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Upregulation of *adamts9* by gonadotropin in preovulatory follicles of zebrafish

Dong Teng Liu[a], Wan Shu Hong[a], Shi Xi Chen[a,**], Yong Zhu[a,b,*]

[a] State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen, Fujian Province, 361102, People's Republic of China

[b] Department of Biology, East Carolina University, 1000 5th Street, Greenville, NC, 27858, USA

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ABSTRACT

Previously we had identified *adamts9* as a downstream target of Pgr, which is essential for ovulation in zebrafish. The primary goal of this study is to determine whether human chorionic gonadotropin (hCG, LH analog) also regulate *adamts9* expression prior to ovulation. The expression of *adamts9* was induced by hCG in a dose and time dependent manner in zebrafish preovulatory follicles *in vitro*. Interestingly, the stimulatory effect of hCG on *adamts9* expression was not blocked in $pgr^{-/-}$ follicles but blocked in $lhcgr^{-/-}$. This effect of hCG was via Lhcgr and its associated cAMP and PKC signaling pathways. Reduced fecundity and reduced expression of *adamts9* were also found in $lhcgr^{-/-}$ females *in vivo*. Therefore, we have provided the first evidence of gonadotropin (hCG) regulated *adamts9* in zebrafish, which could be important for ovulation.

1. Introduction

Ovulation, the release of a mature and fertilizable oocyte from a follicle, is a principle function of ovary that ensures the preservation of a species. In mammals, this complex process starts when a preovulatory follicle receives a surge of luteinizing hormone (LH) from the anterior pituitary gland. By binding to the LH/chorionic gonadotropin receptor (LHCGR) located on the supporting cells of an oocyte destined to ovulate, LH triggers increases of progesterone (P4), prostaglandins (PGs) and multiple paracrine factors such as EGF and proteolytic enzymes, resumes meiosis and culminates in follicular rupture (Espey and Richards, 2006). Metalloproteinases including members of matrix metalloproteinases and ADAMTS (a disintegrin and metalloproteinase with thrombospondin-like motifs) have been suggested to play an important role in the follicular rupture (Takahashi et al., 2019; Duffy et al., 2019); however, our knowledge on the LH regulation of these proteinases and involved signaling pathways is still lacking.

Recently, LH has also been demonstrated to be essential for final oocyte maturation and ovulation in zebrafish (*Danio rerio*). Knockout of LH ($lhb^{-/-}$) in zebrafish did not affect folliculogenesis, but blocked oocyte maturation and ovulation likely due to the lack of maturation-inducing hormone synthesis that should be induced by LH (Chu et al., 2014; Zhang et al., 2015a; Takahashi et al., 2016). Surprisingly, the Lhcgr knockout female fish ($lhcgr^{-/-}$) were fertile because of a promiscuous ligand-receptor interaction between LH and follicle-stimulating hormone receptor (Fshr) in zebrafish (Chu et al., 2014; Zhang et al., 2015b). It was also reported that ovulation but not maturation was slightly affected in $lhcgr^{-/-}$ fish (Chu et al., 2014). However, it remains to be determined whether Lhcgr plays any role in continuous ovulation.

In our previous study, we demonstrated that the nuclear progestin receptor (Pgr), a key downstream target of LH, is essential for ovulation in zebrafish (Zhu et al., 2015). Similar as in mice and rats, oocytes in Pgr knockout zebrafish ($pgr^{-/-}$) mature normally but are trapped in ovaries after maturation (Lydon et al., 1995; Zhu et al., 2015; Kubota et al., 2016). Subsequent studies reveal a dramatic Pgr-dependent up-regulation of several metalloproteinases including *adamts1*, *adamts9*, *adam8b* and *mmp9* in the zebrafish preovulatory follicular cells (Liu et al. 2017, 2018). Compared to other metalloproteinases, *adamts9* expresses specifically in the follicular cells in which both Pgr and Lhcgr are induced prior to ovulation (Liu et al. 2017, 2018).

We hypothesize that *adamts9* should also be regulated by LH if *adamts9* is involved in the ovulation. The main goal of this study is to determine whether human chronic gonadotropin (hCG, LH analog) and Lhcgr regulate the expression of *adamts9* and involved signaling pathways in zebrafish preovulatory follicles. We found hCG treatment increased *adamts9* expression via Lhcgr signaling pathways in preovulatory follicles *in vitro*. This stimulatory effect is independent of Pgr

* Corresponding author. Department of Biology, East Carolina University, 1000 E 5th Street, Greenville, NC, 27858, USA.
** Corresponding author.
E-mail addresses: chenshixi@xmu.edu.cn (S.X. Chen), zhuy@ecu.edu (Y. Zhu).

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and capable of further potentiating *adamts9* expression in the presence of progestin. Interestingly, we found *lhcgr*$^{-/-}$ female fish exhibited disrupted expression of *adamts9* and ovulation. Therefore, we provided the first evidence that *adamts9* expression is regulated by LH and Lhcgr in zebrafish, which also has a consequence in ovulation.

2. Materials and methods

2.1. Zebrafish husbandry

The wild-type (*wt*) zebrafish used in the present study are a Tübingen strain initially obtained from the Zebrafish International Resource Center and propagated in our lab. The knockout lines of *pgr* gene (*pgr*$^{-/-}$) used were generated and characterized previously (Zhu et al., 2015; Wang et al., 2016). Lhcgr knockout fish (*lhcgr*$^{-/-}$) were kindly provided by Dr. Wei Ge (Zhang et al., 2015b). Pgr and Lhcgr double knockout fish (*lhcgr*$^{-/-}$*pgr*$^{-/-}$) were obtained by crossing these two lines. Fish were kept under a photoperiod of 14 h (hrs) light and 10 h dark (lights on at 08:00) in recirculating freshwater (pH 7.2–7.6) at around 28.5 °C in the zebrafish facility (ESEN, Beijing, China). Fish were fed three times daily to satiation with a commercial food (Otohime B2, Reed Mariculture, CA, USA) containing high protein content and supplemented with newly hatched brine shrimp. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Xiamen University.

