Candidate gene identification of ovulation-inducing genes with in vivo assay in zebrafish

静岡大学博士論文

Candidate gene identification of ovulation-inducing genes with in vivo assay in zebrafish

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LIST OF ABBREVIATIONS

17, 20b-DHP	:	17, 20b-dihydroxy-4-pregnen-3-one
AMPK	:	The adenosine monophosphate-activated kinase
cAMP	:	Cyclic adenosine monophosphate
cDNA	:	Complementary DNA
COX	:	Cyclooxygenase
cRNA	:	Complementary RNA
DES	:	Diethylstilbestrol
E2	:	17β-estradiol
ECM	:	Extracellular matrix
EDCs	:	Endocrine disrupting chemicals
EP4	:	Prostaglandin E2 receptor 4
ER	:	Endoplasmic reticulum
ERS	:	Endoplasmic reticulum stress
EtOH	:	Ethanol
FSH	:	Follicle stimulating hormone
G6P	:	Glucose-6-phosphate
G6PT	:	Glucose-6-phosphate transporter
Gi	:	Inhibitory G-protein
GVBD	:	Germinal vesicle breakdown
hCG	:	Human chorionic gonadotropin
KO	:	Knock-out fish
KO	:	knock-out
LH	:	Luteinizing hormone
Lhr	:	Luteinizing hormone receptor

Lhβ	:	Luteinizing hormone subunit beta
MIH	:	Maturation-inducing hormone
MPF	:	Maturation-promoting factor
mPR	:	Membrane progestin receptor
mRNA	:	MessengerRNA
nPR	:	Nuclear progesterone receptor
P4	:	Progesterone
PGE2	:	Prostaglandin E2
PGES-2	:	Prostaglandin synthase-2
PKA	:	Protein kinase A
ptger4b	:	Prostaglandin E2 receptor EP4b
qPCR	:	Quantitative polymerase chain reaction
RNA-seq	:	RNA sequencing
siRNA	:	Small interfering RNA
StAR	:	Steroidogenic acute regulatory protein
stm-l	:	Starmaker-like
T3	:	Triiodothyronine
T4	:	Tetraiodothyronine
Tes	:	Testosterone

ABSTRACT

Two essential processes, oocyte maturation and ovulation, are independently induced but co-operatively activated at the final step in oogenesis. Even though these two processes are induced by same maturation-inducing steroid, 17, 20β-dihydroxy-4-pregnen-3-one (17, 20β-DHP), in teleost, the receptor for each pathway is suggested to be different and thus signal transduction pathways are different. There was no way to separate ovulation-inducing pathway from maturation-inducing pathway. It was difficult to identify ovulation-inducing genes.

In vivo induction technique which enables to induce oocyte maturation and ovulation in living zebrafish (referred to *in vivo* assay) have been applied to select highly up-regulated genes (ovulation-inducing genes) in this study. Living fishes, which treated by 17, 20β-DHP *in vivo*, can be induced both maturation and ovulation. It have been demonstrated that 17, 20β-DHP bind to membrane progestin receptor (mPR) and act via non-genomic pathways to induce oocyte maturation, in addition, ovulation can be activated by 17, 20β-DHP with binding to nuclear progesterone receptor (nPR) via genomic pathways. On the other hand, a synthetic estrogen, diethylstilbestrol (DES) can induce only maturation. DES binds on mPR and activates non-genomic pathways to induce oocyte maturation. Thus, it became possible to prepare ovarian samples that were induced only maturation and both induced maturation and ovulation. By comparing the gene expression profile in these samples, it became possible to distinguish maturation-inducing genes from ovulation-inducing genes for the first time.

Gravid female fishes were exposed to ethanol (EtOH) (none-activated group), diethylstilbestrol (DES) and testosterone (Tes) (maturation induced group), and 17, 20β-DHP

(maturation and ovulation induced group) by *in vivo* assay. Using genome wide microarray of zebrafish, gene expression levels in ovaries of these 4 groups were compared. Specifically up-regulated genes during induction of ovulation was selected by Subio Platform. Fold change of expression in 17-20β-DHP treated sample against EtOH, DES and Tes were calculated. The venn diagram analysis was performed to select genes that specifically up-regulated for 1.8 times in 17-20β-DHP treated samples. ANOVA was performed across all groups to detect significantly change among all treated groups. Candidate genes (33 genes) for ovulation-inducing pathway were selected as overlapping genes in fold change analysis and ANOVA. The mRNA expression levels of the first 20 of 33 have been checked by quantitative polymerase chain reaction (qPCR). However only 2 genes, slc37a4a and zgc:65811, showed ovulation specific up-regulation. Thus, we tried to select genes by just fold-change analysis. Again only one gene, zgc:92184, was selected as a candidate for ovulation-inducing gene. Although our microarray analysis resulted in a large number of false-positive, it is indicated that *in vivo* assay will be a new way to select ovulation-inducing genes. Therefore, we were going to apply newly developed technique, RNA-sequencing, for selection of ovulation-inducing genes. Then we applied a novel gene expression analysis technique, RNA-sequencing, by using a genome sequencer. By RNA-seq analysis, 9 another candidates, ctrb1, prss59.1, ctsbb, stm, adamts15a, sik1, pax2a rbm47 and ptgs2a were selected. qPCR confirmed its specific up-regulation during ovulation. New molecular pathway for fish ovulation has been identified here.

INTRODUCTION

1.1 general introduction to fish ovulation

Maturation-inducing pathway can be separated into three-steps induced by three factors , luteinizing hormone (LH), the maturation- inducing hormone (MIH) and the maturation-promoting factor (MPF). The LH which is released from the pituitary stimulates the production of MIH in the ovarian follicle layers. The MIH acts on oocyte MIH receptor. Inhibitory G-protein (Gi) coupled with MIH receptor is activated by binding with MIH and resulting in decrease of intracellular cyclic adenosine monophosphate (cAMP) and read reducing the acitivity of protein kinase A (PKA). The MIH signal induces the *de novo* synthesis of cyclin B from the stored mRNA. Immature oocytes contain monomeric 35 kDa cdc2 kinase. A preexisting 35 kDa cdc2 is activated by cyclin B and transformed to 34 kDa active cdc2 or active MPF. The MPF is an essential factor to induce germinal vesicle breakdown and oocyte maturation (Figure 1).

The 17α,20β-dihydroxy-4-pregnen-3-one (17, 20β-DHP), a steroid hormone is reported as the MIH in the most of fishes (Nagahama and Yamashita, 2008). In zebrafish, the 17, 20β-DHP can induce maturation *in vitro* (Tokumoto *et al.*, 2004) (Figure 2). An endocrine-disrupting chemical (EDC), diethylstilbestrol (DES), a non-steroidal estrogen also triggers the oocyte maturation in zebrafish. The germinal vesicles breakdown have been reported in oocytes treated by DES *in vitro* (Figure 3A). The cyclin B, which is an important factor leading to synthesis of MPF has been detected in oocytes treated by DES *in vitro* (Tokumoto *et al.*, 2004) (Figure 3C). The *in vitro* effects of DES upon fish oocytes maturation imply that the DES potentially binds to the MIS receptor. Ability of DES to interact with the membrane steroid receptor mediating

oocyte maturation has been confirmed by the receptor binding assays (Tokumoto *et al.*, 2007). It indicated that the membrane steroid receptor is a possible new target for endocrine disrupting chemicals (EDCs).

The i*n vivo* bioassay has been established to understand the effect of EDCs and steroid hormones on oocyte maturation and ovulation in living zebrafish (Tokumoto *et al.*, 2011). Effects of DES upon fish oocyte maturation have been tested by treatment *in vitro* (Tokumoto *et al.*, 2004) and a direct interaction between DES and MIS receptor with steroid binding assays (Tokumoto, Tokumoto, and Thomas, 2007). It was necessary to understand the effects of DES in the surrounding aquatic environment since the effects of chemicals should be evaluated when the chemicals contaminate in the environments for environmental assessment. A simple method has been created for assessment that need only adding a solution of DES into the water. This method is called i*n vivo* bioassay (Tokumoto *et al.*, 2011). Expectedly, DES induces maturation in living zebrafish *in vivo*. The 17, 20β-DHP can induce maturation within 2 hour and prolonged incubation of 17, 20β-DHP can induce ovulation within 3 hour. While, DES can induce oocyte maturation within 3 hour but it cannot induce ovulation. It indicates that DES can terminate oocytes development at maturation (Figure 4).

Ovulation refers to expel of the matured oocyte through the ruptured follicle wall and release of a matured oocyte from its follicle layer (Figure 5). This is a critical biological process to prepare fertilizable eggs prior to spawning. Ovary during ovulation in zebrafish possesses unique characteristics. Enlarge deep yellow ovaries are present almost the entire body cavity. Especially in roy zebrafish, transparent oocytes which undergo GVBD can be detected with the naked eye. Abdomen of gravid fish becomes a round shape because of ripe ova popped inside. The ovulated eggs don’t be released till male performs courtship to female. Ovulated eggs

produce fertilization membrane immediately after expose to water. Ovulation can be induced *in vivo* (Tokumoto *et al.*, 2011).

Induction of fish ovulation requires multiple factors and it varies between organisms (Fujimori *et al.*, 2012; Goetz and Garczynski, 1997; Joy and Chaube, 2015; Tang *et al.*, 2016; Zhu *et al.*, 2015). Vasotocin is a prerequisite factor necessary for the resumption of meiosis and ovulation in trout and catfish (Bobe *et al.*, 2006; Joy and Chaube, 2015; Rawat *et al.*, 2015; Singh and Joy, 2011). Binding of prostaglandin E2 (PGE_2) on prostaglandin E2 receptor 4 (EP4) is shown to be required for ovulation in medaka (Fujimori *et al.*, 2011; Fujimori *et al.*, 2012).

In zebrafish, it has been revealed that ovulation is mediated by nuclear progestin receptor (nPR) through the genomic steroid signaling mechanisms (Tang *et al.*, 2016; Zhu *et al.*, 2015). Recently, the nPR becomes more important research topic for understanding ovulation in zebrafish. It has been demonstrated that 17, 20β-DHP also binds to a membrane-bound progestin receptor (mPR) and acts via the non-genomic steroid signaling pathway to induce oocyte maturation. 17, 20β-DHP not only binds to mPR but it also binds to nPR and triggers the genomic steroid signaling pathway to induce ovulation (Figure 6). Progestin has been reported as a signaling steroid for inducing maturation and ovulation (Fujimori *et al.*, 2011; Fujimori *et al.*, 2012; Lister and Van Der Kraak, 2008; Zhu *et al.*, 2015). The role of nPR in term of control ovulation has been studied in nPR knock-out zebrafish (nPR-KO) (Tang *et al.*, 2016; Zhu *et al.*, 2015). Homozygous nPR-KO females cannot undergo ovulation and oocyte development stops at maturation; oocytes cannot be released from follicle cells even after treated by the 17, 20β-DHP (Figure 7). Thus it has been shown that ovulation is mediated by nPR through genomic steroid signaling pathway, not non-genomic steroid signaling pathway (Zhu *et al.*, 2015).

Blocking the steroid signaling pathway via nPR-KO is not the only way to block ovulation, but prevent the LH signaling pathway also interrupts ovulation (Chu *et al.*, 2014; Tang *et al.*, 2016). Relative expression of nPR in follicles in luteinizing hormone subunit beta (lhβ) and luteinizing hormone receptor (lhr) deficient fishes had been compared with mPRα, mPRβ (membrane progesterone receptor) expression levels (Figure 8). The nPR expression is lower than mPRα, mPRβ. It indicated that nPR expression is regulated by LH signaling pathway. To understand the relationship between nPR and ovulation in zebrafish, human chorionic gonadotropin (hCG) was injected to wild type and nPR-KO to imitate endogenous LH elevation in order to induce ovulation (Tang *et al.*, 2016). It has been reported that hCG is able to induce ovulation within 3 hour in wild type but not in the nPR-KO zebrafish. It means that interruption of nPR leads to ovulation deficiency in zebrafish through the LH signaling pathway. Relative expression of ovulation-related genes in the nPR-KO zebrafish follicles after hCG injection has been observed to reveal the genes that could be regulated by LH signaling pathway in the nPR-dependent or nPR-independent manner (Tang *et al.*, 2016). Ovulation-related genes have been reported to consist in the prostaglandin biosynthesis (cpla2, ptgs1, ptgs2a, ptgs2b and ptger4b), matrix metalloproteinases gene (mmps), tissue inhibitor of metalloproteinases (timp2b), genes involved in steroidogenesis (3β-hsd, 17β-hsd3, cyp19a1a), a disintegrin and metalloprotease with thrombospondin motifs1 (adamts1) and cathepsin L (ctsl). Expression of two genes (ptger4b and adamts1) significantly up-regulate in the wildtype fish but not in the nPR-KO. It implies that these genes are induced by LH signaling pathway through activation of nPR. Other genes including cpla2, ptgs2a and timp2b up-regulate in both the nPR-KO and wildtype fish, indicating that these genes may be induced by LH signaling pathway in an nPR-independent manner.

1.2 Scientific problems

Fish oocyte maturation can be initiated by maturation-inducing hormone (MIH), 17, 20β-DHP. This hormone binds to mPR and acts via non-genomic pathways to induce oocyte maturation (Tokumoto *et al.*, 2006; Zhu *et al.*, 2003). While, ovulation is induced by 17, 20β-DHP through a nuclear isoform of nPR (Zhu *et al.*, 2015). However, these two signaling pathways are overlapping. Oocyte maturation and ovulation are coordinately induced. It is difficult to specify the beginning or the end event of each development stages (Lubzens *et al.*, 2010). In zebrfish oocyte maturation is induced in 2 hours and matured oocytes undergo ovulation 1 hour later (Tokumoto *et al.*, 2011). Thus actual signaling pathways for induction of oocyte maturation and ovulation were coordinated between genomic and non-genomic pathways induced by 17, 20β-DHP (Tokumoto, 2014).

To examine only ovulation–associated genes in ovarian tissue is almost impossible. Both maturation- and ovulation–associated genes are mixed in an ovarian sample. This obstacle limited the study of ovulation. Most of researches regarding to fish reproduction have been focused only on primary growth and maturation. Transcriptional profile of fathead minnow (*Pimephales promelas*) ovary in different ovarian stages (atretic, previtellogenic, vitellogenic, and post-ovulatory follicles) has been tested (Villeneuve *et al.*, 2010). They provided a list of genes that shift over gross ovarian stage. The transcriptional levels in pre-ovulatory rainbow trout (*Oncorhynchus mykiss*) ovaries revealed a number of genes linked to maturational competence (Bobe *et al.*, 2003; Bobe *et al.*, 2006). There is a study of expression of some interesting genes from primary growth to mature follicles and effect of hCG on the expression of steroidogenic enzymes and steroidogenic acute regulatory protein (StAR) during those follicles development (Ings and Van Der Kraak, 2006). Ovarian steroidogenesis that initially induces

oocyte maturation and ovulation is well known, while downstream pathways during ovulation are still mystery. Up-regulated genes only in ovulated eggs may possibly shed a light on molecular mechanism to regulate ovulation.

Recent method to induce ovulation *in vivo* by Tokumoto *et al.* (2011) lights up the way to understand this final step before spawning. This procedure makes it possible to prepare ovarian samples, which contain matured oocytes but not ovulated eggs (treatment of the DES or Tes *in vivo*) and both matured oocytes and ovulated eggs (treatment of the 17, 20β-DHP *in vivo*) (Tokumoto *et al.*, 2011). Matured oocytes consist of maturation-related genes, whereas ovulated eggs have both maturation- and ovulation- related genes. Thus, it became possible to select the specific up-regulated genes in ovulation by comparing the gene expression profiles between matured and ovulated eggs (Figure 9).

Screening of gene profile is an available technique for identifying a number of genes, which express at the given time. Microarray is a useful tool used in many research areas including fish reproduction (Bobe *et al.*, 2003; Bobe *et al.*, 2006; Villeneuve *et al.*, 2010; Bonnet *et al.*, 2007). Although, Microarray is a traditional approach to observe gene expression profile over decade, many discrepancies have been reported such as error of probe hybridization. We have been identified candidates of ovulation-inducing genes via microarray (Klangnurak and Tokumoto, 2017). However, expression levels of most genes showed inconsistent expression tendency against the qPCR analysis. To determine specific ovulation–associated genes, it was necessary to check cellular transcriptome via more reliable screening method. Up to date method, RNA sequencing, has been established to reveal precise cellular transcriptome by sequencing-based methods of the expressed gene at the particular period of time. Both

microarray and RNA sequencing should performed to reveal expression profile of matured and ovulated ovaries.

1.3 Aim of this study

In this study, I conducted fine selection of ovulation-inducing genes which is not related to maturation –inducing pathway. The *in vivo* bioassay has been conducted to prepare matured and ovulated ovarian samples. Using genome-wide microarray of zebrafish and RNA sequencing, gene expression levels were compared among an ethanol-treated group (none-activated group), a 17, 20β-DHP-treated group (maturation and ovulation induced group), and DES- or Tes-treated group (maturation induced group). Up-regulated genes only in 17, 20β-DHP treatment were selected from microarray analysis and RNA sequencing. The mRNA abundance of up-regulated genes was confirmed by qPCR analysis.

MATERIALS AND METHODS

2.1 Experimental animals

Zebrafish were received from our experimental aqualab at Shizuoka University, Japan. Fish were raised in proper chamber with a recirculating water system which was maintained at 28.5 ˚C under a 14 h light: 10 h dark cycle (Westerfield, 1995) and fed a diet of brine shrimp in the morning and fish feed pellet (Croma, Kobe, Japan) in the evening. Female fish were raised until they possessed full-grown immature oocytes before used in this experiment. All experiments were conducted in accordance with procedures approved by the Shizuoka University Animal Care Committee.

2.2 Preparation of confirmed female

Ovulation assessment has been tested to selected females who can ovulate. Individuals that failed to ovulate would not use in this study. Female fish possessed full-grown immature oocytes were selected from a mixed group of 10-50 males and females held in 20 cm x 25 cm x 25 cm square acryl case with the standard water system in our aqualab. Females were transferred into a glass case containing 100 ml of water per fish. Fish were exposed to agents *in vivo* by adding 10 µl of 1 mM 17, 20β-DHP into water for each fish. Fish were incubated at 28.5 ˚C for 5 hour. Confirmed females display transparent oocytes at posterior part of ovary which can be detected with the naked eye (Figure 10). Ovulated eggs can be squeezed from ovaries. All confirmed females were kept together in the same tank under the standard water system for 5

days before using for experiments. Each bio-replicate for the transcriptome analyses obtained from the same batch of confirmed females.

