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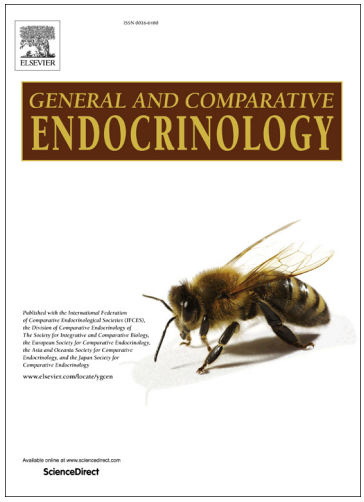

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The role of PDE5a in oocyte maturation of zebrafish

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Abstract

The importance of cyclic guanosine monophosphate (cGMP) signaling pathway in oocyte maturation has recently attracted much attention in vertebrates. Previously, using zebrafish as a model, we have revealed the role of cGMP and the action of cGMP protein kinase (PKG) in oocyte maturation. In the present study, the function of a cGMP specific phosphodiesterase (PDE5a) is further analyzed in oocyte maturation in zebrafish. Two distinct PDE5a coding genes (named PDE5aa and PDE5ab) were identified in zebrafish, and expressed in most adult tissues including ovary. Both *pde5aa* and *pde5ab* mRNA are predominantly expressed in the oocyte but not in follicular cells. Two commercial antibodies targeted to mammalian PDE5a and phosphorylated PDE5a were validated in zebrafish, and we found both antibodies can be used to detect PDE5ab and phosphorylated PDE5ab of zebrafish, respectively. Using both antibodies, we found PDE5ab is only expressed in the oocyte and the phosphorylation of PDE5ab in oocyte could be activated during oocyte maturation induced by human chronic gonadotropin. Intriguingly, we found that the oocyte maturation could be stimulated by treatment of either two different PDE5a inhibitors, sildenafil or tadalafil， and such effects could be completely blocked by a PKG inhibitor KT5823 and two gap junction blockers, respectively. All of these results clearly demonstrate the importance of PDE5a in maintaining the oocyte maturation of zebrafish. When compared with mammals, the functional model of PDE5a is different in zebrafish, suggesting the function of PDE5a might shift from the oocyte in fish to the granulosa cell in mammals during evolution.

Introduction

Normal female fertility depends on proper development of the oocyte. The oocyte maturation is the final stage of oocyte development before ovulation. The precisely regulated meiotic progression during oocyte maturation is essential for normal ovulation and subsequent fertilization, and involves changes in the delicate balance between factors promoting meiotic arrest and others that inhibiting oocyte maturation [1-4].

In recent years, cyclic nucleotides have been demonstrated to be essential for oocyte maturation. The actions of LH on oocyte meiotic resumption are believed to be mediated in large part through increasing the production of cyclic adenosine 3′,5′-monophosphate (cAMP) [5]. The role of another cyclic nucleotide cyclic guanosine 5′-monophosphate (cGMP) in oocyte maturation has recently attracted much attention in mammals [1, 6, 7]. In mouse preovulatory follicles, meiotic arrest is maintained by cGMP that is produced in the granulosa cells and diffuses through gap junctions into the oocyte [8, 9]. Within the oocyte, cGMP inhibits the cAMP phosphodiesterase PDE3A, maintaining a high level of cAMP in the oocyte, which acts on cell cycle proteins to prevent the progression of meiosis [8]. LH induces a rapid reduction of cGMP in follicular cells, resulting in diffusion of cGMP out of oocyte. This relieves the inhibition of PDE3A in the oocyte, lowering cAMP content and allowing meiosis to resume [1].

In mural granulosa and cumulus cells, cGMP is produced by the membrane guanylyl cyclase natriuretic peptide receptor (NPR2), which can be activated by phosphorylation or by the binding of its ligand, C-type natriuretic peptide (CNP or NPPC) [7, 10]. In addition to the CNP/NPR2 system, cGMP phosphodiesterase (PDE) is also involved in regulating the cGMP

level in ovarian follicles after LH receptor activation. For example, in rat follicles, part of the basal cGMP-hydrolytic activity is explained by PDE5 [11]. LH induces the phosphorylation of PDE5 on serine 92 in rat follicles, which is associated with an ~70% increase in cGMP-hydrolytic activity [11, 12]. Inhibition of PDE5 activity significantly and reversibly inhibits spontaneous maturation of cumulus-oocyte complexes [11-13].