2.2. Chemicals and reagents

Human chorionic gonadotropin (hCG) was obtained from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel); forskolin (FSK) and H89 were purchased from APExBIO (Houston, TX, USA); db-cAMP, Phorbol 12-myristate 13-acetate (PMA), and H7 were purchased from Sigma-Aldrich (St. Louis, MO, USA); 4-pregnen-17, 20β-diol-3-one (DHP), a maturation and ovulation inducing progestin in zebrafish, was obtained from Steraloids (Newport, RI, USA), and Leibovitz's L-15 medium was from Thermo Fisher Scientific (Waltham, MA, USA).

2.3. Isolation and preparation of ovarian follicles for in vitro incubation

The experiments usually started in late afternoon. Typically, experimental zebrafish had completed spawning and released mature oocytes in the morning, and immature oocytes would continue to develop into stage IVa follicles in late afternoon (Liu et al., 2018). Stage IVa follicles are fully-grown (> 650 μm, opaque) but immature follicles with an enlarged nucleus (germinal vesicle) in the center of oocyte. Fully-grown follicles will undergo germinal vesicle breakdown (GVBD, i.e. meiosis resumption) to become Stage IVb follicles (> 690 μm, translucent) once stimulated by hormones such as LH or progestin appropriately. Gravid female zebrafish were deeply anesthetized in a lethal dose of MS-222 (300 mg/L buffered solution) for 10 min. To ensure death, the spinal cord and blood supply behind the gill cover were cut off using sharp scissors. Ovaries were removed immediately and placed in a 60-mm petri dish containing 60% L-15 media (in 15 mM HEPES, pH 7.2). With the aid of fine forceps, ovaries were teared into approximately 4 mm^3 pieces, and then transferred to a 15-mL centrifuge tube. Individual follicles were separated by pipetting up and down using a disposable glass pipette with a polished 3 mm opening. Thereafter, washed and clean follicles were transferred back to a 60-mm petri dish containing fresh 60% L-15 media. The diameters of ovarian follicles were measured with an ocular micrometer under a dissecting microscope. Intact stage IVa follicles with visible germinal vesicles (GV) were carefully collected using a curve-neck glass pipette with a polished 1 mm opening. The whole preparation process was finished within 2–3 h and the selected follicles were treated with different hormones or drugs immediately.

2.4. Hormone and drug treatments of stage IVa immature follicles in vitro

Follicles collected as described in section 2.3 were transferred into a 24-well tissue culture plate containing 1 mL of 60% L15 medium (20 follicles per well). To determine signaling pathways involved in the LH regulation of *adamts9*, we treated Stage IVa follicles with hCG (LH analog), steroid (progestin), activators or inhibitors of cAMP and PKC signaling pathways. Our previous study found a transient increase of *adatms9* transcript that reached maximum at 2 h following treatments of hormones or drugs (Liu et al., 2018). Therefore, an exposure time of 2 h was selected to conduct the treatments of hormones or drugs unless indicated otherwise. These follicles were incubated for 2 h at 28 °C with hCG (5, 15, 50 IU/mL), db-cAMP (0.1, 0.5, 1.5 mM), FSK (0.1, 1, 10 μM), PMA (0.1, 1, 10 μM), H89 (10, 20, 50 μM), H7 (10, 20, 50 μM), and DHP (100 nM), alone or in combination. Excluding broken follicles, all the follicles were collected and homogenized immediately in RNAzol for qPCR analyses of gene expression.

2.5. Collection of preovulatory follicles

Oocyte maturation, ovulation and spawning in zebrafish are synchronized with lights and occur sequentially every morning. Typically, Stage IVa (> 650 μm, opaque) fully grown immature follicles will undergo oocyte maturation (meiosis resumption) to become Stage IVb (> 690 μm, translucent) preovulatory mature follicles, and subsequently ovulate to become Stage V fertilizable oocytes (> 720 μm, translucent) within an hour before lights on. Spawning occurs after going through complex reproductive behavior with stimulation from males within 1 h after lights on (Yong et al., 2017). Stage IVb follicles are still enclosed within follicular cells and individually scattered around an ovary (Fig.6D), while Stage V oocytes will be grouped together and located in posterior of an ovary (Fig.6A). More detailed information on collecting preovulatory Stage IVb mature follicles can be found in our previous publications (Liu et al. 2017, 2018). Briefly, approximately three months old gravid females of *wt*, *lhcgr*$^{-/-}$, *pgr*$^{-/-}$ and *lhcgr*$^{-/-}$*pgr*$^{-/-}$ zebrafish were individually paired with a *wt* male in the night before sampling. Then, these female fish were sacrificed an hour before lights on in the morning, and ovaries containing healthy preovulatory follicles with a translucent appearance were immediately removed and transferred to petri dishes containing 60% L-15 medium. Thereafter, ovarian follicles were isolated as abovementioned in section 2.3, and 30 stage IVb follicles were collected from each fish, homogenized in RNAzol and stored in a −80 °C freezer until RNA extraction. Sampling were conducted at the same time points (06:45–07:10) over a three-day period, and 20 fish in total were sampled (6 *wt*, 7 *lhcgr*$^{-/-}$, 3 *pgr*$^{-/-}$ and 4 *lhcgr*$^{-/-}$*pgr*$^{-/-}$). At least one fish individual for each genotype was sampled in each collection.

