Hormone-induced thrombosis is mediated through non-canonical fibrin(ogen) aggregation and a novel estrogen target in zebrafish

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ABSTRACT

Venous thrombosis is a well-known complication of sex hormone therapy, with onset typically within weeks to months after initiation. Worldwide, more than 100 million pre-menopausal women use combined oral contraceptives, with tens to hundreds of thousands developing thrombosis annually, resulting in significant morbidity and mortality. Although it is known that estrogens can alter expression of coagulation factors, the pathways and mechanisms that connect the two systems, as well as the proteins involved in progression to thrombosis, are poorly understood. Identification of these mediators are central to any comprehensive understanding of hormone-induced pathophysiology, could help ascertain patients at higher risk for thrombosis, and may also pinpoint future therapeutic targets. The zebrafish is a powerful genetic model in which the hemostatic system is almost entirely conserved with humans. Its external development, ability to generate thousands of offspring at low cost, and optical transparency all make it a powerful tool to study the genetics of coagulation disorders. We previously produced a transgenic line (*fgb-egfp*) that generates GFP-tagged fibrinogen that labels induced and spontaneous fibrin-rich thrombi. Here we show rapid onset of thrombosis after exposure to various estrogens, but not progestins or testosterone. Thrombi are localized to the venous system, develop broadly along the posterior cardinal vein, and show evidence for clot contraction. Thrombosis is only partially impeded by anticoagulants, occurs in the absence of factor X and prothrombin, but is completely blocked in the absence of fibrinogen. Furthermore, although an estrogen receptor antagonist is partially inhibitory, targeted knockout of all known estrogen receptors does not eliminate thrombosis. These data suggest that zebrafish can be used to model human estrogen-induced thrombosis, although the lack of dependence on the canonical coagulation cascade is surprising. The inability to completely inhibit thrombosis through genetic/pharmacologic anticoagulation or estrogen receptor disruption suggests that the mechanisms may be multifactorial. We hypothesize that thrombi are composed of fibrin(ogen) aggregates rather than purely fibrin. Results of further studies could lead to novel therapeutic targets and ascertain patients at higher risk for thrombosis.

INTRODUCTION

Venous thromboembolism (VTE) is caused by the formation of pathologic blood clots in major veins, resulting in deep vein thrombosis and/or pulmonary embolism. According to the Centers for Disease Control, VTE affects ~900,000 people/year in the U.S., and accounts for 60,000-100,000 deaths[1]. Major causes are inherited or acquired hypercoagulable states and excess estrogen, the latter particularly from combined oral contraceptives (COCs)[2-4]. Excess estrogen is a longstanding well-known risk factor for VTE[5-7], especially in combination with other acquired risk factors, such as smoking and obesity[8]. Despite multiple clinical studies, the mechanisms of estrogen-induced thrombosis are minimally understood[9].

Estrogens are a class of molecules that are structurally similar and target multiple receptors with various agonistic affinities for nuclear hormone receptors (ESRs)[9] and a G-protein coupled estrogen receptor (GPER1)[10]. In premenopausal women, 17α-ethinylestradiol (EE2), a synthetic estrogen with four to five times the potency of natural estrogens, is most commonly used in COCs. Activities are also dependent on the crosstalk between various hormone receptors, including progestins, androgens, and estrogens[11]. For example, some androgens, such as 19-nortestosterone, have weak estrogenic activities[12], and some progestins are known to have weak androgenic

activities[13]. Estrogen functions through two mechanisms, genomic and non-genomic[14]. ERα and ERβ (genes *ESR1* and *ESR2*) are the two nuclear hormone receptors, with zebrafish orthologs Esr1, Esr2a, and Esr2b[15,16]. After binding estrogen, these dimerize and translocate to the nucleus to bind estrogen response elements (EREs) and regulate transcription[17]. Non-genomic effects are mediated by GPER1[18]. There is also evidence for additional receptors[19], as well as new functions for known ones[20,21].

Over the years, studies have shown that estrogen is able to induce upregulation of gene expression of multiple blood coagulation factors in humans, mice, and zebrafish[9,22-24] and downregulation of anticoagulants[25,26]. Recent progress has been made through *in vitro* and *ex vivo* studies. In endothelial cells 17β-estradiol (E2) activated exocytosis through ERα[27], but COCs did not affect endothelial procoagulant activity, even with overexpression of the *ESR* genes[28]. *Ex vivo*, estradiol appeared to affect clot dynamics, including formation, propagation, strength, structure, and fibrinolysis. However, *in vivo* murine studies have been largely unsuccessful as ethinylestradiol increased thrombin generation, but there was no increase in tissue fibrin deposition or a visible thrombotic phenotype[29]. Additionally, these studies showed suppression of thrombosis by ethinylestradiol[30]. This absence of an animal model has contributed to a continued lack of mechanistic understanding.

As a vertebrate model, zebrafish (*Danio rerio*) exhibits unique characteristics such as external fertilization, rapid development, high fecundity, and optical transparency, and possesses all the key coagulation factors found in humans[31-33]. The transparency of zebrafish larvae allows for real-time observation of blood circulation and fluid dynamics. Our previous research utilizing genome editing in zebrafish has revealed remarkable similarities between the pro- and anticoagulant factors in zebrafish and those in humans[34-39]. Zebrafish also demonstrate the preservation of major estrogen receptors and estrogen-regulated gene activities[40-42]. In this study, we demonstrate that zebrafish develop venous thrombosis in response to multiple estrogens, but not progestins or testosterone. These thrombi form even in the absence or inhibition of the coagulation cascade, as well as absence of all known estrogen receptors.