2.3 Experimental design for collecting ovary sample: artificial induction *in vivo*

To investigate ovulation-inducing genes, artificial induction of maturation and ovulation was conducted by *in vivo* bioassay following Tokumoto *et al.* (2011) (Figure 11). Confirmed females were transferred into a glass case containing 100 ml of water per fish. Fish were exposed to agents *in vivo* by adding each agent, a 10,000-fold stock in ethanol (EtOH), (final concentration; 0.01% EtOH, 0.1 μM 17, 20β-DHP, 5 μM DES, 10 μM Tes) into water and incubated at 28.5 ˚C. After 3 hours of incubation, female zebrafish were killed by spinal severance followed by abdomen dissection. Ovarian samples were cut from the body cavity under sterile conditions. One side of ovary was put in 1.5 ml microtube and immediately frozen in liquid nitrogen to preserve RNA until extraction. To assess oocyte development stage and define the good ovary for RNA extraction, another side of ovary was placed in fresh zebrafish Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.2) and observed under a stereomicroscope.

Maturation-induced ovaries have been prepared by treating DES or Tes *in vivo* and ovulation-induced ovaries have been prepared by 17,20β-DHP treatment. Criteria to describe oocyte stage have followed Tokumoto *et al*. (2011). Briefly, samples treated with DES and Tes, oocyte showed dramatically morphological changes comparing with control (EtOH) (Figure 12).

Oocytes underwent germinal vesicle breakdown and became transparent that indicate a morphological characteristic of matured oocytes (Patino and Sullivan, 2002; Tokumoto *et al.*, 2011). While oocytes form 17, 20β-DHP treated-fish expelled from their follicles layer and a fertilization membrane developed after contact with water that indicate a morphological characteristic of ovulated eggs (Tokumoto *et al.*, 2011). Good ovaries contain low percentage of oocytes at early follicular growth stages and low percentage of die oocytes. Another side of matured and ovulated ovaries used for transcriptome analysis.

2.4 Experimental design for collecting ovary samples: Time course

To collect a bio-replicate sample of 17, 20β-DHP treatment before treating (0 hr.), 3 and 5 hr. after treating, at least 10 confirmed females were put in the same breaker. The treatment was conducted by an *in vivo* assay as described above. A fish was randomly collected at each time point. Ovarian samples collection method was the same as artificial inducing samples. Three bio-replicates of the sample were taken.

2.5 Experimental design for collecting ovary sample: natural induction of maturation and ovulation

To observe changes of gene expression levels of candidate genes in natural oocyte maturation and ovulation, ovarian samples during natural pairing were collected. A male and confirmed female were crossed in the pairing case on the evening of the day before sample collection. Ovaries for matured samples were collected at 5 a.m. on the next day. Ovarian

samples were collected by the same method as artificially induced samples. Ovulated females that showed some transparent eggs at the posterior part of body cavity were collected at 8 a.m. immediately after light turning on (Figure 13). Ovarian samples were collected and kept at -80 ˚C until RNA extraction. Three bio-replicates of the samples were collected for both naturally matured and ovulated samples.

2.6 RNA extraction

Total RNA was extracted from ovarian tissue using ISOGEN (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's protocol. ISOGEN is a phenol based pre-made reagent for RNA extraction.

2.7 Microarray

Complementary DNA (cDNA) was prepared from 500 ng of total RNA from each replicate as described in the agilent low RNA input linear amplification kit manual (Agilent Technologies, Palo Alto, CA, USA). Double-stranded cDNA was synthesized using the reagents from this kit, and Cy3-labeled cRNA was prepared by cDNA *in vitro* transcription in the presence of cyanine 3-CTP dyes. Fluorescently labeled RNA was then purified with the qiagen RNeasy spin columns, in accordance with the manufacturer's protocol (Qiagen, Hilden, Germany). After purification, cRNA was stored at -80˚C until use.

The cRNA was fragmented and used to hybridize to the zebrafish G2519F 4X44 microarray (Agilent Technologies, Palo Alto, CA, USA). Hybridization, washing, and scanning

were performed in accordance with the manufacturer's protocol. Microarrays were scanned on a DNA microarray scanner (Agilent Technologies, Palo Alto, CA, USA) at five micron resolution. Raw digitized expression values from each probe set were extracted using feature extraction software, and features were flagged manually for poor quality. Data were then analyzed with Gene Spring ver. 11.0 (Agilent Technologies, Palo Alto, CA, USA). Triplicate probe sets on each array were considered as technical replicates for the analysis. The 3 biological replicates on separated arrays (Figure 14).

2.8 Gene Selection by microarray analysis

To remove maturation-inducing genes from the 17, 20β-DHP-treated group which contains both maturation- and ovulation-inducing genes, highly expressed genes only in the 17, 20β-DHP-treated group but not in EOH, DES, and Tes, were selected. The median signal from microarray analysis of ovary from fish treated by EtOH, DES, Tes and 17, 20β-DHP were compared by Subio Platform ver. 1.18.4667 (Subio Inc., Amami, Japan) (Figure 15). Analysis of variance (ANOVA) was performed to detect significantly different genes among 4 treated-groups by using the basic plug-in. Moreover, the 17, 20β-DHP-treated group was separately compared with the DES, Tes, and control, EtOH treated group. Any genes which exhibited at least 1.8 times higher expression in the 17, 20β-DHP-treated group were selected. Finally, the significantly different genes from ANOVA and genes which only dominant expression in the 17, 20β-DHP-treated group when compared with others treated-groups were overlapped in Venn diagram analysis. Only central overlapping genes were selected as candidates for ovulation-inducing genes. This method to select the genes called statistical selection. Another

uncomplicated and high-impact method was performed to selected ovulation-related gene, named non-statistical selection. Any genes that expression level was 10 times higher in the 17, 20β-DHP-treated group were selected (Figure 15).

2.9 RNA sequencing

RNA clade from the ovaries of the treated fishes (EtOH, DES, Tes and 17, 20β-DHP) were prepared for screening their expression profile by RNA sequencing. RNA purification was performed using RNEasy® mini kit. cDNA library of each treated sample was prepared from 10 μg of RNA according to the manufacturers' instructions of the TruSeq RNA-seq kit (Illumina). Using Illumina GAIIx platform, all libraries were sequenced on a single lane of 36-bp single-end sequencing according to the manufacturer's protocol (Illumina, Inc.). To count the number of transcript, RNAseq reads were aligned to the genome sequence of Zebrafish using the BWA mapping software (Figure 16).

2.10 Gene Selection for RNA sequencing

Gene expression profile of the ovaries treated by EtOH, DES, Tes and 17, 20β-DHP from the RNA sequencing was analyzed by Subio Platform ver. 1.18.4667 (Subio Inc., Amami, Japan). The non-statistical selection method has been performed to select ovulation-related genes. Any genes revealing its expression at least a 2-fold higher in the 17, 20β-DHP-treated group were selected by Venn diagram analysis. Central overlapped genes were target genes associated with ovulation. (Figure 17). A number of genes, which show interesting role related to

ovulation and enormously express in the 17, 20β-DHP-treated group have been selected to investigate their quantitative expression by qPCR.

2.11 Quantitative RT-PCR

The mRNA abundance of all selected genes was assessed by qPCR to confirm their expression level in the EtOH-, DES-, Tes- and 17, 20β-DHP-treated samples (Figure 18). Specific primers were designed via Primer 3 (Rozen and Skaletsky, 2000) (Table 1 and Table 2). To prevent genomic DNA contamination, primers were designed to cover exon-exon boundary from nucleotide sequences available in GeneBank. Gradient annealing temperature was investigated in all primers to find its suitable temperature for qPCR amplification (data not show). A widely used reference gene, elongation factor 1 alpha (ef1α), was amplified using the same sample sets to validate the normalization procedure.

Total RNA (1μg) was reverse transcribed using illustra Ready-To-Go RT-PCR Beads (GE Healthcare Life Sciences, Buckinghamshire, UK) according to manufacturer's instruction to prepare cDNA. The qPCR reactions were performed in 20 μl volume, containing 5 μl of 10 time-diluted cDNA, 1 μl of each primer (10 μM), and 10 μl of SYBR green PCR Master Mix (Roche Applied Science, Mannheim, Germany). Real time qPCR was conducted by LightCycler® Nano System (Roche Applied Science, Mannheim, Germany). The thermal cycle began with an initial denaturation step at 95°C for 6 minute, followed by 45 cycles of denaturation at 95°C for 10 second, annealing at Ta °C (Table 1 and Table 2) for 10 second, and extension at 72°C for 15 second. Final melting curve analysis was observed at 65°C for 20 second following by 95°C for 20 second. The qPCR products were assert by 1.5% agarose gel electrophoresis to confirm size

of amplified products. The melting curve was observed to check the unspecific amplicons. The mRNA abundance of each target gene was calculated from a serially diluted cDNA and was normalized against the expression level of the reference gene. Triplicates were performed for each cDNA samples. Normalized mRNA abundances were presented as the mean ± SE.

2.12 Statistical analyses

The mRNA abundances of each gene among different treated fish were computed using Kruskal-Wallis, one-way ANOVA nonparametric test, since all data set didn't match with ANOVA assumptions. Mann-Whitney *U* test was calculated to compare whether any difference of expression levels in each gene between treated fish and also to detect whether any difference between natural matured and ovulated sample. Significance was assigned as $P \leq 0.05$.

RESULTS

3.1 Microarray

Transcriptome analysis was conducted using microarrays to identify genes up- and down-regulated during ovulation. We tried to compare the expression levels of mRNAs from ovaries in the fish treated with EtOH, DES, Tes, and 17,20β-DHP by using a conventional microarray containing probes of genome wide variety (Agilent 4x44k). Signals of ovaries from fish treated with DES, Tes, or 17,20β-DHP were significantly higher for almost all genes than the samples treated with ethanol.

3.1.1 Ovulation-related genes selected from microarray

We compiled a list of highly abundant transcripts unique for ovulated ovaries prepared from the fish treated with 17, 20β-DHP by 2 method for selection, the statistical and non-statistical selection as described in Materials and Methods. A total of 5448 genes were selected by ANOVA (category A in Figure 15). Subsequently, genes that up-regulated more than 1.8-fold in the 17, 20β-DHP-treated group against EtOH-, DES and Tes-treated groups, 8237, 443 and 768 genes were selected, respectively. Overlapping genes among these three groups were selected by venn diagram analysis (category B in Figure 15). Finally, overlapping genes in categories A and B were selected. Thirty-three genes were selected as candidates for genes associated with ovulation (Table 3). For non-statistical gene selection, 53, 5 and 14 genes that were 10 times higher in 17, 20β-DHP compared with the EtOH-, DES-, and Tes-reated groups, respectively, were analyzed by venn diagram. Only 2 genes were selected as candidates for genes associated with ovulation by this method (Table 4). One of them (cyp11a1) was also identified by statistical gene selection method.

3.1.2 Expression of genes in the ovaries among treated fish

Expression levels of the first 20 genes from the statistical selection and a gene from the non-statistical selection in the different treated samples were confirmed by qPCR analysis (Figure 19). Three of them (api5, gbx2 and rhbdl3) couldn't be amplified. Absolute quantification analysis and melting temperature profiles of other 18 genes showed excellent amplification without any unspecifically amplified product. Three genes, slc37a4a, zgc:65811 and zgc:92184 showed significantly greater expression ($p<0.001$) in the 17, 20β-DHP-treated group. A discordant tendency of expression was presented between the microarray detectability and qPCR analysis in the other genes. Therefore, we noted that only three genes, slc37a4a, zgc:65811 and zgc:92184, were candidates for ovulation-related genes by the microarray analysis platform. Expression level of a positive control gene, prostaglandin-endoperoxide synthase 2 (ptgs2), was significantly high in the ovaries from the 17, 20β-DHP-treated fish (Figure 19). The ptgs2 gene is a prostaglandin biosynthesis genes which was reported to be prior up regulated for ovulation (Knight and Van Der Kraak, 2015). Therefore, these ovarian samples obtained from *in vivo* bioassay could be suitable representatives of ovary at maturing and ovulating point of time.

3.1.3 Changes in expression of genes before and after of induction of ovulation

Time course analysis of the expression levels of three selected genes and ptgs2a during the 17, 20β-DHP treatment has been investigated. As expected, transcriptional levels of all four genes were elevated after three hours. The zgc:65811 was still strongly up-regulated at five hours (Figure 20). We suggested zgc:65811 and zgc:92184 as the genes most likely to be associated with ovulation.

3.2 RNA sequencing

Illumina-based RNA-sequencing (RNA-seq) was done on the ovarian RNA from EtOH, DES, Tes, or 17,20β-DHP-treated fish. Three sets of sequencing for each treatment were carried out. Number of RNA-seq reads varied between 19 to 42 million reads (Table 5). Over 75% of reads were mapped onto the reference genomes. A set of 15,865 genes have been identified. Raw read data were submitted to the DNA data bank of Japan (DDBJ). Bio project is PRJDB5490 (PSUB006856).

3.2.1 Ovulation-related gene selected from RNA-sequencing

RNA-sequencing have been done to observe gene expression profile in ovary over the four treated fish (EtOH, DES, Tes and 17, 20β-DHP). Ovulation-inducing genes have been expected to be up-regulated with the 17, 20β-DHP. Therefore, 297 genes which showed 2 times higher expression in 17, 20β-DHP comparing with EtOH, DES and Tes were identified including one of positive control gene for ovulation-inducing study, ptgs2a (Figure 17). Eight most interesting genes, which potentially relate to ovulation and didn't show too low expression detected via RNA sequencing were selected to check their ovarian mRNA abundance by qPCR (Table 6).

3.2.2 Expression of genes in the ovaries of treated fish

Ovarian mRNA levels were quantified by qPCR in the EtOH, DES, Tes and 17, 20β-DHP-treated fish. Relative expression of all eight genes and a positive control gene was significantly different among treatments ($P \leq 0.05$) and specifically up-regulated in 17, 20β-DHP (Figure 21). Expression of the prss59.1, stm and pax2a was slightly high in ovary from EtOH-treated fish, however, it was over 2 times lower than the 17, 20β DHP-treated fish.

3.2.3 Expression of genes in samples from natural induction

The mRNA abundance of 9 genes (including positive gene, ptgs2a) has been checked in naturally matured and ovulated ovaries (Figure 22). As expected, all genes showed high expression in ovulated ovaries and were significantly different against the matured ovaries. Expression of ctsbb, adamts15a, sik1, rbm47 and ptgs2a were almost undetectable in the matured ovaries.

DISCUSSION

4.1 General discussion

In vivo bioassay established by Tokumoto *et al.* (2011) provides a novel way to distinguish signaling pathways to induce ovulation which is known to be induced by genomic actions of steroids from other pathways involved in the induction of oocyte maturation, non-genomic action of steroids. It is a practical technique to prepare matured and ovulated ovarian samples. Theoretically, high expression of maturation-inducing genes is detected in mRNAs extracted from matured oocytes. Likewise, both maturation-inducing and ovulation-inducing genes are highly expressed in ovulated eggs. Therefore, genes that highly expressed in ovulated eggs but low expression in matured oocytes were our candidates for ovulation-inducing genes. This *In vivo* assay have been previously developed to understand the influence of EDCs and steroid hormones on oocyte maturation and ovulation in living zebrafish (Tokumoto *et al.*, 2011). Both DES, one of the EDCs, and natural occurring steroid hormone, 17, 20β-DHP were effective when these compounds were externally applied to water column of living fish. Result showed

that fish kept in water containing the 17, 20β-DHP induced both maturation and ovulation. While, fish treated with DES successfully underwent oocyte maturation and oocytes stopped development in this stage.

Mechanism of the ovulation-inducing pathway in early development has been study for over two decades (Goetz and Garczynski, 1997) and provided many evidence to understand the reproductive system. Specifically regulated genes in pre-ovulatory, peri-ovulatory, and peri-spawning have been identified which shed some light on ovulation-inducing genes (Knight and Van Der Kraak, 2015). However, none of these studies eliminated putative maturation-inducing regulator which might be presented in ovulated ovarian tissues when they studied ovulation-inducing pathway, even though, it is well known that maturation and ovulation are an overlapping event (Patino *et al.*, 2003). This is the first observation that eliminated the maturation-inducing genes from ovulation-regulated study. Importantly, it can reveal specific ovulation-inducing genes.

The microarray analysis identified a number of up-regulated genes. A solute carrier family 37 (the glucose-6-phosphate transporter) member 4a (slc37a4a) and the zgc:65811 were selected from statistical selection method and the zgc:92184 was selected from non-statistical selection method. Unfortunately, inconsistent expression levels between the microarray and qPCR analyses have been detected in many genes. The cross hybridization of mRNA must be considered. It caused an inaccurate detection of signals in the microarray analysis. This weakness can lead to the misinterpretation of the gene expression profile (Gunnarsson *et al.*, 2007). RNA sequencing, which is a modern approach to detect transcriptome profiling, has been done to check more plausible gene expression profile. More eight genes (ctrb1, prss59.1, ctsbb, stm, adamts15a, sik1, pax2a, and rbm47) and one positive gene (ptgs2a) were identified.

Ptgs2a is a positive control as a gene which up-regulated during ovulation (Klangnurak and Tokumoto, 2017). It encodes prostaglandin-endoperoxide synthase 2a protein. Ptgs2a expression in ovarian tissue rises up at full-grown stage and it is significantly high in matured oocytes. Comparison between mRNA expression between oocytes and follicular cell layers noted that this gene is located in follicular cell layers (Tang *et al.*, 2017). Expression of ptgs2a is up-regulated after 2 hour of hCG treatment *in vitro* (Tang *et al.*, 2017) and by 17, 20β-DHP treatment *in vivo* (Knight and Van Der Kraak, 2015). This is the first study that reported Ptgs2a expression level in ovulated eggs. Elevation of expression during ovulation rather than maturation supported a key role of this gene to regulate ovulation. From reported function of eleven ovulation-related genes, I hypothesized several molecular signaling pathways during fish ovulation. Candidate genes have been classified into 3 groups. First group is associated with apoptosis. Apoptosis is one of the mechanisms that are involved in induction of fish ovulation. Four out of 11 candidate genes in this study (slc37a4a, zgc:92184, ctsbb and sik1) can trigger apoptosis. Second group of three candidates (zgc:65811, adamts15a and rbm47) might play the role in fertilization and embryogenesis. Last group of four genes are new players in ovulation inducing pathway (pax2a, stm, ctrb1, and prss59.1).

