before ovulation and cultured at 26 °C in 90% M199 medium (pH 7.4) with or without 10 µM GW627368X, 0.1-2.5 µg/mL Rho inhibitor (Cytoskeleton, Inc.; Denver, CO), 10 µM ML 141 (Tocris Bioscience; Ellisville, MO), 50 µM Rac1 inhibitor (Wako, Osaka, Japan), 1 µg/mL Rho activator (Cytoskeleton, Inc.), and 0.1-10 µM Y-27632 (Rock inhibitor, Wako). After culturing for 3, 4, 6, or 8 h, the follicles were collected and used for subsequent experiments. The ovulation rate was determined by counting the number of oocytes that successfully ovulated 8 h after incubation.

2.3. Cloning

As the nucleotide sequence of medaka *rock1* was different from that currently available from the National Center of Biotechnology Information (NCBI) database

(<https://www.ncbi.nlm.nih.gov/>), the gene was subjected to cDNA cloning. The nucleotide sequence of the *rock1* coding region was determined from follicle cDNA using reverse transcription-polymerase chain reaction (RT-PCR) with KOD Fx DNA polymerase (Toyobo; Osaka, Japan). The primers used were Rock1 pET SS and Rock1 cds-AS (Supplementary Table S1). The PCR products were phosphorylated, gel-purified, and ligated into pBluescript SK vector (Agilent Technologies; Santa Clara, CA). The nucleotide sequence of the resulting vector was confirmed by sequencing. The determined sequence was deposited in the DDBJ/GenBank/NCBI database (Accession number: LC726226).

2.4. Reverse-transcription and real-time PCR

Total RNA isolation, and real-time quantitative RT-PCR (qRT-PCR) were performed as previously described (Ogiwara and Takahashi, 2016). The primer pairs used are listed in Table S1. To determine the expression level, KOD SYBR qPCR Mix (Toyobo) or KAPA Fast qPCR Kit (Nippon Genetics Co., Ltd.; Tokyo, Japan) was used. To normalize the transcript levels of the target genes, we tested the housekeeping genes, such as cytoplasmic actin (*actb*), 18S rRNA (*rn18s1*), ribosomal protein L7 (*rpl7*), and elongation factor 1a (*eef1a*). The most stably expressed gene in the examined tissues was *eef1a*;

therefore, *eef1a* mRNA expression was used for normalization. The qPCR threshold cycle (Ct) value was automatically determined using the instrument' software (Thermal Cycler Dice, Real-Time System II software Ver.5.1.1, TAKARA BIO Inc., Shiga, Japan). The Δ Ct value (Ct value of the reference sample minus Ct of the sample minus) was calculated, and $2^{\Delta Ct}$ was calculated. The relative expression data were generated using the resulting values.

2.5. Antibody preparation

Recombinant antigens were produced using *E. coli*. expression system. The coding regions of Rhoa-a and Rhoa-c, or the partial coding region of Rock1 were amplified using PCR with KOD Fx Neo DNA polymerase (Toyobo), and the primer pairs are listed in Table S1. The PCR products were ligated into the pET 30a vector (Novagen; Madison, WI), and the nucleotide sequences were confirmed by sequencing. The recombinant proteins were expressed and purified as previously described (Ogiwara and Takahashi, 2007). The purify of the purified proteins was confirmed using SDS-PAGE. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue (CBB) in 30% methanol, 10% acetic acid, and 40% H₂O. After staining for 20 min, the gel was destained with 30% methanol, 10% acetic acid, and 40% H₂O. Specific antibodies were produced

in mice (Ogiwara et al., 2013) and purified (Ogiwara et al., 2012), according to previously described methods. Mouse anti-medaka Rpl7 antibody was prepared as previously described (Ogiwara and Takahashi, 2019).

2.6. Tissue extract preparation, immunoprecipitation (IP), and western blot analysis

Follicle extract for IP was prepared from 30 ovarian follicles per sample as previously described (Ogiwara et al., 2021). The follicle layer extract was prepared as previously described (Ogiwara et al., 2005). The protein concentration was determined using a BCA kit (Thermo Fischer Scientific, San Jose, CA). IP and western blotting were performed as previously described (Ogiwara et al., 2021), except that anti-medaka Rhoa-c or anti-medaka Rock1 antibodies were used. We examined the protein expression of Actb, Glyceraldehyde-3-phosphate dehydrogenase, and Rpl7 to select an appropriate protein as a reference using the follicle layer and preovulatory follicle extract. The most stably expressed protein in the examined tissues was Rpl7; therefore, Rpl7 was used as the reference.

2.7. Primary granulosa cell (pGC) preparation

GCs were isolated from -1 h follicles, and pGCs were prepared according to a previously

described method (Ogiwara and Takahashi, 2016).

2.8. Immunohistochemistry

Paraffin sections (5 µm thick) were prepared as previously described (Ogiwara et al., 2015). The sections were dewaxed twice in xylene for 10 min and then placed in 99% ethanol for 2 min. The samples were hydrated using a graded ethanol series and then rinsed with pure water for 5 min. The specimens were then placed in phosphate-buffered saline (PBS) for 2 min and incubated in PBS containing 3% H₂O₂ for 10 min. The sections were then incubated in PBS containing 1% bovine serum albumin (Wako) at room temperature for 60 min and with anti-medaka Rhoa-c or Rock1 antibody diluted in PBS at room temperature for 60 min. They were then washed three times with PBS for 20 min and incubated with a DAKO envision mouse (Agilent Technologies Inc.) for 60 min. After washing three times with PBS for 20 min, the signal was detected using the ImmPACT™ AEC HRP Substrate kit (Vector Laboratories, Burlingame, CA). As a negative control, the primary antibody and its antigen (20 µg) were diluted in PBS, preincubated for 16 h at 4°C, and used for immunohistochemistry.

2.9. Active Rhoa-c detection

Active medaka Rhoa-c was detected using a RhoA Pull-Down Activation Assay Biochem Kit (Cytoskeleton Inc.) according to the manufacturer's instructions. For sample preparation, follicle layers from preovulatory follicles or follicles cultured with chemicals were homogenized in the cell lysis buffer supplied in the kit. The sample was then centrifuged at 12,000 ×g for 10 min, and the resulting supernatant was used for the assay. Active Rhoa-c was detected by western blot analysis using an anti-medaka Rhoa-c antibody.

2.10. *rhoa-c*, *rock1*, or *ptger4b* knockout in OLHNI-2 cells

The knockout was performed using CRISPR/Cas9-mediated genome editing according to a previous method (Ogiwara et al., 2021). Briefly, the oligonucleotide pair listed in Table S1 was annealed and ligated to pDR274, and a medaka caudal fin cell line (OLHNI-2) derived from the HNI strain, which was obtained from the RIKEN BioResource Center (Tsukuba, Japan), was co-transfected with the vector and Cas9 nuclease expression vector carrying a hygromycin B resistance gene. After hygromycin B selection, the cells were used for the experiments.

2.11. G-/F-actin detection

The G-/F-actin ratio was measured as previously described (Ogiwara and Takahashi, 2016). Briefly, OLHNI-2 cells and follicle layers from preovulatory follicles or follicles cultured with chemicals were gently homogenized in an actin stabilization buffer to prepare the G-actin and F-actin fractions. The samples were then subjected to western blot analysis. The actin bands were detected using an anti-medaka Actb antibody.