METHODS

Zebrafish

Zebrafish were maintained on a hybrid background of wild-type strains (ABxTL) to minimize strain-specific effects and enhance fecundity. Fish were examined at various developmental stages: embryo (0-2 days post-fertilization, dpf), larvae (3-29 dpf), juvenile (30-89 dpf), and adult (>90 dpf) and housed at 28.5°C, with a 14-hour light/10-hour dark cycle. All procedures were approved by the University of Michigan Animal Care and Use Committee. Previously described transgenic and mutant lines include, *fgb-egfp* (labels fibrin(ogen) with green fluorescence)[43], prothrombin (*f2*)[39], factor X (*f10*)[35], fibrinogen α-chain (*fga*)[36] and *gper1*[44] knockouts, *flk-mcherry* (red endothelium)[45], *ere-gfp* (estrogen response element reporter)[41]. Nearly all experiments were performed in the *fgb-egfp* background.

Small molecule treatments

Chemicals were purchased from Sigma-Aldrich, dissolved in DMSO and then into E3 embryo buffer, followed by submersion of typically 7-10 larvae in 2 ml E3 embryo buffer (Figure 1A). Final DMSO concentrations were always less than 2%. Larvae were treated at 3-5 dpf for 5-48 hours. Warfarin (50 ug/ml, Sigma-Aldrich) was initiated at 3 dpf and then followed until 5 dpf.

Imaging and quantification of thrombosis

After treatment, zebrafish larvae were anesthetized in tricaine and embedded in 0.8% low-melting point agarose on glass cover slips and visualized on an inverted microscope (Olympus IX71, 20x objective). For all experiments evaluating thrombosis, the observer was blinded to treatment condition and/or genotype. A semi-quantitative grade was assigned based on the fluorescence intensity in the posterior cardinal vein (PCV). Grade 0 indicated no fluorescence, grade 1, sparse puncta, prominent accumulation was observed in grade 2, and grade 3 exhibited dense nearly complete coverage (Figure 1B). Mutant larvae were recovered from agarose after evaluation and genotyped if needed. Confocal images were acquired by a Nikon A1 high sensitivity confocal microscope. Fluorescence intensity was measured with ImageJ to calculate corrected total cell fluorescence (CTCF) as described[34].

Measurement of plasma estrogen

After treatment, larvae were washed three times in E3 embryo buffer for 10 minutes, anesthetized, then individually bled in 5 uL of 4% sodium citrate diluted 1:6 in Tyrode's buffer[46], with an estimated whole blood yield of 50 nl giving a 1:100 dilution. Ten larvae were pooled (50 ul), centrifuged at 500g for 10 minutes, and 30 ul supernatant was collected. This was diluted to 0.1 ml and mixed with 0.1-0.2 ml water and deuterium-labeled internal standards in 0.1 mL of 40% aqueous methanol. This mixture was applied to a supported liquid extraction cartridge (Isolute, Biotage) and analyzed by mass spectrometry as previously described[47], except that only the 1.50-2.65 min window from the first dimension chromatography was passed to the resolving column, and only E2, estrone, testosterone, and androstenedione were quantified.

Scanning electron microscopy

After treatment with E2, larvae were immobilized using tricaine anesthesia and euthanized on ice. For scanning electron microscopy (SEM) analysis, sagittal cuts were made in the larvae to open the PCV, rinsed in 50 mM sodium cacodylate buffer (pH 7.5) with 150 mM NaCl, and fixed in 2% glutaraldehyde. Samples were rinsed three times with the cacodylate buffer for 5 minutes and dehydrated in a series of increasing ethanol concentrations, immersed in hexamethyldisilazane, and dried overnight. A thin coating of gold-palladium was layered over the samples using a sputter coater (Polaron e5100, Quorum Technologies). Micrographs were captured using a Quanta 250 FEG scanning electron microscope (Thermo Fisher Scientific).

CRISPR-mediated genome editing

We used CRISPR/Cas9 and designed single guide RNAs (sgRNAs) to target upstream and downstream exons, thereby deleting nearly the entire locus of each *esr* gene (Table 1) as previously

described[48]. The *esr1* and *esr2a* genes are tightly linked, therefore *esr1* was targeted first, followed by *esr2a* once the former line had been established. Once the *esr1;esr2a* double mutants were produced, they were crossed to *esr2b* mutants to produce triple heterozygotes. *esr1;esr2a;esr2b* triple heterozygotes were incrossed to produce triple homozygous mutant larvae.

Transgene expression

The *esr1* overexpression plasmid (p*hs-esr1*-Y549S) was constructed by inserting the cDNA into a vector under control of the heat shock promoter[49] downstream of *mcherry* and a p2A sequence, all flanked by Tol2 repeats. 20-25 picograms plasmid was injected into *fgb-gfp* and *ere-gfp* embryos. At 3 dpf, larvae were incubated at 35ºC and evaluated for thrombosis.

Statistical analysis

Statistical analysis was performed using two-tailed Fisher exact or Mann-Whitney *U* tests. Charts were generated using Prism (GraphPad Software, San Diego, CA, USA).

RESULTS

17β-estradiol causes thrombosis *in vivo*

We incubated *fgb-egfp* larvae in 17β-estradiol (E2) for 5 hours and discovered fluorescent thrombi appearing in the PCV (Figure 1A). We developed a semi-quantitative grading system (Figure 1B) which we found to be highly reproducible. When we compared these results to quantitative measurement of fluorescence through CTCF, there was remarkable consistency between the two methods (Figure 1C). Given this result and its ease of use, all further data were collected using the grading system. The most efficacious results were observed at relatively high concentrations of E2 (50-75 uM), therefore we measured the larval plasma concentration. We found that only a small fraction was absorbed (Figure 1D).