4.2 Apoptosis: one of the mechanisms involved in induction of fish ovulation.

Apoptosis has been shown to be an essential mechanism for follicle rupture and leads to the completion of ovulation (Crespo *et al.*, 2015; Crespo *et al.*, 2010; Murdoch and McDonnel, 2002). Four up-regulated genes found in this study might have the roles in induction of ovulation through the regulation of apoptosis. Apoptosis occurring prior to ovulation becomes an interesting mechanism in term of regulating rupture of oocyte from its follicular layer

(Klangnurak and Tokumoto, 2017). Degradation of some follicular cells to create the rupture site might be mediated by apoptosis.

A solute carrier family 37 (the glucose-6-phosphate transporter) member 4a (slc37a4a)

Expression of the slc37a4a gene up regulated during ovulation in zebrafish detected by qPCR. The mRNA of SLC37A4 expressed ubiquitously in human (Lin *et al.*, 1998), whereas its variant is primarily expressed in the brain, heart, and skeletal muscle (Lin *et al.*, 2000). Human SLC37A4a (also known as the glucose-6-phosphate transporter 1, G6PT1) encodes glucose-6-phosphate translocase protein, which plays a central role as glucose-6-phosphate transmembrane transporter (He *et al.*, 2009; Bartoloni and Antonarakis, 2004). Moreover, human SLC37A4 have been found at the endoplasmic reticulum (ER) via 10 transmembrane domains (Pan *et al.*, 2009). It plays a central role in the translocation of glucose-6-phosphate (G6P) between the cytoplasm and the lumen of ER (Chen *et al.*, 2008). A mutation in the G6PT gene causes a deficiency in the transport of G6P into the lumen of the ER, which leads to type Ib glycogen storage disease (Hiraiwa *et al.*, 1999). This finding supports the idea that SLC37A4 plays an important role in intracellular G6P homeostasis.

In dysregulated myocardial glucose metabolism, the intracellular accumulation of G6P can activate endoplasmic reticulum stress (ERS) (Kundu *et al.*, 2015). The signals from ERS mediated apoptosis by several pathways (Vannuvel *et al.*, 2013) (Figure 23). It could be speculated that a high expression of SLC37A4 (G6PT) results in excess G6P in the cell, thereby inducing ERS, leading to apoptosis that in turn induces ovulation (Tabas and Ron, 2011).

The zgc:92184

Another candidate ovulation-inducing gene, zgc:92184, is still not annotated. Blast analysis showed the highest similarity with the GTPase immune-associated proteins family member 7-like (LOC100005907) (Gimap7) with 82% identity. Based on this high sequence similarity, we assumed that the biological function of the unknown zgc:92184 gene might be analogous to Gimap7. The Gimap7 gene corresponds to GTPase of the immunity-associated protein family (GIMAPs). GIMAPs related to immunological functions, such as lymphocyte survival, thymocyte development and apoptosis regulation in cells of the mammalian immune system (Filen and Lahesmaa, 2010; Krucken *et al.*, 2004). It has been suggested that GIMAPs can play opposite roles to regulate the survival of lymphocyte cells; for example, GIMAP4 and GIMAP5 function differently in the regulation of apoptosis. GIMAP4-deficient rats are resistant to apoptosis (Carter *et al.*, 2007), whereas GIMAP5-deficient mice showed higher rates of apoptosis (Chen *et al.*, 2011). Although the role of GIMAP7 in stimulating apoptosis is still uncertain.

The Cathepsin Bb (ctsbb)

Cathepsin Bb (ctsbb) is an ortholog with human cathepsin B (CTSB) and also widely distributed in many living organisms. It belongs to lysosomal cysteine proteases family. Cathepsin is ubiquitously expressed among human tissues and plays a key role in intracellular proteolysis involving in a normal cellular protein degradation and turnover (Turk *et al.*, 2012). Cathepsin B promotes tumor genesis and cancer progression in many human cells (Ruan *et al.*, 2016; Mirkovic *et al.*, 2015; Bian *et al.*, 2016). Cathepsin can mediate apoptosis by cleavage of

the proapoptotic members (Bid) (Cirman *et al.*, 2004) and induces degradation of antiapoptotic members (Bcl-2, Bcl-xL, or Mcl-1) that promote the mitochondrial pathway of apoptosis (Droga-Mazovec *et al.*, 2008).

The Salt Inducible Kinase 1 (Sik1)

Salt Inducible Kinase 1 (Sik1), which is proposed to be an ovulation-inducing gene in this study, is another gene that can mediate apoptosis (Cheng *et al.*, 2009; Qu and Qu, 2017). Sik1 belongs to the adenosine monophosphate-activated kinase (AMPK) subfamily. The AMPK implicated to cell metabolism (Fu and Screaton, 2008). The AMPK induced cytokineses and cytotoxicitys of CD8(+) T-cells toward islets from mice. These mechanisms involved in regulating the β-cell apoptosis (Riboulet-Chavey *et al.*, 2008). Sik1 provokes P53 protein activity and subsequently induced anoikis, a subtype of apoptosis. Resistance of apoptosis in the cells can be found when cells lack of SIK1 activity (Cheng *et al.*, 2009).

A number of mechanisms explain induction of apoptosis. This cell-death program has been well studied in various cell types. Four candidates for ovulation-related genes show relative function to apoptosis. Molecular signaling pathways to explain potential relationship among 4 candidates have been showed (Figure 23). This hypothesized signaling pathways must be checked, however, it was out of scope of this study.

4.3 An ovulation- related genes play the role in fertilization and embryogenesis.

The zgc:65811

The zgc:65811 was not annotated, but showed relatively high similarity with CD9 antigen-like gene of common carp (64%). CD9 antigen has previously been shown to be involved in fertilization in knockout mice (Kaji *et al.*, 2000). It leads this gene to be a candidate for conserved gene regulating ovulation over vertebrates in order to prepare fertilizable gamete.

A disintegrin-like and metalloproteinase domain with thrombospondin-15 motifs (adamts15a)

The adamts15a gene encodes a protease with disintegrin-like and metalloproteinase domain and thrombospondin-15 motifs (ADAMTS-15). Mammalian genome consists of 19 ADAMTS genes called ADAMTS 1 to 20 (Porter *et al.*, 2005). ADAMTS5 and ADAMTS11 were determined to an identical gene (Kelwick *et al.*, 2015). Various functions of members of the ADAMTS gene have been clearly shown by establish the knock-out animals (Dubail and Apte, 2015). Physiological roles of a lot of genes involve in development of the reproductive organs and fertility (Russell *et al.*, 2015). Seventeen adamts genes have been found in zebrafish (Brunet *et al.*, 2015). The mRNA of adamts15a is expressed ubiquitously in several organs, especially heart, liver, and kidney. Expression levels of all members of the ADAMTS genes were low in oocyte. However, in comparison with other genes in this family, adamts15a is one of highly up-regulated gene since 24 hour post fertilization (hpf) (Brunet *et al.*, 2015) (Figure 24). This gene

plays a key role during embryogenesis. Biological function of this gene during oogenesis is still unclear.

This is the first evidence to report high expression of adamts15a during fish ovulation. On the other hand, a member in this gene family, adamts1, becomes a well-known biomarker to understand ovulation in both invertebrates and mammals. The expression of adamts1 reduced in the nPR-KO fish after hCG injection. The nPR mediated final oocyte maturation and ovulation of zebrafish via LH signaling pathway (Tang *et al.*, 2016). Lack of adamts1 in nPR-KO fish supposes function of this gene to regulate the ovulation. In mammal, role of ADAMTS1 gene for ovulation has been studied in many points of view. Ovulation or regression of follicles in mouse depends on development of extracellular matrix (ECM) in the regions surrounding growing follicles. Incompetence of growing follicles to construct ECM components of the follicle and stromal environments has been reported in ADAMTS1 null mutant mouse. ADAMTS1 is require for follicle development (Brown *et al.*, 2006). Female ADAMTS1 null mice displayed subfertility, while male underwent normal fertility. Deficiency of ADAMTS1 effect normal procession of ovulation and fertility (Mittaz *et al.*, 2004). It guarantees that adamts gene family is involved in some part of ovulation.

The RNA binding motif protein 47 (rbm47)

RNA binding motif protein 47 (rbm47) is an ortholog of RBM47. The RBM47 ubiquitously expresses in many human tissues. This gene plays an essential role during embryogenesis such as head formation and embryonic patterning (Guan *et al.*, 2013). Another function of this gene prior embryogenesis has been speculated in this study. While, up-regulation

of rbm47 has never been reported in ovarian tissue of zebrafish, it described in ovary of matured eel (Burgerhout *et al.*, 2016).

4.4 New players in ovulation inducing pathway.

Some up-regulated genes during ovulation in this study have never reported in reproductive system. It reveals new pathways for induction of ovulation.

The Paired box protein (Pax2a)

The Pax2a gene encodes a transcription factor, paired box protein. This gene is expressed in ovary of catfish and has been speculated to be an up-stream regulator of Wnt signaling pathway. Knockdown of Pax2a mRNA by siRNA reduced the expression of various transcripts related to ovarian development such as signaling molecules including wnt4 and wnt5. Wnt signaling pathway is essential for steroidogenesis and/or ovarian development (Prathibha and Senthilkumaran, 2016).

Pax2a play a role to maintain the primordium or the differentiation of thyroid follicles in zebrafish during early embryogenesis (Wendl *et al.*, 2002). Triiodothyronine (T3) and tetraiodothyronine (T4) secreted from the thyroid gland control FSH and LH production. Low concentration of T4 and T3 were enough to induce the excess amounts of FSH and LH (Liu *et al.*, 2011), which would sequentially induce steroidogenesis. Loss and disorganization of thyroid follicles after treatment by perchlorate result in low fertilization rate of zebrafish (Mukhi and Patino, 2007). Thyroid hormone mainly controls metabolism, growth, and development in vertebrates and is involved in the reproductive system in teleost (Mukhi and Patino, 2007; Liu *et*

al., 2011). This is the first time of detection of high expression of pax2a in oocytes during ovulation. Thyroid receptor is also located on human ovarian surface epithelial cells (Rae *et al.*, 2007). It is possible that pax2a play a role at both the thyroid follicles and thyroid receptors in ovary in term of induction of steroidogenesis.

The Starmaker protein (stm)

Stm encodes Starmaker protein; an essential protein for zebrafish otolith morphogenesis regulating crystal growth and otolith shape. Starmaker strangely expresses at ear in early embryogenesis and subsequently presents at backbone in later stage (Sollner *et al.*, 2003). Expression of this protein becomes a useful bio-indicator to study the process of otolith formation in fish (Sollner *et al.*, 2004).

Interestingly, Pax2, a candidate regulating steroidogenesis in ovary during ovulation though Wnt signaling pathway (Prathibha and Senthilkumaran, 2016), are also involved in otolith formation (Bajoghli *et al.*, 2009). Expression of starmaker-like (stm-l) has been observed in medaka embryos. A heat-inducible pax2 construct was injected to medaka embryos to reveal induction of stm-l by pax2. Embryo possesses greatly stm-l expression after heat shock. Pax2 potentially mediates stm-l expression in medaka embryos at otic vesicle (Bajoghli *et al.*, 2009).

We found that both pax2a and stm exhibited high expression in ovarian tissue during ovulation. This is the first evidence to reveal that pax2a and its downstream target gene, stm, not only play a role in otolith formation but also cooperate in ovulation inducing pathway in reproductive system of zebrafish (Figure 25).

The Chymotrypsinogen B1 (ctrb1)

Chymotrypsinogen B1 (ctrb1) encodes a principal precursor of the pancreatic proteolytic enzymes and a family of the serine protease enzymes synthesized in pancreas and are continuously secreted to small intestine.

Protease, serine, 59, tandem duplicate 1 (prss59.1)

Protease, serine, 59, tandem duplicate 1 (prss59.1) encodes trypsinogen 1a, a precursor for trypsin production in pancreas. This gene is up-regulated in pregnant seahorse brood pouch (Whittington *et al.*, 2015). While function of prss59.1 is unclear, the role of other related gene, the trypsinogen isoform-7 (a paralog with human cationic trypsinogen (PRSS1)), has been observed in knock-out mice. Intra-acinar trypsinogen activation makes injured-cells during early stages of pancreatitis but not lead to inflammation in acute pancreatitis (Dawra *et al.*, 2011).

This study provides a new system for discovering the candidate genes for induction of ovulation. I succeeded to establish the selection method to eliminate the list of maturation-inducing genes from ovulation-inducing pathway and reveal specific ovulation-inducing genes for the first time. Twelve candidate genes reported in this study provides significant new insight for the study of molecular mechanisms regulating ovulation. I classified function of candidate genes into three groups. Firstly, I strongly suggested that ovulation was regulated through apoptosis at follicle layer. Degradation of follicle cell is a critical step allowed matured oocytes rupture from follicle layer and undergo ovulation. Secondly, some genes, which play a role in fertilization and

embryogenesis, potentially express since ovulation. Lastly, I proposed new players to be involved in ovulation. Further, the biological function of all candidates underlying the ovulation should be examined using the recently developed gene knockout technique such as CRISPR/Cas9 system.

Table 1 Primers used for qPCR analyses of candidate genes in the microarray section.

Target gene	Accession #	Forward Primer 5' to 3'	Reverse Primer 5' to 3'	product size, bp	Ta, °C
cyp11a1	NM_152953	AAAGCCTGAAGACGGTGCTA	AGCAGGACGCCATATTTTTG	117	60
zgc:136308	NM_001111227	GCAAACACGACACAACTCCTGC	TGTGTCCTCCATCAGGTCTGTTTAC	130	56
efna1	NM_200783	AGCAGTTGGCGAAGGTGATG	CGGTAATGGAGGAGGCGTTC	99	56
slc37a4a	NM_214738	CTCCAGCAAAAATGAAAGCA	CACCCCAAACACCACCAG	91	56
Slc25a10	NM_201172	TAATATACTCACACACTTCCTG	CTGTATTCTCCTTTAGAGTTC	114	56
Spata	NM_001018678	AGAAATAACACTCAAAGAGG	AGACTACAAGGAGAAACACT	174	55
sox21a	NM_131286	GTTCCCTCATCTTATGTACT	TTAAACTCCACTCATATCGT	92	56
api5	NM_199540	GAGTCAAATCTTACCTTTCA	ACATACATCAGGGCATAATA	86	-
nup85	NM_001003625	CACTCTTACAGACCATGCCCATATT	CCAGTGTCTCCATTTCACATCAAAC	80	60
gbx2	AF288762	CATTAACACAACCATAATCC	GTCACTAACACAGTCTCACAT	106	-
zgc65811	NM_200552	TCCTCATGTTAGTTTAAGGTCACGG	ACAGAAATGAAAGAGAAGCAGAAGT	90	60
cnn2	NM_213349	GGACTACAGATGGGAACAAATAAAT	ATGTGTGACTTGGGATAATACAGAT	92	60
CK139976	CK139976	CAGTATCTGCGATGTTTAATGTCAG	GCTGTCTGAGTCTTCCATTTGA	102	61
bcl3	XM_688922	TGAAGAAAGAGGTGTGAGTTGATAG	TTAAAGAGACACAATGCTGAACGAA	115	61
asic2	NM_214788	AATGTTCTTTGAGGATGGATGGTT	GTGACCTTGTATTTAGATTGAGAGC	80	60
zgc:56525	NM_200279	CAACATTTCCTCCAGTGCTAAAG	CTTCAGTTCACTCTGCATCTTATTC	94	55
asic4b	NM_214786	GAGGAGTACATCAGAGACAACTTTC	CTTTCTTCTGCTCAATCGTTTCATA	82	57
rhbdl3	NM_001017556	TCACTATAACAGAGGTTGTTGTCTT	TTCAGGAAGTATGGCGATGATAC	88	-
LOC100003798	XM_001343224.1	GTACCTCAGTCAATCTCTAATCCTC	GAACCTTTGTTCATCTCTTCTGTTT	85	60
Nptna	NM_001160156	ATACAGGCATTTCCAGGCTTTATTT	AAAGAACTGTCCAACCAGAATCAT	110	60
zgc:92184	NM_001002344	CCCGTGGCGGCGATATGCTT	TCCCCCGCAGCGTCTGATGA	544	56
ptgs2a	NM_153657	ATGTTTGCTTTCTTCGCCCA	AGATCCACTCCATGACCCAG	101	55
ef1α	L47669	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC	358	60

Table 2 Primers used for qPCR analyses of eight candidates and one positive gene in the RNA-seq section.

Target gene	Accession #	Forward Primer 5' to 3'	Reverse Primer 5' to 3'	product size, bp	Ta, °C
ctrb1	NM_212618	CAATGAGAATTGGGTTGTGACTGC	TGTTGTAGTTAGGATGCTTGATGGA	146	55
prss59.1	NM_199605	CTGCTCACTGCTACAAGTCAC	GTCAAGATCCCACGAGTCATAG	134	56
Ctsbb	NM_001110478	GATGAATGTGGGATTGAGAGTGAGA	ATTGTGTTTGAGCGTGATTTAGAGG	120	58
Stm	NM_198817	ATCTCTGATGTCTTTGTCTATGTGG	CAGTCTTTATTAGATTTGCTCCCTG	149	60
adamts15a	NM_001126429	GAGAGCAAAGATAACAAGGCACAAA	TTTTCCACCTTTATTGACTCCACCT	96	56
sik1	NM_001126383	AGAGAAGACCAAAGTTGATT	ATAGGATGGCTAGTATTGTG	154	60
pax2a	NM_131184	TAATGCTTGCGGTCCCTTAAATATG	ATCAGTCCATTCAACGAAGACACG	149	61
rbm47	NM_001114686	CCAGAGAGGCACAATCTTAATGTCA	GACTACAGCACAGAGACGAGTTAAA	109	57
ptgs2a	NM_153657	ATGTTTGCTTTCTTCGCCCA	AGATCCACTCCATGACCCAG	101	55

Table 3 Top twenty candidates for genes associated with ovulation selected by the statistical selection method. The fold changes among treated samples are shown.