2.12. Statistical analysis

To validate our results, all experiments were repeated 3-8 times. The data collected were subjected to statistical analysis using Excel software, and the mean of 3-8 replicates was evaluated as the mean $\pm$ standard error of the mean. Significance was determined using Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's post hoc test, as appropriate, and presented as $P < 0.05$ (*) or $P < 0.01$ (**). Equal variation was confirmed using the F-test or Bartlett's test as appropriate. For western blotting and immunohistochemical analyses, at least three separate experiments were performed to confirm the reproducibility of the findings, and the representative results of all experiments are shown.

3. Results

3.1. Rho inhibitor inhibits in vitro ovulation in medaka

Our previous study reported that Ptger4b activation did not activate or inhibit adenylate cyclase in the OLHNI2 cell line; thus, Ptger4b is strongly suggested to be coupled with G12/13 or Gq/G11 to activate the Ptger4b signaling pathway (Takahashi et al., 2018). This possibility was examined using in vitro follicle culture. It is well established that G12/13 proteins can couple with G-protein-coupled receptors to activate RHO by regulating the activity of RhoGTPase nucleotide exchange factors (Siehler, 2009; Vogt, 2003). We first examined whether RHO family protein(s), including RHO, CDC42, and RAC1 (Tapon and Hall, 1997), are involved in medaka ovulation. When -3 h follicles were cultured with or without each RHO family member inhibitor, in vitro ovulation was significantly inhibited by the RHO inhibitor (Fig. 1A). No significant inhibitory effects were observed in follicles treated with the CDC42 inhibitor ML141 or the RAC1 inhibitor. Ovulation was blocked in a dose-dependent manner by an RHO inhibitor (Fig. 1B). Incubating -3h follicles with GW627368X, an EP4 antagonist, strongly inhibited in vitro ovulation, which was ameliorated by adding the RHO activator (Fig. 1C). These results indicate that Rho acts in the Ptger4b signaling pathway and Rho activation is required for successful medaka ovulation.

3.2. Rho mRNA and protein expression in preovulatory follicles during a 24 h spawning cycle

As Rho is an important factor in the Ptger4b signaling pathway and medaka ovulation, a medaka genome database was searched using the Ensembl genome browser (Ensembl database, <https://www.ensembl.org/index.html>) to identify the *rho* gene(s) responsible for the Ptger4b signaling pathway. Six *rho* genes, *rhoa-a*, *rhoa-b*, *rhoa-c*,

rhob, *rhog*, and *rhov*, were identified. Real-time RT-PCR analysis of mRNAs of these genes revealed that all the RNAs were present, with the highest expression for *rhoa-c* and the second highest for *rhoa-a* in the follicle layers of the -3 h follicles (Fig. 2A). *rhoa-a* and *rhoa-c* mRNA expression patterns in the preovulatory follicle during a 24 h spawning cycle were examined. Both mRNAs were constitutively expressed in the follicles (Fig. 2B and C).

To detect Rho proteins in preovulatory follicles, we generated a mouse polyclonal antibody raised against medaka Rho-a and Rho-c. Recombinant medaka Rho-a and Rho-c were produced in an *E. coli* expression system and used separately as antigens for immunization. The antibody cross-reacted with the respective antigens. However, the antigen was not recognized by the absorbed antibody, indicating that the antibodies were specific (Fig. 2D). The anti-Rho-c antibody detected the protein in the preovulatory follicles; however, no bands were detected with the anti-Rho-a antibody (data not shown). We further examined the expression of Rho-c in preovulatory follicles during a 24 h spawning cycle. The corresponding proteins associated with the preovulatory follicles were detected by IP and western blotting using an antibody. The antibody constitutively detected Rho-c, irrespective of when the extracts were prepared from intact preovulatory follicles in the 24-h spawning cycle (Fig. 2E). Western blot analysis revealed that Rho-c was expressed in GCs (Fig. 2F).

Immunohistochemical experiments using fish ovary sections collected 3 h before ovulation were conducted to determine Rho-c localization, which showed the highest mRNA expression in the -3 h-follicles in PCR analysis. Rho-c was localized in the follicle layer of the largest or post-vitellogenic follicles (stage X; Fig. 2G, arrows and asterisks) and was also expressed in the follicle layer of follicles larger than 150 μ m,

which were in an early-, late-, or post-vitellogenic phase at stages V–IX (Fig. 2 G, arrowheads). No signal was observed in the section stained with a pre-adsorbed antibody/antigen complex, which was the antibody preincubated with its antigen (absorbed antibody), indicating that the signal was specific (Fig. 2 G, lower panel). These results indicate that Rhoa-c is expressed in the follicle layers of both vitellogenic and post-vitellogenic follicles. These results also indicate that the Rho protein plays a role in PGE₂/Ptger4b signaling in follicle layers.

3.3. Active Rhoa-c detection in follicles about to ovulate

To examine whether the PGE₂ pathway mediates Rho activation in medaka preovulatory follicles, a pull-down assay was conducted to detect active Rho in the follicles. Active Rhoa-c was pulled-down from the extracts and the precipitating Rhoa-c was detected by western blot analysis. Active Rhoa-c was detected in the -3 and -1 h follicles but not in the -6 h follicles (Fig. 3A). Rhoa-c was activated after incubating the -3 h follicles with PGE₂ in vitro, which was inhibited by GW627368X. However, this inhibitory effect was abolished by the addition of the Rho activator (Fig. 3B). No inhibitory effects were observed when the follicles were cultured with Rho or Rock inhibitors. These results indicate that Rhoa-c is activated downstream of Ptger4b.

3.4. Rock involvement in medaka ovulation

Rho exerts its biological effects by binding to its downstream effector molecules including Rock, an important downstream factor. As active RhoA-c was detected in follicles about to ovulate, we examined whether Rock might be involved in medaka ovulation. In vitro ovulation was significantly suppressed in a dose-dependent manner after culturing preovulatory follicles with the Rock inhibitor, which was not abolished after culturing the follicles with both the Rock inhibitor and Rho activator (Fig. 4A, B). To predict the timing of Rock activation, we examined the in vitro ovulation rates after exposing the follicles to the inhibitor for various time periods (Fig. 4C). Ovulation was strongly inhibited when the follicles were cultured in the presence of Rock inhibitor for 8 h (-3 to +5 h), 7 h (-2 to +5 h), and 6 h (-1 to +5 h). Exposure of the follicles to the inhibitor for 1 h (-3 to -2 h) or 2 h (-3 to -1 h) did not inhibit ovulation significantly. The above results suggest that Rock is involved in medaka ovulation, and that kinase activity is required for successful ovulation between -1 h and 0 h.

3.5. Rock mRNA and protein expression in the preovulatory follicles during 24 h spawning cycles

A database search for medaka *rock* genes using the Ensembl genome database revealed that the fish possessed three *rock* genes: *rock1*, *rock2a*, and *rock2b*. Among these *rock* genes, *rock1* expression levels were highest in the follicle layers of -3 h follicles (Fig.

5A). Therefore, *rock1* was further analyzed by studying *rock1* mRNA expression in preovulatory follicles using the total RNA extracted from intact follicles obtained from the ovaries at various time points during the 24-h spawning cycle (Fig. 5B). The *rock1* transcript levels were almost constant in the preovulatory follicles during the spawning cycle.