Recently, using zebrafish as a model, we have demonstrated the importance of cGMP in oocyte maturation [14]. We also found cGMP-dependent protein kinase is required for both spontaneous and cGMP-regulated oocyte maturation in zebrafish [15]. Whether cGMP specific PDE is involved in fish oocyte maturation is still largely unknown. In this study, we aimed to investigate the role of PDE5a in oocyte maturation using the zebrafish as a model.

Materials and Methods

Animals

All zebrafish were purchased locally. Zebrafish were kept in a circulated water system with a 14h light and 10h dark cycle at 28.5 °C according to the zebrafish book [16]. Fish were fed twice daily with newly hatched brine shrimp (Brine Shrimp Direct, USA). All of the procedures performed in fish experiments are in accordance to the regulations of the Animal Experimentation Ethics Committee of Northwest Normal University.

Chemicals

AR grade chemicals and hCG were obtained from Sigma-Aldrich (USA), culture media from Gibco and enzymes were from Promega (USA). Both Sildenafil and Tadalafil were purchased from Selleck (USA). The hCG was dissolved in culture media at 5 IU/μl. The other chemicals were all dissolved in DMSO at 100 mM as stock solution.

RNA isolation and RT-PCR

Total RNA samples were isolated using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity and amount of the RNA were determined by a NanoDrop 2000C Spectrophotometer (Thermo, USA). Quantitative real-time PCR was carried out using the SYBR Green Real-Time PCR Mix (Toyobo, Japan) on a real-time detection system (Bio-Rad, USA) as previously described [17]. Whereas semiquantitative RT-PCR analysis, PCR was carried out on a Thermal Cycler 9600 (Eppendorf, Germany). All primers used in this study are listed in Table 1.

Western blot analysis and immunostaining

For Western blot, adult tissues or ovulatory follicles at different time points after treatment were collected and lysed. The concentration of lysates was determined by BCA Protein Assay Kit (Pierce, USA). Western blot was performed as previously described [18]. Since antibodies for zebrafish PDE5as and phosphorylated PDE5as (pPDE5as) are unavailable, two commercial antibodies including anti-human PDE5a (Aviva System biology, USA) and anti-human pPDE5a (GeneTex, USA) were used in this study. The epitope sequence for PDE5a is TACDLSAITKPWPIQQRIAELVATEFFDQGDRERKELNIEPTDLMNREKK. and the epitope sequence for pPDE5a is GTPTRKI(S92)ASEFDR. The specificity of antibody against PDE5 and phosphorylated PDE5 was validated by western blot. Actin (Santa Cruz, USA) was used as loading control. All the primary antibodies were used at a 1:1,000 dilution. The extracted proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto PVDF membranes. The membranes were then incubated in a solution with the appropriate primary antibodies .Horseradish peroxidase (HRP)-labeled secondary antibodies were used to generate a chemiluminescent signal which was detected with a CCD camera-based imager (Chemidoc MP Imaging System, Bio-Rad, USA). The gray density of protein bands was quantified by ImageJ following the procedure as previously described [19].

For immunostaining, ovulatory follicles were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS; pH 7.4). Samples were dehydrated,

infiltrated and embedded in wax. Sections of ovaries were cut at 5 μm. After antigen retrieval with 0.01 M citrate buffer (pH 6.0), the slides were washed with PTW (PBS, 0.1% Tween) and were blocked for 120 min in 5% BSA in PTW. The whole setup was then incubated with PDE5 (1:50 dilution) or pPDE5 (1:100 dilution) antibody overnight at 4°C. The sections were washed and probed with goat anti-rabbit Alexa 488 (Invitrogen, USA) then washed in PBS. Cell nuclei were then visualized using counterstaining reagent 4'-6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA), and photographed on a confocal system with inverted microscope (Olympus FV1000, Japan).

Isolation and incubation of ovarian follicles

The staging system that we have adopted is based on the original definition of Selman [20]. Adult female zebrafish were killed and the stage FG follicles were carefully separated in 60% Leibovitz L-15 medium using a fine forceps without damaging the follicle cell layers as previously described [21]. Follicles that were full grown but immature (FG; about 0.65 mm in diameter) were manually isolated. Follicles were randomly distributed into the wells of a 24-well plate and incubated (around 30 follicles/well) at 28°C. Each group had four to five replicate wells and each experiment was repeated at least three times.

Separation of follicular cells and oocyte from ovarian follicles

The follicular cell layer was carefully peeled off with fine forceps as previously described [14]. The oocytes and follicular cells were collected for RNA extraction, separately.