Gene name	Accession #	Description	Fold change relative to EtOH			Fold change relative to DES			Fold change relative to Tes			Fold change relative to 17, 20β-DHP		
			DES	Tes	17, 20β-DHP	EtOH	Tes	17, 20β-DHP	EtOH	DES	17, 20β-DHP	EtOH	DES	Tes
cyp11a1	NM_152953	Danio rerio cytochrome P450, subfamily XIA, polypeptide 1), mRNA	31.7	1.3	433.7	<0.01	<0.01	13.7	0.8	24.9	340.8	<0.01	0.1	<0.01
zgc:136308	NM_001045247	Danio rerio zgc:136308, mRNA	1.2	4.5	24.5	0.8	3.6	19.7	0.2	0.3	5.4	<0.01	0.1	0.2
efna1	NM_200783	Danio rerio ephrin A1, mRNA	1.7	5.5	16.5	0.6	3.2	9.6	0.2	0.3	3.0	0.1	0.1	0.3
slc37a4a	NM_214738	Danio rerio solute carrier family 37 (glucose-6-phosphate transporter), member 4a, mRNA	7.9	6.4	15.3	0.1	0.8	1.9	0.2	1.2	2.4	0.1	0.5	0.4
slc25a10	NM_201172	Danio rerio solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10, mRNA]	2.5	1.7	12.1	0.4	0.7	4.8	0.6	1.5	7.1	0.1	0.2	0.1
spata18	NM_001018678	Danio rerio spermatogenesis associated 18 , mRNA	4.1	5.2	11.6	0.2	1.2	2.8	0.2	0.8	2.2	0.1	0.4	0.4
sox21a	NM_131286	Danio rerio SRY-box containing gene 21a , mRNA	3.1	3.5	7.9	0.3	1.1	2.6	0.3	0.9	2.3	0.1	0.4	0.4
api5	NM_199540	Danio rerio apoptosis inhibitor 5, mRNA	3.2	3.1	7.0	0.3	1.0	2.2	0.3	1.0	2.2	0.1	0.5	0.4
nup85	NM_001003625	Danio rerio nucleoporin 85	2.3	3.6	7.0	0.4	1.6	3.0	0.3	0.6	1.9	0.1	0.3	0.5
gbx2	NM_152964	Danio rerio gastrulation brain homeo box 2	1.4	2.3	6.9	0.7	1.7	5.0	0.4	0.6	3.0	0.1	0.2	0.3
zgc:65811	NM_200552	Danio rerio zgc:65811 , mRNA	2.7	2.8	6.4	0.4	1.0	2.4	0.4	1.0	2.3	0.2	0.4	0.4
cnn2	NM_213349	Danio rerio calponin 2 , mRNA	2.5	1.5	5.6	0.4	0.6	2.2	0.7	1.7	3.6	0.2	0.5	0.3
CK139976	CK139976	AGENCOURT_16876226 NCI_CGAP_ZEmb3 Danio rerio cDNA clone IMAGE:7059552 5', mRNA sequence]	1.4	1.4	5.4	0.7	1.0	3.9	0.7	1.0	3.7	0.2	0.3	0.3
bcl3	XM_688922	PREDICTED: Danio rerio B-cell CLL/lymphoma 3, mRNA	1.3	1.6	4.5	0.8	1.2	3.5	0.6	0.8	2.9	0.2	0.3	0.3
asic2	NM_214788	Danio rerio acid-sensing (proton-gated) ion channel 2, mRNA	2.2	2.1	4.4	0.4	0.9	2.0	0.5	1.1	2.1	0.2	0.5	0.5
zgc:56525	NM_200279	Danio rerio zgc:56525, mRNA	1.9	2.3	4.2	0.5	1.2	2.2	0.4	0.8	1.8	0.2	0.5	0.5
asic4b	NM_214786	Danio rerio acid-sensing (proton-gated) ion channel family member 4b, mRNA	0.8	1.7	4.2	1.3	2.2	5.5	0.6	0.4	2.5	0.2	0.2	0.4
rhbdl3	NM_001017556	Danio rerio rhomboid, veinlet-like 3 (Drosophila), mRNA [NM_001017556]	0.6	1.3	3.7	1.7	2.2	6.3	0.8	0.5	2.9	0.3	0.2	0.3
LOC100003798	XM_001343224	PREDICTED: Danio rerio hypothetical protein LOC100003798, mRNA.	1.1	0.5	3.1	0.9	0.4	2.9	2.2	2.4	6.8	0.3	0.3	0.1
nptna	NM_001160156	Danio rerio neuroplastin a, mRNA	1.5	1.6	3.1	0.7	1.1	2.1	0.6	0.9	1.9	0.3	0.5	0.5

Table 4 Two genes associated with ovulation selected by the non-statistical selection method. The fold changes among treated samples are shown.

Gene name	Accession #	Description	Fold change relative to EtOH			Fold change relative to DES			Fold change relative to Tes			Fold change relative to 17, 20β-DHP		
			DES	Tes	17, 20β-DHP	EtOH	Tes	17, 20β-DHP	EtOH	DES	17, 20β-DHP	EtOH	DES	Tes
cyp11a1	NM_152953	Danio rerio cytochrome P450, family 11, subfamily A, polypeptide 1	31.7	1.3	433.7	0.0	0.0	13.7	0.8	24.9	340.8	<0.01	0.1	<0.01
Zgc:92184	NM_001002344	Danio rerio zgc:92184	1.4	0.9	20.7	0.7	0.6	14.9	1.1	1.6	23.6	<0.01	0.1	<0.01

Table 5 Summary of the RNA-seq read information and detail of represented genes of each library.

Biological replicate	Sample name	No. of Reads	Uniq map (seed 32 , mismatch2)	% of uniq map	No. of represented genes (Total)	No. of represented genes (RPKM > 0)	No. of represented genes (RPKM > 1)	Reference Genome
1	EtOH_1	21,986,129	17,371,766	79%	15865	13,372	12,407	danRer7
	DES_1	31,568,904	24,827,164	79%	15865	13,624	9,205	
	Tes_1	19,925,529	15,951,889	80%	15865	13,259	9,240	
	17, 20β-DHP_1	25,196,538	20,090,159	80%	15865	13,637	9,351	
2	EtOH_2	32,000,909	24,959,619	78%	15865	13,755	9,401	danRer7
	DES_2	42,557,042	33,294,822	78%	15865	13,581	9,025	
	Tes_2	38,989,411	30,741,391	79%	15865	13,839	9,386	
	17, 20β-DHP_2	42,422,071	33,424,477	79%	15865	13,912	9,328	
3	EtOH_3	40,466,435	30,016,408	74%	15865	13,889	9,308	danRer7
	DES_3	22,925,036	17,487,847	76%	15865	13,320	9,117	
	Tes_3	38,410,087	29,143,517	76%	15865	13,761	9,178	
	17, 20β-DHP_3	26,935,561	20,626,033	77%	15865	13,747	9,412	

Table 6 Eight ovulation-related gene candidates selected by RNA seq platform . Fold-change difference obtained from comparison between the 17, 20β-DHP treated sample and other indicated samples are shown.

Gene name	Accession#	Description	Fold change relative to EtOH			Fold change relative to DES			Fold change relative to Tes			Fold change relative to 17, 20β-DHP		
			DES	Tes	17, 20β-DHP	EtOH	TES	17, 20β-DHP	EtOH	DES	17, 20β-DHP	EtOH	DES	TES
ctrb1	NM_212618	chymotrypsin B1 precursor	25.7	2.0	248.8	0.0	0.1	9.7	0.5	12.9	124.4	0.0	0.1	0.0
prss59.1	NM_199605	Danio rerio protease, serine, 59, tandem duplicate 2 (prss59.2), mRNA/trypsinogen precursor	10.7	2.0	76.0	0.1	0.2	7.1	0.5	5.5	38.7	0.0	0.1	0.0
ctsbb	NM_001110478	capthepsin B, b precursor	2.9	0.3	53.6	0.4	0.1	18.8	3.5	10.0	187.7	0.0	0.1	0.0
stm	NM_198817	protein starmaker precursor	0.6	1.5	9.4	1.7	2.6	16.3	0.7	0.4	6.4	0.1	0.1	0.2
adamts15a	NM_001126429	A disintegrin and metalloproteinase with thrombospondin motifs 15 precursor	2.3	2.5	8.5	0.4	1.1	3.8	0.4	0.9	3.4	0.1	0.3	0.3
sik1	NM_001126383	salt-inducible kinase1	0.9	1.6	6.9	1.1	1.8	7.7	0.6	0.6	4.3	0.1	0.1	0.2
pax2a	NM_131184	paired box protein Pax-2a	0.6	0.8	4.6	1.6	1.3	7.6	1.2	0.7	5.7	0.2	0.1	0.2
rbm47	NM_001114686	RNA-binding motif protein 47, transcript variant X3	0.5	0.9	2.1	2.0	1.9	4.3	1.1	0.5	2.3	0.5	0.2	0.4
ptgs2a	NM_153657	prostaglandin-endoperoxide synthase 2 precursor	0.5	0.8	34.6	2.2	1.8	75.0	1.2	0.5	40.9	0.0	0.0	0.0

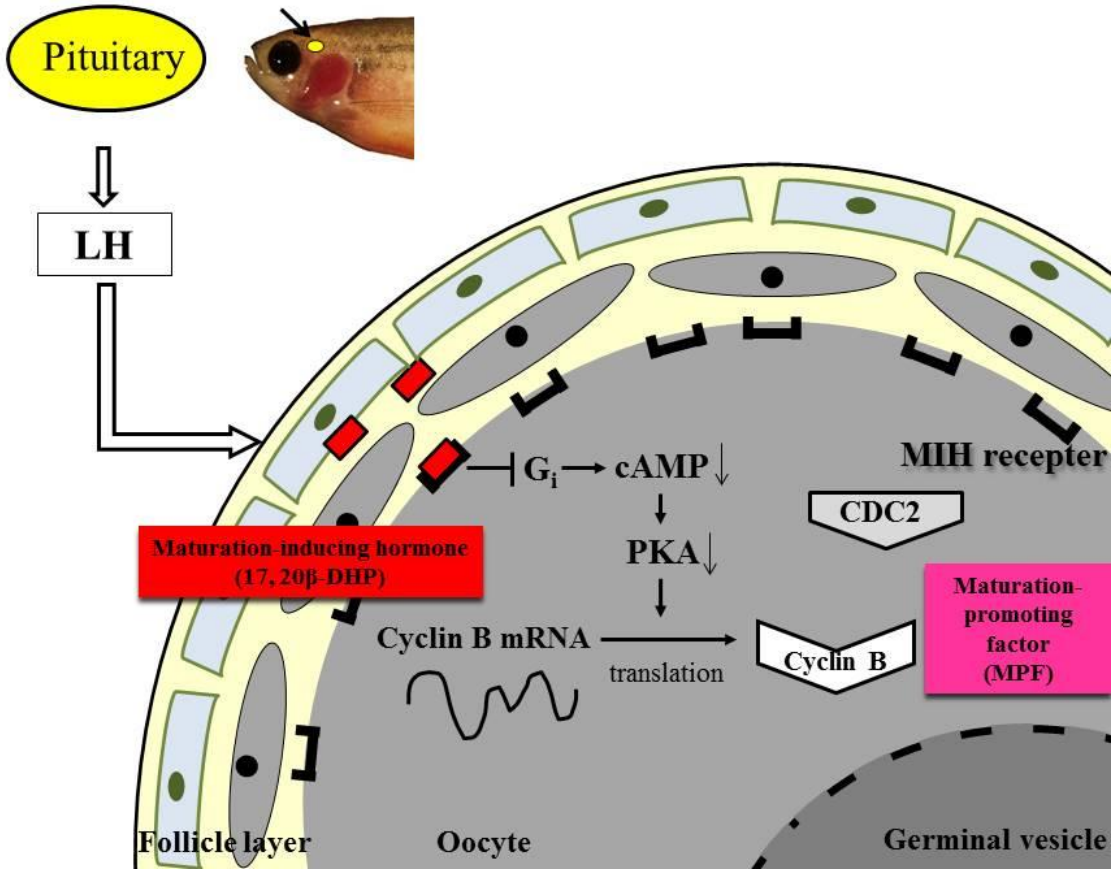

Figure 1 Hormonal control of oocyte maturation in teleosts. Luteinizing hormone (LH) acts on the ovarian follicle layer to produce maturation-inducing hormone (MIH) such as the 17, 20β-DHP. MIH directly acts on maturation-inducing hormone receptor (MIH receptor) to induce final maturation by stimulating the formation of maturation-promoting factor (MPF) – a complex of cyclin B and cdc2 kinase (CDC2).

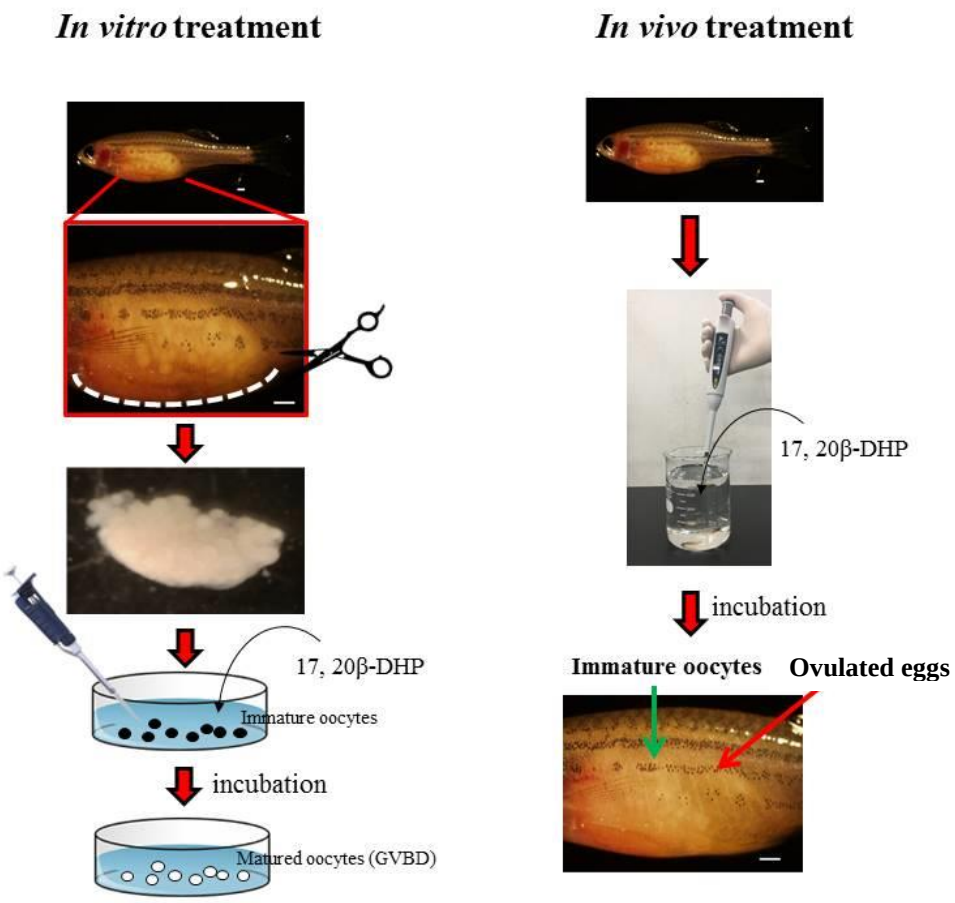

Figure 2 Summarized protocols for the *in vitro* and *in vivo* treatments. *In vitro* treatment, fish was killed and its ovary was taken to zebrafish ringer's solution (116 mM NaCl/2.9 mM KCl/1.8m $MCaCl_2$/5mM Hepes, pH 7.2) before treatment. While *in vivo* treatment, fish was kept in water containing chemicals such as the 17, 20β-DHP. It allows us to observe the effect of the target chemicals in living fish. Scale bars indicate 1 mm.

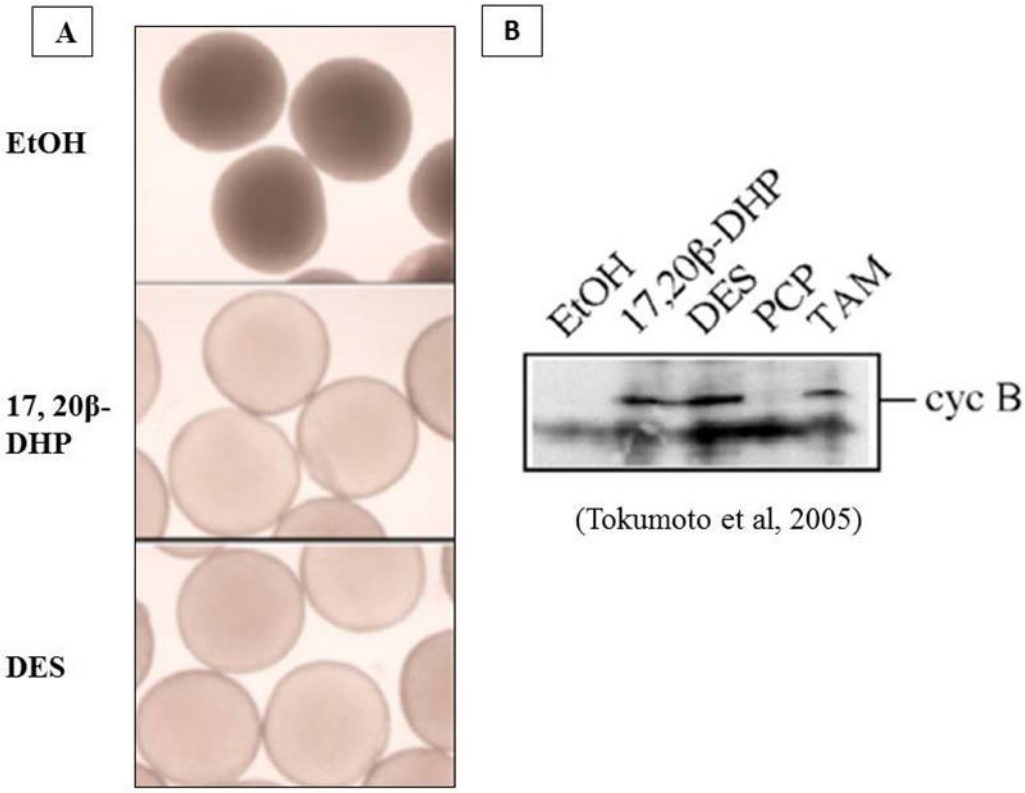

Figure 3 DES and 17,20β-DHP induce oocyte maturation. (A) The morphology of oocytes after in vitro treatment were photographed. Germinal vesicles still exist after EtOH treatment. On the other hand, they break after 17,20β-DHP and DES treatments. (B) To observe cyclinB (cyc B), the extracts of 20 oocytes after incubation with EtOH, 17,20β-DHP, or DES were prepared. Extracts of each treatment were electrophoresed and stained with the coomassie brilliant blue (CBBR) or immunostained with anti-goldfish cyc B polyclonal antibody after electroblotting (cyc B). The 48-kDa band of cyc B is indicated by an arrow.