Estrogen analogs induce *in vivo* thrombosis

We next compared the potency of multiple estrogen derivatives, which all showed dose-dependent effects (Figure 2). Although E2 and ethinylestradiol both showed similar data (Figure 2B,C), these results were inconsistent between experiments, and what is shown are the "representative" best data. Mestranol and quinestrol, which are metabolized to EE2 *in vivo*, demonstrated greater potency and efficacy, and were more consistent across multiple experiments, although they required longer incubations (24-48 hours, Figure 2C,D).

Testosterone derivatives and progestins do not cause thrombosis

Testosterone has been shown to have an association with increased VTE[50], while progestins are considered to be relatively safe when taken independently[9]. We tested three testosterone derivatives and four progestins (at the maximum tolerated doses) across different generations (Figure 3A-D), but neither group triggered thrombosis.

Static and time-lapse imaging of estrogen-induced thrombosis

To confirm the localization and composition of estrogen-induced thrombi, we used confocal and electron microscopy. Confocal imaging was performed in a double transgenic that included the *flk-mcherry* line, labeling endothelial cells with red fluorescence (Figure 4A,B). GFP was not colocalized with endothelial cells nor present in the dorsal aorta (DA), but rather was present within the PCV. This indicates that the thrombosis was not generated within nor internalized by endothelial cells. Increasing doses or duration of incubation did not lead to arterial thrombosis.

In humans, it is well-know that thrombi often originate at a point of injury, and then extend through the vasculature. However, given the spontaneous and unpredictable nature of estrogen-induced thrombosis, it is unknown how this occurs in patients. Time-lapse imaging was performed over a six-hour period after initiation of treatment with E2. The results show that thrombosis occurs simultaneously across the venous endothelium, rather than initiating and spreading from a single location (Figure 4C-F). Scanning electron microscope images revealed the morphology of these thrombi in estradiol-treated larvae (Figure 4G,H). We observed polyhedrocytes (Figure 4H), consistent with the shape change that occurs in erythrocytes after clot contraction[51,52]. We have previously shown this occurs in zebrafish thrombi that develop after induced endothelial injury[53]. Sparse fibers were also observed (Figure 4H), which we hypothesize could be composed of fibrinogen or fibrin.

Estrogen-induced thrombi are not dependent on the canonical coagulation cascade

To further determine the origins of the thrombi, we evaluated coagulation using inhibitors and factor knockout lines. We tested multiple clinical anticoagulants, including warfarin, rivaroxaban (factor Xa inhibitor), and dabigatran (thrombin inhibitor), and knockouts of factor X (*f10*) and prothrombin (*f2*)[35,39]. We have previously shown both the inhibitors and knockouts result in complete loss of thrombosis secondary to endothelial injury[34,35,39]. The anticoagulants significantly reduced E2-induced thrombosis, but did not completely block it (Figure 5A,B). Surprisingly, in the *f10* and *f2* knockouts, there was no inhibition of thrombosis in the homozygous mutants (Figure 5C,D). Given these results, we evaluated whether fibrinogen itself is a component in the observed thrombi by examining estrogen-induced thrombosis in an α-chain (*fga*) knockout. Absence of the α-chain has proven to result in loss of fibrinogen in mammals[54-56] and zebrafish[36,57]. We have shown that the *fga* knockout also has complete absence of endothelial injury-induced thrombosis. Siblings from $fga^{+/-}$ incrosses were incubated with estrogens and there was no thrombosis detected (Figure 5E,F). The *fgb-egfp* line is labeled with GFP on the β-chain, therefore loss of fluorescent thrombi in the α-chain knockout confirms that these are fibrin(ogen)-containing structures rather than non-specific GFP accumulation. Overall, these data suggest that estrogen-induced thrombi may be composed of both fibrin and fibrinogen.

Estrogen-induced thrombosis is mediated through a non-canonical target

Next, we explored whether the effect is mediated through known estrogen receptors. We first examined fulvestrant[58], an estrogen antagonist with similar binding affinity to E2. It is simply a 7α-alkylsulphinyl analog of E2 that prevents nuclear hormone receptor dimerization which is

essential for downstream signaling and facilitates receptor degradation. We treated zebrafish larvae with E2 or mestranol and fulvestrant, and observed significant reduction of thrombosis (Figure 6A,B). We proceeded to examine the effect of loss of the various estrogen receptors, starting with the nuclear hormone receptors (*esr1*, *esr2a*, and *esr2b*). Previous studies with small indels have found that double and triple, but not single mutants have effects on reproduction[59], so we chose to produce complete locus deletions of each gene to ensure complete ablation. Since *esr1* and *esr2a* are tightly linked, we had to produce these double mutants through iterative targeting. Using a triple heterozygous mutant incross, we were able to evaluate single *esr2b*, double *esr1/2a*, and triple homozygous mutants (Figure 6C), all of which developed equivalent levels of estrogen-induced thrombosis. The same was true of our *gper1* line[44], which also exhibited persistent thrombosis (Figure 6D).