2.6. Consecutive spawning test

Spawning of zebrafish was optimized under enhanced feeding condition (Liu et al., 2018). Six months old mature females of *wt*, *lhcgr*$^{+/-}$ and *lhcgr*$^{-/-}$ fish were individually housed with a *wt* male in multiple spawning tanks. Everyday each pair of fish were fed twice in the afternoon with adequate food in an interval of about 5 h. Around 21:00 (1 h prior to lights off), water in spawning tanks was replaced with clean water, and a mesh inner tank was inserted into each spawning tank to prevent the eating of eggs by the adults. The number of fertilized eggs were recorded for each pair of fish every morning. The *wt* and *lhcgr*$^{+/-}$ females were continuously monitored for two to three weeks, whereas the *lhcgr*$^{-/-}$ females were monitored for at least a month for each individual due to increased spawning intervals observed in these fish. Embryo number and spawning interval were averaged from data of at least five times of spawning. Six individual females of each genotype were examined.

2.7. RNA extraction, reverse transcription and real-time quantitative PCR

Total RNA from ovarian follicle samples was extracted using RNAzol reagent (MRC, Cincinnati, Ohio, USA). Briefly, the RNAzol lysed follicle samples were centrifuged (12,000 rpm, 10 min) to remove undissolved yolk fractions before the first precipitation step with water. BAN solution (4-bromoanisole) was added to purify the RNA and eliminate genomic DNA in the second precipitation step. Then, an equal volume of cold 100% isopropanol was added. The precipitated pellet was washed three times with 500 μL 75% ethanol and dissolved in 25 μL RNase-free water. The approximate concentration and purity of samples were determined using a Nanodrop 2000 Spectrophotometer. Same amount of total RNA (0.5 μg) from each sample was used for synthesis of the first strand cDNAs in a 10 μL reaction using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). All quantitative PCRs (qPCRs) were carried out in a 20 μL reaction on the qTOWER 2.2 Real-Time PCR detection system (Analytik Jena AG, Jena, Germany) at default settings using PowerUp SYBR Green Master Mix (Applied Biosystems, USA). The absolute transcript levels, expressed as copies/μg total RNA, were determined using Ct values of samples and standard curve corresponding to the target gene generated from known serial plasmid concentrations (10^2-10^7 copies/μL). The specificity and efficiency of the specific primers for *adamts9* have been described and validated in previous studies (Liu et al., 2018).

2.8. Statistical analyses

Data were presented as means ± SEM. Depending on the experimental setup, data were analyzed using either Student' s *t*-test or one-way ANOVA followed by Fisher' s PLSD post hoc test to assess statistical differences among the individual groups using the SPSS (version 23.0) statistical software package. All experiments were repeated three times, and the results of one representative experiment is shown.

3. Results

3.1. hCG upregulates the expression of adamts9 in stage IVa follicles in vitro

HCG is commonly used as LH analog to induce ovulation in various species including zebrafish (Chu et al., 2014). Because progestin and its receptor (Pgr) act as downstream targets of LH to induce *adamts9* expression prior to ovulation (Liu et al., 2018), we hypothesized that hCG could also increase *adamts9* expression. We carried out a follicle incubation assay using preovulatory (stage IVa) follicles from sexually mature zebrafish that have maximal expression of Lhcgr (Kwok et al., 2005). Our results showed that exposure to hCG significantly increased *adamts9* expression in a dose dependent manner in stage IVa follicles from wild-type fish (*wt*, Fig. 1A). This hCG induced *adamts9* upregulation was transient, peaked around 2 h post incubation (hCG, 50 IU/mL), then decreased sharply (Fig. 1B).

3.2. hCG upregulates adamts9 expression via Lhcgr signaling pathways

LH is signaling through Lhcgr, and activates downstream genes primarily through cAMP-PKA pathway or PKC pathway (Choi and Smitz, 2014). To determine whether Lhcgr and its downstream signaling pathways involved in the upregulation of *adamts9*, we examined the effects of activators or inhibitors of cAMP-PKA pathway or PKC pathway on the *adamts9* expression in *wt* stage IVa follicles *in vitro*. Treatment of *wt* stage IVa follicles with db-cAMP, a membrane-permeable analog of cAMP, mimicked the effect of hCG and increased *adamts9* expression in a dose-dependent manner (Fig. 2A). In agreement with the effect of db-cAMP, treatment with the diterpene forskolin (FSK), a potent stimulator of adenylate cyclase (Hedin and Rosberg, 1983), also upregulated the expression of *adamts9* dose-dependently (Fig. 2B). FSK exhibited stronger stimulatory effect on *adamts9* expression than db-cAMP, possibly because FSK could activate all intracellular adenylate cyclases (ACs) except AC9 (Fig. 2A&B). Similarly, treatment with PMA, a PKC activator, significantly increased *adamts9* expression in *wt* stage IVa follicles at a dose of 10 μM (Fig. 2C). Conversely, both inhibitors for PKA (H89, Fig. 2D) and PKC (H7, Fig. 2E) effectively blocked the hCG-induced *adamts9* expression in *wt* stage IVa follicles. Importantly, hCG-induced *adamts9* expression was completely blocked in stage IVa follicles from Lhcgr knockout fish ($lhcgr^{-/-}$, Fig. 2F).

3.3. DHP and hCG jointly upregulate adamts9 expression in vitro

Surprisingly, hCG also significantly induced *adamts9* expression in stage IVa follicles from Pgr knockout zebrafish ($pgr^{-/-}$) compared to those in control treatment *in vitro* (Fig. 3A), suggesting at least some effect of hCG on *adamts9* expression is likely Pgr-independent. To verify this, we examined the effect of combined treatment of DHP and hCG. As expected, the expression of *adamts9* was significantly higher in *wt* stage IVa follicles exposed to a mixture of DHP (100 nM) and hCG (50 IU/mL) than in those follicles exposed to any of two hormones alone (Fig. 3B).