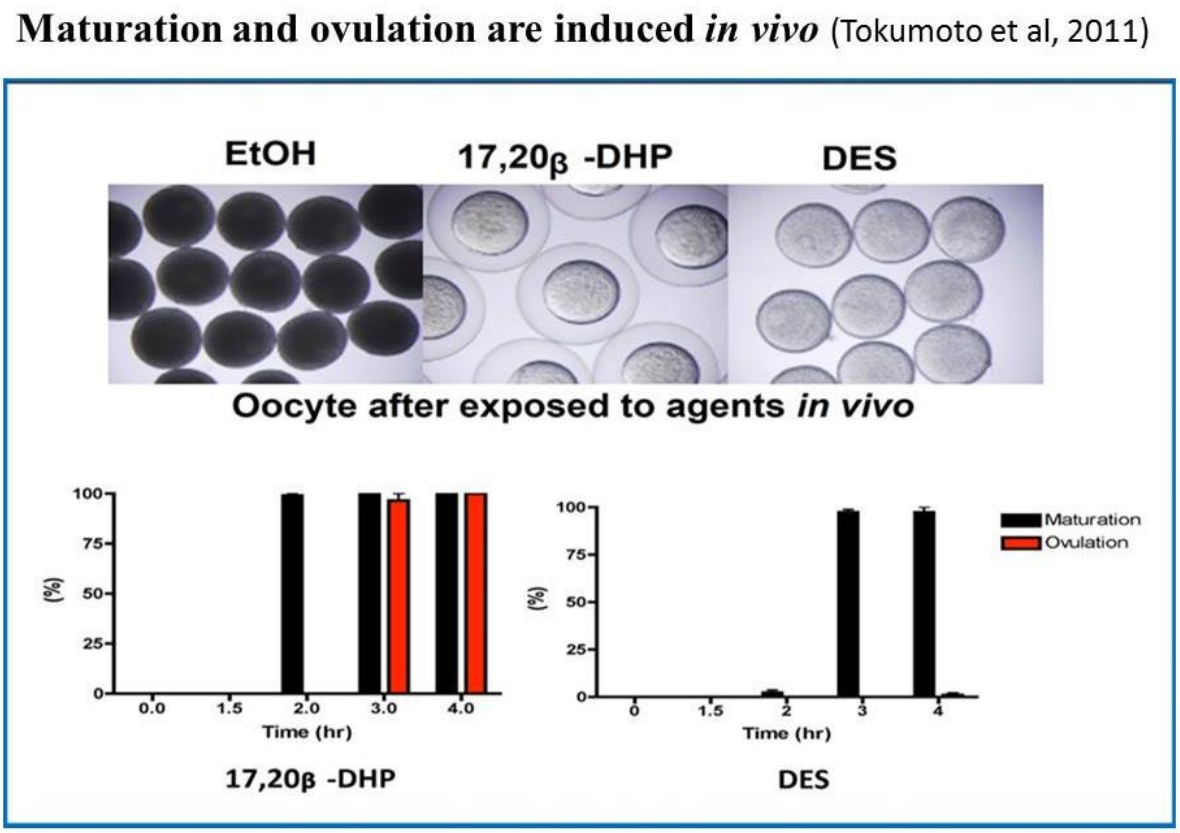

Figure 4 Induction of fish maturation and ovulation *in vivo*. Morphology of oocytes after 17, 20β-DHP- and DES-treatment is clearly different. Oocytes undergo maturation after 2 hour and subsequently ovulation after 3 hour by 17, 20β-DHP-treatment *in vivo*. On the other hand, DES induces only maturation. This figure was modified from Tokumoto *et al*. (2011).

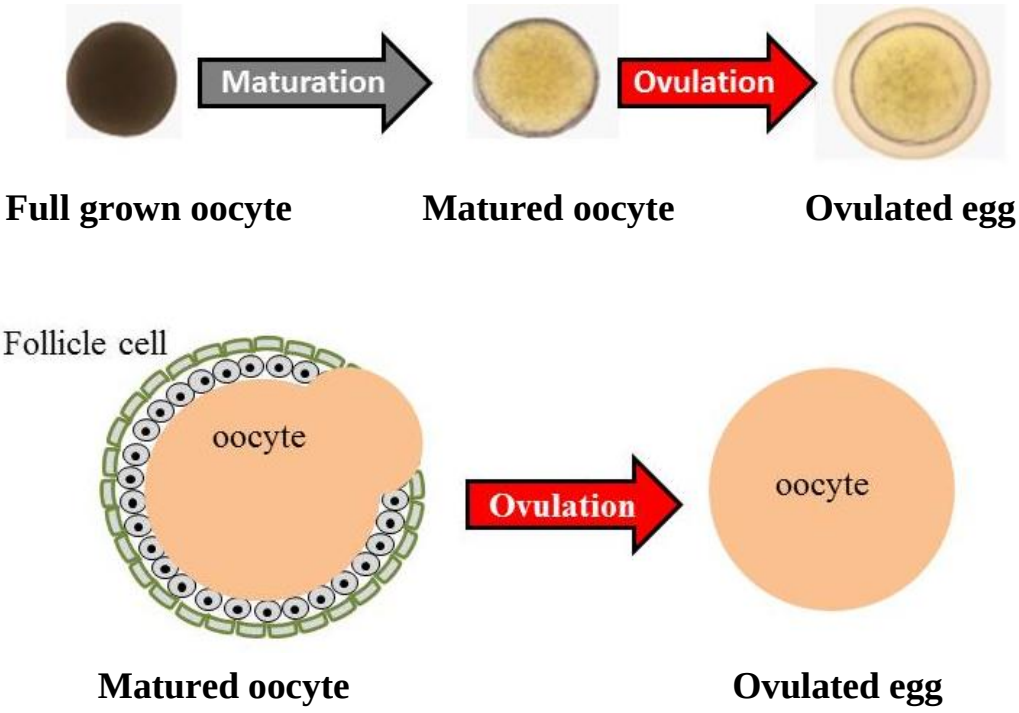

Figure 5 Changes in morphology of oocytes during ovulation. The follicle cell layers rupture after oocyte maturation and matured oocyte is released from the rupture site.

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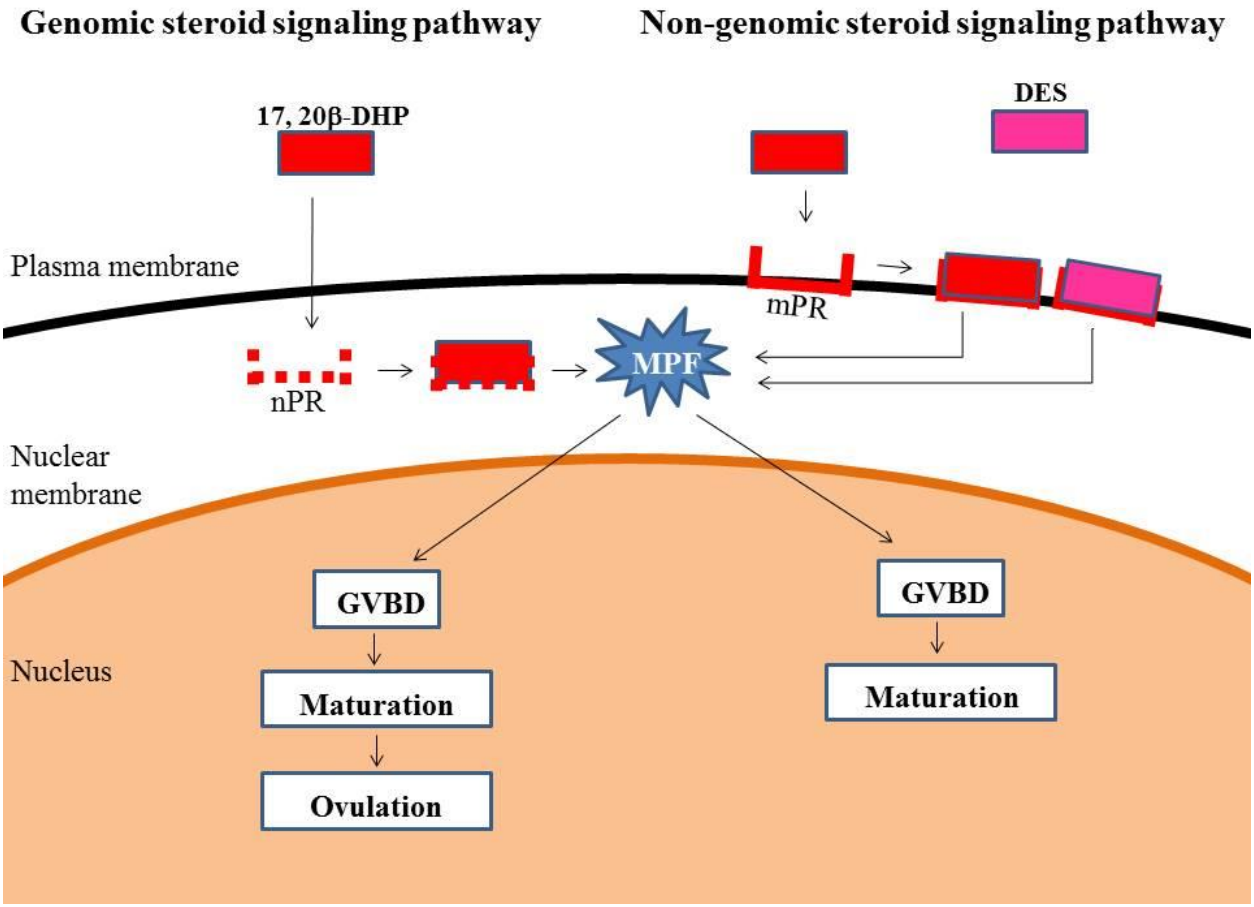

Figure 6 Genomic and non-genomic steroid signaling pathways. 17, 20β-DHP binds to a membrane progestin receptor (mPR) and acts via the non-genomic steroid signaling pathway to induce oocyte maturation. Moreover, 17, 20β-DHP also acts on the nuclear progestin receptor (nPR) and mediates the genomic steroid signaling pathway to induce maturation and ovulation. While, DES only binds to mPR and induces oocyte maturation in zebrafish via the non-genomic steroid signaling pathway.

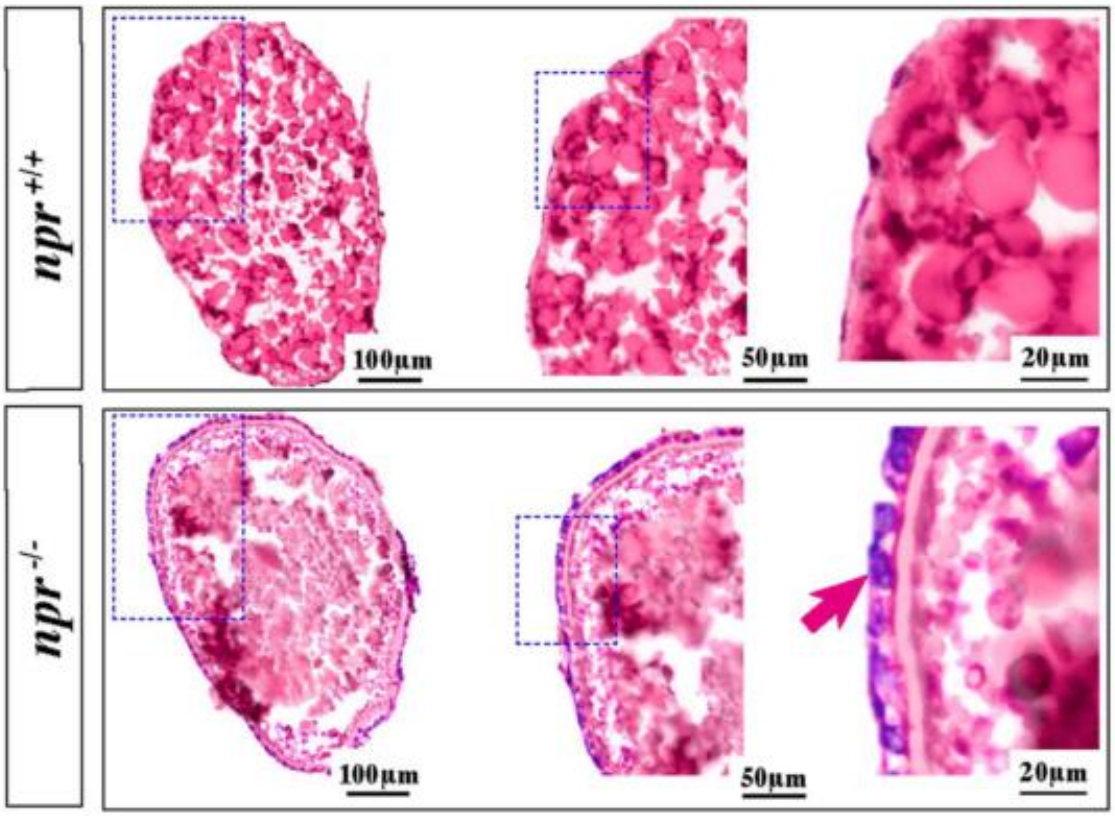

Figure 7 Oocyte of homozygous nPR-KO females cannot rupture from follicle layer.

Morphology of the outer layer cells of wild type fish (the npr+/+) and nPR-KO females **(**npr−/−) is different. The follicle layer of the nPR-KO is indicated by arrow. The follicle layer remains (Tang *et al.*, 2016).

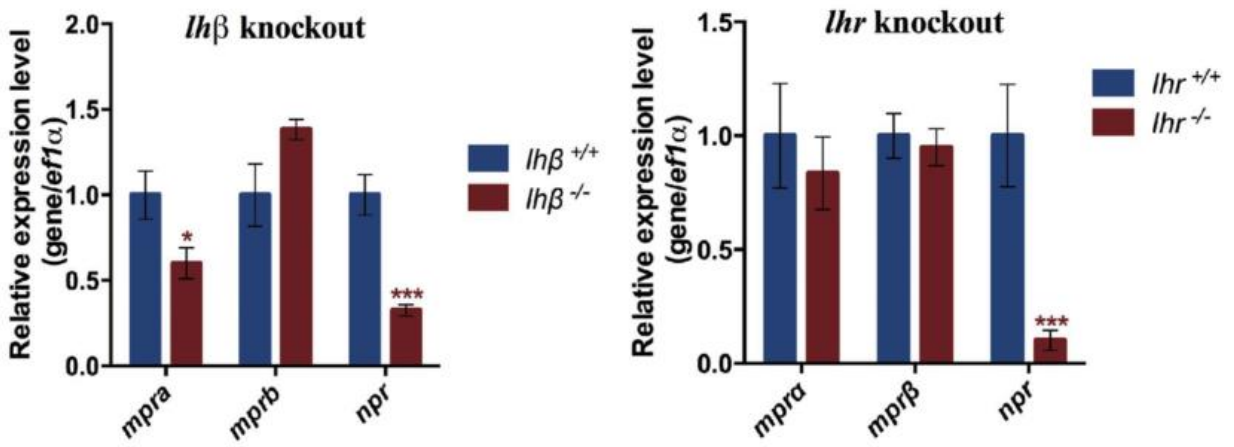

Figure 8 Relative expression of mPRα, mPRβ and nPR of the lhβ and lhr knockout fish.

The mRNAs of the full-grown stage follicles were prepared for qPCR. Expression of the nPR is lower than mPRα, mPRβ. It indicated that nPR expression is controlled by LH signaling pathway (Tang et al. 2016).

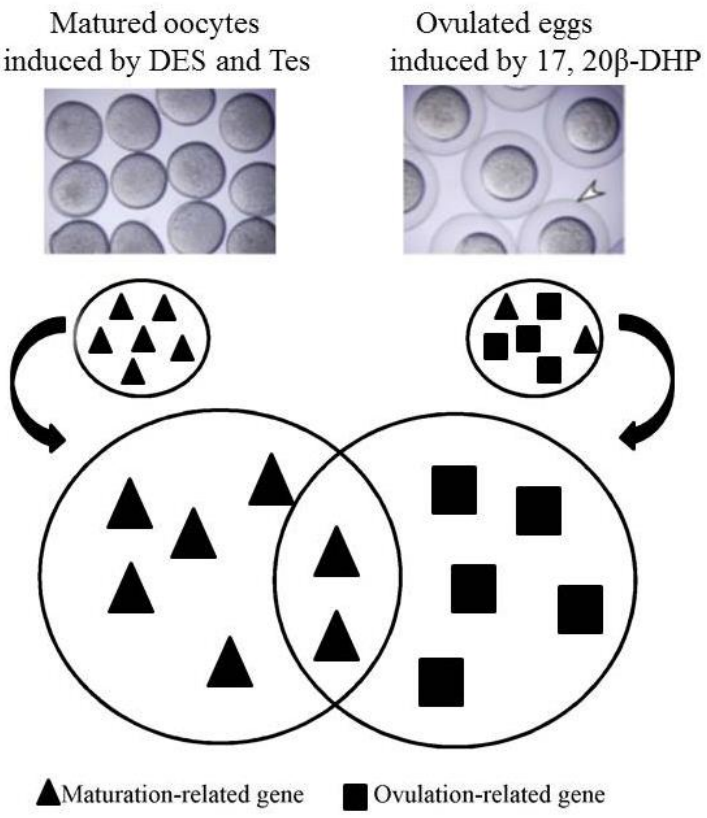

Figure 9 Proposed idea to select ovulation-related genes. Fish kept in water containing 17, 20β-DHP induced both maturation and ovulation. While, fish treated with DES or testosterone (Tes) successfully underwent oocyte maturation and its oocyte development was stopped at this stage. Matured oocytes obtained from DES and TES-treated fish contain only maturation- related genes, while ovulated eggs obtained from 17, 20β-DHP -treated fish contain both maturation- and ovulation-related genes. Up-regulated genes presented in ovary of 17, 20β-DHP-treated fish but not up-regulated in other treatment were candidate genes in regulation of ovulation.