It is well known that 30-40% of zebrafish genes are duplicated due to an ancient event[60]. The whole genome has been sequenced[61] and it is believed that there are only three *esr* genes, but the possibility remains that additional ones may exist. A Y549S substitution has been shown to render zebrafish Esr1 constitutively active[62]. We cloned *esr1* under control of a ubiquitous promoter[63] and engineered this substitution, but transgenic embryos did not survive (data not shown). Therefore, we swapped in a heat shock promoter to enable activation of *esr1* at selected timepoints, along with a fluorescent *mcherry* reporter (Figure 7A). We injected this construct into the *ere-gfp* transgenic line which has been shown to be responsive to activated estrogen receptors[41]. After heat shock, both *mcherry* and *gfp* signals were observed indicating successful expression of the mutant *esr1* and activation of the *ere-gfp* transgene (Figure 7B). However, when injected into the *fgb-egfp* background, no thrombosis was observed in either group. Taken together, these data suggest that estrogen-induced thrombosis is mediated through a novel target.

DISCUSSION

Thrombosis has been a well-known consequence of estrogen therapy for decades, yet we still know little about the underlying mechanisms. One of the major factors is the lack of an animal model. Blood coagulation is a complex process that relies on circulating cells and proteins in the context of an intact endothelial system, which is not easily modeled *in vitro*. Despite the aforementioned *in vitro* and *in vivo* murine studies, there remain many unanswered questions. We have developed a reliable and genetically tractable model using zebrafish, which has yielded some potential answers. Furthermore, this highly penetrant model that does not require weeks or months to develop a phenotype is a major advantage for future studies. Zebrafish treated with estrogens replicate key characteristics consistent with the majority of human estrogen-induced thrombosis. These include venous thrombosis and lack of response to progesterone. Due to the transparency of zebrafish, we were also able to address a question that is essentially impossible to answer in mammals, i.e., the nature of the initiating event. In humans, thrombosis is often triggered by localized endothelial injury, e.g. trauma, surgery, and vascular access devices. Frequently, these thrombi subsequently spread through the vasculature. Since estrogen-induced thrombosis occurs weeks to months after initiation of therapy, it is impossible to determine if the etiology is similar. Through time-lapse imaging we found that thrombosis occurs simultaneously along the venous endothelium. We hypothesize that estrogen has a direct effect on the endothelium, altering gene expression programs[64] that increase the risk of thrombosis.

Given what is known about estrogen signaling, we believe that induction of thrombosis is preceded by alteration of transcription or cytoplasmic signaling in endothelial cells. We initially thought it would be easy to determine the first step through knockout of known receptors. However, through knockout and overexpression studies, we found that this effect is not mediated through nuclear hormones or the known G-protein receptors. Despite this, our data indicate that this is a specific effect of estrogen. First of all, it does not occur with other steroid hormones. Additionally, the receptor blocker fulvestrant, which is simply a modified version of E2, partially blocks thrombosis. Finally, there is precedence for alternative targets or receptors. In elegant studies, Kelly and colleagues have clearly demonstrated the existence of a novel estrogen-responsive G-protein receptor, although the gene itself remains undiscovered[19]. There is also evidence for other additional targets, including ion channels[11,65]. It is tempting to speculate that estrogen-induced thrombosis may be mediated through one of these targets.

The most surprising results from our studies were the observations that estrogen-induced thrombosis persisted despite treatment with potent anticoagulants, as well as loss of common pathway procoagulant factors. We have previously shown that all of these medications and mutations completely block thrombosis in response to endothelial injury[34,35,38,39]. Taken together, these data suggest that the fluorescent thrombi are not solely composed of fibrin secondary to thrombin-mediated cleavage of fibrinogen. However, our data using the α-chain knockout prove that the thrombi are indeed dependent on circulating fibrinogen. This is not a fluorescence artifact since it is known that ablation of *fga* results in absence of fibrinogen[36,57], and our GFP tag is on the β-chain.

The next question we plan to address in future studies is the structure of these thrombi. Our data suggest several possibilities. The anticoagulants do inhibit thrombosis to a degree, so fibrin may be a partial component. Additionally, we found polyhedrocytes, which are known to form upon clot contraction[53]. However, complete loss of the *f2* and *f10* genes should block all fibrin formation. Thus, there could be an alternative means of generating fibrin independent of thrombin, but this seems unlikely as there is no evidence for such a protease. A more likely hypothesis is that estrogen-induced thrombi are composed of crosslinked fibrinogen. It has previously been shown that non-factor XIII transglutaminases have this capability *ex vivo*[66]. More recently, tissue transglutaminase-2 (TGM2) was shown to crosslink fibrin(ogen) in the setting of experimental liver fibrosis[67]. Most intriguingly, injection of a Tgm2 fluorescent competitive substrate into zebrafish larvae yielded a labeling pattern[68] that bears a striking similarity to what we observe for estrogen-induced thrombosis. Tgm2 activity in zebrafish is primarily composed of two ohnologs (*tgm2a* and *b*), which were shown to be expressed during the larval period (1-5 dpf)[68].

We have discovered a model of estrogen-induced thrombosis that shares many features consistent with human disease, as well as others that are unexpectedly different. Identification of the novel estrogen target/receptor as well as mechanisms for fibrin(ogen) crosslinking could lead to insights into hormone-induced thrombosis. Future studies could develop novel therapeutic targets as well as predict those at the highest risk for thrombosis.

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AUTHORSHIP CONTRIBUTIONS

X.Y. designed and performed research, analyzed data, and wrote the manuscript; Q.Y.Z., M.Y., S.M.E, J.K.L, H.S., performed research and analyzed data. A.C.F., C.N., S.C., performed research, W.G., J.W.W., R.A. designed and supervised research and analyzed data, J.A.S. designed, performed, and supervised research, analyzed data, and wrote the manuscript. All authors reviewed the manuscript.