A mouse polyclonal antibody for recombinant medaka Rock1 was prepared and its specificity was confirmed (Fig. 5C). Western blot analysis was performed using follicle layer extracts to detect Rock1 protein. The corresponding protein associated with the follicle layer of the preovulatory follicles was detected using the antibody at position 158 kDa in all the extracts examined, corresponding to the size predicted for the medaka Rock1 protein (Fig. 5D). Western blot analysis revealed that the Rock1 protein was expressed in the GCs (Fig. 5E). In the immunohistochemical analysis of the fish ovary sections 3 h before ovulation, the medaka Rock1 antibody detected positive signals associated with the follicle layer of the largest follicle (stage X) (Fig. 5F, arrows and asterisks). Signals were also detected in the follicle layer of follicles larger than 150 μ m, corresponding to the early-, late-, or post-vitellogenic phases (stages V–IX) (Fig. 5 F, arrowheads). In stage X follicles, positive signals for Rock1 protein were localized in GCs (Fig. 5 F, lower right panel). No signal was observed in the section stained with a

pre-adsorbed antibody/antigen complex, which was the antibody pre-incubated with the antigen (absorbed antibody) (Fig. 5 F, upper right panel).

3.6. PGE₂ modulates actin filament polymerization in the follicle cells of follicles about to ovulate via the Rho/Rock pathway.

Our previous studies suggested that the PGE₂/Ptger4b signaling cascade is involved in intracellular actin cytoskeleton rearrangement in follicle cells of follicles about to ovulate (Ogiwara and Takahashi, 2016; Takahashi et al., 2018). To examine whether the Rho/Rock pathway is involved in actin filament rearrangement, we analyzed the actin cytoskeleton polymerization status in follicle cells of follicles cultured with reagents that affect the Rho/Rock pathway. The G-/F-actin ratio increased significantly when follicles were cultured without any inhibitor. This increase was inhibited by GW627368X; however, this inhibition was abolished by adding the Rho activator together with the antagonist. The G-/F-actin ratio did not increase in follicles treated with Rho or Rock inhibitor (Fig. 6A).

OLHNI-2 cells, which express Ptger4b, Rhoa-c, and Rock, were treated with PGE₂ to examine the effects of these proteins on changes in rearrangement (Fig. 6B). The G-/F-actin ratio significantly increased at 6 h after PGE₂ treatment. Next, we analyzed the ratio of Rhoa-c-, Rock1-, and Ptger4b-deficient OLHNI-2 cells. No bands for Rhoa-c or Rock1 were detected in the knockout cells (Fig. 6C). We could not examine Ptger4b protein expression in knockout cells, because a Ptger4b-specific antibody was not available. The G-/F-actin ratio was significantly increased in PGE₂-treated cells, but not in PGE₂-treated knockout cells (Fig. 6D).

These results indicate that PGE₂/Ptger4b and its downstream Rho/Rock system are involved in intracellular actin cytoskeleton rearrangement.

4. Discussion

We previously reported that the PGE₂/Ptger4b system is involved in medaka ovulation (Fugimori et al., 2011; Fugimori et al., 2012) and that PGE₂ exerts its effects through Ptger4b between -1 and 0 h of ovulation (Fugimori et al., 2012). Moreover, we have previously suggested that PGE₂/Ptger4b-mediated intracellular actin cytoskeleton rearrangement in follicles about to ovulate is important for medaka ovulation (Ogiwara and Takahashi, 2016; Takahashi et al., 2018). This study was conducted to further substantiate the role of the PGE₂/Ptger4b system in follicle ovulation and clarify the signaling cascade of the system. The results of this study strengthen the notion that intracellular actin cytoskeleton rearrangement in the GCs of ovulating follicles is necessary for successful ovulation in medaka. This study also revealed that PGE₂/Ptger4b system activation-induced changes in the cytoskeleton during ovulation are mediated via the Rho/Rock pathway.

The rationale for our conclusion that PGE₂/Ptger4b induces intracellular actin cytoskeleton rearrangement via the Rho/Rock pathway in the GCs of preovulatory follicles at ovulation is as follows: (i) RhoA-c and Rock1 proteins were expressed in the

GCs of preovulatory follicles (Fig. 2F and 5E). (ii) Rhoa-c was activated in follicle cells of follicles immediately before ovulation (Fig. 3). (iii) The G-/F-actin ratio increased in the GCs of the follicles immediately before ovulation, and at or around the time of ovulation in the control follicles. In contrast, treatment of preovulatory follicles with a Ptger4b antagonist, Rho inhibitor, or Rock inhibitor suppressed in vitro follicle ovulation and inhibited the increase in the G-/F-actin ratio (Fig. 1, 4, and 6). (iv) Rhoa-c was activated and the G-/F-actin ratio increased during control follicle ovulation; however, opposite results were noted in both events after GW627368X treatment. GW627368X-mediated ovulation inhibition was abolished by further treatment with Rho activator (Fig. 1, 3, and 6). (v) The G-/F-actin ratio significantly increased in control OLHNI-2 cells following treatment with PGE₂. However, this PGE₂-induced G-/F-actin ratio increase was not detected in Ptger4b-, Rhoa-c-, or Rock1-deficient cells (Fig. 6). Based on these results and our previous findings (Ogiwara and Takahashi, 2016), we proposed a pathway for the induction of actin cytoskeleton rearrangement. PGE₂, secreted by GCs (Ogiwara and Takahashi, 2016; Takahashi et al., 2018), activates Rhoa-c. Currently, the pathway through which active Rock1 causes intracellular actin cytoskeleton rearrangement remains unknown. In mammals, the LIM-Kinase (LIMK)/cofilin pathway (Venessa T Chin et al., 2015), myosin light chain kinase (MLCK) (Sharanek et al., 2016), and ERM

(ezrin, radixin, moesin) proteins (Jiao et al., 2017), are reported to be activated by the Rho/Rock pathway to regulate intracellular actin cytoskeleton rearrangement. We examined whether these factors are involved in PGE₂-induced intracellular actin cytoskeleton rearrangement in medaka ovulatory follicles. However, in vitro follicle ovulation was not significantly inhibited when follicles were cultured with LIMK or MLCK inhibitors (data not shown). Ogiwara and Takahashi (2016) reported that moesin A is involved in the regulation of intracellular actin cytoskeleton rearrangement. In the present study, GW627368X did not inhibit moesin A phosphorylation (data not shown), suggesting that moesin A is not a downstream target of Rock1 (Ogiwara and Takahashi, 2016). We speculate that there is a novel pathway for inducing actin filament rearrangement that involves factor(s) other than LIMK/Coffin, MLCK, and ERM proteins downstream of Rock1 in medaka ovarian follicles.

Pendergrass and Schroeder (1976), Jalabert and Szöllösi (1975), and Trubnikova (2003) reported that cytochalasin B, an actin polymerization inhibitor, inhibits ovulation in medaka, trout, and sturgeons, respectively. Thus, actin cytoskeleton rearrangement accompanying ovulatory processes may be common among teleosts. However, the mechanism underlying cytochalasin-B-mediated teleost ovulation inhibition remains unclear. However, the results reported by Ogiwara and Takahashi (2016) and those of the

present study provide information that answers this long-standing question. An alteration in the extent of actin polymerization within follicle cells, particularly in GCs, is required for LH-induced ovulation; therefore, a disturbance of actin cytoskeleton rearrangement by cytochalasin B treatment most likely causes anovulation. Studies on follicle rupture during ovulation in medaka have established that successful ovulation is assured by the activation of two events induced after the LH surge: ECM protein hydrolysis in the follicle layers of ovulating follicles (Ogiwara et al., 2005; Takahashi et al., 2019) and actin cytoskeleton rearrangement in GCs (Fujimori et al., 2011; Fujimori et al., 2012; Hagiwara et al., 2014; Ogiwara and Takahashi, 2016). The removal of ECM proteins surrounding the GCs by proteolytic enzymes and changes in the intracellular actin cytoskeleton in GCs disintegrates cell-cell interactions in GCs, oocytes, and neighboring GCs. This probably increases GC mobility, facilitating the formation of openings in the apical region through which the oocyte can escape from the periovulatory follicles.