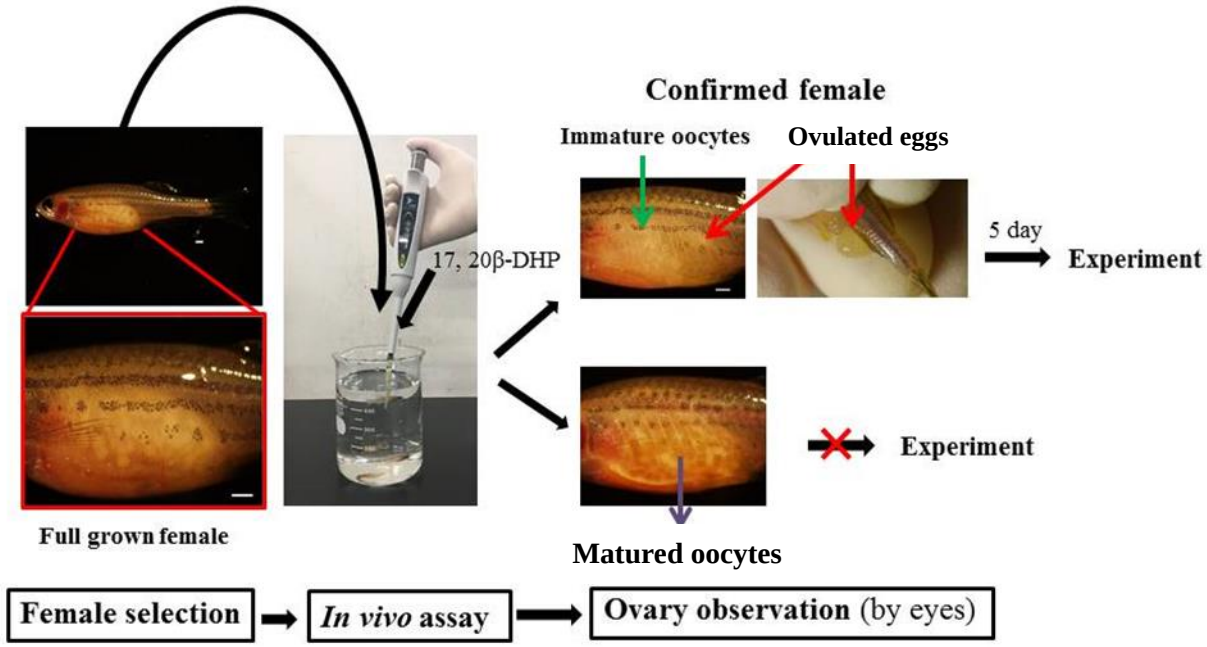

Figure 10 The outline to prepare the confirmed female. Full-grown females are treated by 17, 20β-DHP *in vivo*. The confirmed female is females that can produce ovulated eggs. Activity of reproduction is confirmed by that those oocytes can be squeezed from ovaries. The confirmed females were kept for 5 days before using for experiment. Scale bars indicate 1 mm.

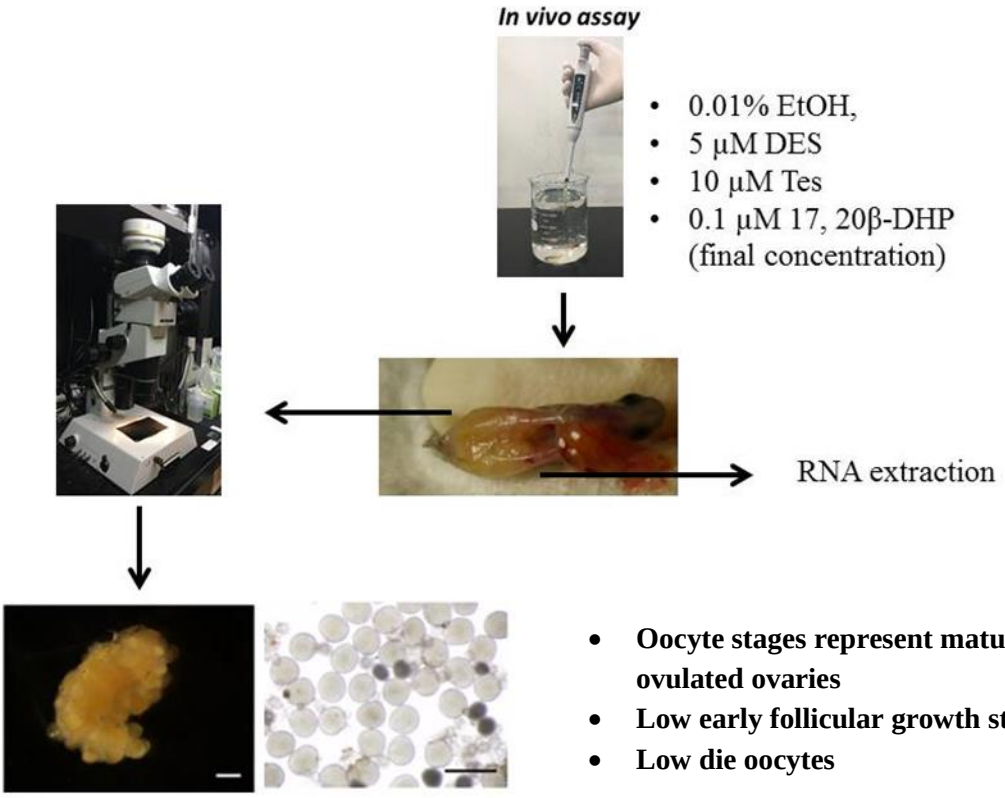

Figure 11 Summary of the process to prepare ovarian samples. Confirmed females were exposed to agents *in vivo* and incubated at 28.5 ˚C for 3 hours. Female zebrafish were killed by spinal severance. Ovaries were cut from the body cavity under sterile conditions. One side of ovary was collected for RNA extraction. Another side of ovary was assessed morphology and quality of oocytes under a stereomicroscope. Scale bars indicate 1 mm.

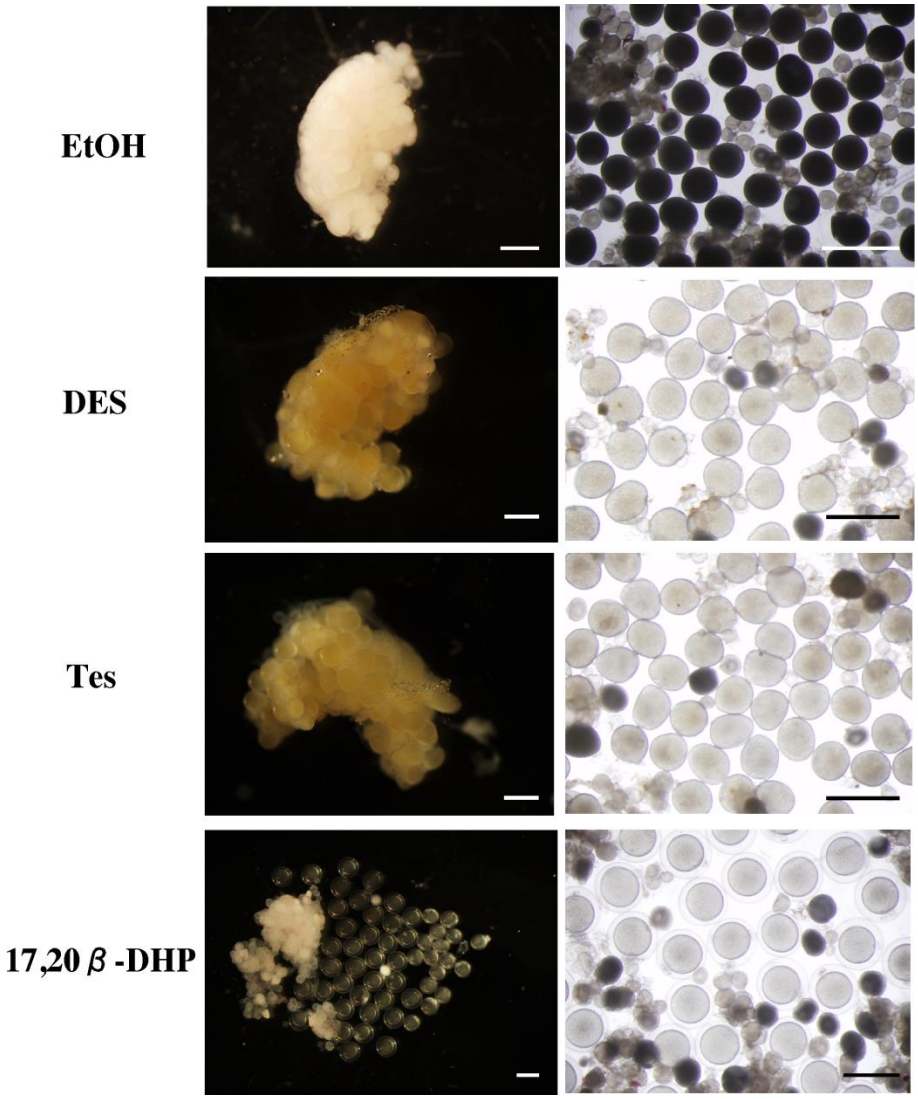

Figure 12 The in vivo bioassay. Fishes were treated with a final concentration of 5 μM of DES, 1 μM of Tes or 0.01 μM of 17, 20βDHP *in vivo*. One side of the ovary was observed by stereomicroscopy. The morphologies of the ovarian samples after 3 hours of treatment were photographed. Ovaries before (left panels) and after (right panels) splitting are shown. After treatment with EtOH, the oocytes remained opaque and showed no morphological change after exposure to water. Oocytes after treatment with DES or Tes became transparent. A fertilization membrane developed in ovulated eggs by 17, 20β-DHP treatment after exposure to water. Scale bars indicate 1 mm.

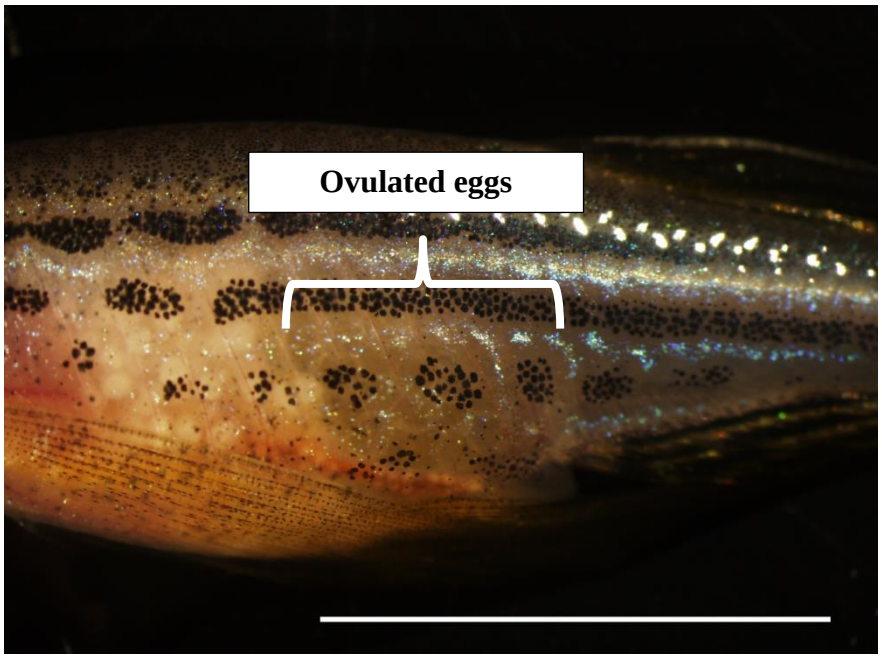

Figure 13 Ovary of ovulated sample achieves from natural induction. Natural paring was set the day before sample collection. A female from natural paring condition was collected at 8.00 a.m. Abdomen of gravid fish become round shape because of ripe ova popped inside. Transparent oocytes indicate that germinal vesicles breakdown (GVBD) was induced. Opaque oocytes remain in the immature stage. The scale bars indicate 1 cm.

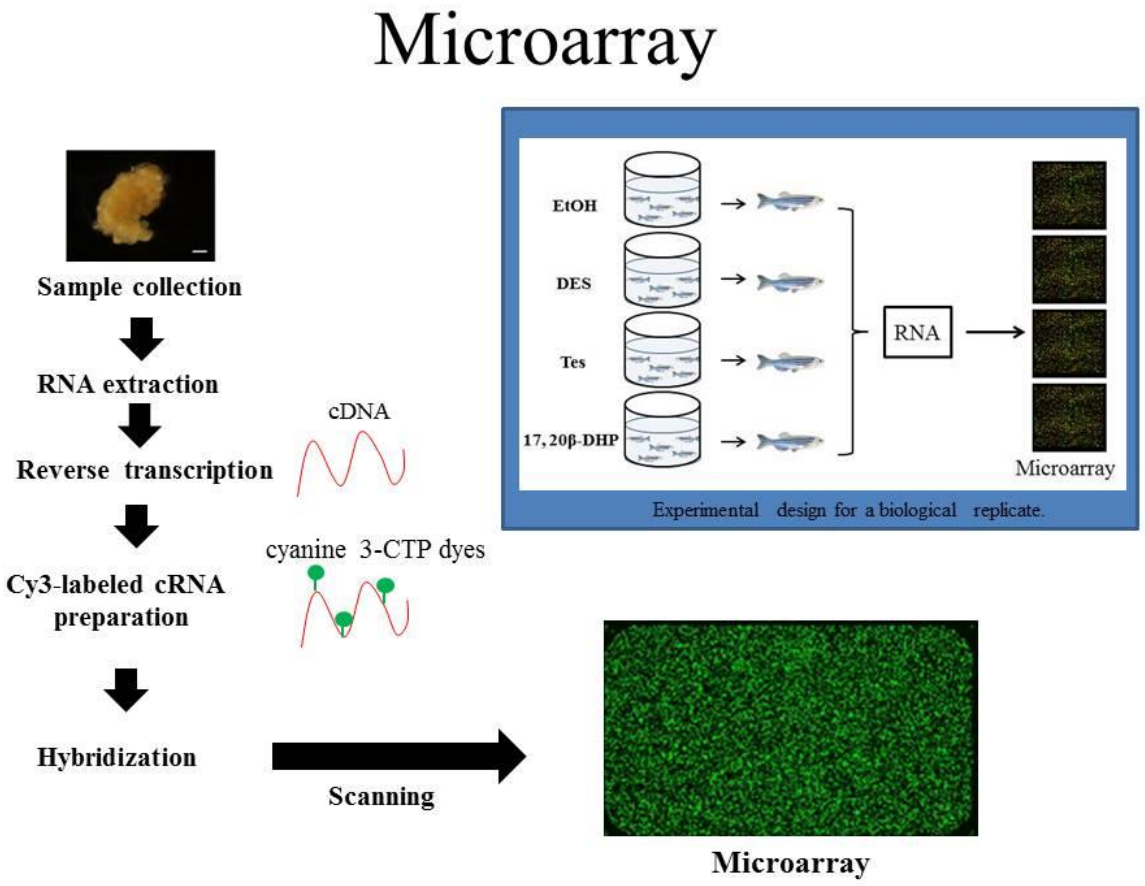

Figure 14 Outline of microarray analysis to compare gene expression profile in ovarian samples. Experimental design to prepare a biological replicate is presented. The *in vivo* bioassay was performed at a final concentration of 5 μM of DES, 1 μM of Tes or 0.01 μM of 17, 20β-DHP. Five fish were treated together in the same beaker. The best specimen was selected from each treatment for each array. Three bio-replicates have been analyzed.

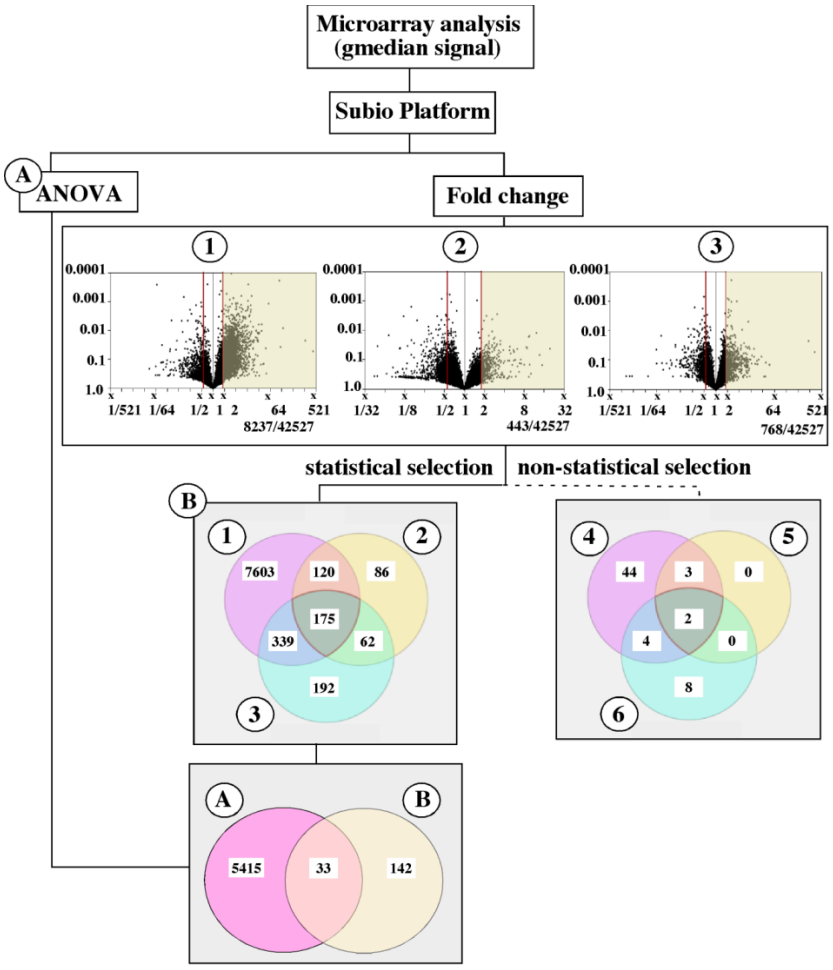

Figure 15 A diagram showing the process for selecting candidates of genes associated with ovulation by the Subio platform (Subio Inc., Amami, Japan). In standard statistical selection, ANOVA was performed across all groups to detect significant differences in both up- and down-regulated genes among all treated groups ($p \leq 0.05$) (A). More than 1.8-fold greater expressions in 17, 20β-DHP than in EtOH (1), DES (2) or Tes (3) have been reported. Overlapping genes among these three groups were selected by Venn diagrams analysis (B). Finally, overlapping genes in categories A and B were selected. Thirty-three genes were selected as candidates for genes associated with ovulation. For non-statistical gene selection, more than 10-fold greater expressions in the 17, 20β-DHP-treated sample compared with the EtOH- (4) DES- (5) or Tes- (6) treated samples were analyzed by Venn diagrams.