Statistical analysis

All data were expressed as mean values ± SEM with a one-way ANOVA was run with statistic significance at $p<0.05$, Fisher's least significant difference test was used to detect treatment differences all statistics were run using GraphPad InStat software (GraphPad Software, USA). Statistical comparisons of the *pde5as* expression levels between follicular cell and oocyte was conducted using an unpaired two-tailed Students's t-test.

Results

1. Two distinct PDE5 coding genes are identified in zebrafish

By searching available databases, we have identified two distinct PDE5 coding genes in zebrafish, including PDE5aa (on Chromosome 1) and PDE5ab (on Chromosome 7). The phylogenetic analysis grouped both zebrafish PDE5s genes into the PDE5 clade, indicating that they are PDE5 coding genes (Fig. 1A). Both PDE5s consisted of two GAF domains and PDEase motif. A serine which could be phosphorylated by PKA/PKG, is present at site 88 in PDE5aa and at site 143 in PDE5ab proteins (Fig. 1B). However, by comparing the sequence and phosphorylated site, PDE5ab is more similar to PDE5a in other species (Fig. 1C). The spatial expression patterns of *pde5a* genes in adult zebrafish shows that both *pde5aa* and *pde5ab* expression are detected in most tissues examined in both male and female fish (Fig. 1D). The expression of both *pde5a* genes could be detected in the ovary, suggesting the role of PDE5 in the ovary development.

2. Both mRNA and protein of PDE5as are predominantly expressed in the oocyte

Using real time PCR, we found both *pde5a* genes exhibit different expression profiles during folliculogenesis (Fig. 2A). Transcripts of both *pde5a* genes were highly expressed in oocytes but not follicular cells (Fig. 2B). To analyze the expression of PDE5a and phosphorylated PDE5 (pPDE5a), two commercial antibodies targeted human PDE5a and pPDE5a were validated in zebrafish. The predicted homologies are 74% of zebrafish PDE5aa and 82% of zebrafish PDE5ab to immunogen sequence of PDE5a, respectively; 0% of zebrafish PDE5aa and 92% of zebrafish PDE5ab to immunogen sequence of pPDE5a,

respectively. To validate both antibodies, three different adult tissues including ovary, liver from females, and testis were employed. The predicted weight of both zebrafish PDEaa and PDEab is around 100kD; a band at corresponding size could only be visualized in the ovary sample but not in liver and testis samples using both PDE5 and pPDE5 antibodies (Fig. 2C). Considering the homology based on immunogen sequence and the fact that *pde5aa* mRNA but not *pde5ab* mRNA is expressed in adult testis (Fig. 1D), both antibodies seem to only specifically recognize zebrafish PDE5ab and phosphorylated PDE5ab, respectively. Using PDE5a antibody, the PDE5ab protein was found to be highly expressed in oocytes but not follicular cells by western blot (Fig. 2D). Using immunostaining, the expression of PDE5ab protein in ovarian follicles was further detected. Consistently, the signal is highly located in the oocyte (Fig. 2E). All these data indicate that both *pde5aa* and *pde5ab* mRNA and PDE5ab protein are predominantly expressed in the oocyte.

3. The phosphorylation of PDE5ab in the oocyte is induced during oocyte maturation

Previously we have established a platform for artificial induction of oocyte maturation in zebrafish [22]. Oocyte maturation can be achieved by 2.0 h after injection of hCG (15 IU/fish) (Fig. 3A). Using this platform, the protein expression and phosphorylation of PDE5ab during oocyte maturation was assessed by western blot. Using western blot analysis, the level of phosphorylated PDE5ab but not total PDE5ab increased (Fig. 3B). The quantitative analysis on western blot showed that the signal of phosphorylated PDE5ab was significantly higher compared to the control (Fig. 3C). This result was further confirmed by the immunostaining analysis that the signal of phosphorylated PDE5ab is increased during oocyte maturation after

injection of hCG. These results indicate that the phosphorylation of PDE5ab in the oocyte is stimulated during oocyte maturation by LH signaling.

4. The basal activity of PDE5a is required for maintaining meiotic arrest in zebrafish

In order to analyze the action of PDE5a in oocyte maturation, FG stage follicles were treated with two different PDE5a inhibitors, sildenafil and tadalafil. The induction of oocyte maturation by both inhibitors exhibits a clear dose-dependence (Fig. 4A). Such stimulatory effect could also be found by treatment with another PDE5a inhibitor, avanafil (data not shown). The stimulatory effects of both sildenafil and tadalafil on oocyte maturation can be totally blocked by a PKG specific inhibitor KT5823 (Fig. 4B). While the effects of tadalafil on oocyte maturation could be largely blocked by two different gap junction blockers, lindane and 18α-Glycyrrhetinic acid, respectively (Fig. 4C). All these results suggest that basal activity of PDE5a is required for maintaining meiotic arrest in zebrafish.