CONFLICT OF INTEREST DISCLOSURES

J.A.S. has been a consultant for Sanofi, NovoNordisk, Biomarin, Takeda, Pfizer, Genentech, CSL Behring, and Medexus. The other authors declare no relevant conflicts of interest.

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Table 1. sgRNA sequences

Description	Sequence (5' → 3')
esr1 sgRNA (exon 2)	CCGTATCTGAGCCATCACGG
esr1 sgRNA (exon 9)	GGAGAAAAGTTACTCTTAGA
esr2a sgRNA (exon 2)	ACACAGACCTTGGCCACGAC
esr2a sgRNA (exon 10)	CTCAAGTACTCACCAGTTCA
esr2b sgRNA (exon 1)	GTGTCCGAAAGCCTCGTCCC
esr2b sgRNA (exon 9)	TTGGCTGAGACAGTTACATA

FIGURE LEGENDS

Figure 1: Induction and quantification of thrombosis in zebrafish larvae. (A) Compounds such as E2 were dissolved in DMSO and added into a multiwell plate with E3 embryo buffer. *fgb-egfp* transgenic larvae, which generate GFP-tagged fibrinogen, were added at 5 dpf. Estrogen concentrations ranged from 5 to 75 uM. After incubation, larvae were briefly washed with system water and then mounted in 0.8% agarose and evaluated with a compound microscope and 20x objective. After the incubation, estrogen-treated fish developed GFP-labeled thrombosis in the PCV, indicating fibrin(ogen) deposition. (B) Representative images for the semi-quantitative grading system. Scoring was always performed by an observer blinded to condition and/or genotype as follows: Grade 0, no fluorescence accumulation. Grade 1, small sporadic fluorescent puncta seen scattered in the PCV. Grade 2: fluorescence is continuous and spreads across the PCV. Grade 3: strong fluorescence visualized across the PCV. (C) Quantification of fluorescence by CTCF in comparison with semi-quantitative grading. n=9-12 at each concentration. (D) Quantification of plasma levels of E2 after treatment in 25 and 50 uM E2 and controls. Larvae were bled into buffer and then pooled for mass spectrometry. "E2 wash" larvae were washed with 50 uM E2 and then system water, but not incubated in E2. Each point represents a pool of 10 larvae. "50 uM solution" is quantification of the E2 containing E3 embryo buffer without larvae.

Figure 2: Estrogen analogs induce *in vivo* thrombosis. Dose-dependent increases in thrombosis were observed among multiple estrogens. (A) 17β-estradiol (E2) treatment for 5 hours at 5 dpf. n=41, 234, 33. (B) 17α-ethinylestradiol (EE2) treatment for 24 hours at 4 dpf. n=36, 50, 8, 17. (C) Mestranol treatment for 24 hours at 4 dpf. n=24, 38, 39, 39, 20. (D) Quinestrol treatment for 24 hours at 4 dpf. n=10-19 in each group. All data were collected using the *fgb-egfp* transgenic background by an observer blinded to condition.

Figure 3: Testosterone derivatives and progestins do not cause thrombosis. (A) Testosterone incubations were for 24 hours starting at 4 dpf. (B) 19-nortestosterone incubations were for 48 hours starting at 3 dpf. (C) Metribolone incubations were for 24 hours starting at 4 dpf. n=10-30 for each testosterone concentration. (D) Multiple progestins were tested at the maximum tolerated dose for 5 hours on 5 dpf. Norethisterone 10 uM, levonogestrel 25 uM, desogestrel 1 uM, drospirenone 25 uM. n=30-65 in each group. All data were collected using the *fgb-egfp* transgenic background by an observer blinded to condition.

Figure 4: Static and time-lapse imaging of estrogen-induced thrombosis. (A-B) Fish larvae were derived from an *fgb-egfp* cross to *flk-mcherry*, resulting in green and red fluorescent thrombi and vasculature, respectively. Confocal microscopy demonstrates that the GFP-labeled thrombi

are contained within the posterior cardinal vein (PCV), and not the dorsal aorta (DA) or intersegment vessels (ISV). Scale bar in (A) 100 um, (B) 20 um. (C-F) Compound microscopy time-lapse images of thrombosis formation over 6 hours reveal initiation simultaneously along the PCV, rather than from a single location. Scale bars, 100 um. (G-H) Scanning electron microscopic images comparing the PCV of a DMSO (control) treated fish (G) and a grade 2 larvae treated with E2 (H). In the control group, erythrocytes exhibit typical flat and oval morphology. In the treated larva, scattered fibers that may be fibrin(ogen) are seen attached to erythrocytes (arrows). Polyhedrocytes (arrowheads) are observed, suggestive of clot contraction. Scale bars in (G-H) 10 um. All data were collected using the *fgb-egfp* transgenic background by an observer blinded to condition.