In summary, the results of this study revealed that actin cytoskeleton rearrangement is regulated by the PGE₂/Ptger4b system via the Rho/Rock pathway. Although several studies have implicated intracellular actin cytoskeleton in teleost ovulation, its physiological relevance remains unclear. This study is the first to report that Rhoac/Rock1 regulates actin cytoskeleton rearrangement during ovulatory processes in

medaka. Further studies should focus on identifying additional factors involved in PGE₂-induced actin cytoskeleton rearrangement during ovulation to fully understand the signaling pathways and regulatory mechanisms of this process.

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674 Figure legends

675 **Fig. 1. Effects of RHO inhibitor, ML141, and RAC inhibitor on in vitro ovulation in**
676 **preovulatory follicles.** (A) The -3 h follicles were cultured for 8 h in the presence of Rho
677 inhibitor (Rho-i), ML 141, or Rac1 inhibitor (Rac1-i), and the ovulation rates were
678 determined. **P<0.01 (ANOVA and Dunnett's post hoc test, N =6). (B) The -3 h follicles
679 were cultured with Rho inhibitor at the indicated concentration. The ovulation rates were
680 determined after incubating for 8 h. *P<0.05, **P<0.01 (ANOVA and Dunnett's post hoc
681 test, N=6). (C) The -3 h follicles were cultured with GW627368X (GW) or both GW and
682 Rho activator (Rho-act). The ovulation rates were determined after incubating for 8 h.
683 **P<0.01 (ANOVA and Dunnett's post hoc test, N=6).

684

685 **Fig. 2. *rho*/Rho expression in medaka ovary.** (A) Real-time RT-PCR analysis was
686 conducted for *rho* genes using total RNA purified from the follicle layers isolated from
687 the preovulatory follicles 3 h before ovulation. (N=3). (B and C) Real-time RT-PCR
688 analysis was conducted for (B) *rhoa-a* and (C) *rhoa-c* using total RNA purified from the
689 preovulatory follicles isolated from ovaries at the indicated time points (N=5-7). (D)
690 Specificity of the antibodies for medaka Rhoa-a (left) and Rhoa-c (right) were examined.
691 Recombinant proteins for antigen were analyzed using CBB staining (CBB) or western
692 blot analysis using purified (WB) or recombinant protein-pretreated (WB(abs))
693 antibodies (left panels in each set). (E) Rhoa-c was detected by
694 immunoprecipitation/western blotting. As a negative control, -3 h follicle extract was
695 immunoprecipitated using normal mouse IgG bound to protein G-sepharose. The arrow
696 indicates Rhoa-c, and the asterisk denotes the band corresponding to the antibody used

for immunoprecipitation. As a positive control, Rpl7 was detected using the input fractions. (F) Rhoa-c was detected by western blotting using the extracts prepared from follicular layer (FL) and pGC. Rpl7 was used as a loading control. (G) Immunohistochemical analyses were performed in sections of the -3 h ovary. Purified anti-medaka Rhoa-c antibody (upper and middle panels) or the antibody previously treated with recombinant Rhoa-c (absorbed antibody) was used as a control (lower panel). The area indicated by a box in the upper panel is displayed at higher magnification in the middle panel. The follicle layer (arrows) of large preovulatory follicles (*) and the follicle layer (arrowheads) of medium-size follicles are positively stained. A representative result of four independent experiments is shown. Bars =300 μ m in the upper and lower panels and 100 μ m in the middle panel.

Fig. 3. Detection of active Rhoa-c in the follicle layer of preovulatory follicles and the follicles cultured with GW, Rho inhibitor, Rho-associated protein kinase inhibitor, and Rho-activator. (A) Active Rhoa-c was detected in follicle layer extracts prepared at the indicated time points. (B) The -6 h follicles were cultured with no additives for 0 h (-6 h layer), 3 h (Cont (3 h)), or 6 h (Cont (6 h)). The -6 h follicles were cultured with GW, Rho inhibitor (Rho-i), Rho-associated protein kinase (Rock) inhibitor (Rock-i), or both GW and Rho-activator for 6 h and then used to detect active Rhoa-c. Extracts of (A) -1 h or (B) -6 h follicle layers were boiled for 10 min and used as a negative control (Rtn). Active Rhoa-c was pulled-down from the extracts and the precipitating Rhoa-c was detected by western blot analysis. Arrows indicate signals for active Rhoa-c. Total Rhoa-c protein (control) was detected using the input fractions.

Fig. 4. Effects of Rho-associated protein kinase (Rock) inhibitor on in vitro ovulation

in preovulatory follicles. (A) The -3 h follicles were cultured with Rock inhibitor Y-27632, and the ovulation rates were determined after incubating for 8 h. $**P<0.01$ (ANOVA and Dunnett's post hoc test, N=6). (B) The -3 h follicles were cultured with Rock inhibitor or both the inhibitor and Rho activator (Rho-act). The ovulation rates were determined after incubating for 8 h. $**P<0.01$ (ANOVA and Dunnett's post hoc test, N=6). (C) The -3 h follicles were cultured with the inhibitor. Black bars and straight lines denote the duration of follicle incubation with or without the inhibitor, respectively (left). The ovulation rates were determined after incubation for 8 h. $**P<0.01$ (ANOVA and Dunnett's post hoc test, N=6).

Fig. 5. *rock1*/Rock1 expression in the medaka ovary. (A) Real-time RT-PCR analysis was conducted for *rock* genes using total RNA purified from the follicle layers isolated from the follicles 3 h before ovulation. (N=3). (B) Real-time RT-PCR analysis was conducted for *rock1* using total RNA purified from the preovulatory follicles isolated from ovaries at the indicated time points (N=5-7). (C) Specificity of the antibody for medaka Rock1 were examined. Recombinant protein for antigen was analyzed using CBB staining (CBB) or western blot analysis using purified (WB) or recombinant protein-pretreated (WB(abs)) antibodies. (D) Rock1 was detected by western blot analysis using the extract purified from the follicle layer of preovulatory follicles isolated at the indicated time points. Rpl7 was used as a loading control. (E) Rock1 was detected by western blotting using the extracts prepared from follicular layer (FL) and pGC. Rpl7 was used as a loading control. (F) Immunohistochemical analyses were performed in sections of the -3 h ovary. Purified anti-medaka Rock1 antibody (two left and lower right panels) or the

antibody previously treated with recombinant Rock1 (absorbed antibody) was used as a control (upper right panel). The area indicated by a box in the upper left panel is displayed at higher magnification in the lower left panel. The area indicated by a box in the lower left panel is displayed at further higher magnification in the lower right panel. The follicle layer (arrows) of large preovulatory follicles (*) and the follicle layer (arrowheads) of medium-size follicles were positively stained. TC, theca cell; GC, granulosa cell; OM, oocyte membrane. A representative result of four independent experiments is shown. Bars =300 μ m in the two upper panels and 100 μ m in the lower left panel.