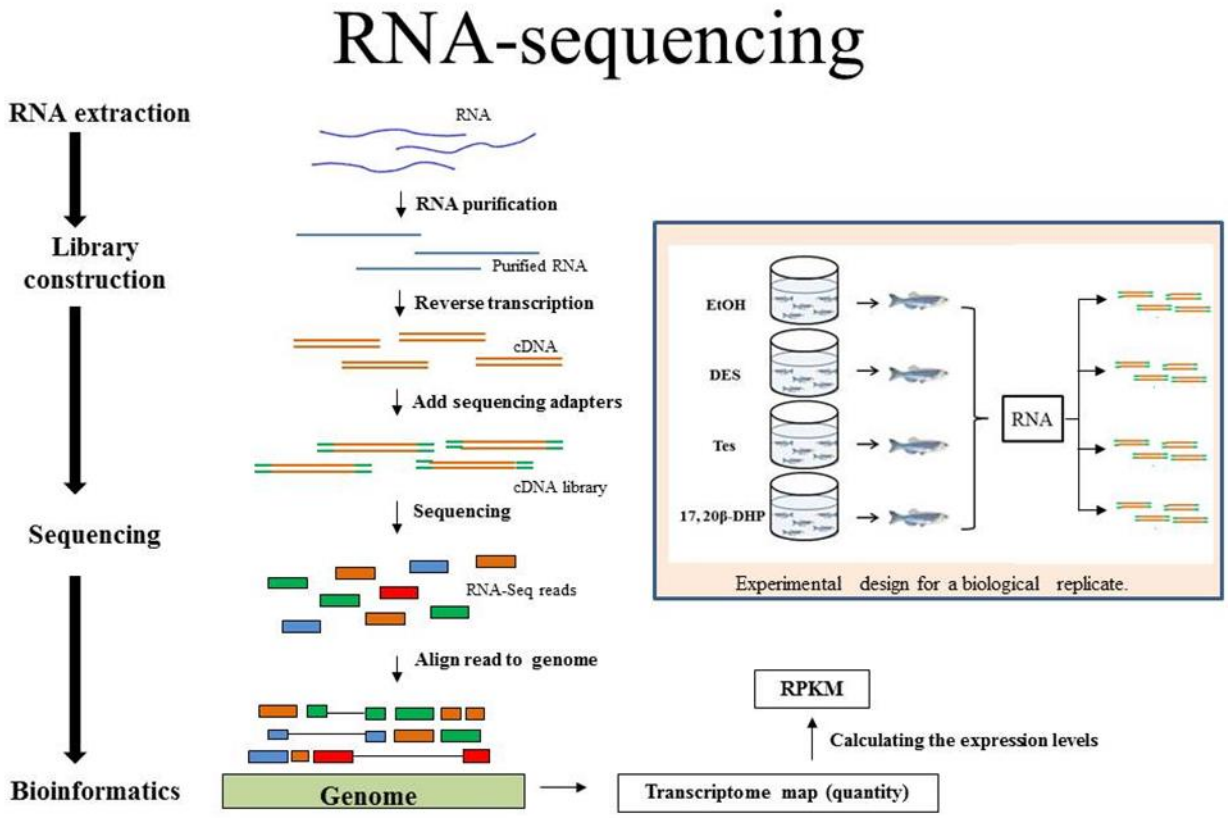

Figure 16 Summary of RNA-sequencing to observe gene expression profile. Experimental design to prepare a biological replicate is presented. The best specimen among each treatment was selected to prepare a cDNA library. Three bio-replicates have been prepared and sequenced.

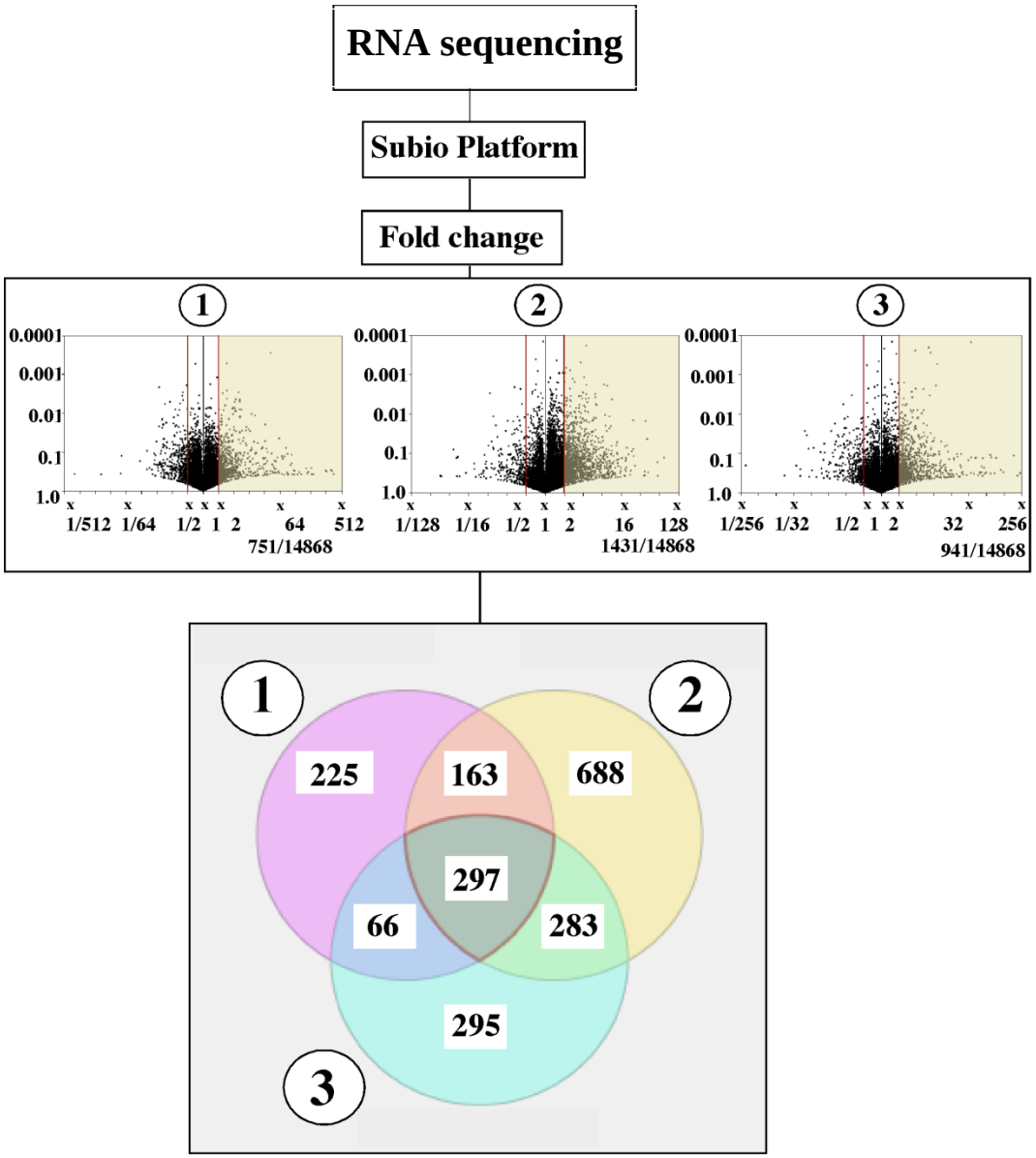

Figure 17 A diagram showing the process for selecting candidates of genes associated with ovulation by the Subio platform (Subio Inc., Amami, Japan). Genes showed more than 2-fold greater expressions in 17, 20β-DHP than in EtOH (1), DES (2) or Tes (3) were selected. Overlapping genes among these three groups were selected by Venn diagrams analysis. Number of genes selected as candidates for genes associated with ovulation is 297.

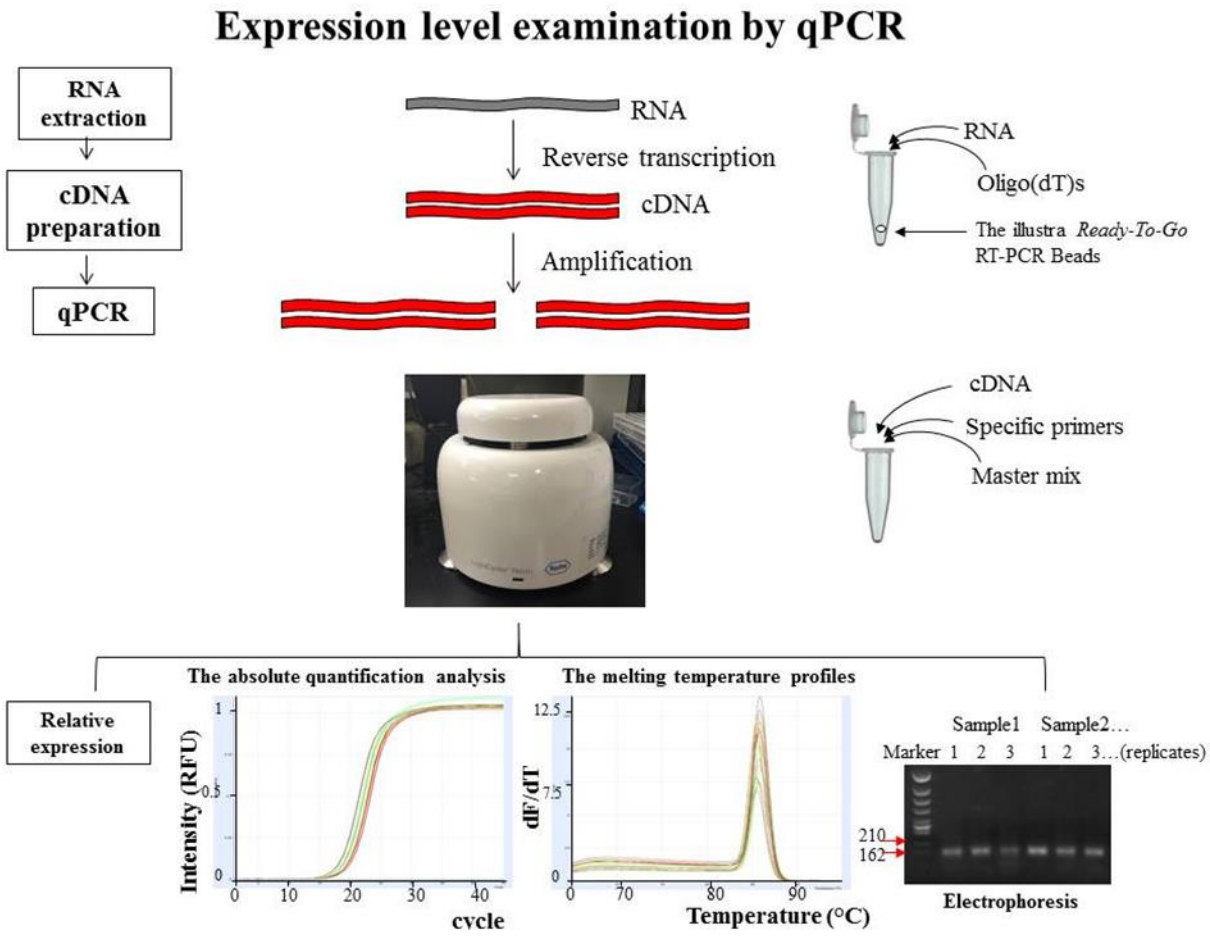

Figure 18 Summary of steps of qPCR to detect the gene expression level. qPCR products were evaluated by 1.5% agarose gel electrophoresis, the absolute quantification analysis and melting temperature profiles have been evaluated.

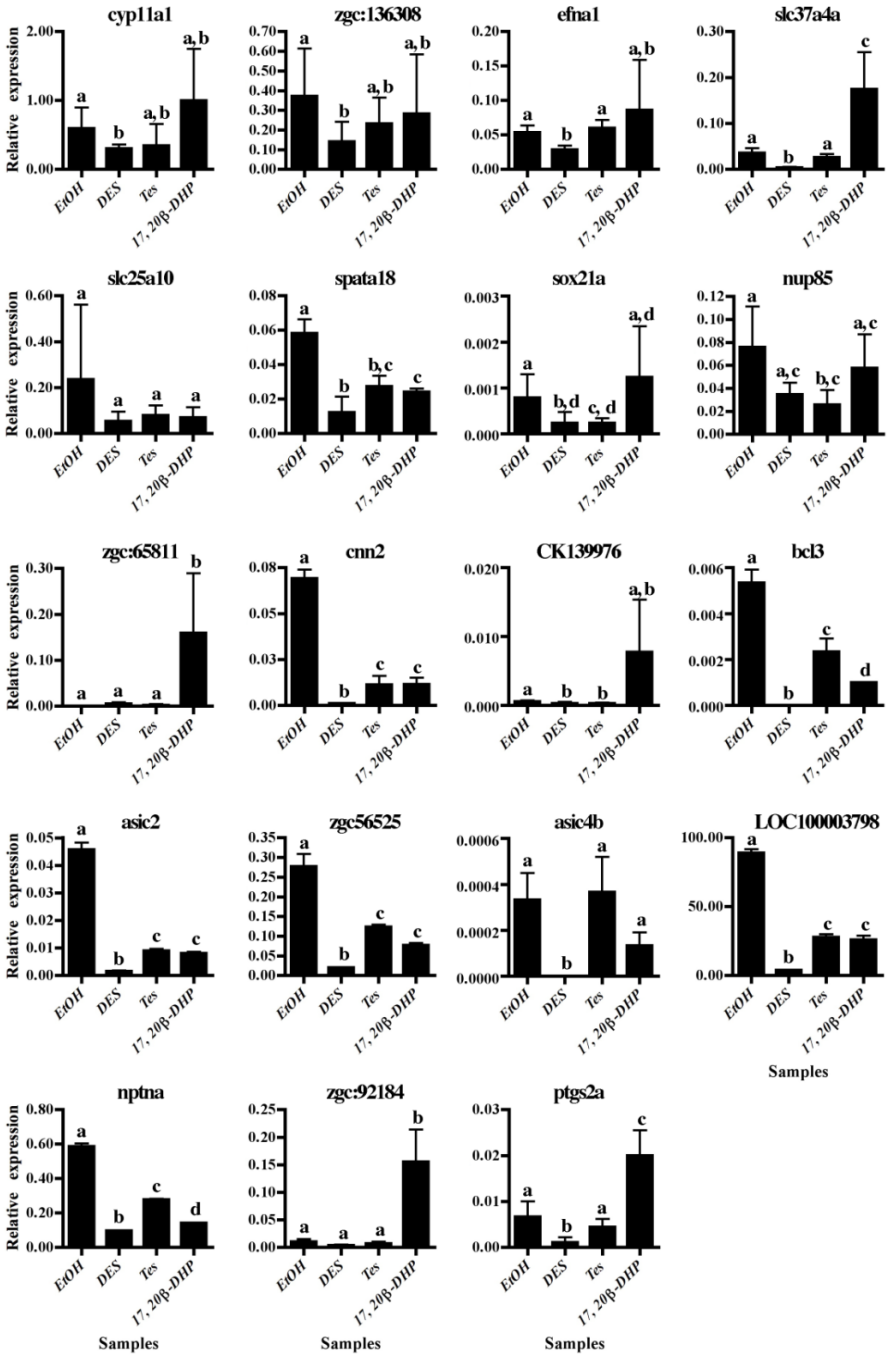

Figure 19 Gene expression profiles of candidates for ovulation-related genes selected by microarray analysis. The qPCR analysis of selected 18 genes and a positive control gene, ptgs2a, were conducted using cDNAs prepared from ovaries from fish treated by EtOH, DES, Tes or 17, 20β-DHP. The mRNA abundance was observed in triplicate for each sample and all data were normalized with the number of transcripts of elongation factor 1α (EF1α) in each sample. Relative expression values are represented as the mean ± SE. Different letters represent significant differences among the data ($p \leq 0.05$).

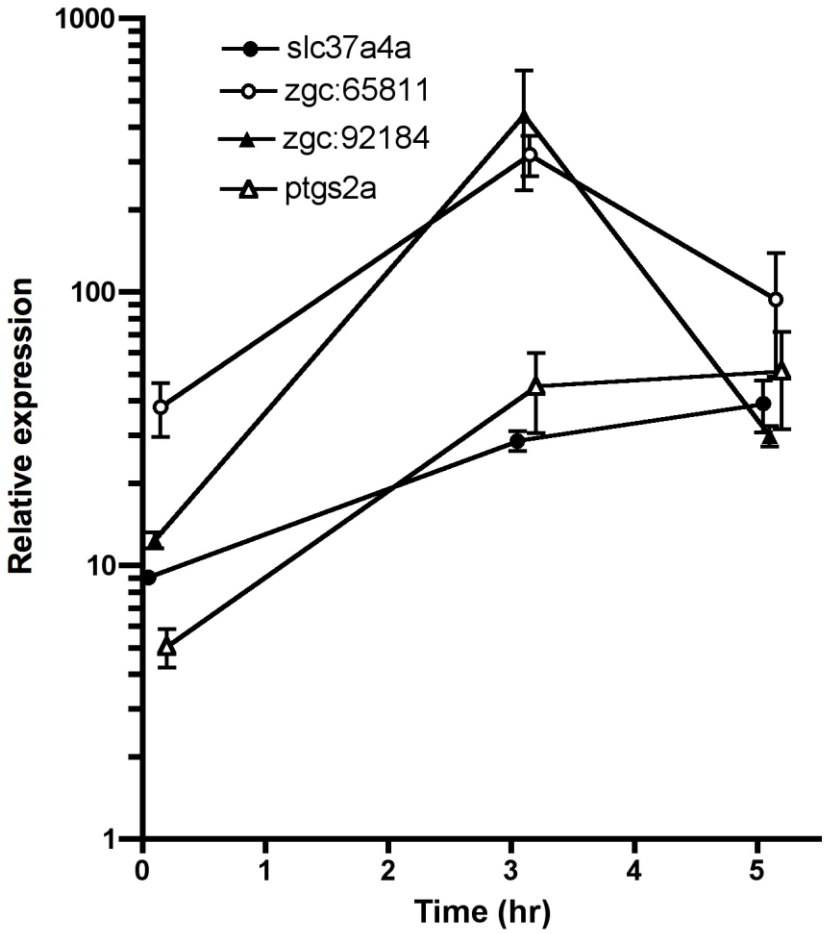

Figure 20 Changes in expression of genes over the time of treatment. Total mRNAs were prepared from samples at 0, 3, 5 hour treated with 17, 20β-DHP. The qPCR analysis of slc37a4a, zgc:65811, zgc:92184 and ptgs2a were conducted. The mRNA abundance was analyzed in triplicate for each sample and all data were normalized with the number of transcripts of elongation factor 1α (EF1α) in each sample. Expression values are represented as the mean ± SE in three independent samples.