3.4. The upregulation of adamts9 in preovulatory follicles is disrupted in $lhcgr^{-/-}$ fish in vivo

Previously we have shown upregulation of *adamts9* specifically occurs in preovulatory follicles in zebrafish (Liu et al., 2018). To investigate whether the expression of *adamts9* is affected by Lhcgr knockout, we compared the mRNA level of *adamts9* in naturally mature

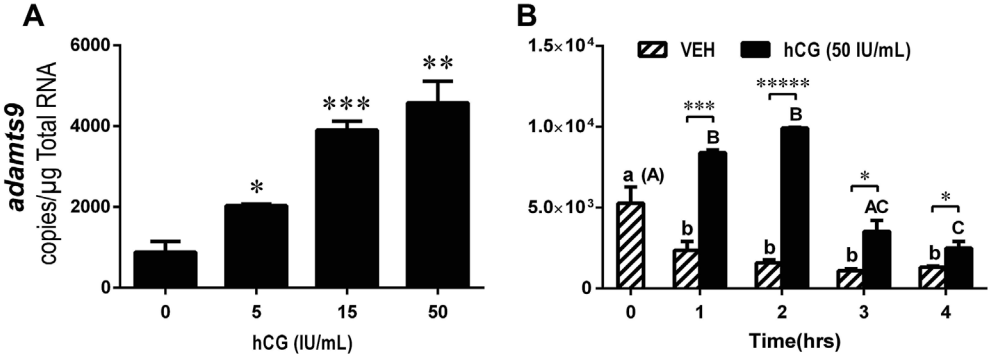

Fig. 1. Upregulation of a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 9 (*adamts9*) transcripts by human chorionic gonadotropin (hCG) in wildtype (*wt*) zebrafish preovulatory (stage IVa) follicles *in vitro*. The *adamts9* transcript was determined and expressed as absolute values according to a standard curve established with known concentrations of serially diluted plasmids using real-time quantitative PCR (qPCR). A) Dose dependent upregulation of *adamts9* transcript in stage IVa follicles after 2-hrs exposure to various concentrations of hCG *in vitro*. B) A transient increase of *adamts9* expression in stage IVa follicles exposed to hCG (50 IU/ml) *in vitro*. Asterisks indicate significant difference in hCG treated samples compared to vehicle treatment at the same time point. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; *****: $p < 0.00001$. Different letters (uppercase for hCG treatment; lowercase for vehicle treatment) indicate significant differences among different time points of exposures. Experiment was repeated three times with results of one representative experiment presented as mean ± SEM. N = 3.