Fig. 6. The G-/F-actin ratio in the GCs of the preovulatory follicle or OLNI-2 cell.

(A) The -4h follicles were incubated with GW627368X (GW), Rho inhibitor (Rho-i), Rock inhibitor (Rock-i), or with both GW and Rho-activator for 4 h. Follicles cultured without additives (-4 h layer and Cont) were also used as controls. After incubation for 4 h, G-actin and F-actin in the follicular layers were detected (lower two panels), and the signal intensities of the bands were densitometrically quantified to determine the G-/F-actin ratio. * $P < 0.05$ (ANOVA and Dunnett's post hoc test, $N=4$). (B) OLHNI-2 cells were treated with 100 nM PGE₂, and G-/F-actin ratio was determined. * $P < 0.05$ (ANOVA and Dunnett's post hoc test, $N=4$). (C) Rhoa-c (left) and Rock1 (right) were detected by western blotting using the extracts of OLHNI-2 cell (Cont), or cells without medaka Rhoa-c (Rhoa-c KO) or Rock1 (Rock1 KO). Rpl7 was used as a loading control. (D) OLHNI-2 cells without medaka Rhoa-c (Rhoa-c KO), Rock1 (Rock1 KO), or Ptger4b (Ptger4b KO) were cultured with or without 100 nM PGE₂ and G-/F-actin ratio was determined as in (A). * $P < 0.05$ (t-test, $N=3$).

