Nuclear Progestin Receptor Mediated Linkage of Blood Coagulation and Ovulation

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Author Contributions:

JH performed most experiments, analyzed the data, and wrote the draft with the assistance of CS, DTL and NNZ. JAS generated the *f5* zebrafish knockout, provided protocols and experimental suggestions, discussed experimental results, and revised the manuscript. YZ supervised the project, conducted the fertility experiment, and revised the manuscript. SXC conceived and supervised the project and revised the manuscript.

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Abstract

Ovulation is a dramatic remodeling process including rupture of blood capillaries and clotting, but coagulation is not thought to directly regulate this process. Herein, we report remarkable increases of coagulation factors V (*f5*, ~3145-fold) and tissue factor (*f3a*, ~120-fold) in zebrafish ovarian follicle cells during ovulation. This increase was mediated through the nuclear progestin receptor (Pgr), which is essential for ovulation in zebrafish. This increase was totally abolished in ovarian follicular cells from $pgr^{-/-}$ mutants. In addition, promoter activities of *f5* and *f3a* were significantly enhanced by progestin (DHP) via Pgr. Similar regulation of human *F5* promoter activity was induced via human PGRB, suggesting a conserved mechanism. Site-directed mutagenesis of the zebrafish *f5* promoter further demonstrated a direct regulation of coagulation factors via progestin response elements. Moreover, a stark increase of erythrocytes occurred in capillaries meshed in wildtype preovulatory follicles, but was absent in $pgr^{-/-}$ mutants. Interestingly, the anticoagulant heparin inhibited ovulation *in vitro*. Furthermore, reduced fecundity was observed in $f5^{+/-}$ female zebrafish. Taken together, our study provides plausible evidence for steroid regulation of coagulation factors, and a new hypothesis for blood clotting triggered ovulation in vertebrates.

1. Introduction

Ovulation is a tissue remodeling process involving the rupture of follicular layers, basal membrane, and many blood capillaries intertwined in the follicular cells. This dramatic remodeling process share several features with inflammatory responses (Duffy, Ko, Jo, Brannstrom, & Curry, 2019). Blood coagulation was suggested to be part of the innate defense in response to inflammation during ovulation (Esmon, 2005). Formation of blood clots at apical vessels was found shortly before follicle rupture in several animal species including sheep, rabbits, and rodents (Cavender & Murdoch, 1988; Duffy et al., 2019; Kanzaki et al., 1982; Zackrisson et al., 2011). Cessation of blood flow likely due to blood coagulation was also observed in apical vessels of ovarian follicles during the late ovulatory phase in women (Brannstrom et al., 1998). In addition, an increase of activity and expression of thrombin (a protease essential for fibrin formation) and its receptors were also reported in luteinized granulosa cells and follicular fluid in human (Gentry et al., 2000; Kim et al., 2006). Increased fibrinogen secretion in bovine granulosa cells was thought to be related to the proteolytic activity needed for follicle rupture. Circulating F3 (also known as tissue factor) was elevated in women with polycystic ovary syndrome that is characterized by chronic oligo-ovulation or anovulation (Gonzalez, Kirwan, Rote, & Minium, 2013). Although these reports imply possible association between blood coagulation and the ovulation process, the mechanisms for this role in ovulation are still unclear.

Progesterone (P4) and its nuclear progesterone receptor (PGR) are well-established initiators for vertebrate ovulation (Hagiwara, Ogiwara, Katsu, & Takahashi, 2014; Kubota et al., 2016; Lydon et al., 1995; Natraj & Richards, 1993; Tang et al., 2016;

Y. Zhu et al., 2015). Recently, we identified coagulation factors *f3a* and *f5* as potential downstream targets of Pgr in a transcriptomic analysis of preovulatory follicles from zebrafish (Liu, Brewer, Chen, Hong, & Zhu, 2017). Interestingly, changes in these coagulation factors during ovulation appear to be conserved among human, mice, and zebrafish (Carletti & Christenson, 2009; Liu et al., 2017; Wissing et al., 2014). Possible involvements of P4 or PGR in the regulation of coagulation have been hinted from several studies using progestin contraceptives. F3 was upregulated in endometriosis and at endometrial bleeding sites in women using long-term progestin-only contraception (Runic et al., 2000). Orally administered progesterone reduced spontaneous miscarriages and improved fertility among women who had a history of recurrent miscarriages due to abnormal bleeding in early pregnancy (Haas, Hathaway, & Ramsey, 2019). Increased risk of venous thromboembolic events was associated with combined oral contraceptives, particularly those containing the progestin desogestrel (Farley, Meirik, Chang, Marmot, & Poulter, 1995; Jick, Jick, Gurewich, Myers, & Vasilakis, 1995). A recent genetic study indicated that the present-day carriers of the Neandertal variant of PGR had less bleeding during early pregnancy because of high levels of PGR expression (Zeberg, Kelso, & Paabo, 2020). However, evidence of P4 induced or PGR mediated regulation of coagulation factors, especially in ovarian follicles during ovulation, is lacking.

In this study, we examined the expression, hormonal regulation, and potential function of coagulation factors (*f5* & *f3*) in preovulatory follicles of zebrafish. The *f3* gene is duplicated in zebrafish (*f3a* and *f3b*) (Stein, Caccamo, Laird, & Leptin, 2007). We first demonstrated the expression and localization of *f5, f3a,* and *f3b* in preovulatory

follicular cells. We found that progestin (DHP, 17α, 20β-dihydroxy-4-pregnen-3-one, a native ovulation-inducing ligand in zebrafish) via Pgr regulated *f5* and *f3a* expression in preovulatory follicles. We also found that follicular cells with strong Pgr expression were adjacent to blood capillaries, and inhibition of blood coagulation reduced ovulation. Finally, fertility and ovulation were reduced in $f5^{+/-}$ zebrafish mutants *in vivo*. Our study provides credible evidence that coagulation factors (*f5* & *f3a*) in preovulatory follicular cells are regulated by progestin (DHP) via Pgr, which are well-known upstream initiators for ovulation. Our results also suggest a previously unknown role of these coagulation factors in ovulation and female fertility.

2. Results

2.1 Dramatic increase of *f5* and *f3a* transcripts in follicular cells of preovulatory follicles during a natural spawning cycle *in vivo*

Expression of *f5* and *f3a* mRNA was exclusively restricted to the follicular cells of mature follicles (IVb) and was not found in the germ cells (oocytes) (Fig. 1B). In contrast, *f3b* mRNA was expressed in both follicular cells and oocytes (Fig. 1B). The expression of *f5*, *f3a*, and *f3b* in mature follicles (IVb), just prior to ovulation, collected at 06:40 were ~3,145-fold, ~120-fold, and ~2-fold higher than those in immature but fully-grown follicles (IVa, collected at 12:00, Fig. 1C). Since the increase in *f3b* expression was modest, we focused the rest of our efforts on *f5* and *f3a*. Results from whole mount *in situ* hybridization (WISH) showed that both *f5* and *f3a* were nearly undetectable in immature follicles (IVa, Fig. 1D, E). In contrast, strong signals of both *f5* and *f3a*

transcripts were detected in the follicular cells of the mature follicles (IVb) (Fig. 1D, E), while no signal was detectable using sense probes (data not shown).

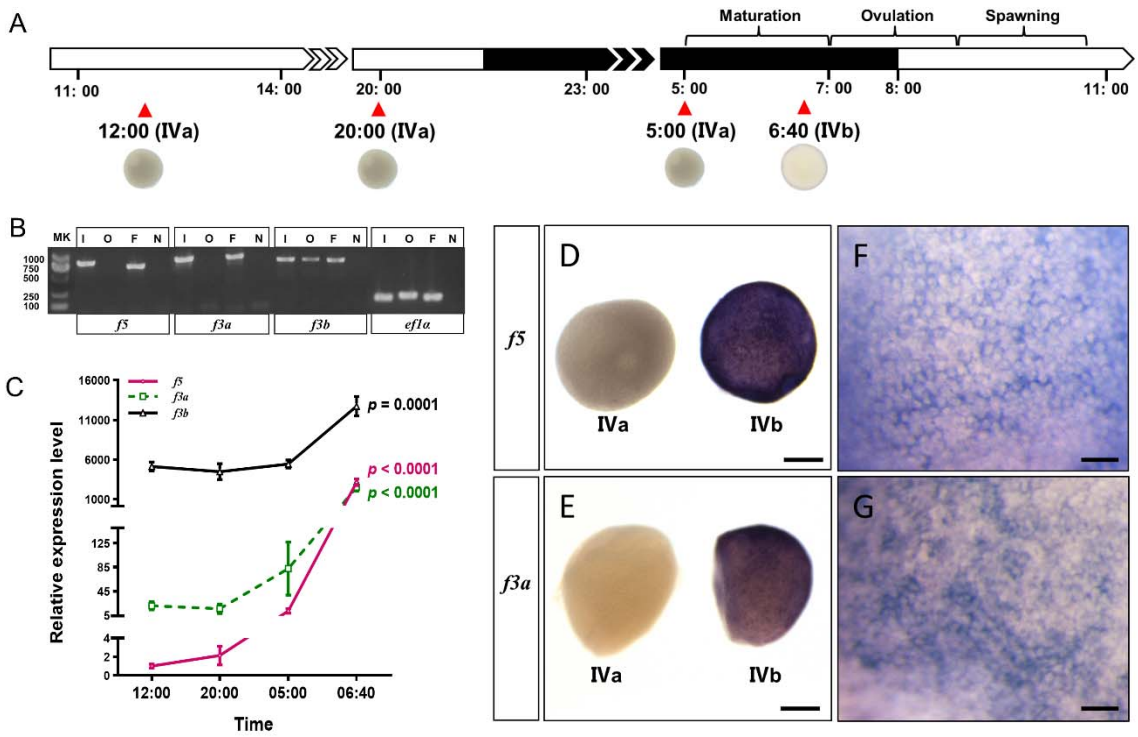

Figure 1. Dramatic increase of *f5* and *f3a* transcripts in follicular cells of preovulatory follicles during a daily spawning cycle *in vivo*.