Figure 5: Estrogen-induced thrombi are not dependent on the canonical coagulation cascade. (A) Larvae were pretreated with warfarin at 50 ug/ml at 3 dpf, followed by E2 for 5 hours at 5 dpf. n= 39, 25, 43, and 52. p=0.0001 for the 50 uM treatment, p=0.002 for the 75 uM treatment, by Fisher exact testing. (B) Larvae were co-treated with E2 and rivaroxaban (anti-Xa) or dabigatran etexilate (anti-IIa) at 250 and 50 uM, respectively, 5 hours at 5 dpf. n=103, 74, 50, and 51. p=0.03 (anti-Xa), 0.03 (anti-IIa), 0.001 (combination) by Fisher exact testing. (C) 5 uM mestranol (n=38, 51, and 50 at 3 dpf for 48 hours) were applied to offspring from *f10*$^{+/-}$ incrosses. (D) 25 uM mestranol (n= 42, 55, and 22 at 3 dpf for 48 hours) were applied to offspring from *f2*$^{+/-}$ incrosses. (E) 50 uM E2 (n=14, 42, and 28 at 5 dpf for 5 hours) and (F) 25 uM mestranol (n= 18, 41, and 27 at 3 dpf for 48 hours) were applied to offspring from *fga*$^{+/-}$ incrosses. All data were collected using the *fgb-egfp* transgenic background by an observer blinded to condition and/or genotype.

Figure 6: Estrogen-induced thrombosis is mediated through a non-canonical target. (A) Co-treatment of E2 (50 uM) and fulvestrant (50 uM) at 5 dpf for 5 hours. n=130, 110, 8. p<0.0001 between estradiol and estradiol + fulvestrant, by Fisher exact testing. (B) Co-treatment of mestranol (2.5 uM) and fulvestrant (25 uM) at 3 dpf for 48 hours. n=69 and 156. p<0.05 by Fisher exact testing. (C) Triple heterozygous *esr1*$^{+/-}$*;esr2a*$^{+/-}$*;esr2b*$^{+/-}$ mutants were incrossed and larvae treated with 5 uM mestranol at 3 dpf for 48 hours. Genotyping was performed after thrombosis quantification. Fisher exact testing revealed no significant differences. (D) Larvae from *gper*$^{+/-}$ incrosses were treated with 5 uM mestranol at 3 dpf for 48 hours. Genotyping was performed after thrombosis quantification. No difference was detected by Fisher exact test, n= 40, 62, and 37. All data were collected using the *fgb-egfp* transgenic background by an observer blinded to condition and/or genotype.

Figure 7: Constitutively active nuclear hormone estrogen receptor does not induce thrombosis. (A) p*hs-esr1*-Y549S plasmid with constitutively active *esr1* (Y549S), driven by the heat shock promoter (HS). *mcherry* signals expression, followed by p2A and the mutant *esr1* cDNA. (B) p*hs-esr1*-Y549S was injected to *fgb-gfp/ere-gfp* transgenic embryos, heat shocked, and evaluated at 5 dpf. GFP indicates activation of the estrogen response element (ERE), and mCherry indicates expression from p*hs-esr1*-Y549S. (i-ii) Uninjected controls show no ERE activity *esr1* expression. (iii) Injected larvae show expression from p*hs-esr1*-Y549S by activation of ERE and GFP signal. (iv) Injected larvae show expression from p*hs-esr1*-Y549S by expression of mCherry. Despite evidence of constitutive *esr1* activity, no thrombosis was observed. All data were collected using the *fgb-egfp* transgenic background by an observer blinded to condition.