Fig. 1

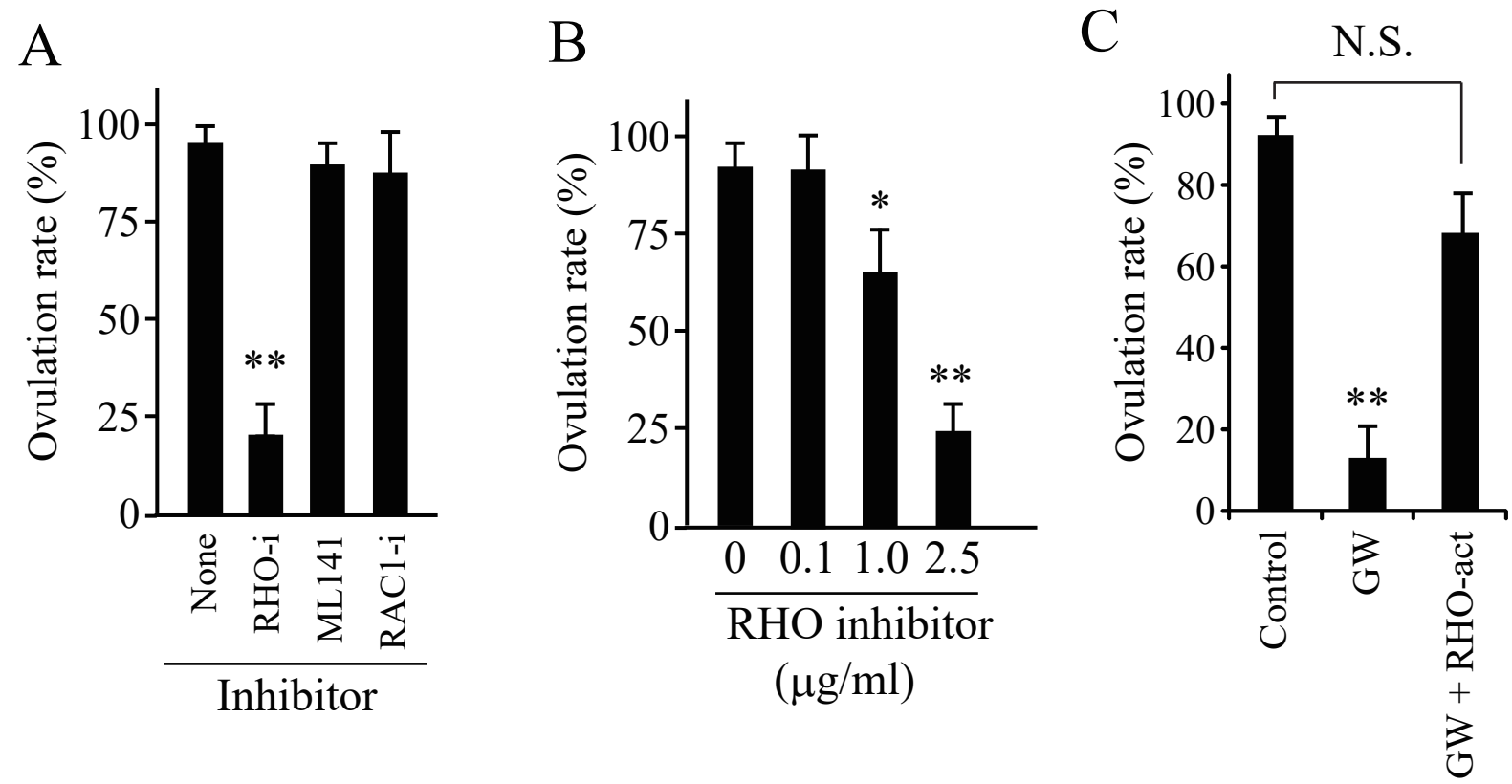


Fig. 2

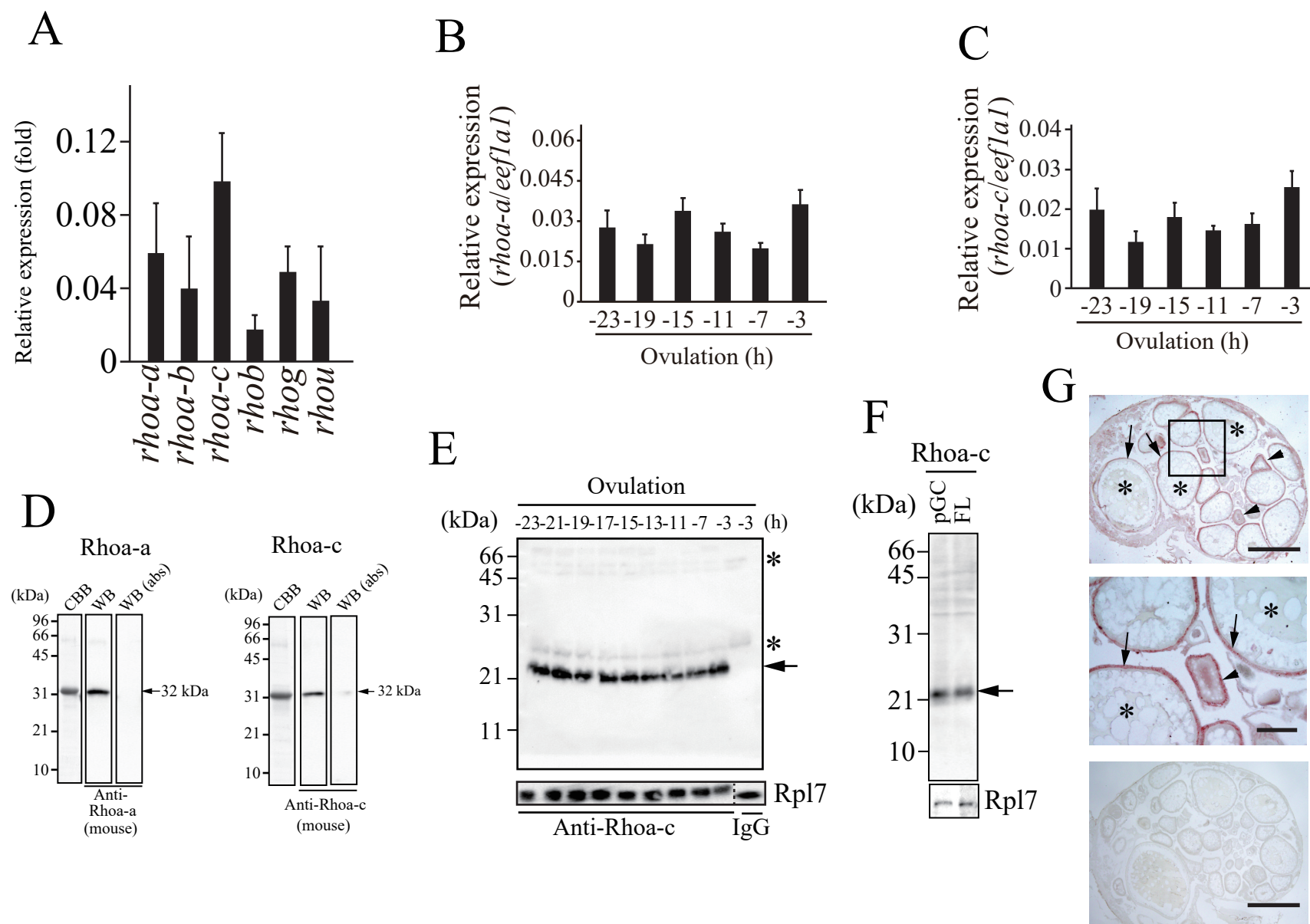


Fig. 3

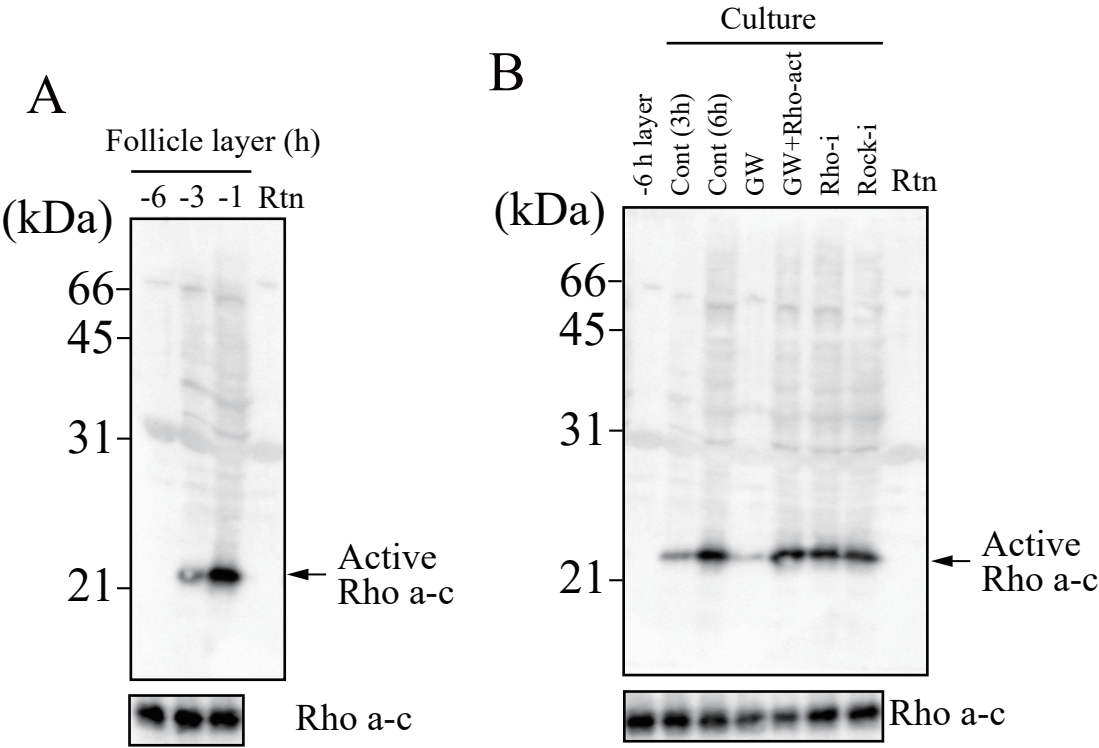