(**A**) Schematic illustration of daily spawning cycle in zebrafish synchronized with lights on. Oocyte maturation in zebrafish typically occurs around 06:40, while ovulation occurs around 07:00.

(**B**) Cell-specific expression of *f5*, *f3a, and f3b* in mature follicles examined by RT-PCR. Transcripts of *f5* and *f3a* were expressed in the follicular cells but not in the oocytes, while the transcript of *f3b* was expressed in both follicular cells and oocytes of mature preovulatory follicles. MK: molecular makers; I: intact follicles including both surrounding follicular cells and germ cell; O: denuded oocyte (without follicular cells); F: follicular cells (with no germ cell); N: no template control.

(**C**) Dramatic increase of *f5*, *f3a*, and *f3b* transcripts in preovulatory follicles during a daily spawning cycle *in vivo*. Fully-grown ovarian follicles (stage IVa or IVb) were collected from wildtype mature female zebrafish at four representative timepoints during a daily spawning cycle (see Fig. 1A). Expression of *f5*, *f3a*, and *f3b* mRNA were determined by qPCR and normalized to the internal housekeeping gene (*ef1α*). Data are expressed as the mean ± SEM (N = 4) relative to the respective transcript levels of *f5* in IVa

follicles collected at 12:00. P-values were calculated by one-tailed one-way ANOVA followed by Dunnett's test against respective controls (samples collected at 12:00).

(**D-G**) Dramatic increase of *f5* and *f3a* transcripts in the follicular cells of mature follicles. Fully-grown but immature (stage IVa) and mature follicles but prior to ovulation (stage IVb) were collected at 20:00 and 06:40, respectively. Strong signals of *f5* and *f3a* transcripts were only observed in mature follicles (IVb) by whole-mount *in situ* hybridization (WISH), while no signals were observed in the immature follicles (IVa, D, E). The positive ISH signals were localized in the follicular cells (F, G). Scale bar, 250 μm (D, E) and 50 μm (F, G).

2.2 Progestin upregulates *f5* and *f3a* transcripts through Pgr

2.2a Transcripts of *f5* and *f3a* were primarily upregulated by progestin in preovulatory follicles

The progestin (DHP) is a well-established native ligand for Pgr and an upstream hormonal inducer for ovulation in zebrafish (Chen et al., 2010; Hanna et al., 2010; Y. Zhu et al., 2015). As predicted, the increase of *f5* and *f3a* transcripts were dose- and time-dependent in fully grown immature follicles (IVa) exposed to external progestin (Fig. 2A-D).

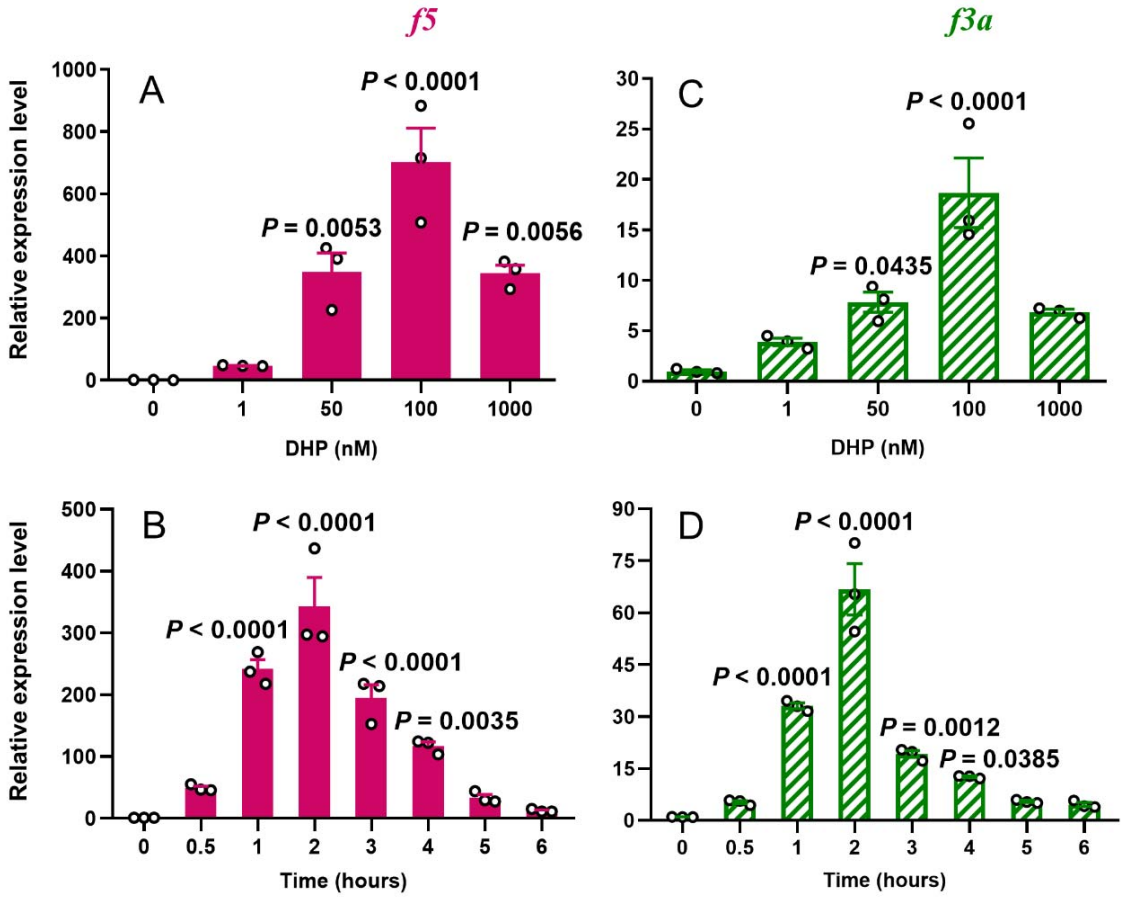

Figure 2. Transient increase of *f5* and *f3a* expression in preovulatory follicles in response to stimulation of progestin *in vitro*.

(**A, C**) Effects of various doses of progestin (DHP) (2 hours exposure) on *f5* and *f3a* expression in fully grown immature preovulatory follicles (stage IVa) *in vitro*. (**B, D**) Effects of DHP (100 nM) on *f5* and *f3a* expression in preovulatory follicles (IVa) at various timepoints *in vitro*. P-values were calculated by one-tailed one-way ANOVA followed by Dunnett's test against respective controls (0 timepoint or vehicle control).

Progestin increased *f5* expression in vitellogenesis follicles (III, ~7 fold) and fully-grown immature follicles (IVa, ~337 fold) (Fig. 3A). In comparison, only fully-grown immature follicles (IVa) responded to progestin with increase in *f3a* transcripts, but not the early developing follicles (stage I-III, Fig. 3B). LH is the primary endocrine

factor for inducing ovulation in all animals including zebrafish (Takahashi, Hagiwara, & Ogiwara, 2019). As expected, increased *f5* expression (~4 fold) was observed in IVa immature follicles exposed to hCG (50 IU/mL, Fig. 3C), but not for *f3a* (Fig. 3D). In comparison, progestin (DHP) had much stronger stimulatory effects on the expression of *f5* (~318 fold) and *f3a* (~10 fold) than hCG (Fig. 3C, D).

Significantly reduced *f5 and f3a* expression was found in mature preovulatory follicles (IVb) collected from *pgr*$^{-/-}$ fish compared to those in wildtype siblings (Fig. 3E). Importantly, DHP-induced *f5* and *f3a* expression were blocked in fully-grown immature follicles (IVa) from *pgr*$^{-/-}$ fish (Fig. 3F).