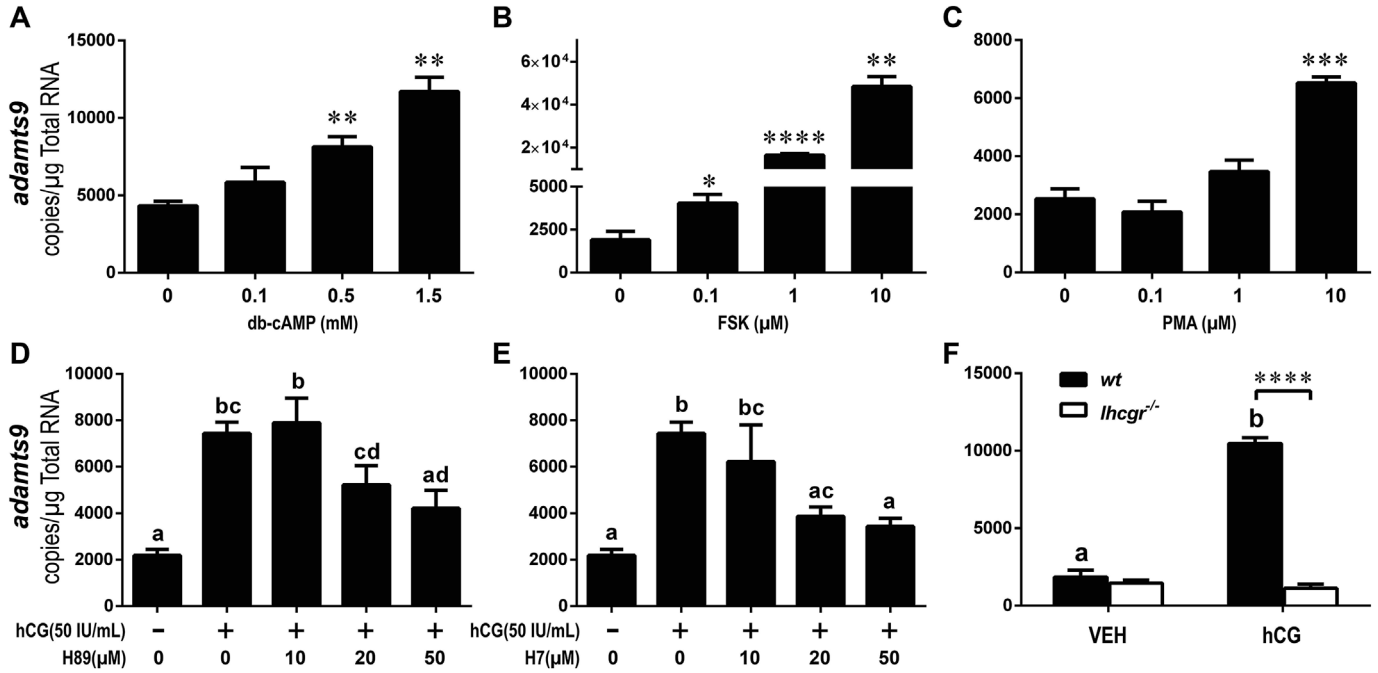

Fig. 2. Regulation of *adamts9* transcript via Lhcgr signaling pathways in preovulatory (stage IVa) follicles from *wt* zebrafish *in vitro*. A) Dose dependent upregulation of *adamts9* transcript after 2-hrs of exposure to various concentrations of db-cAMP (an analog of cAMP) *in vitro*. B) Dose dependent upregulation of *adamts9* transcript after 2-hrs of exposure to various concentrations of forskolin (FSK, an activator of adenylate cyclases) *in vitro*. C) Dose dependent upregulation of *adamts9* transcript after 2-hrs of exposure to various concentrations of PMA (an PKC activator) *in vitro*. D) Dose dependent inhibitory effect of H89 (a selective PKA inhibitor) on the hCG induced expression of *adamts9* transcript *in vitro*. E) Dose dependent inhibitory effect of H7 (a PKC inhibitor) on the hCG-induced *adamts9* expression *in vitro*. F) Complete inhibition of hCG induced *adamts9* expression in stage IVa follicles from Lhcgr knockout ($lhcgr^{-/-}$) zebrafish *in vitro*. Asterisks indicate significant difference in drug treated samples compared to vehicle treatment (A, B&C), or significant difference in hCG treated $lhcgr^{-/-}$ samples compared to hCG treated *wt* samples (F). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. Different letters indicate significant differences among different treatments of *wt* follicles. Experiment was repeated three times with results of one representative experiment presented as mean ± SEM. N = 3.

yet not ovulated Stage IVb follicles among *wt*, $lhcgr^{-/-}$, $pgr^{-/-}$ and $lhcgr^{-/-}pgr^{-/-}$ fish. Expectedly, both $pgr^{-/-}$ and $lhcgr^{-/-}pgr^{-/-}$ fish had significantly reduced *adamts9* expression and are infertile in comparison with *wt* fish (Fig. 4). No difference of *adamts9* expression was observed between $pgr^{-/-}$ and $lhcgr^{-/-}pgr^{-/-}$ fish (Fig. 4). The expression of *adamts9* in $lhcgr^{-/-}$ fish varied greatly among individuals and was not significantly different from those in *wt*, $pgr^{-/-}$ or $lhcgr^{-/-}pgr^{-/-}$ fish (Fig. 4). Interestingly, the expressions of *adamts9* in different $lhcgr^{-/-}$ individuals were clearly separated into two groups, in one of which *adamts9* mRNA levels were relatively normal and comparable to those in *wt* while in the other group their levels were low and similar as those in $pgr^{-/-}$ or $lhcgr^{-/-}pgr^{-/-}$ (Fig. 4).

3.5. Lhcgr knockout fish had impaired ovulation and reduced fertility

Ovulation was reported to be somehow disrupted in the $lhcgr^{-/-}$ zebrafish (Chu et al., 2014; Zhang et al., 2015b). To verify this phenotype, we performed a consecutive spawning test, a method we established previously to optimize the spawning of zebrafish (Liu et al., 2018; Wu et al., 2018), on $lhcgr^{-/-}$ fish in comparison with $lhcgr^{+/-}$ and *wt* fish. The spawning frequency of $lhcgr^{-/-}$ female fish in two weeks were significantly reduced by ~60% when compared to those of *wt* or $lhcgr^{+/-}$ female fish (Fig. 5A). One $lhcgr^{-/-}$ individual only spawned twice in two weeks (Fig. 5A). The average embryo number per spawning of $lhcgr^{-/-}$ female fish was also dramatically reduced in comparison with either *wt* or $lhcgr^{+/-}$ female fish (Fig. 5B). Importantly, the spawning of $lhcgr^{-/-}$ female fish became unpredictable with varying spawning intervals (about 4.5 days on average); in contrast, all tested *wt* and $lhcgr^{+/-}$ female fish spawned almost daily (Fig. 5C).

Analysis of ovaries from unmated gravid females 2 h after lights on in the morning showed that transparent mature oocytes were present in all genotypes of fish (Fig. 6). However, unlike in *wt* or $lhcgr^{+/-}$ fish

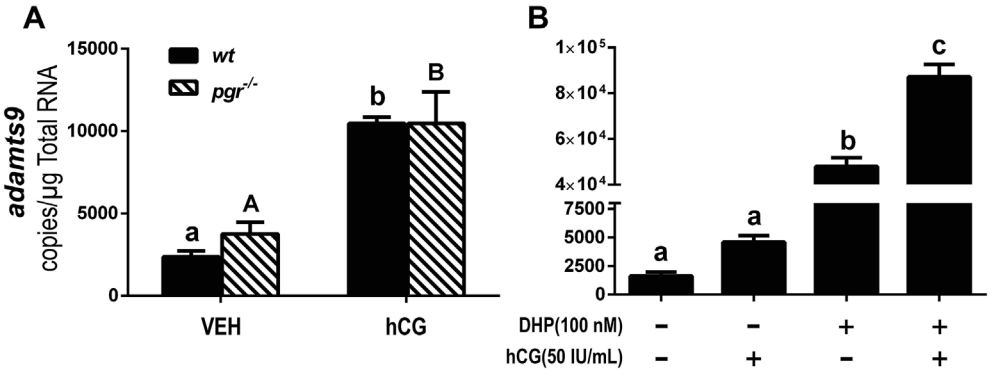

Fig. 3. Upregulation of *adamts9* transcript in Stage IVa follicles by hCG was via Pgr-independent signaling pathway *in vitro*. A) Pgr-independent upregulation of *adamts9* transcript in stage IVa follicles exposed to hCG (50 IU/mL) for 2 h. Letters on the bars (lowercase for *wt*; uppercase for $pgr^{-/-}$) indicate significant differences ($p < 0.05$). B) Synergistic increase of *adamts9* transcript in *wt* stage IVa follicles by hCG and 4-pregnen-17, 20β-diol-3-one (DHP, an oocyte maturation and ovulation inducing progestin in zebrafish). Experiment was repeated three times with results of one representative experiment presented as mean ± SEM. N = 3.