Fig. 4

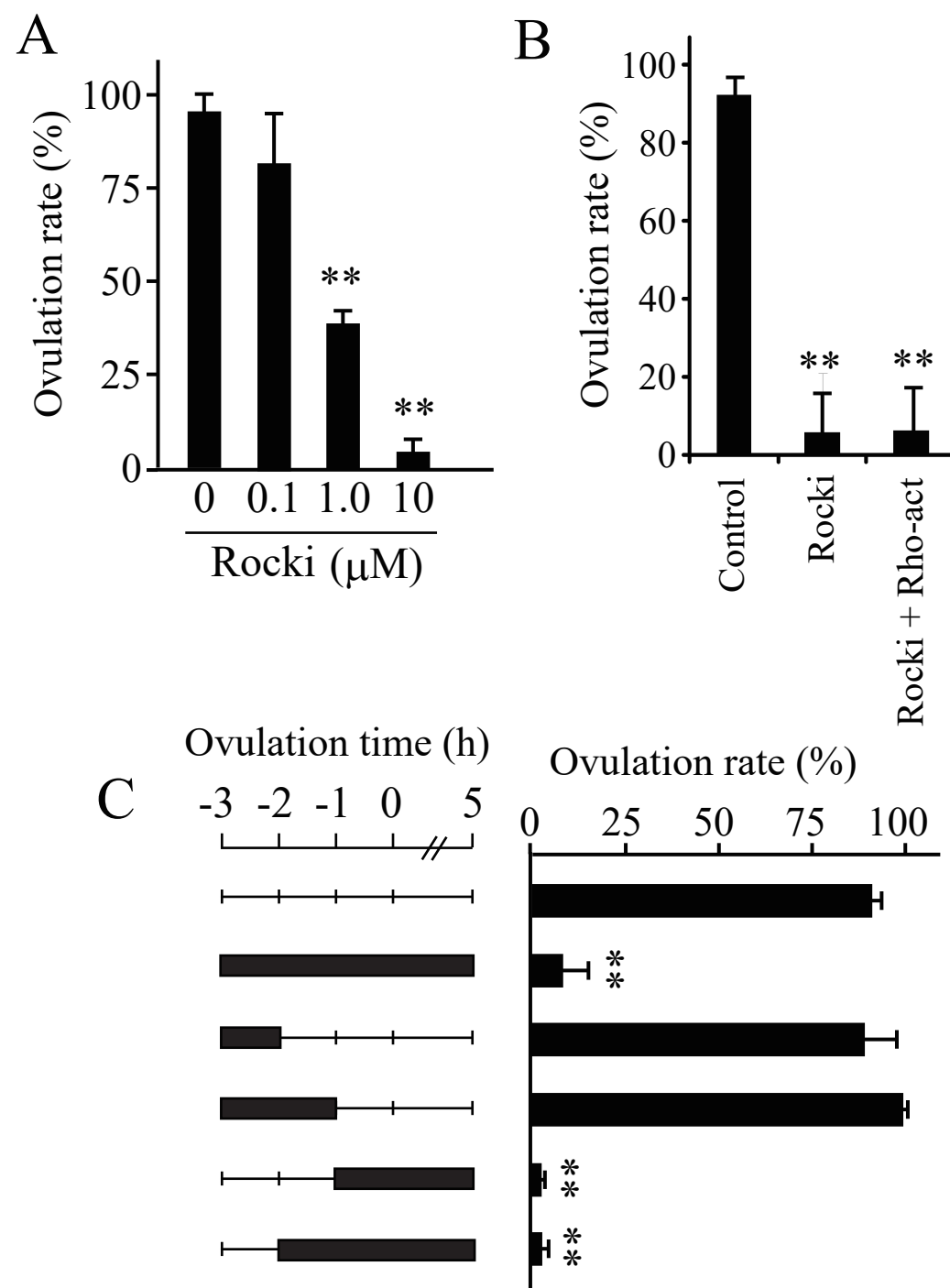


Fig. 5

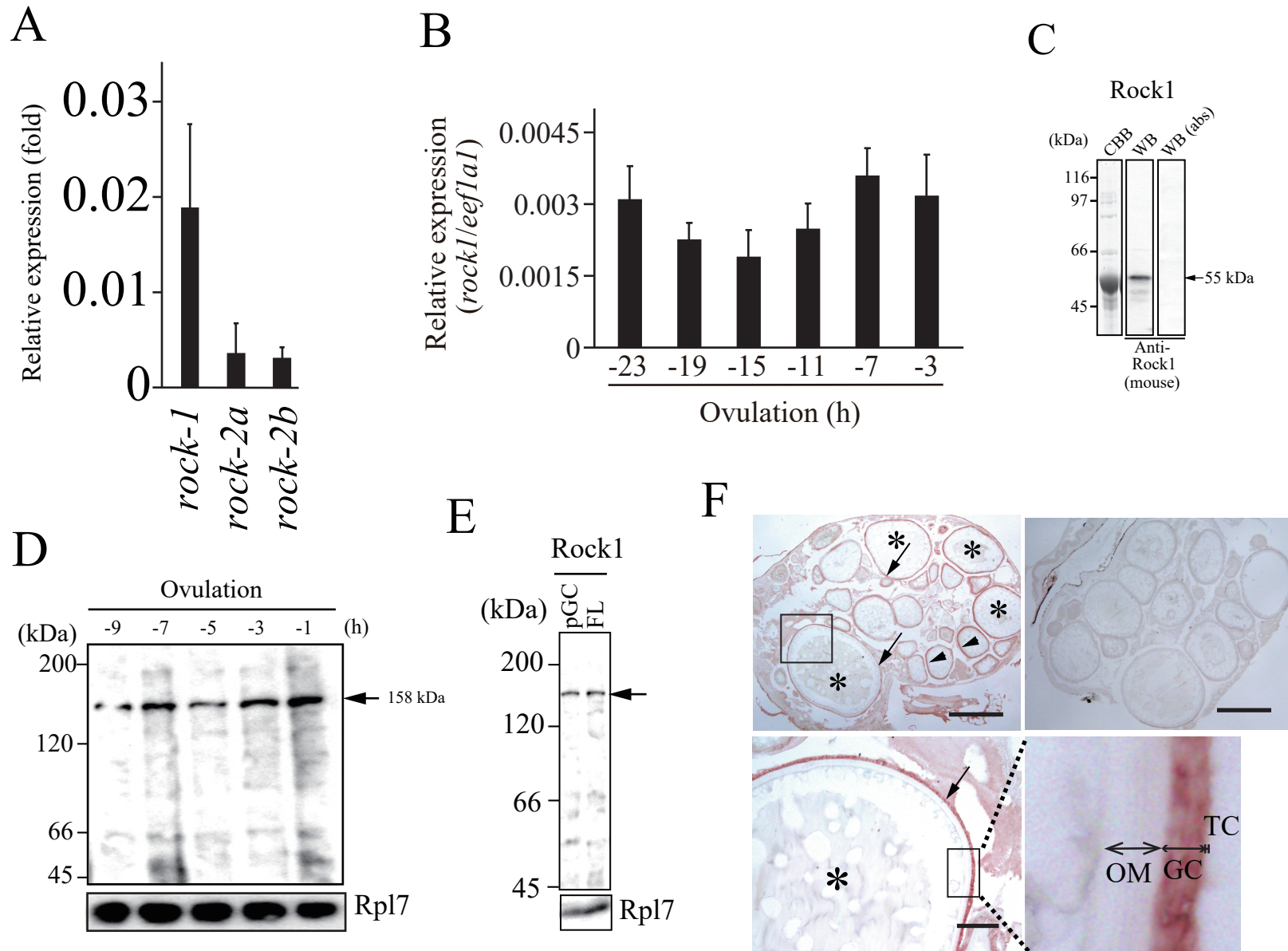
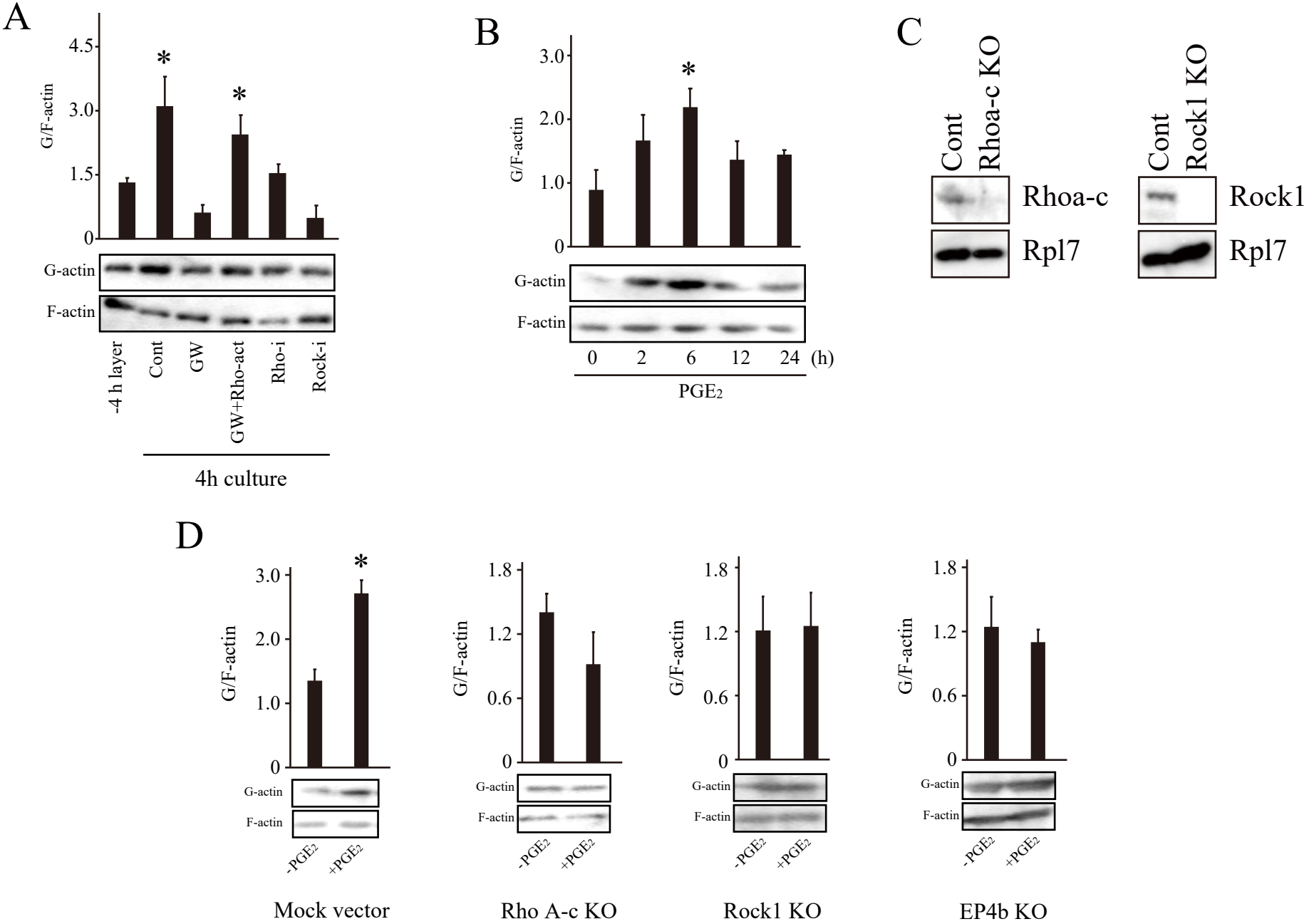