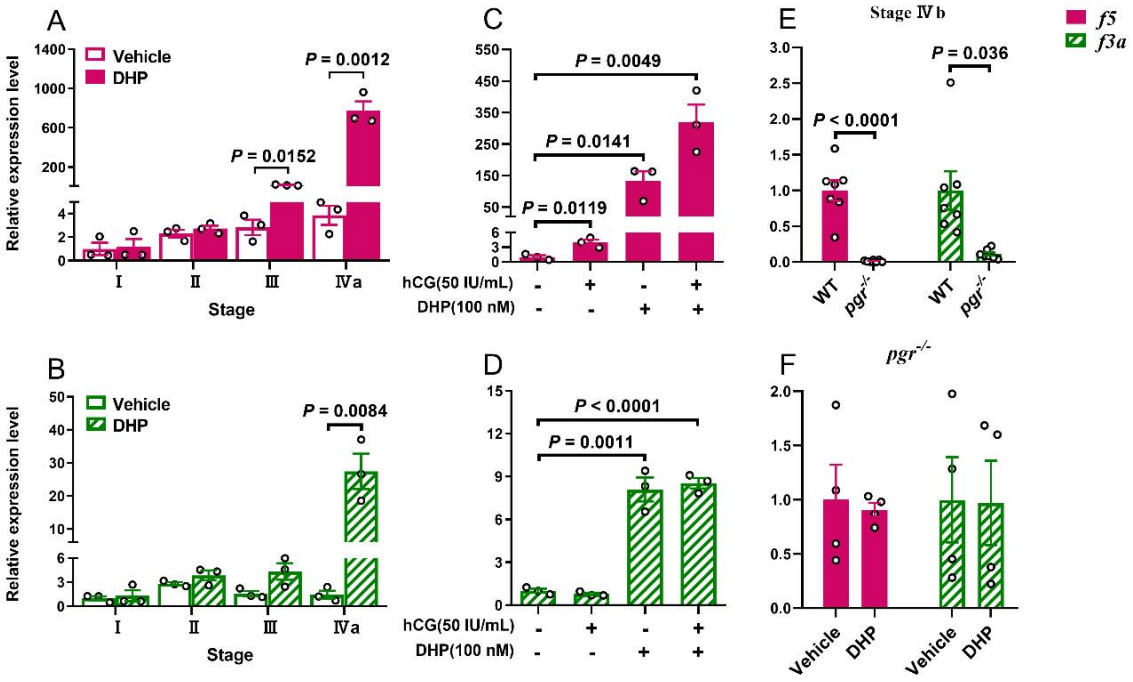

Figure 3. Increase of *f5* and *f3a* transcripts in late-stage follicles is mediated by Pgr.

Follicles were incubated with progestin (DHP, 100nM), or LH (hCG, 50 IU/ml) alone, or in combination for 2 hrs at 28 ℃ *in vitro* before qPCR analysis. (**A**) An increase of *f5* transcript was observed in late-stage (III and IVa) follicles from wildtype (*pgr*$^{+/+}$) zebrafish, but not in early-stage follicles (I or II) from

wildtype zebrafish, in response to progestin stimulation. (**B**) An increase of *f*3a expression was observed only in fully-grown immature follicles (IVa) from wildtype ($pgr^{+/+}$) zebrafish, but not in developing follicles (I-III) from wildtype fish, in response to progestin stimulation. (**C**) Synergistic increase of *f5* mRNA by hCG and DHP in fully grown immature follicles (IVa) from wildtype zebrafish. (**D**) Progestin but not hCG stimulated the expression of *f3a* mRNA in full-grown immature follicles (IVa) from wildtype zebrafish. (**E**) Both *f3a* and *f5* transcripts in preovulatory follicles (stage IVb) of $pgr^{-/-}$ were significantly lower than that of $pgr^{+/+}$ (WT). (**F**) Stimulatory effect of progestin (DHP) for *f3a* and *f5* expression was abolished in the stage IVa follicles of $pgr^{-/-}$ mutants. The expression of *f5* and *f3a* transcripts were determined by qPCR and normalized to an internal control (*ef1α*). Data are expressed as the mean ± SEM (N = 3) relative to the respective transcript levels of target gene measured in wildtype, or vehicle treatment group. P-values were calculated by two-tailed Students' t-test.

2.2b Progestin modulates promoter activity of *f5* and *f3a* via Pgr

As expected (Chen et al., 2010), progestin induced dose-dependent, Pgr-mediated activation of the MMTV promoters (Fig. 4A). In the presence of Pgr, progestin significantly increased the *f5 and f3a* promoter activities, and this was dose-dependent (Fig. 4B, C). No change of promoter activity was observed when pcDNA3.1(+)-Pgr was replaced by a control vector (pcDNA3.1(+), Fig. 4B, C). The increase for *f5* was much bigger than that of the *f3a*. In the presence of human PGRB, P4 also significantly increased human *F5* promoter activity in a dose-dependent manner. No change in promoter activity was observed when pCMV-HA-hPGRB was replaced by control (pCMV-HA, Fig. 4D). Moreover, site-directed mutagenesis of four predicted progesterone response elements (PRE) significantly decreased zebrafish *f5* promoter activity (Fig. 4E, Supplemental Table 5). A PRE (5'-AGGACATTTTTTA-3') localizing at -1438 to -1425 of zebrafish *f5* appears to be the primary mediator of Pgr regulation (Fig. 4E).

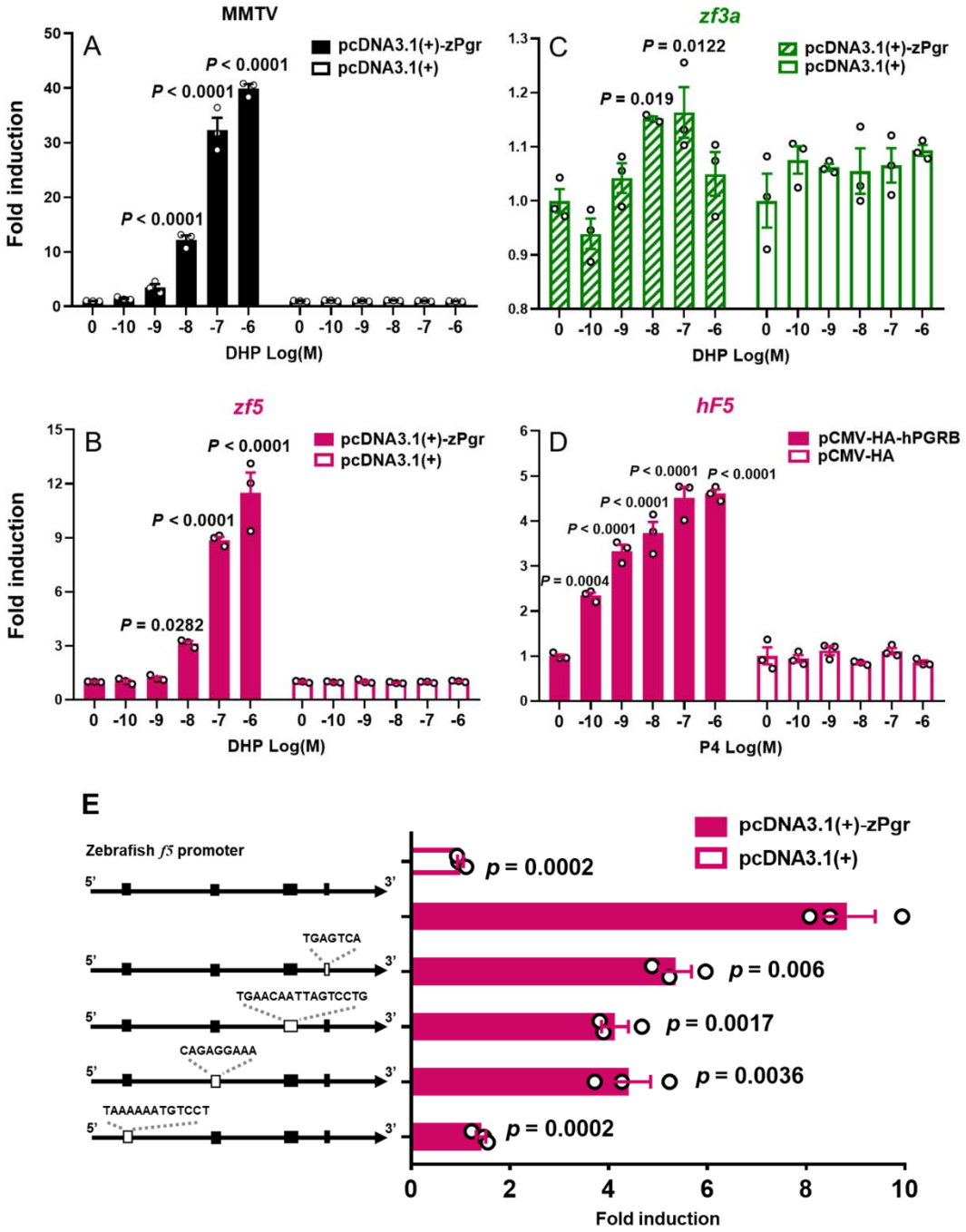

Figure 4. Progestin enhances promoter activity of *f5* and *f3a* via Pgr.

HEK293T cells were transiently co-transfected with a firefly luciferase reporter vector containing putative Pgr binding elements (3 μg/vector/60mm petri dish), a pRL-TK vector (150 ng/60mm petri dish) containing the Renilla luciferase reporter gene (as a control for transfection efficiency), and a Pgr expression vector (1.5 μg/vector/60mm petri dish) or an empty control expression vector (1.5

μg/vector/60mm petri dish). In the presence of zebrafish Pgr (zPgr), progestin significantly increased MMTV (panel **A**), *zf5* (panel **B**), and *zf3a* (panel **C**) promoter activities. This increase was dose-dependent. In the presence of human PGRB (hPGRB), P4 also significantly increased human *hF5* promoter activity, and the increase was dose-dependent (Panel **D**). Site-directed mutagenesis of four likely progestin receptor response elements (PRE) decreased the *zf5* promoter activity (Panel **E**). HEK293T cells were incubated for 24 hrs with increasing concentrations of progestins (DHP or P4: 100 pM to 1 μM). Extracts of the treated cells were assayed for luciferase activity. Values are shown relative to the luciferase activity of the vehicle treatment group. Data are expressed as mean ± SEM (N = 3). P-values were calculated by one-tailed one-way ANOVA followed by Dunnett's test against respective controls (vehicle controls, zero dose).