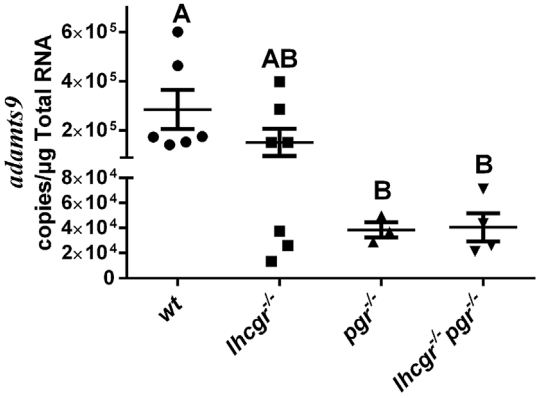

Fig. 4. Expression of *adamts9* in Stage IVb preovulatory follicles in *lhcgr*$^{-/-}$ fish were separated into two distinct groups *in vivo*. In the night before sampling, *wt*, *lhcgr*$^{-/-}$, *pgr*$^{-/-}$ and *lhcgr*$^{-/-}$/*pgr*$^{-/-}$ gravid female fish were individually paired with a fertile male fish. The transcript level of *adamts9* was determined *in vivo* in mature preovulatory follicles (stage IVb) collected an hour before lights on. Sampling were conducted at the same time points over a three-day period to minimize the sampling time for each collection, and data was presented as mean ± SEM. Different letters indicate significant differences among different genotypes of preovulatory follicles.

showing mature oocytes were all ovulated (stage V, Fig. 6A&B), in *lhcgr*$^{-/-}$ fish mature oocytes were either partially or completely trapped within ovary (stage IVb, Fig. 6C&D). Additionally, severe anovulatory oocytes (Fig. 6D) were also observed in the gravid *lhcgr*$^{-/-}$ females that did not spawn for more than two days (data not shown).

4. Discussion

We reported recently that progestin (DHP) and Pgr were essential for upregulation of several metalloproteinases including *adamts9* in preovulatory follicles during ovulation in zebrafish (Liu et al., 2018). Herein we show for the first time that the gonadotropin (hCG) directly increases the expression of *adamts9* via Lhcgr signaling pathways in zebrafish preovulatory follicles *in vitro*; this Lhcgr-mediated effect is Pgr-independent and may coordinate with DHP in the upregulation of *adamts9*. We also show that *lhcgr*$^{-/-}$ female zebrafish have reduced fertility including reduced spawning frequency and number of eggs, which may partly due to reduced expression of *adamts9* that impaired ovulation.

Both LH and hCG act on the same receptor Lhcgr to elicit intracellular activation of PKA and PKC pathways and are generally considered interchangeable regarding inducing ovulatory genes and ovulation in various animals (Salvador et al., 2002; Casarini et al., 2012; Gupta et al., 2012; Choi and Smitz, 2014). During ovulation, the expression of ovulatory genes after the LH surge typically exhibit a signature pattern of transient increase immediately followed by downregulation (Russell and Robker, 2007). In bovine, macaque and human, *ADAMTS9* transcript is induced in preovulatory follicular cells after administration of hCG or LH (Fortune et al., 2009; Peluffo et al., 2011; Rosewell et al., 2015). In the present study, we showed that hCG induced a transient increase of *adamts9* expression in zebrafish preovulatory follicles *in vitro*, suggesting this gene has a potential role in ovulation across vertebrates.

LH upregulates ovulatory proteases through multiple signaling pathways and mediators such as progestin and prostaglandin (Kim et al., 2009; Robker et al., 2009). Progestin (DHP) and Pgr are known to be critical mediators for LH action on ovulation and are essential for the preovulatory upregulation of *adamts9* in zebrafish (Zhu et al., 2015; Liu et al., 2018). However, in our previous study, we also observed a small increase of *adamts9* in preovulatory follicles of *pgr*$^{-/-}$ zebrafish *in vivo* suggesting Pgr-independent LH regulation of *adamts9* (Liu et al., 2018). In this study, we showed that hCG also induced an increase of *adamts9* in *pgr*$^{-/-}$ zebrafish preovulatory follicles *in vitro*, which is consistent with our previous findings *in vivo*. It is surprising to observe that the hCG-induced increase of *adamts9* in *pgr*$^{-/-}$ follicles was comparable to that in *wt* follicles *in vitro* as hCG could also increase progestin (DHP), which could act on Pgr in *wt* follicles. It is probably due to weak activity of hCG in progestin synthesis in zebrafish follicles *in vitro*. Typically, a relatively long incubation time is required (approximate 24 h) for the induction of oocyte maturation by hCG *in vitro* when compared to that *in vivo* (~2 h) (Selman et al., 1994; Chu et al., 2014). Nevertheless, we showed that the increase of *adamts9* was greater in combined exposure of DHP and hCG than those in DHP or hCG treatment alone in zebrafish preovulatory follicles, further supporting our hypothesis that both Pgr-dependent and -independent pathways are important for the optimal expression of *adamts9* in zebrafish preovulatory follicles. The synergistic effect of DHP and hCG on *adamts9* in zebrafish agrees with the observation in mice granulosa cells that a synergistic increase of *Adamts1* promoter activity is induced by LH and PGR (Doyle et al., 2004). Based on our results and others', we propose that a coordinate action between LH and progestin signals is essential for the maximizing expression of ovulatory genes during ovulation.