Figure 1

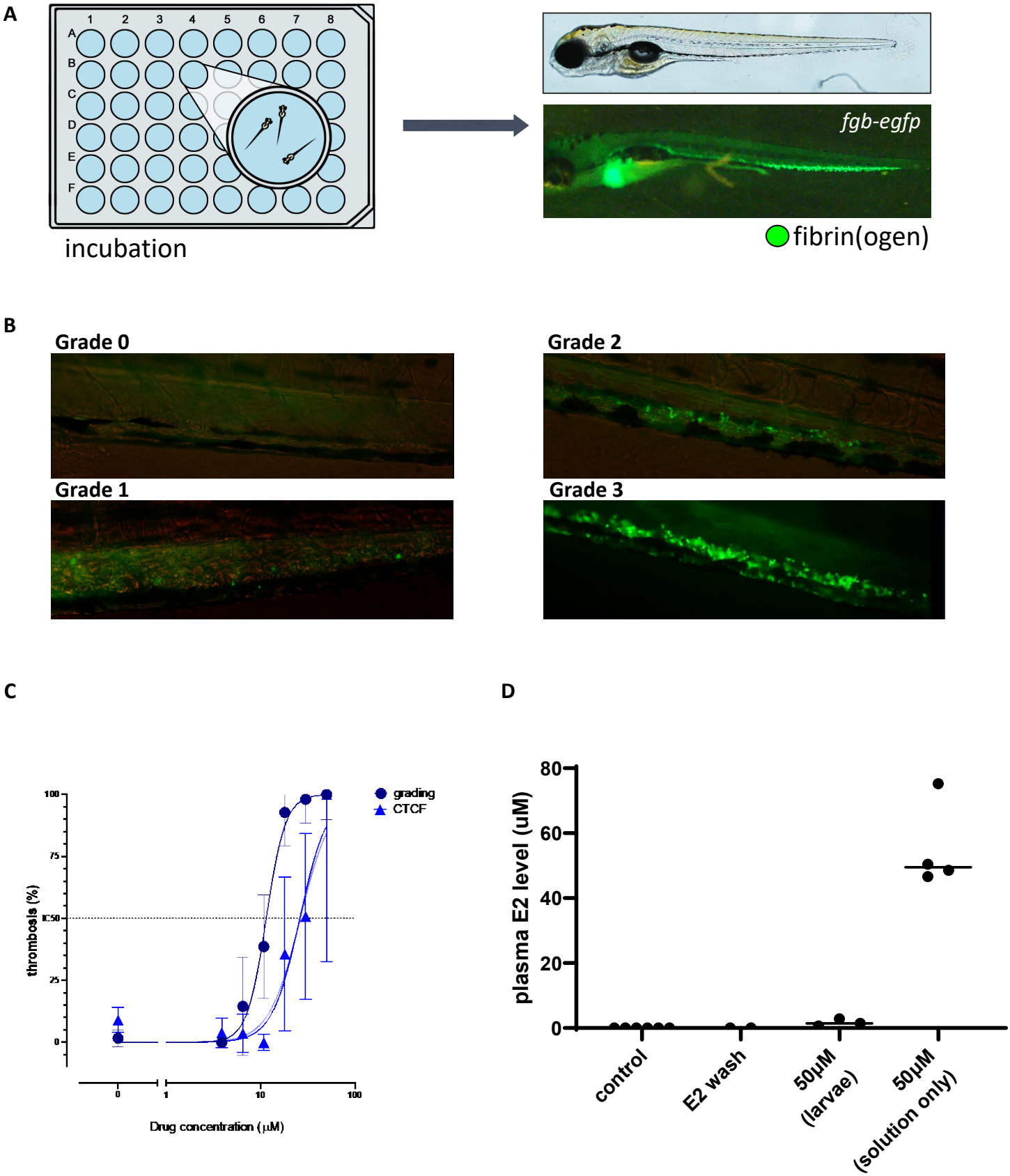

A
1 2 3 4 5 6 7 8
A B C D E F
incubation
fgb-egfp
fibrin(ogen)
B
Grade 0
Grade 1
Grade 2
Grade 3
C
grading
CTCF
thrombosis (%)
100
75
IC50
25
0
0
1
10
100
Drug concentration (μM)
D
plasma E2 level (uM)
80
60
40
20
0
control
E2 wash
50μM (larvae)
50μM (solution only)