2.3 Coagulation factor contribution to ovulation

2.3a Follicular cells with strong Pgr expression localize adjacent to blood vessels

Intriguingly, we found almost all the follicular cells with strong Pgr expression were located adjacent to capillary vessel networks on the surface of the IVa follicles in a dual transgenic zebrafish line *Tg(pgr:eGFP/ fli1:DsRed)* (Fig. 5).

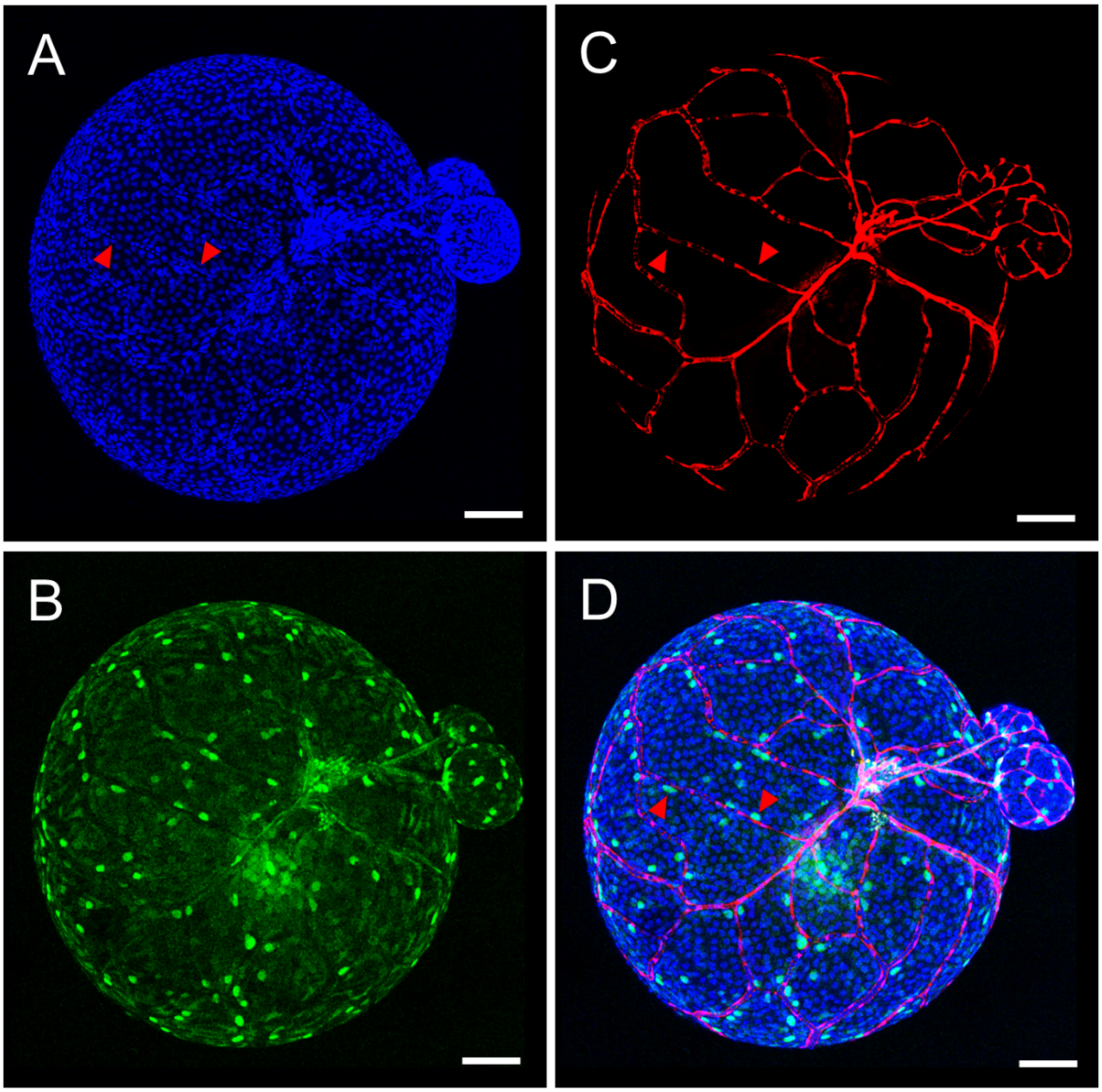

Figure 5. Follicular cells with strong Pgr expression are adjacent to blood capillaries. Images of a representative stage IVa follicle from a double transgenic zebrafish line *Tg(pgr:eGFP/ fli1:DsRed)* are shown. (**A**) Nucleus staining of follicular cells by Hoechst 33342. (**B**) Pgr promoter drives GFP expression in follicular cells. (**C**) Red fluorescence-labeled vascular endothelial cells on the follicles. (**D**) Merged images to show that follicular cells with strong GFP expression driven by the Pgr promoters are adjacent to blood capillaries. Red arrowheads indicate follicular cells with strong GFP expression driven by the Pgr promoters. Scale bars: 100 μm.

2.3b Rupture of blood vessels during ovulation

The ovulation process started with the appearance of a small hole in follicular cells and a ruptured blood vessel (indicated by a red arrow in Fig. 6A, supplemental video clip A) with a concomitant appearance of the ovulating oocyte on the surface of the ovaries. When the hole of the broken follicular cell layer reached a certain size, the oocyte was quickly released from the follicular layer (Fig. 6B-E, supplemental video clip A).

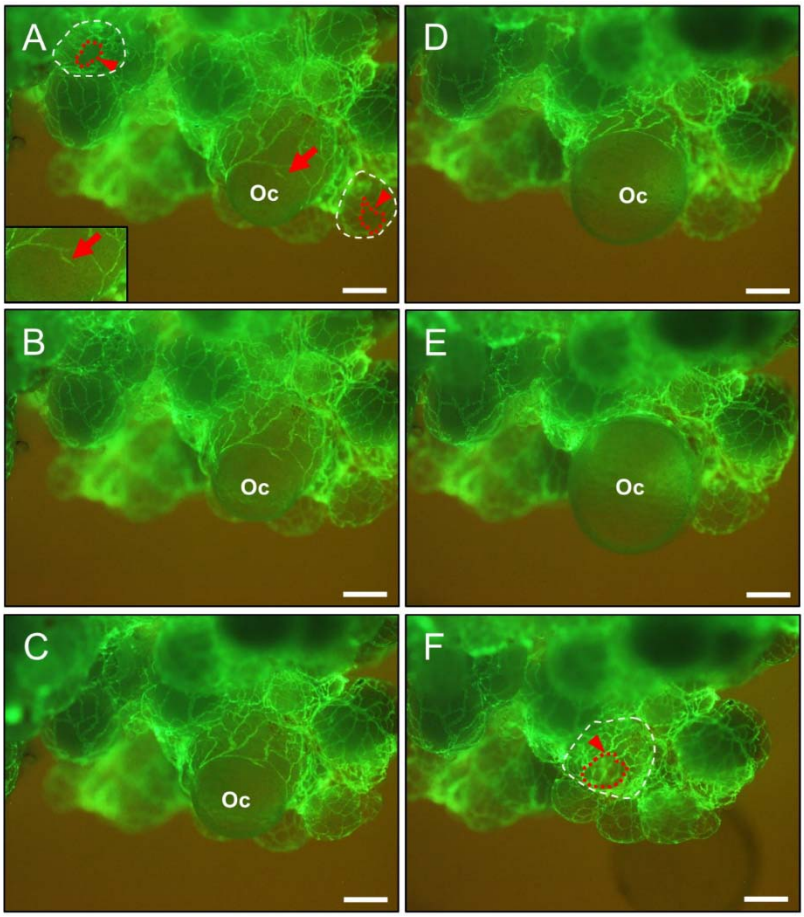

Figure 6. Rupture of blood capillaries and follicular cells during ovulation.
Ovaries undergoing ovulation were collected from *Tg(fli1:eGFP)* females at 07:00. (**A-F**), a series of images were taken when a mature (stage IVb) ovarian follicle started ovulation. Interconnected blood

vessels labeled with GFP were observed easily on the surface of ovarian follicles. A red arrow indicates a ruptured blood vessel on the surface of an ovulating follicle. Red arrowheads and red dotted lines indicate a hole left in the remaining follicular cells after ovulation. The white dotted line represents the remaining follicular cells. Scale bars: 250 μm. Oc, oocyte. (Please also see supplemental video clip A for additional details).

2.3c Increased numbers of erythrocytes appear in the lumen of blood vessels of mature preovulatory follicles as ovulation proceeds

The numbers of erythrocytes in capillaries of mature preovulatory follicles (IVb) increased as they proceeded to follicular rupture, from ~06:40, (Fig. 7A, D, C) to ~07:00 (Fig. 7B, E, G). This effect was absent in the same mature follicles (IVb) collected from anovulatory *pgr*$^{-/-}$ female siblings around 07:00 (Fig. 7C, F, G).