LH plays an essential role in triggering final oocyte maturation and ovulation in zebrafish as demonstrated by two recent studies using *lhb*$^{-/-}$ fish (Chu et al., 2014; Zhang et al., 2015a). In contrast, *lhcgr*$^{-/-}$ female fish appears fertile because of the promiscuous activation of Fshr by LH in zebrafish (So et al., 2005; Chu et al., 2014; Zhang et al., 2015b). However, these studies did not detailly evaluate spawning frequency that reflects ovulatory cycle in zebrafish; moreover, zebrafish skip spawning frequently in suboptimal conditions such as group

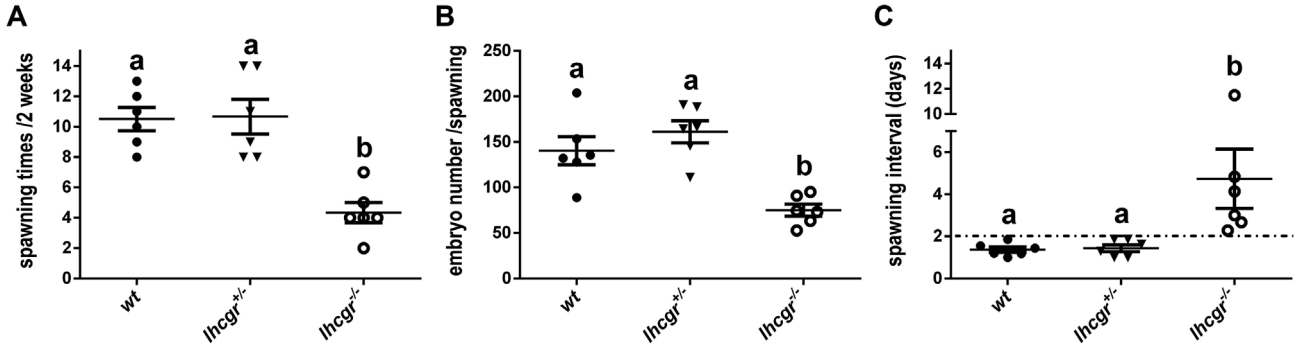

Fig. 5. Reduced fecundity in *lhcgr*$^{-/-}$ females which spawned unpredictably under consecutive spawning test. A) Reduced spawning times in two weeks in *lhcgr*$^{-/-}$ females in comparison with *wt* or *lhcgr*$^{+/-}$ females. B) Reduced embryo numbers per spawning in *lhcgr*$^{-/-}$ females in comparison with *wt* or *lhcgr*$^{+/-}$ females; embryo number was averaged from data of at least 5 times of spawnings of each individual fish. C) Increased spawning intervals in *lhcgr*$^{-/-}$ females in comparison with *wt* or *lhcgr*$^{+/-}$ females; spawning interval was averaged of at least 5 times of spawning intervals of each individual fish; dash line indicates maximum spawning interval in fish with normal fecundity under consecutive spawning test. Letters indicate significant differences ($p < 0.05$). N = 6.