Discussion

In recent years, many studies have demonstrated that cGMP is a critical factor in regulating the oocyte maturation in several species [1, 23, 24]. Using zebrafish as model, we have recently revealed the dual role of cGMP in regulation of oocyte maturation [14], and the importance of PKG in mediating the action of cGMP on oocyte maturation [15]. In the present study, we have further revealed the function of a cGMP specific phosphodiesterase (PDE5a) in oocyte maturation of zebrafish.

Mammals only have one type of PDE5 coding gene, PDE5a [25]. Unlike in mammals, we

identified two different PDE5 coding genes including PDE5aa and PDE5ab. By searching the genomic database, both PDE5aa and PDE5ab are found only in some teleost species, indicating both genes might be generated by a third genome duplication (3R) that occurs in most teleost fish [26]. Based on the phylogenetic tree and alignment analysis, we found PDE5ab is closer to PDE5a from other species compared to PDE5aa, implying that the function of PDE5ab in zebrafish is more similar to PDE5a. In this study, we found the both *pde5as* mRNA and PDE5ab protein is predominantly expressed in oocyte but not in follicular cells of zebrafish. This result is quite different from the findings in mammals. In mouse, PDE5a protein is highly expressed in the thecal cell but low in the oocyte [13], and PDE5a mRNA can be detected by real-time PCR in granulosa cell of both rat and mouse [11]. Here, we further found that in zebrafish the phosphorylation of PDE5ab takes place in the oocyte but not follicular cells, which could be activated during oocyte maturation by LH signaling. Although the distribution of phosphorylated PDE5a in ovarian follicles has not been investigated in mammals, the phosphorylation of PDE5a in the granulosa cells has been demonstrated and found to be increased in response to LH through PKA pathway [11]. However, since the antibodies employed in this study seems to only recognize PDE5ab but not PDE5aa in zebrafish, the expression of PDE5aa protein and its phosphorylation during oocyte maturation is still unknown, which warrants more studies in the future.

One of interesting findings in this study is the effect of PDE5a inhibitors on oocyte maturation in zebrafish. Since the phosphorylation of PDE5a/PDE5ab can be activated during oocyte maturation in both mammals and zebrafish, respectively, we believed that similar to the mammals, inhibition of PDE5a activity by inhibitors might block the spontaneous oocyte

maturation in zebrafish. Surprisingly, we found the oocyte maturation was not blocked but significantly stimulated in intact and full grown follicles by treatment of three different PDE5a inhibitors. This finding is in significant contrast to the results in mammals. In both mouse and rat, PDE5a inhibitors could significantly decrease the LH-induced or spontaneous oocyte maturation [11-13]. This difference between fish and mammals might be due to the different function of cGMP in regulation of oocyte maturation. PDE5a is the cGMP specific phosphodiesterase. In mammals the decrease in cGMP production in granulosa cells leads to release of meiotic arrest [1]. However, in zebrafish, we previously proposed the dual role of cGMP in oocyte maturation, that cGMP increased in follicular cells or decreased in the oocyte can induce oocyte maturation [14]. Therefore, the stimulatory effect of PDE5s inhibitors on oocyte maturation in zebrafish might be explained the induction of oocyte maturation by increasing oocyte cGMP after PDE5 inhibition. To investigate this speculation, the role of cGMP protein kinase (PKG) in PDE5 inhibitors-regulated oocyte maturation was analyzed. We have previously revealed that PKG activity in follicular cells is essential for cGMP-induced oocyte maturation [15]. In this study, we found the stimulatory effects of PDE5s inhibitors-induced oocyte maturation could be totally blocked by a PKG specific inhibitor, suggesting that PDE5a inhibitors induce the oocyte maturation through the cGMP/PKG pathway. Moreover, the expression and phosphorylation of PDE5a is restricted in the oocyte of zebrafish. The cGMP increased by PDE5a inhibitors might diffuse from oocyte to follicular cell through gap junctions. Here, we found that such stimulatory effect of PDE5a inhibitors on oocyte maturation could be totally blocked by two different gap junction blockers, suggesting the gap junction is important for PDE5a action on oocyte maturation and

this further confirm our speculation on action of PDE5a inhibitors. Consistently, we found PDE5a inhibitors could induce the oocyte maturation in intact follicles. Therefore, it can be explained that in intact follicles, the cGMP increased in the oocyte after treatment of PDE5a inhibitors, can move into follicular cells by gap junctions to induce oocyte maturation by regulating some unknown factors in follicular cell through PKG. However, we found that cGMP levels in intact follicles and denuded oocytes did not significantly increase in response to PDE5a inhibitors sildenafil and tadalafil (data now shown). This result is similar to the mammals that sildenafil didn't largely affect the concentration of cGMP [13]. It is possible that, after treatment with PDE5a inhibitors, the change of cGMP level is too transient or the quantity of cGMP changed is too small to be detected by ELISA assay.