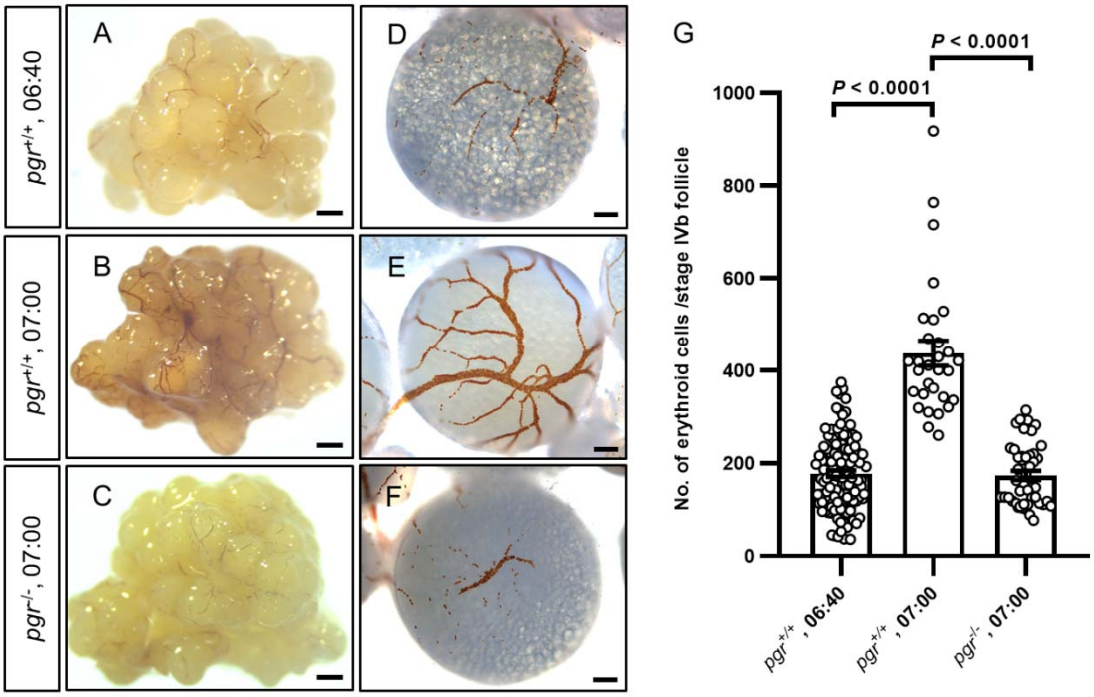

Figure 7. Increase of erythrocytes in capillaries on the surface of mature follicles during progression but prior to rupture.

(**A, D, B, E**) Erythrocytes within the surface capillaries of mature follicles (IVb) from wildtype (*pgr*$^{+/+}$) female zebrafish collected at 6:40 or 7:00 were visualized using *o*-dianisidine staining as follicles progressing towards rupture and ovulation. (**C, F**) Erythrocytes were also visualized in similar mature follicles that were collected at 7:00 from *pgr*$^{-/-}$ females, in which ovulation never occurs (Y. Zhu et al., 2015). (**G**) At least 30 mature follicles were randomly chosen from at least 3 different females of each condition, and the number of erythrocytes in capillaries were counted by a blinded observer. Data are expressed as mean ± SEM (N ≥ 30). P-values were calculated by two-tailed Student's t-test. Scale bars: 500 μm (A-C) or 100 μm (D-F).

2.3d Heparin inhibits ovulation *in vitro*

The intact IVb follicles or ovulated follicles from *Tg(fli1:eGFP)* were easily recognizable by the interconnecting blood vessels with strong GFP expression and dramatic changes in size (Fig. 8A-D). As expected, the ovulation rate decreased to 41 % when follicles were exposed to EDTA (2 mM), an inhibitor of metalloproteases that are required for ovulation (Ogiwara, Takano, Shinohara, Murakami, & Takahashi, 2005). Intriguingly, heparin (100 μg/mL), an anticoagulant, significantly reduced the rate of ovulation to 66% (Fig. 8E). Another anticoagulant, warfarin (50 μg/mL), had a reduced but not significant adverse effect on ovulation rate (83 %) (Fig. 8E).

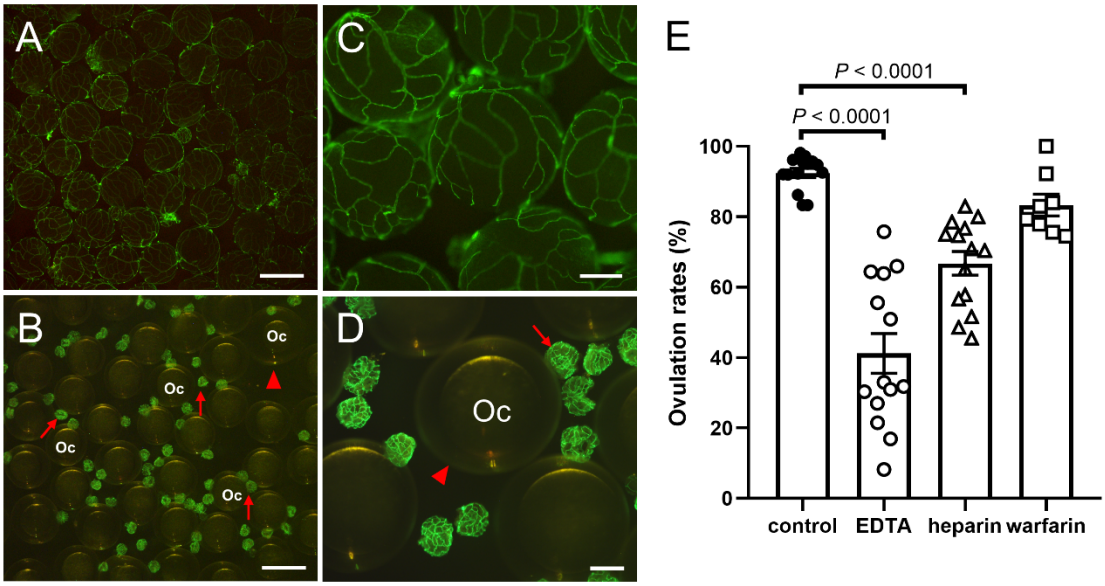

Figure 8. Heparin inhibits ovulation *in vitro*.

Ovulation rate was determined using mature follicles (IVb) isolated from transgenic female zebrafish (*Tg(fli1:eGFP*). The intact IVb follicles were easily recognizable by the interconnecting blood vessels with strong GFP expression (**A, C**). After ovulation, oocytes were easily recognizable by the absence of interconnecting GFP-positive vessels and expansion of the vitelline envelope (chorion) (**B, D**). Follicular layers separated from ovulated oocytes could also be easily distinguished due to interconnecting blood vessels and a dramatic size reduction (**B, D**). (**A, C**) Mature follicles (IVb) were collected from *Tg(fli1:eGFP)* female zebrafish. (**B, D**) Representative images of ovulated mature follicles were taken 2 hrs after incubation at 28°C. Arrows indicate the remaining follicular layer following ovulation. Arrowheads indicate expanded vitelline envelopes. Oc, oocyte. (**E**) mature follicles were incubated in a medium containing EDTA (2 mM), heparin (100 µg/mL), warfarin (50 µg/mL), or vehicle control, and rates of ovulation were determined 2 hrs following incubation. Data are expressed as the mean ± SEM (N ≥ 7). P-values were calculated by two-tailed Student's t-test. Scale bars: 1 mm (A, B) or 250 µm (C, D).

2.3e Reduced fertility in *f5*$^{+/-}$ zebrafish females *in vivo*

Although *f5* homozygous mutants live to early adulthood, most die by 4-6 months of age and do not breed well (Weyand et al., 2019). Therefore, we examined female fertility in *f5*$^{+/-}$ mutants in a consecutive spawning test. We observed significantly

reduced numbers of embryos per spawning and total embryos over two weeks in comparison to wildtype sibling females. (Fig. 9A, B). The spawning frequency of *f5*$^{+/-}$ female fish also significantly decreased compared to that of wildtype siblings (Fig. 9C). One *f5*$^{+/-}$ female only spawned once in two weeks (Fig. 9C). The spawning intervals of *f5*$^{+/-}$ females were significantly longer in comparison to that of wildtype siblings (Fig. 9D).