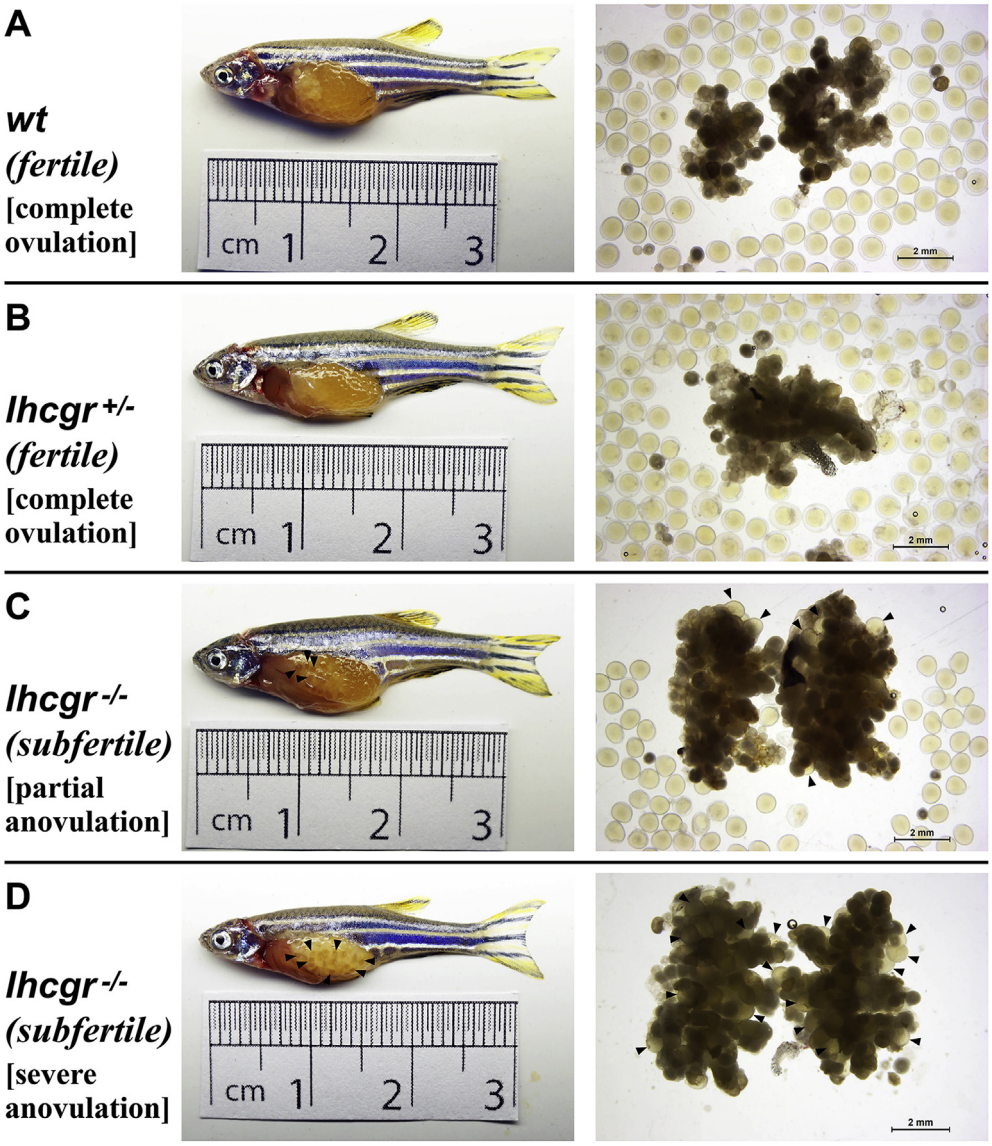

Fig. 6. Anovulatory ovaries from *lhcgr*$^{-/-}$ females sampled in the morning 2 h after the onset of lights. A) Normal ovulation with all mature oocytes ovulated in the ovary of *wt* fish. B) Normal ovulation with all mature oocytes ovulated in the ovary of *lhcgr*$^{+/-}$ fish. C) Partial anovulation with a portion of mature oocytes trapped within the ovary of *lhcgr*$^{-/-}$ fish. D) Severe anovulation with all mature oocytes trapped within the ovary of *lhcgr*$^{-/-}$ fish. Arrow heads indicate anovulatory oocytes.

housing or deficiency in nutrition. It is unclear whether the compensation of Fshr by LH would compromise in ovulation when *lhcgr*$^{-/-}$ female fish were subjected to optimal conditions. In the present study, we carefully monitored spawning of several individuals over a consecutive period with adequate food nutrition and optimal pairing. We have used this method to examine the fecundity of several zebrafish mutant lines (Liu et al., 2018; Wu et al., 2018; Carter et al., 2019). Interestingly, we found that *lhcgr*$^{-/-}$ female fish were actually subfertile and exhibited reduced fecundity due to reduced ovulation; the morphology of ovary from these fish was similar to that from *pgr*$^{-/-}$ fish. These results reveal the difference between Fshr and Lhcgr in response to LH surge during zebrafish ovulation, and that Lhcgr is important for normal ovulation in zebrafish. It is interesting to investigate what downstream genes are responsible for the anovulation in *lhcgr*$^{-/-}$ fish in the future.

The first adult Adamts9 global knockout (*adamts9*$^{-/-}$) model established in our lab demonstrated that Adamts9 is required for ovary development in zebrafish (Carter et al., 2019). The *adamts9*$^{-/-}$ female fish are infertile and have no preovulatory mature follicle in the ovary (Carter et al., 2019). Intriguingly, in the present study, we observed that *lhcgr*$^{-/-}$ females had two distinct groups in terms of *adamts9* expression. Average of low *adamts9* expression group in preovulatory follicles of *lhcgr*$^{-/-}$ females was similar as those in infertile *pgr*$^{-/-}$ and *lhcgr*$^{-/-}$*pgr*$^{-/-}$ fish, while average of high *adamts9* expression group in preovulatory follicles of *lhcgr*$^{-/-}$ females was similar as those in fertile *wt*. We also observed *lhcgr*$^{-/-}$ females did not spawn daily, whereas *wt* females spawn almost every day under our experimental setting. It is likely those *lhcgr*$^{-/-}$ females with low *adamts9* expression would skip spawning, while those *lhcgr*$^{-/-}$ females with high *adamts9* expression would spawn on the sampling day. LH is essential whereas Lhcgr is dispensable for female fertility in zebrafish (Chu et al., 2014; Zhang et al., 2015a, 2015b; Takahashi et al., 2016). It is suggested that LH could also signal via follicle-stimulating hormone receptor (Fshr), which led to no apparent deficiency in female fertility in *lhcgr*$^{-/-}$ zebrafish (Chu et al., 2014; Zhang et al., 2015b). It is possible that LH signaling via Fshr in *lhcgr*$^{-/-}$ females may not be as effective or sufficient enough as LH signaling via Lhcgr in *wt*, which is likely cause frequent skip of ovulation and spawning in *lhcgr*$^{-/-}$ females. Further

studies are required to understand the difference in signaling mechanisms of LH-Lhcgr and Lh-Fshr in zebrafish, which provides an excellent model for studying evolution of LH signaling.

Knockout of Adamts9 ortholog, gon-1, in *C. elegans* leads to infertility due to mis-migration and mis-formation of gonad but has no obvious effects in survival (Blelloch and Kimble, 1999; Blelloch et al., 1999). Knockout of Adamts9 ortholog (Adamts-A) in *Drosophila* caused mis-migration of collective cells including germ cells (Ismat et al., 2013). Unfortunately, our knowledge of Adamts9 functions in reproduction in adult vertebrates is lacking mainly due to embryonic lethality in ADAMTS9 knockout mice (Kern et al., 2010). Limited correlation studies have indicated increased expression of *Adamts9* during ovulation (Peluffo et al., 2011; Rosewell et al., 2015; Lussier et al., 2017). However, the role of ADAMTS9 in mammalian ovulation is still unclear. Clearly, further studies are required to elucidate functions of ADAMTS9 in vertebrate ovaries, which may provide us new insights into reproduction success and ovarian diseases.

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Author contributions

DTL performed experiments, analyzed results and drafted the paper. WSH supervised the project and discussed the results. SXC and YZ conceived and supervised the project, analyzed results and wrote the paper.

Declaration of competing interest

The authors have nothing to disclose.

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