In this study, we found the phosphorylation of PDE5a could be activated in the oocyte of zebrafish during oocyte maturation by hCG. What is the physiological function of this activation? The decrease of cGMP in the oocyte during oocyte maturation of zebrafish has been demonstrated by us [14]. We speculate that the activation of PDE5 is involved in decrease of cGMP level in oocyte to regulate oocyte maturation. However, we found the oocyte maturation of denuded oocytes was not largely affected by treatment of both PDE5a inhibitors for 6 h (data now shown), suggesting the activation of PDE5a in the oocyte might contribute to but redundant in regulation of oocyte maturation through cGMP. It is possible that some other cGMP specific PDEs are also involved in the regulation of oocyte maturation in zebrafish.

Taken together, using zebrafish as a model, we have revealed for the first time in fish the function of PDE5a in regulation of oocyte maturation. Two PDE5a homologous genes are

highly expressed in the oocyte of zebrafish. The phosphorylation of PDE5a in the oocyte could be activated during oocyte maturation by LH signal. Different from the role of PDE5a in oocyte maturation revealed in mammals, we conclude that in zebrafish that a basal activity of PDE5a is required for maintaining meiotic arrest in zebrafish.

Acknowledgement

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Figure legends

Fig. 1 Two distinct PDE5as are identified in zebrafish

(A) Phylogenetic analysis of the vertebrate PDE5a family members. Full length of PDE5s were analyzed by the neighbor-joining method using the MEGA3 program. Bootstrap values derived from 1,000 runs are shown. Human PDE6 is used as an outgroup. Accession numbers for the sequences used are: zebrafish (PDE5aa, XP_009305135.1; PDE5ab, NP_001116732.1), tilapia (PDE5aa, ENSONIT000000011200.1; PDE5ab, ENSONIT00000006868.1), medaka (PDE5aa, ENSORLT000000039837.1; PDE5ab, ENSORLT000000038028.1), mouse (PDE5a, NP_700471.2), human (PDE5a, NP_001074.2; PDE6, NP_000431.2), chicken (PDE5a, NP_001305949.1) and *Xenopus* (PDE5a, NP_001072812.1). (B) The conserved domains of both zebrafish PDE5aa and PDE5ab. The boxes in different colors indicate functional domains: GAF and PDEase_1. Serine can be phosphorylated by PKA/PKG is marked by arrow. (C) The alignment of sequences from different species around the phosphorylated serine. Conserved phosphorylated serine is marked by white bracket. (D) RT-PCR analysis on the expression of both *pde5aa* and *pde5ab* mRNA in different adult zebrafish tissues.

Fig. 2 Expression of PDE5as in ovary of zebrafish.

(A) Temporal expression of both *pde5aa* and *pde5ab* mRNA in the follicles of different stages isolated from the ovaries of adult fish. PG, primary growth; PV, previtellogenic stage; EV, early vitellogenic stage; MV, midvitellogenic stage; LV, late vitellogenic stage; FG, full grown stage. (B) The expression of both *pde5aa* and *pde5ab* mRNA in follicular cells and

oocytes compartments. (C) Validation of PDE5 and phosphorylated PDE5 antibody in zebrafish. The expression of total PDE5a, phosphorylated PDE5 (pPDE5a) and actin was detected in adult ovary, liver and testis of zebrafish by western blot. (D) The expression of PDE5ab protein in follicular cells and oocytes compartments, using actin as a reference gene. (E) The immunostaining analysis on the PDE5ab expression in the ovarian follicles.

Fig. 3 The activation of PDE5ab phosphorylation during oocyte maturation in zebrafish

(A) Oocyte maturation could be dynamically induced by administration of hCG (15 IU/fish). (B) The expression of phosphorylated PDE5ab and total PDE5ab protein during oocyte maturation induced by hCG. (C) Quantitative of the ratio of phosphorylated PDE5ab/total PDE5ab signal during oocyte maturation. Each value represents the mean value ± SEM from three independent experiments (*P<0.05 vs. control). (D) The immunostaining analysis on the expression of phosphorylated PDE5ab in the preovulatory follicles during oocyte maturation.