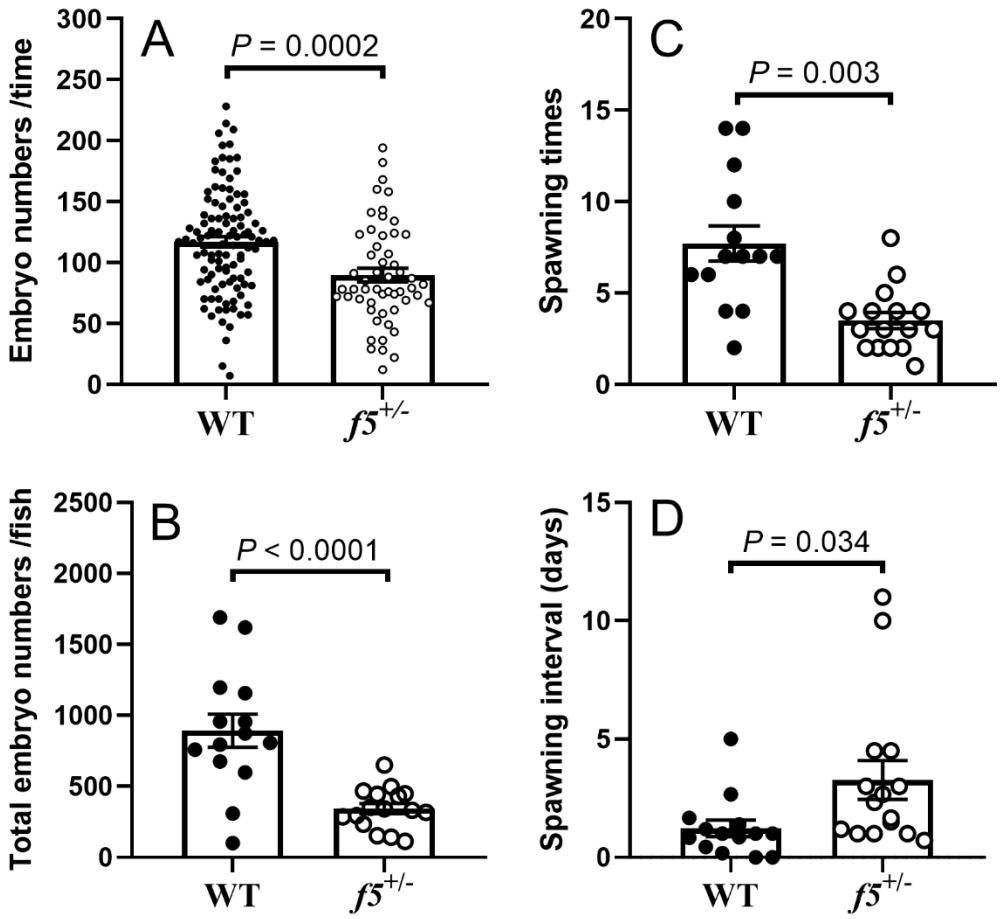

Figure 9. Reduced fecundity in *f5*$^{+/-}$ female zebrafish.

(**A**) Reduced numbers of live embryos per spawning for each *f5*$^{+/-}$ female in comparison to that of wildtype (*f5*$^{+/+}$) females. (**B**) Reduced total numbers of embryos for each *f5*$^{+/-}$ female fish in comparison to wildtype (*f5*$^{+/+}$) females. (**C**) Reduced spawning times in *f5*$^{+/-}$ females in comparison to that of wildtype (*f5*$^{+/+}$)

females. (**D**) Increased spawning intervals in *f5*$^{+/-}$ females in comparison to wildtype (*f5*$^{+/+}$) females. Data are expressed as the mean ± SEM (N ≥ 14). P-values were calculated by two-tailed Student's t-test.

3. Discussion

We have provided comprehensive evidence demonstrating that the expression of coagulation factors *f5* and *f3a* in preovulatory follicles is controlled by progestin and its cognate receptor Pgr. Increased expression of these coagulation factors is likely critical for the regulation of ovulation and female fertility in addition to their well-established roles in hemostasis. Our conclusions are based on the following evidence: 1) Coagulation factors, *f5* and *f3a*, are expressed in the follicular cells and increase dramatically prior to ovulation during natural spawning cycles in zebrafish; 2) Expression of these cofactors in preovulatory follicles is controlled by progestin via Pgr, which are essential and upstream regulators for ovulation; 3) Follicular cells with strong Pgr expression are adjacent to blood capillaries; 4). As follicular cells near rupture, a significant increase in the numbers of erythrocytes in blood vessels suggests blood clotting which is correlated with a dramatic increase of *f5* and *f3a* in preovulatory follicular cells; 5) Inhibition of coagulation leads to reduced ovulation; 6) Low fertility is found in *f5*$^{+/-}$ zebrafish female mutants.

3.1 Progestin regulates coagulation factors via Pgr

A major finding of the present study is the regulation of *f5* and *f3a* by progestin via Pgr in the preovulatory follicles during ovulation (Fig.10). F3 is expressed in a wide

array of tissues (Osterud & Bjorklid, 2006). It has been demonstrated that physiological concentrations of P4 could increase both F3 mRNA and protein in breast cancer cell lines ZR-75 and T47D (Kato et al., 2005). Increased F3 expression was correlated with an increase of procoagulant activity, which was hypothesized to be responsible for the increased risk of thrombosis in breast cancer patients (Richer et al., 2002). Studies of the uterus have also shown that RU486, an antagonist of PGR, not only blocks but also reverses progestin-enhanced expression of F3 mRNA and protein in stromal cells (Lockwood et al., 1994). In line with these studies, PGR binding sites were identified in the promoter region of the human F3 gene (Supplemental Table 5) (Bravo et al., 2015). The increase of *f3a* expression in the preovulatory follicles is primarily controlled by Pgr (current study). A difference in the weak activity of *f3a* promoters observed in a human immortal cell line (HEK293T) *in vitro* in comparison to progestin-induced strong *f3a* promoter activities found in zebrafish follicles both *in vivo* and *in vitro* may be due to missing core *cis*-regulatory elements in the cloned promoter sequence of zebrafish *f3a* (Supplemental Table 5). Nevertheless, our results demonstrate that like *f5*, *f3a* expression is also controlled by progestin via Pgr (Supplemental Table 5, Fig.10).

Until now, no information was available for progestin regulation of F5. Estrogen and progesterone receptors regulate *F5* expression in mouse bone marrow/bone (Cleuren et al., 2010; Moverare, Skrtic, Lindberg, Dahlman-Wright, & Ohlsson, 2004). The present study demonstrates that a new extrahepatic site of *f5* expression is regulated by progestin through Pgr in zebrafish. Moreover, progestin enhances the promoter activities of *f5* in zebrafish and F5 in humans via PGR. Several putative PREs were predicted in the

promoter regions of human, mouse, and zebrafish *F5* (Supplemental Table 6). Our site-direct mutation analyses of these PREs in the zebrafish *f5* promoter suggests direct regulation of progestin/Pgr via binding to and activating these a PREs. One PRE site (5'-TGACTCA-3') in the *f5* promoter is also conserved between zebrafish, mice and humans (Supplemental Table 6). Whether P4 and/or PGR regulate *F5* expression in human ovaries *in vivo* will be an interesting topic for further investigation.

3.2 Speculation of how ovarian produced coagulation factors regulate ovulation

The functions of coagulation factors in hemostasis are well established (Esmon, 2004; Hathcock, 2006; Spronk, Govers-Riemslag, & ten Cate, 2003). However, the role of coagulation factors during ovulation is significantly less studied. Formation of blood clots at apical blood vessels was found shortly before follicular rupture in several mammalian species (Brannstrom et al., 1998; Cavender & Murdoch, 1988; Duffy et al., 2019; Kanzaki et al., 1982; Zackrisson et al., 2011). In addition, the permeability of blood vessels increased 3-fold prior to ovulation in rats (Mitsube, Brannstrom, & Haraldsson, 2013), which likely facilitates material exchange between the follicles and blood vessels. The increase of material exchange might be important for anticipating local but avoiding systemic activation of blood clotting. Our observation of ruptured blood vessels on the surface of ovulating follicles further supports this hypothesis. In addition, we also found a significant increase of erythrocytes in capillaries on the surface of ovulating follicles as they progress to rupture/ovulation, suggestive of blood clotting. Lack of functional F5 or

F8 caused various bleeding symptoms, including recurrent and serious ovulation-related blood loss in women (Spiliopoulos & Kadir, 2016). Occurrence of blood clotting in advance of follicle rupture could preemptively stop excessive bleeding caused by ovulation-induced capillary rupture. The granulosa cells appear to be the primary cells for synthesizing F5 and F3a, as these account for most that express Pgr (this study & (Hanna et al., 2010; Natraj & Richards, 1993)). Intriguingly, we also found that strong Pgr expressing theca cells were localized near capillaries on the follicles. Whether these theca cells are key switchers of F3a-initiated hemostasis in the capillaries around ovarian follicles will be an interesting topic to examine in the future.

The dramatic induction of *f5* and *f3a* expression in preovulatory follicles via Pgr, an essential mediator for activation of various ovulation-related genes, suggests that locally produced F5 and F3a may be required for ovulation. Activation of multiple metalloproteases within the ovulating follicles at the right times is a critical step for sequential digestion of extracellular matrix proteins of the follicular cells that are required for ovulation (Duffy et al., 2019; Takahashi et al., 2019). Their activation and substrate binding are precisely regulated by the timing of prodomain cleavage of the enzyme and removal of endogenous tissue inhibitors of metalloproteases (Timps) (Yong Zhu, 2021).. Protease inhibitors (Timps and other inhibitors) are typically present at high concentrations in the serum to prevent metalloproteases from acting accidentally (Travis & Salvesen, 1983). Vasoconstriction of blood vessels within the thecal layer at the apex of the follicles was suggested to be critical for the removal of protease inhibition from the circulation (Migone et al., 2016). We hypothesize that coagulation that occurs shortly