Fig. 6



Supplemental Table S1. Primers used in this study.

Primer name	gene	Sequence	Accession No.
<u>Real-time PCR</u>			
Rho A-a ss	<i>rhoa-a</i>	5'-CAACGACTGACGCTAACAAAG-3'	XM_004086108
Rho A-a as	<i>rhoa-a</i>	5'-CCGACTATGACCAGCTTTTTC-3'	XM_004086108
Rho A-b ss	<i>rhoa-b</i>	5'-ACTGCAAGGTCACATACATA-3'	XM_023956423
Rho A-b as	<i>rhoa-b</i>	5'-CAGGGAAGTATCCTTACTG-3'	XM_023956423
Rho A-c ss	<i>rhoa-c</i>	5'-CTCAGCGAAACGCAGTTAGA-3'	XM_004070475
Rho A-c as	<i>rhoa-c</i>	5'-CCCAACAATCACCAACTTCTT-3'	XM_004070475
Rho B ss	<i>rhob</i>	5'-CTCCTAGGAAAAGGCAGGAAT-3'	XM_004083452
Rho B as	<i>rhob</i>	5'-CTCGTCCCTGCTAAACACAAT-3'	XM_004083452
Rho G ss	<i>rhog</i>	5'-ACTCATCCATCAGCATCTAG-3'	XM_004076673
Rho G as	<i>rhog</i>	5'-AGGCTCCGGTGGTGTAGGAA-3'	XM_004076673
Rho U ss	<i>rhou</i>	5'-GTTCTGGTCGGGACACAGTG-3'	XM_004067248
Rho U as	<i>rhou</i>	5'-TCCGATCTCCACTGCACAAT-3'	XM_004067248
Rock1 ss	<i>rock1</i>	5'-TCTCTGGTTGGGACTTACAG-3'	XM_004079418
Rock1 as	<i>rock1</i>	5'-GCAGACTCTCCAGATGACTT-3'	XM_004079418
Rock2a ss	<i>rock2a</i>	5'-TGGAGGACATGCTCAGAGAC-3'	XM_023951816
Rock2a as	<i>rock2a</i>	5'-CGTAGCGCCGGGTAGTCCAA-3'	XM_023951816
Rock2b ss	<i>rock2b</i>	5'-AGGCCTTCCTCAACAGATAT-3'	XM_023952796
Rock2b as	<i>rock2b</i>	5'-CCAGCTGTACTTCTCCAAAT-3'	XM_023952796
EF1a SS	<i>eef1a</i>	5'-CACCGGTCACCTGATCTACA-3'	AB013606
EF1a AS	<i>eef1a</i>	5'-GCTCAGCCTTGAGTTTGTCC-3'	AB013606
<u>Recombinant protein</u>			
RhoA-a pET SS	<i>rhoa-a</i>	5'-ATGGCAGCGATCAGGAAAAAG-3'	XM_004086108
RhoA-a pET AS	<i>rhoa-a</i>	5'-TTACAGTAGGACGCATCTATT-3'	XM_004086108
RhoA-c pET SS	<i>rhoa-c</i>	5'-ATGGCCGCACTCAGAAAGAAG-3'	XM_004070475
RhoA-c pET AS	<i>rhoa-c</i>	5'-TCACAGTAGCTGGCAGCCCCC-3'	XM_004070475
Rock1 pET SS	<i>rock1</i>	5'-ATGTCCTCCGAGATAAACATG-3'	LC726226
Rock1 pET AS	<i>rock1</i>	5'-TTACAGACTCTCCAGATGACTT-3'	LC726226
<u>Cloning</u>			
Rock1 cds-AS	<i>rock1</i>	5'-TTAGCTCAGCTTTCCCGTGTTG-3'	LC726226

Table S1. (continued)

Primer name	gene	Sequence	Accession No.
<u>Crsper-Cas9</u>			
EP4b sgRNA-SS	<i>ptger4b</i>	5'-TAGGGAATGTCATCGCCATCG-3'	AB563504
EP4b sgRNA-AS	<i>ptger4b</i>	5'-AAACCGATGGCGATGACATTG-3'	AB563504
Rhoa-c sgRNA-SS	<i>rhoa-c</i>	5'-TAGGAACTACATTGCTGACATTG-3'	XM_004070475
Rhoa-c sgRNA-AS	<i>rhoa-c</i>	5'-AAACCAATGTCAGCAATGTAGTT-3'	XM_004070475
Rock-1 sgRNA-SS	<i>rock1</i>	5'-TAGGCTGGATGGCTTGGATGCTC-3'	LC726226
Rock-1 sgRNA-AS	<i>rock1</i>	5'-AAACGAGCATCCAAGCCATCCAG-3'	LC726226
<u>RT-PCR</u>			
EP4b-RT-SS	<i>ptger4b</i>	5'-CTGTGAGAAGGTCTTCCTAG-3'	AB563504
EP4b-RT-AS	<i>ptger4b</i>	5'-CCACCCCCTTACATCTGATT-3'	AB563504
Rhoa-c-RT-SS	<i>rhoa-c</i>	5'-ATGGCCGCACTCAGAAAGAAG-3'	XM_004070475
Rhoa-c-RT-AS	<i>rhoa-c</i>	5'-TCACAGTAGCTGGCAGCCCCC-3'	XM_004070475
Rock-1-RT-SS	<i>rock1</i>	5'-CTGAAGCTCAGCCTCTCTAA-3'	LC726226
Rock-1-RT-AS	<i>rock1</i>	5'-TGTCACCGTCAGATCCAAC-3'	LC726226
b-actin-RT-SS	<i>actb</i>	5'-CAGACACGTATTTGCCTCTG-3'	D89627
b-actin-RT-AS	<i>actb</i>	5'-CAAGTCGGAACACATGTGCA-3'	D89627