Fig. 4 The function of PDE5a in oocyte maturation of zebrafish

(A) Effects on the rate of oocyte maturation by treatment with two different PDE5a inhibitors, sildenafil (left panel) or tadalafil (right panel), at different doses for 16 hours. (B) Effects on the rate of sildenafil- or tadalafil-induced oocyte maturation by treatment with a PKG inhibitor (10 μM KT5823). (C) Effects on the rate of tadalafil-induced oocyte maturation by treatment with two different gap junction blockers, lindane and carbenoxolone for 16 hours. Each value represents the mean value ± SEM of quintuplicate assays from three independent experiments (***, P<0.001 vs. control).

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Fig. 1

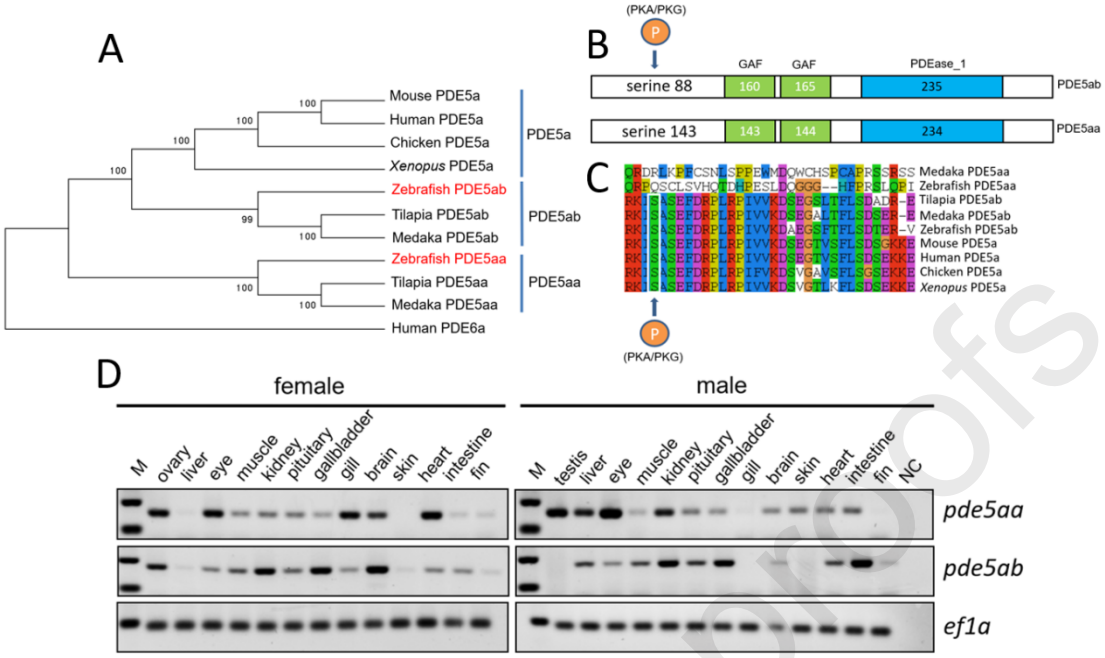


Fig. 2

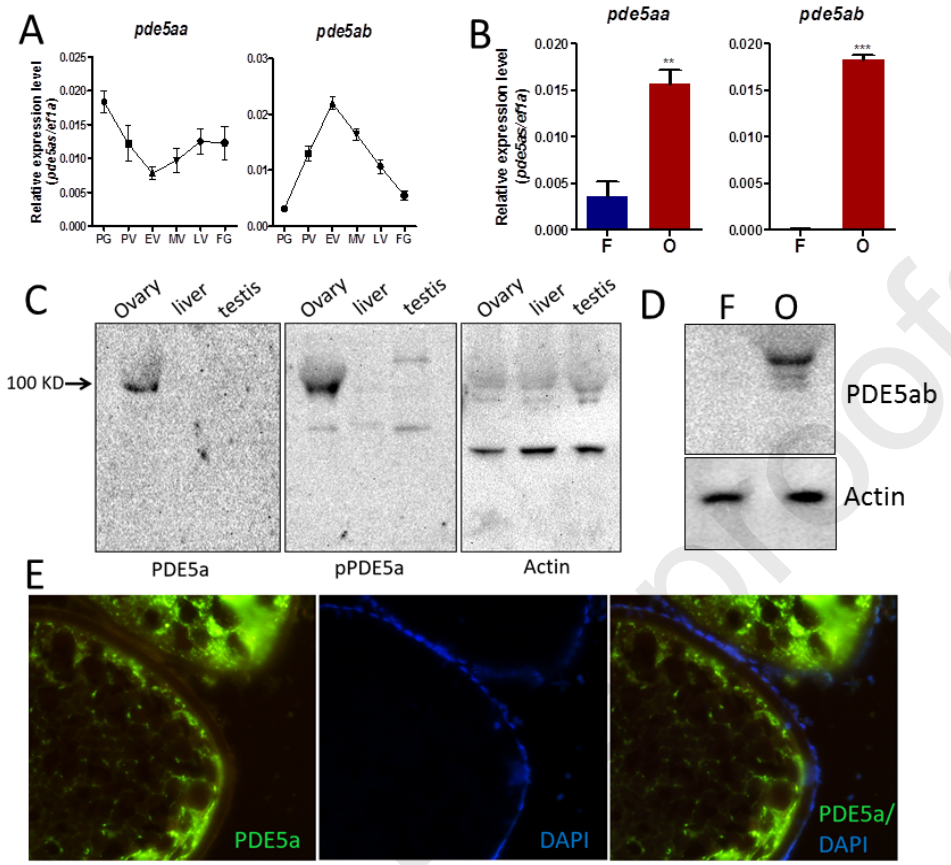


Fig. 3

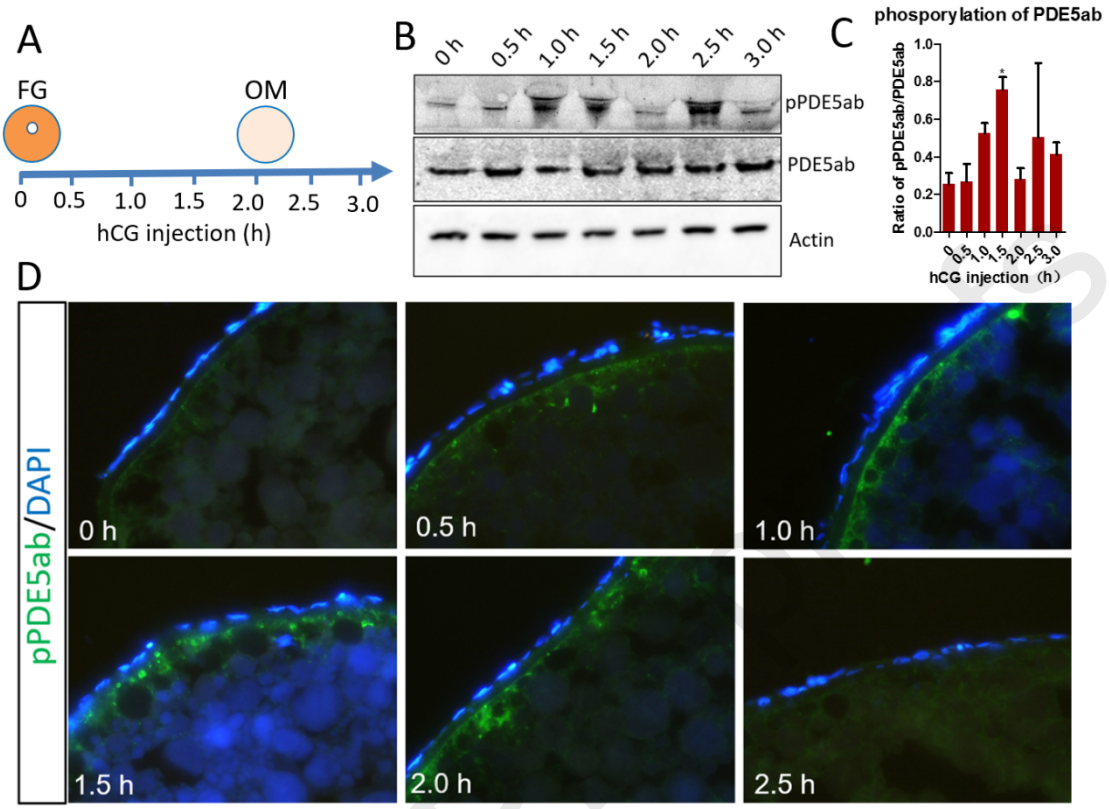


Fig. 4

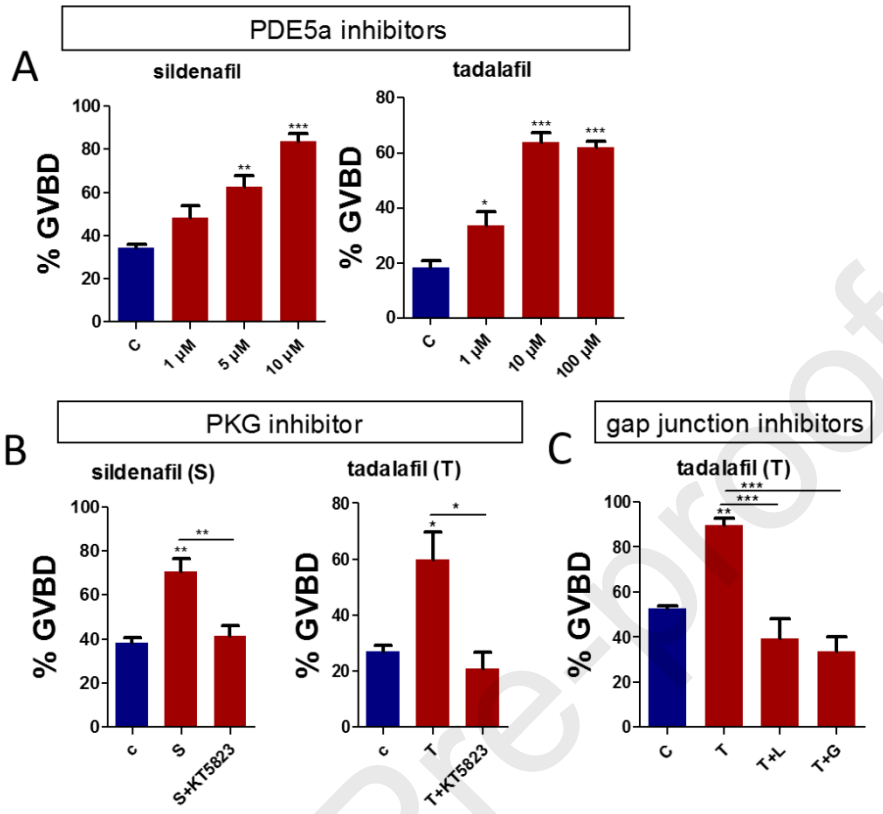
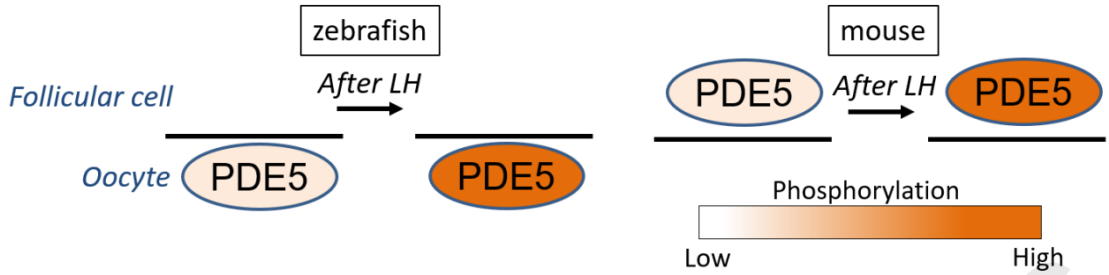


Table. 1 Primers used in this study.

Gene	Sequence(5' to 3' direction)	Strand	Application
pde5aa	GACCGTGAAAATGCGTCTCG	S	Real-time PCR
	TCGTCCACCATTTCTTGCCA	AS	
pde5ab	GACAGATGCTGAGACGACCC	S	Real-time PCR
	AACATGCACTGGGAGGTGTT	AS	
ef1a	CTGGAGGCCAGCTCAAACAT	S	Real-time PCR
	ATCAAGAAGAGTAGTACCGCTAGCATTAC	AS	

Graphic Abstract



Working hypothesis for the regulation of PDE5a during oocyte maturation in both zebrafish and mouse: the diagram integrates our current findings and studies in mammals as discussed in the text. In zebrafish, the PDE5ab in the oocyte can be phosphorylated after LH surge. However, the phosphorylation of PDE5a in the follicular cell can be activated in mouse.

Highlights:

1. Two PDE5a coding genes (PDE5aa and PDEab) were identified in zebrafish

2. Both PDE5a mRNA and protein are predominantly expressed in the oocyte of zebrafish

3. The phosphorylation of PDE5ab in oocyte could be activated during oocyte maturation

4. PDE5a activity is important for maintaining the oocyte maturation of zebrafish