before ovulation in the follicles may have a similar function. In addition to the removal of protease inhibitors from serum, the cleavage of propeptide from latent form of metalloproteinase is required for activation and thus ovulation (Fig.10). An F3-dependent pathway of thrombin generation was also shown in bovine, equine, and human follicular fluid (Gentry et al., 2000; Roach, Petrik, Plante, LaMarre, & Gentry, 2002; Yamada & Gentry, 1995). Granulosa cells were shown to express and secrete fibrinogen, which is catalyzed by thrombin to become fibrin (Parrott, Whaley, & Skinner, 1993). Fibrin formation could stimulate activities of plasmin/plasminogen activators (plat and plau) signal, which is well-established for plasminogen (plg) activation (Medved & Nieuwenhuizen, 2003). Furthermore, the function of Plat, Plau, and Plg in metalloproteinase activations during ovulation is also recognized (Beers, Strickland, & Reich, 1975; Ogiwara, Minagawa, Takano, Kageyama, & Takahashi, 2012). Therefore, we hypothesize that a dramatic increase of F3a and F5 expression in the follicular cells may facilitate the formation of fibrin, followed by the activation of Plat, Plau, and Plg, which further activate metalloproteinases. Taken together, as blood coagulation occurs, blood flow decreases, then stops completely, effectively terminating inhibitory effects of protease inhibitors from the serum, triggering metalloprotease activations to start the degradation of the follicular extracellular matrix for preparing the release of a mature oocyte (Fig.10). A patient diagnosed with severe F5 deficiency had to undergo bilateral ovariectomies because of the presence of hemorrhagic anovulatory follicles and life-threatening hemorrhage in the pouch of Douglas (Montefusco et al., 2003). Circulating F3 has been shown to be elevated in women with polycystic ovary syndrome, the most common form of anovulatory infertility (Gonzalez et al., 2013). Consistent with findings

from medaka (Ogiwara et al., 2005), the rate of ovulation was suppressed by EDTA treatment in the current study likely via inhibition of metalloproteases, or possibly inhibition of coagulation. EDTA is a known anticoagulant, acting through chelation of calcium, as many coagulation factors depend on this ion for function. Indeed, the known anticoagulant heparin also suppressed ovulation. The anticoagulant warfarin did not, but this was unsurprising since its mechanism of action usually requires days of treatment. Another finding in the present study is that reduced fecundity and impaired ovulation were observed in *f5*$^{+/-}$ female zebrafish *in vivo*. Together, these results argue for conserved functions of coagulation factors in the regulation of ovulation in vertebrate ovaries. Long-term efforts in development of new instruments and new techniques/methods, with suitable *in vivo* imaging and examining the association of blood flow and timing of metalloproteases activation, are required for further elucidation of underlying mechanisms and testing our hypothetical model (Fig.10).

In summary, our results demonstrate that *f5* and *f3a* expression are dramatically induced by progestin via a Pgr-dependent mechanism in preovulatory follicles (Fig. 10). Moreover, these coagulation factors may regulate ovulation and female fertility in addition to their roles in hemostasis. Future studies on the regulation and functions of coagulation factors in other organisms including mammalian follicles or ovarian fluid during ovulation will likely lead to developments of diagnostic and therapeutic modalities for patients with gynecological problems associated with mutations and/or dysregulation of coagulation.

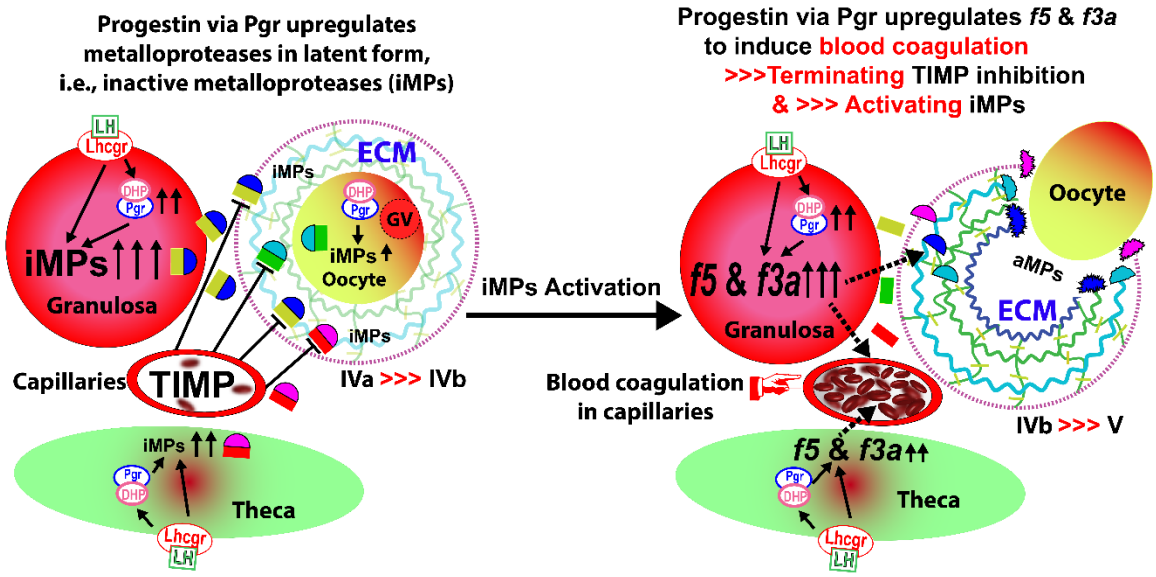

Figure 10. A proposed model for nuclear progestin receptor (Pgr) mediated upregulation of *f5* and *f3a* in blood coagulation that facilitates ovulation in zebrafish.

Luteinizing hormone (LH) induces ovulation via luteinizing hormone receptor (Lhcgr) in preovulatory follicles, namely in stage IV follicles in zebrafish. LH signaling is mainly mediated by progestin (DHP, i.e., 17α,20β-dihydroxy-progesterone, a native ovulation inducing steroid in zebrafish) and its receptor (Pgr) in the production and activation of metalloproteases. The illustration on the left side is showing the upregulation of metalloproteases in preovulatory follicles, to be more specific, during the transition from stage IVa to IVb follicles, which is an important preparation step for ovulation (Liu et al., 2017; Liu et al., 2018; Liu, Hong, Chen, & Zhu, 2020). However, these proteases are produced in a latent form, i.e., inactive form of metalloproteases (iMPs, see Yong Zhu, 2021 for detail), and inhibited by tissue inhibitors of metalloproteases (TIMP) presented at high levels in and delivered by blood capillaries to the follicles. The illustration on the right side is showing a dramatic increase of coagulation factors *f5* and *f3a* shortly before ovulation, specifically in the late stage of IVb follicles, which are also mainly upregulated by progestin via Pgr. These ovarian follicles derived, and locally produced coagulation factors (F5 and F3a) are imported into inside blood vessels messed in the follicles and activated via yet to be defined mechanisms. Once F5 and F3a are activated, these coagulation factors accelerate blood coagulation, which in turn stops blood flow and therefore effectively terminates TIMP inhibition of metalloproteases from the bloodstream. In

addition, the production of fibrin (not shown) due to the formation of blood clots stimulates activities of plasmin/plasminogen activators, which are well-known inducers for metalloprotease activation. The combined force of these two pathways triggers rapid activation of metalloproteases, breakdown of extracellular matrix and basal membrane, rupture of follicular cell layers, the final release of a mature oocyte.

4. Materials and Methods

4.1 Animals & Reagents

Zebrafish used are offspring of Tübingen strain unless stated otherwise. Detailed information for knockouts, transgenic lines, key chemicals, and reagents are listed in Supplemental Table 1 & Table 2. Fish were fed three times daily with a commercial food (Otohime B2; Marubeni Nisshin Feed, Tokyo, Japan), supplemented with newly hatched brine shrimp, and were maintained in recirculating system (ESEN, Beijing, China) at 28 ± 0.5 □ under a controlled photoperiod (lights on at 08:00, off at 22:00). The daily ovulation cycle in mature adult zebrafish is established according to an established protocol (Liu et al., 2018). Briefly, each mature adult female and male zebrafish were paired in a spawning tank. These fish typically start spawning soon after lights on in the morning. Around noon, fish water was replaced, and fish were fed at least three times daily with the commercial food and supplemented with newly hatched brine shrimp. Around one hour prior to lights off, fish water was replaced again, a mesh inner tank was inserted into each spawning tank to prevent cannibalism typical for zebrafish adults. After one week, most fish form a daily ovulation cycle. When applicable, siblings from heterozygous in-cross were used for comparison. All experimental protocols were

approved by the Institutional Animal Care and Use Committee (IACUC) at Xiamen University.

4.2 Collection of fully-grown follicles (stage □) during a daily spawning cycle

Fully-grown immature follicles (stage IVa, >650 μm, with visible germinal vesicles, i.e., GV, appears opaque) or mature follicles (stage IVb, GV disappears, appears transparent) were collected at four representative timepoints during a daily spawning cycle (Fig. 1A): 12:00 (IVa follicles); 20:00 (IVa follicles); 05:00 (the onset of oocyte maturation, IVa follicles); and 06:40 (completion of oocyte maturation but prior to ovulation, IVb follicles). To be simple and accurate in our description, we will use "IVa" to indicate fully-grown immature stage IVa follicles; "IVb" to indicate mature but yet ovulated follicles (prior to ovulation) throughout this manuscript.

To sacrifice fish humanely, the spinal cord and blood supply behind the gill cover were cut off swiftly using sharp scissors following a lethal dose of anesthetization (MS-222, 300 mg/L buffered solutions). Ovaries were immediately placed in 60 % L-15 media containing 15 mM HEPES at pH 7.2. Individual follicles were obtained according to an established procedure (Liu et al., 2018; Liu et al., 2020). At each timepoint, a total of 25 □a or IVb follicles (containing both germ cell and surrounding follicular cells) from 4 females were pooled as one sample. In addition, the follicular cells (∼100 follicles) or their enclosed oocytes (∼20 follicles) were collected separately from IVb follicles according to an established method (Hanna & Zhu, 2011). These samples were

immediately homogenized in RNAzol solution (Molecular Research Center, Cincinnati, USA) and stored at a -80 ℃ freezer until further processing.