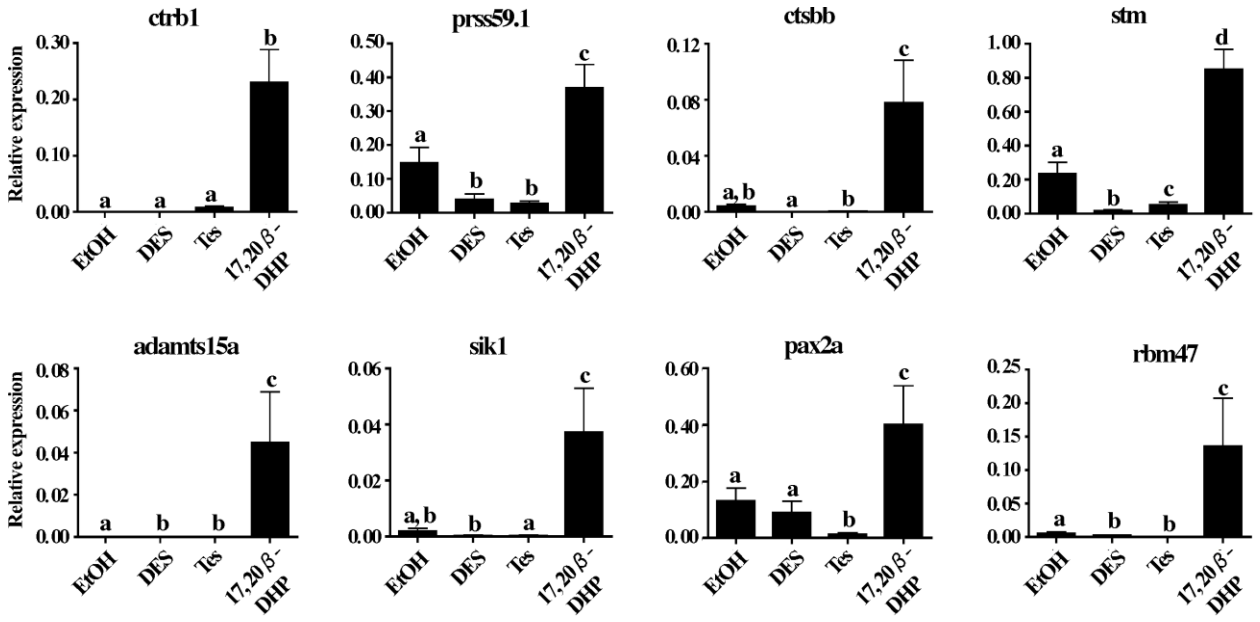

Figure 21 Candidates for ovulation-related gene selected by RNA-sequencing analysis. Relative expression of 8 genes (a positive control gene, ptgs2a, described in above), were analyzed by qPCR. mRNA abundance in ovarian tissues treated by EtOH, DES, Tes or 17, 20β-DHP are presented. All data sets were normalized with the number of transcripts of elongation factor 1α (EF1α) in same sample. Expression values are represented as the mean ± SE. Different letters represent significant differences among the data ($p \leq 0.05$).

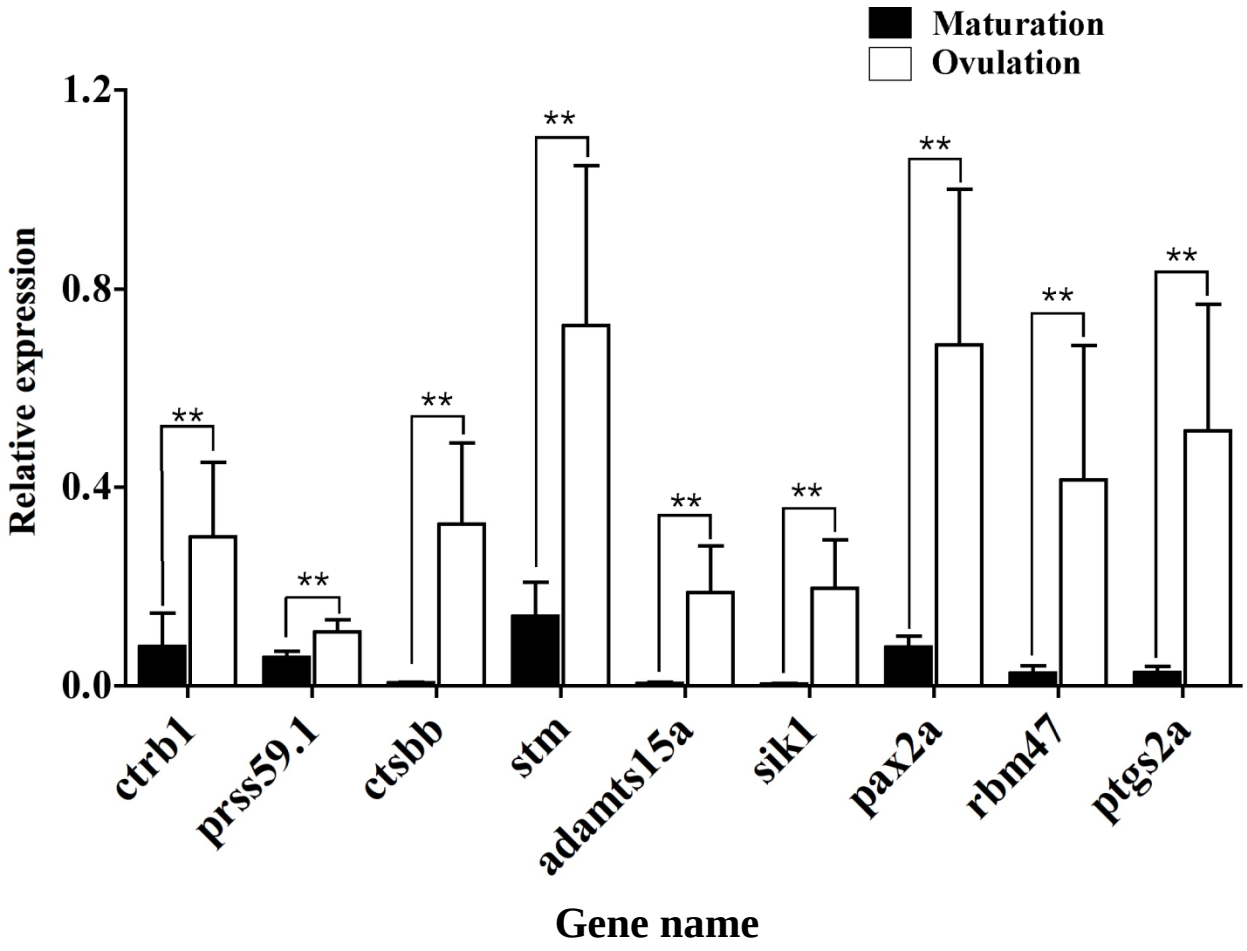

Figure 22 Relative expression of candidate genes in matured and ovulated samples obtained from natural induction. Expression levels of all genes in ovulated samples are higher than in matured samples. Asterisks represent significant difference between the samples. ($P \leq 0.05$).

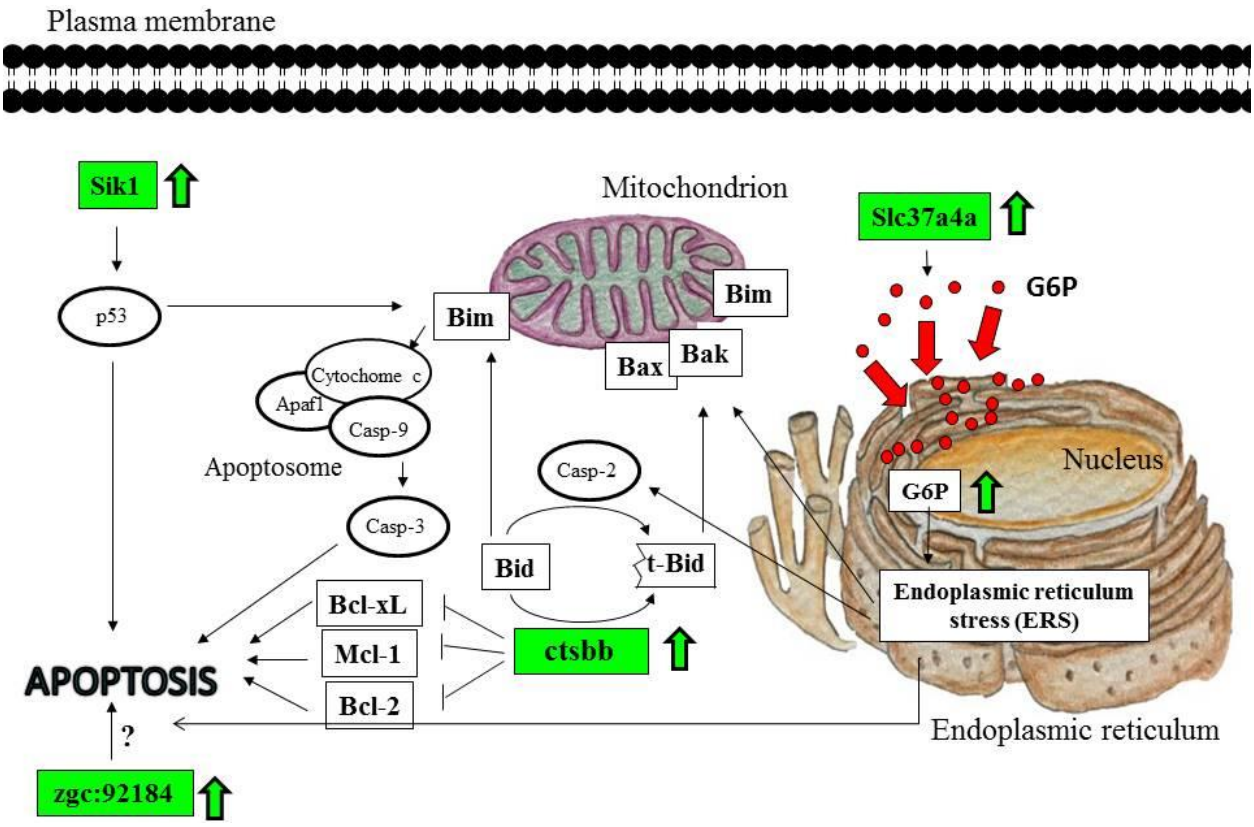

Figure 23 Diagrammatic presentation of hypothesized signaling pathways for apoptosis induced by 4 candidates of the ovulation-related genes. Expression of the slc37a4a gene induces accumulation of intracellular G6P in ER. Excessive amount of intracellular G6P triggers ERS. Prolonged ERS leads to several cell death pathways, for example, (I) the signals from ERS activate several proteins located on the ER–mitochondria interface such as Bax, Bak, and Bim. The BAX/BAK proteins promote the release of pro-apoptotic factors such as the cytochrome c. An apoptosome, a complex structure protein including the cytochrome c, apaf1 and casp-9, stimulates casp3 leading to apoptosis. (II) ERS mediates Bid cleavage. The t-Bid leads to production of pro-apoptotic factors. (III) ERS is a direct cause of apoptosis. The cathepsin gene (ctsbb) inhibits several antiapoptotic factors. It blocks pro-survival pathways. Moreover, ctsbb involves in the cleavage of Bid. Up-regulation of sik1 stimulates P53 protein, a well-known factor to induce apoptosis. The Bim expression is induced by the phosphorylation of transcription factors, such as p53. The P53 also acts as a part of a mitochondrial complex allowing Bax activation and cytochrome c release. The zgc:92184 has shown the 82% identity to Gimap7. Some of members in GIMAP family involve in apoptosis, however, inducing pathways are obscures.

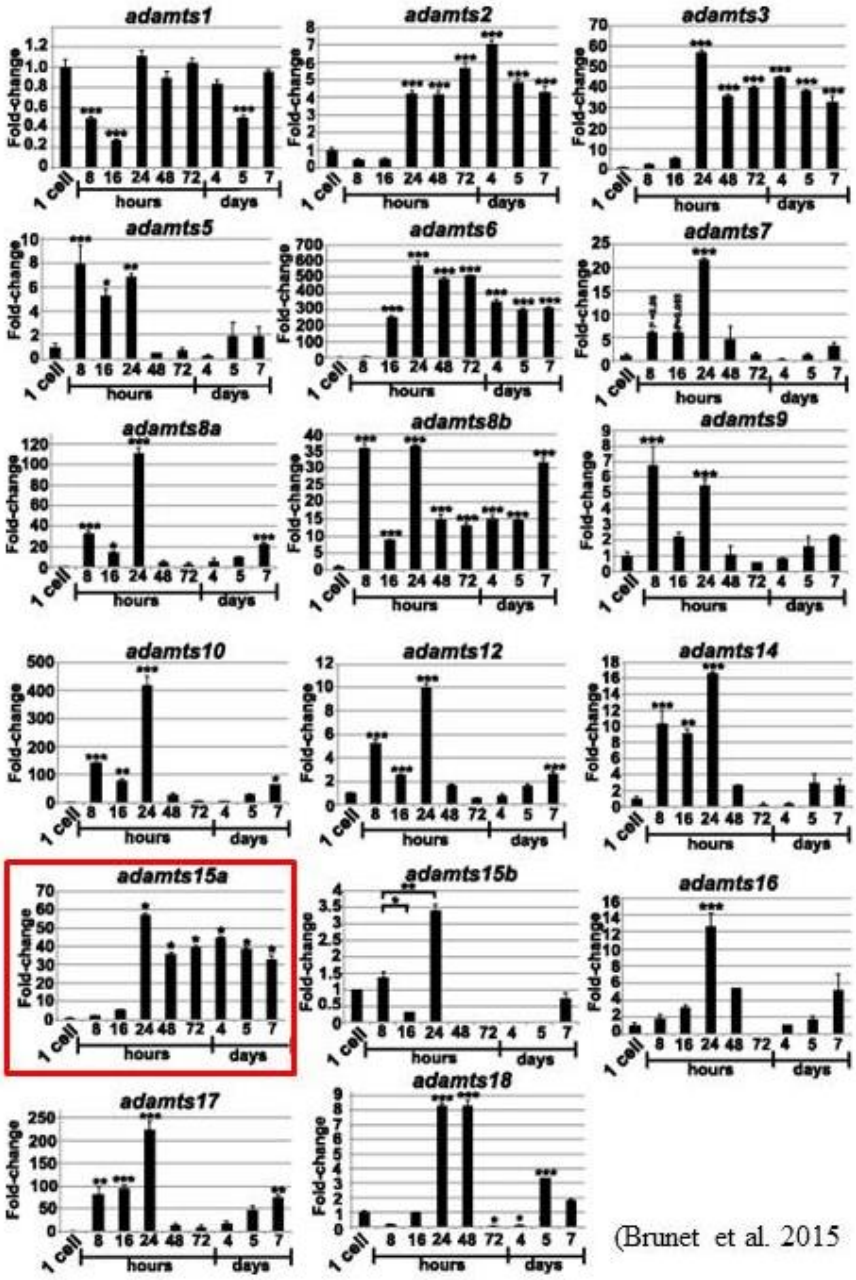

Figure 24 Expression of adamts genes family during zebrafish development. Total RNA were prepared from several zebrafish embryo stages from the first cell development to 7 days post fertilization stages. Expression levels were evaluated by qPCR. The data are normalized to the 1 cell stage (zygote). *P < 0.05, **P < 0.01, ***P < 0.001. Expression levels of adamts15a soar since 24 hour post fertilization (hpf).

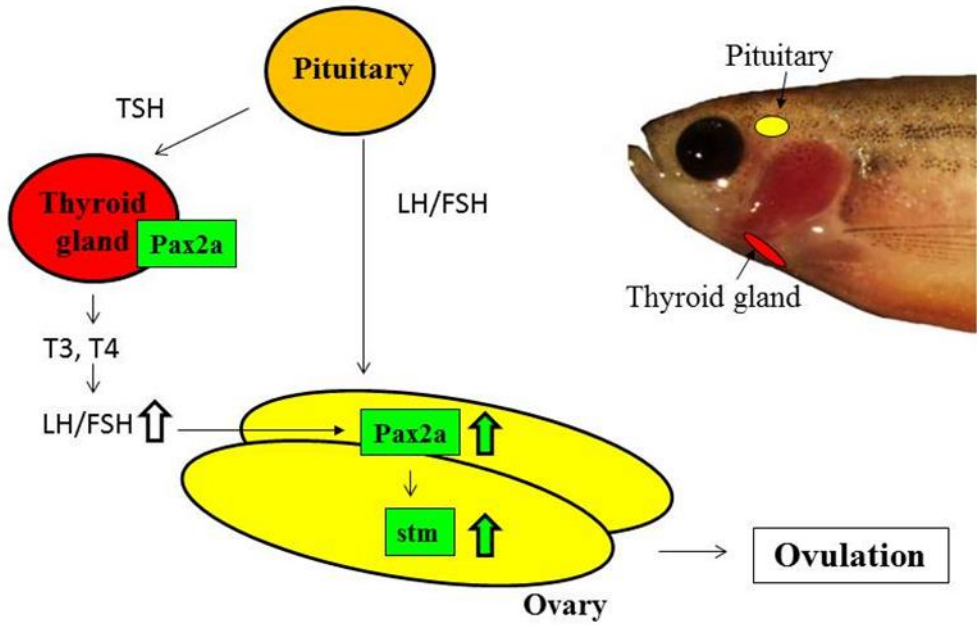

Figure 25 Pax2a and stm genes involve in ovulation. This is a hypothesized signaling pathway to explain relationship of pax2a and stm in ovaries during ovulation. Pax2a plays a role in the production of LH/FSH in the thyroid gland. It might has a potential function responded to LH in ovaries. Pax2a mediates expression of stm in the otolith morphogenesis. This signaling pathway is hypothesized as the ovulation-inducing pathway.

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