4.3 Hormone treatments

According to established follicle classification system (Liu et al., 2018), follicles at the different stages were collected at 14:00 from several females as described above. Intact follicles with no damage were transferred into a 24-well culture plate containing 60 % L-15 medium. These follicles were incubated at 28 □ with various doses of hormones or various exposure times. Several progestin concentrations (DHP, 1-1,000 nM) with a 2 hrs incubation or several exposure times (0.5-6 hrs) with 100 nM DHP were examined. Based on the result of dose- and time-dependent experiments, follicles at different stages were incubated with DHP (100 nM) for 2 hrs. Besides, stage IVa follicles were incubated with hCG (50 IU/mL) (Liu et al., 2020) or DHP (100 nM) alone or in combination for 2 hrs. Stage IVa follicles collected from *pgr*$^{-/-}$ were incubated with DHP (100 nM) for 2 hrs. After incubation, minimal 25 follicles with no damage were collected and homogenized in RNAzol, stored in a -80 □ freezer until RNA purification. Minimal three replicates were collected at each timepoint.

4.4 Real-time quantitative PCR (qPCR)

RevertAid cDNA synthesis kit (Thermo Scientific, Waltham, USA) was used for reverse transcription (500 ng total RNA/sample). All qPCRs were carried out in a 20 µL reaction on the qTOWER 2.2 real-time PCR system (Analytik Jena AG, Jena, Germany) using the PowerUp SYBR green master mix (Applied Biosystems, USA). Each PCR primer of a pair was designed to target different exons of the gene (Supplemental Table 3). Expression of the target genes was determined as relative values using the comparative C_t method (Schmittgen & Livak, 2008) with the *ef1α* gene serving as an internal control.

4.5 Whole-mount *in situ* hybridization (WISH)

A DNA fragment corresponding to partial *f5* or *f3a* cDNAs was amplified from ovarian cDNAs by PCR using the gene-specific primers (see Supplemental Table 3 for detail) and cloned into the pGEM-T Easy vector (Promega, USA). Correct sequence and orientations of inserts were validated by Sanger sequencing. Antisense or sense digoxigenin-labeled cRNA probes for *f3a* or *f5* were synthesized by PCR using the plasmid cDNA templates, SP6 or T7 RNA polymerase depending on the insert orientation. IVa or IVb follicles were collected at 20:00 and 06:40 (immediately prior to ovulation), respectively, and fixed in 4 % PFA overnight at 4 ℃. Following washing with PBST, the follicles were treated with proteinase K (5 µg/mL) for 2 min at 37 °C, then washed and subsequently hybridized with digoxigenin-labeled *f5* or *f3a* sense or antisense probes at 65 °C overnight using a previously established protocol (Hauptmann

& Gerster, 2000). Following WISH, follicles were photographed using an M165FC dissecting microscope and DFC 550 digital camera (Leica, Wetzlar, Germany).

4.6 Dual-luciferase reporter assay

The Pgr expression and reporter assay plasmid constructs were listed in Supplemental Table 4. Site-directed mutagenesis of four potential Pgr response elements (PRE, i.e., Pgr binding sites) in zebrafish *f5* proximal promoter sequences (Fig. 4E, Supplemental Table 6) was conducted using a Q5 site-directed mutagenesis kit (NEB, Ipswich, MA, USA) according to the manufacturer's protocol. Human embryonic kidney cells (HEK 293T) were used for the transactivation assays (Chen et al., 2010; Zhang et al., 2018). Briefly, cells were maintained at 37 °C with 5 % CO_2 in phenol red-free Dulbecco's modified eagle's medium (Hyclone, USA) containing penicillin/streptomycin (Meilunbio, Dalian, China) and supplemented with 10 % fetal bovine serum (FBS, Gibco, Brazil). Lipofectamine 3000 (Life Technologies) was used for transient transfection according to manufactures' protocols. Cells were transiently transfected with a firefly luciferase reporter plasmid (3 μg/vector/60 mm petri dish), the pRL-TK vector (150 ng/60 mm petri dish) containing the Renilla luciferase reporter gene (as an internal control for transfection efficiency), and a Pgr expression vector or a control vector with no insert (1.5 μg/vector/60 mm petri dish). After an overnight incubation at 37 °C, the medium was replaced with luciferase assay medium (DMEM without phenol red, supplemented with 10 % charcoal-stripped FBS) containing various concentrations of progestins (100 pM-1 μM DHP for zebrafish Pgr, P4 or human PGRB, respectively) or

vehicle. Following additional 24 hrs of incubation at 37 °C, the cells were washed with PBS and harvested to determine firefly and Renilla luciferase activities according to the Dual-Luciferase reporter assay system (Promega, USA) on a luminometer (GloMax 20/20, Promega, USA). Firefly luciferase activities were normalized using Renilla luciferase data. After normalization for transfection efficiency, induction factors were calculated as the ratios of the average luciferase value of the steroid-treated samples versus vehicle-treated samples.

4.7 Blood capillaries in stage □ follicles

IVa immature follicles were obtained from paired female fish (*Tg(pgr:eGFP/ fli1:DsRed)*). In this line, the blood capillaries are labeled with DsRed, while the follicular cells are labeled with eGFP. The nucleus of follicles was co-stained with Hoechst 33342 in a 29-mm glass-bottom dish (Cellvis, Hangzhou, China). Images were captured using an SP8 confocal laser scanning microscope (Leica, Germany). IVb mature follicles were obtained from ovaries undergoing ovulation from faired female fish (*Tg(fli1:eGFP)*) at 7:00. A series of images during ovulation was recorded using a Leica digital camera and fluorescent dissecting microscope described previously.

4.8 Erythrocyte numbers inside the capillaries on the surface of preovulatory follicles.

Erythrocytes inside the capillaries on the surface of ovarian follicles were stained with *o*-dianisidine according to an established protocol (Detrich et al., 1995). Ovaries

with IVb follicles were collected at 6:40 or 7:00 from siblings of *pgr*$^{+/-}$ in-cross, producing both wildtype (*pgr*$^{+/+}$) and *pgr*$^{-/-}$. As sampling moves closely toward 7:00, IVb follicles will be further in the ovulation process in *pgr*$^{+/+}$ females. In contrast, IVb follicles in *pgr*$^{-/-}$ females will never initiate ovulation (Tang et al., 2016; Y. Zhu et al., 2015), which serves as an excellent control. After collection, the whole ovaries were immediately stained for 15 min at room temperature in the dark in 0.6 mg/ml *o*-dianisidine, 0.01 M sodium acetate (pH 4.5), 0.65 % H_20_2, and 40 % (vol/vol) ethanol. Stained ovaries were washed 3 times for 2 min each with PBST. At least 3 ovary samples were collected at each timepoint for each genotype. Minimal 10 IVb follicles were randomly chosen from each sample for image capture using DM 2500 LED microscope and DFC 7000T digital camera (Leica), then the number of erythrocytes under the field of view were counted by a reviewer who was blinded from sampling conditions and genotypes.

4.9 *In vitro* ovulation assay

Ovaries with IVb mature follicles from *Tg(fli1:eGFP)* zebrafish were collected at 06:40, 20 min before ovulation, and were placed in 60 % L-15 medium. Each ovary was immediately cut into several small pieces and then transferred into a 12-well plate with medium containing 2 mM EDTA, 100 µg/mL heparin, or 50 µg/mL warfarin, to examine their inhibitory effect on ovulation. The dose of each drug was chosen based on previous reports (Hanumanthaiah, Thankavel, Day, Gregory, & Jagadeeswaran, 2001; Ogiwara et al., 2005). Follicles were immediately dispersed by pipetting using a glass pipette (~ 1

mm in diameter). At least 50 IVb preovulatory follicles per well were used in each experiment. The follicles were cultured at 28 °C in a 2 mL culture medium for 2 hrs. Ovulation was continuously monitored until 09:00 under a fluorescent dissecting microscope (Leica M165FC), and the numbers of oocytes that had successfully ovulated were recorded. Minimal replicates for each treatment were eight.

4.10 Consecutive spawning test

The spawning of zebrafish was optimized under enhanced feeding conditions as described previously (Liu et al., 2018). Multiple pairs and spawning tanks were set up. Ten to twenty mature females (three months old) of *f5*$^{+/-}$ or wildtype (*f5*$^{+/+}$) female siblings were used. Each female was individually paired and housed with a fertile wildtype male. Around 21:00 (1 hrs prior to lights off), water in spawning tanks was replaced, 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 was recorded for each pair of fish around noon of the next day. Then, water would be replaced, and fish would be fed at least three times a day. The *f5*$^{+/-}$ and wildtype (*f5*$^{+/+}$) females were continuously monitored for at least 3 weeks. Data from the last two weeks were used for analyses.

4.11 Statistical analyses

Data analyses were performed using GraphPad Prism version 8. Depending on the experimental setup, either independent (unpaired two-tailed) Student's t-test or one-way

ANOVA (one-tailed) followed by the Dunnett's post hoc test was used to assess statistical differences in comparison to the control group. Each experiment (e.g. qPCR, reporter assay, WISH, drug exposure, reporter assay, erythrocytes count, *in vitro* ovulation, fertility test) was repeated at least once.

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