Figure 2

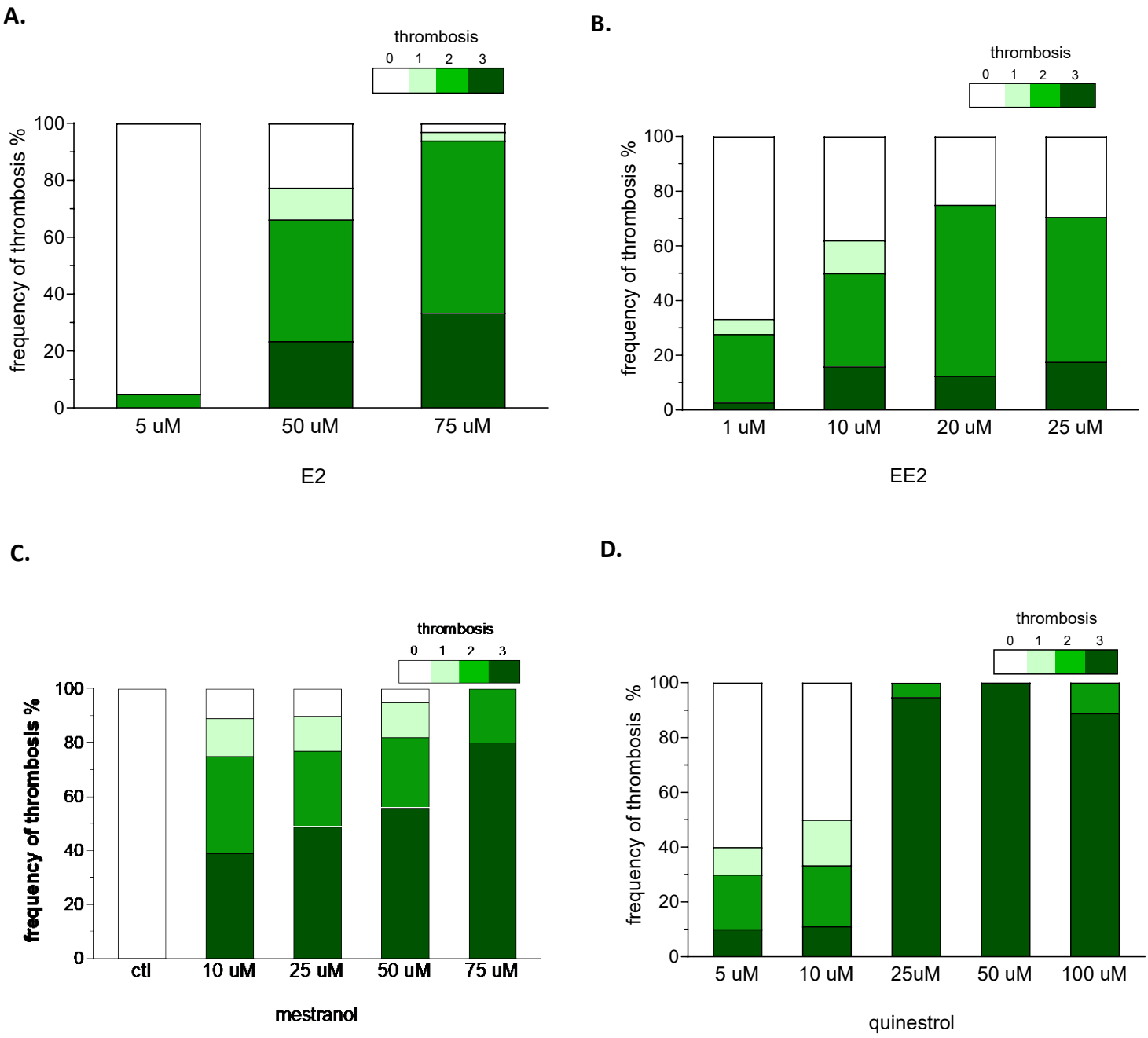

A.
thrombosis
0 1 2 3
frequency of thrombosis %
100
80
60
40
20
0
5 uM
50 uM
75 uM
E2
B.
thrombosis
0 1 2 3
frequency of thrombosis %
100
80
60
40
20
0
1 uM
10 uM
20 uM
25 uM
EE2
C.
thrombosis
0 1 2 3
frequency of thrombosis %
100
80
60
40
20
0
ctl
10 uM
25 uM
50 uM
75 uM
mestranol
D.
thrombosis
0 1 2 3
frequency of thrombosis %
100
80
60
40
20
0
5 uM
10 uM
25uM
50 uM
100 uM
quinestrol

Figure 3

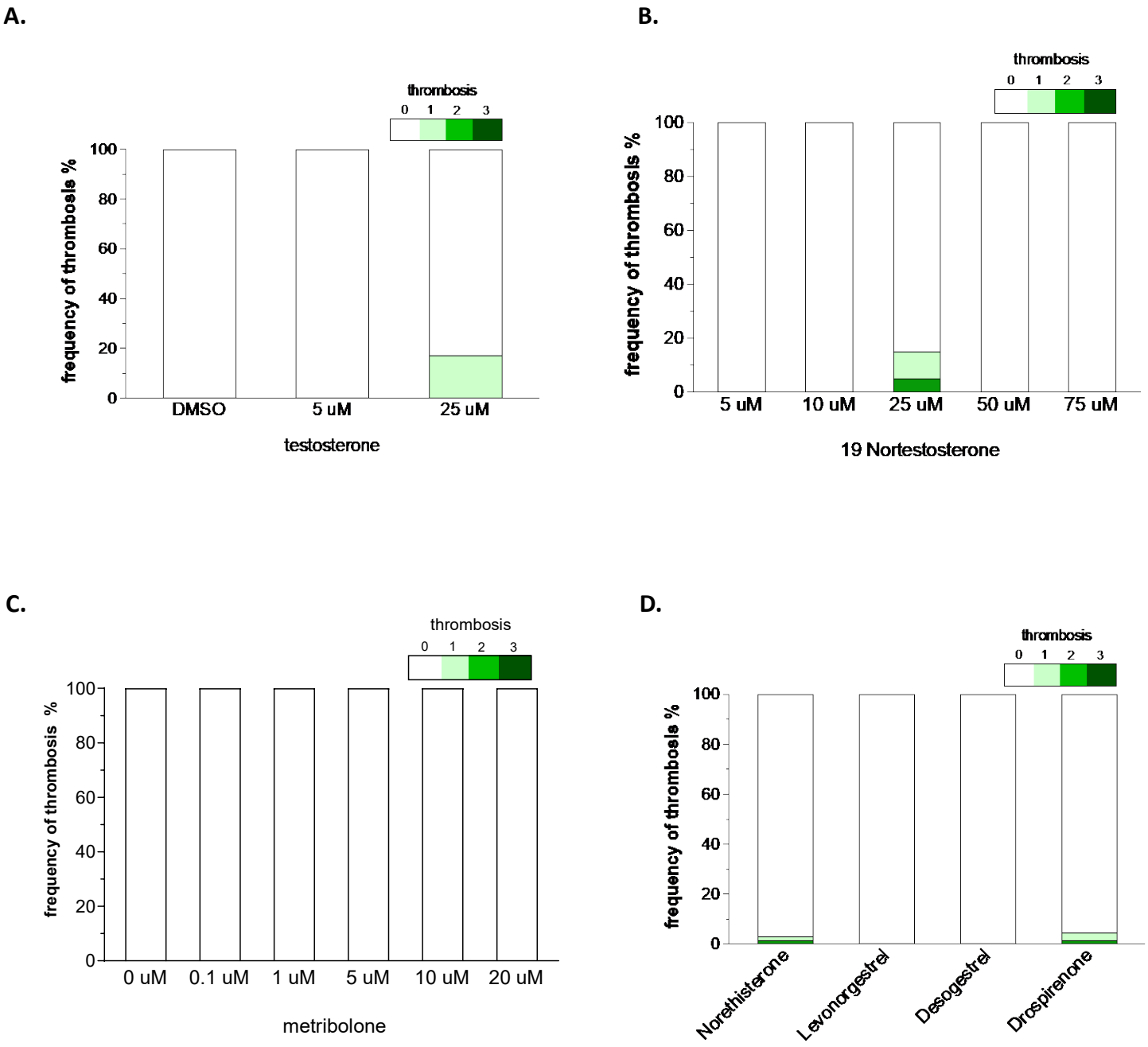

A.
thrombosis
0 1 2 3
frequency of thrombosis %
100
80
60
40
20
0
DMSO
5 uM
25 uM
testosterone
B.
thrombosis
0 1 2 3
frequency of thrombosis %
100
80
60
40
20
0
5 uM
10 uM
25 uM
50 uM
75 uM
19 Nortestosterone
C.
thrombosis
0 1 2 3
frequency of thrombosis %
100
80
60
40
20
0
0 uM
0.1 uM
1 uM
5 uM
10 uM
20 uM
metribolone
D.
thrombosis
0 1 2 3
frequency of thrombosis %
100
80
60
40
20
0
Norethisterone
Levonorgestrel
Desogestrel
Drospirenone

Figure 4

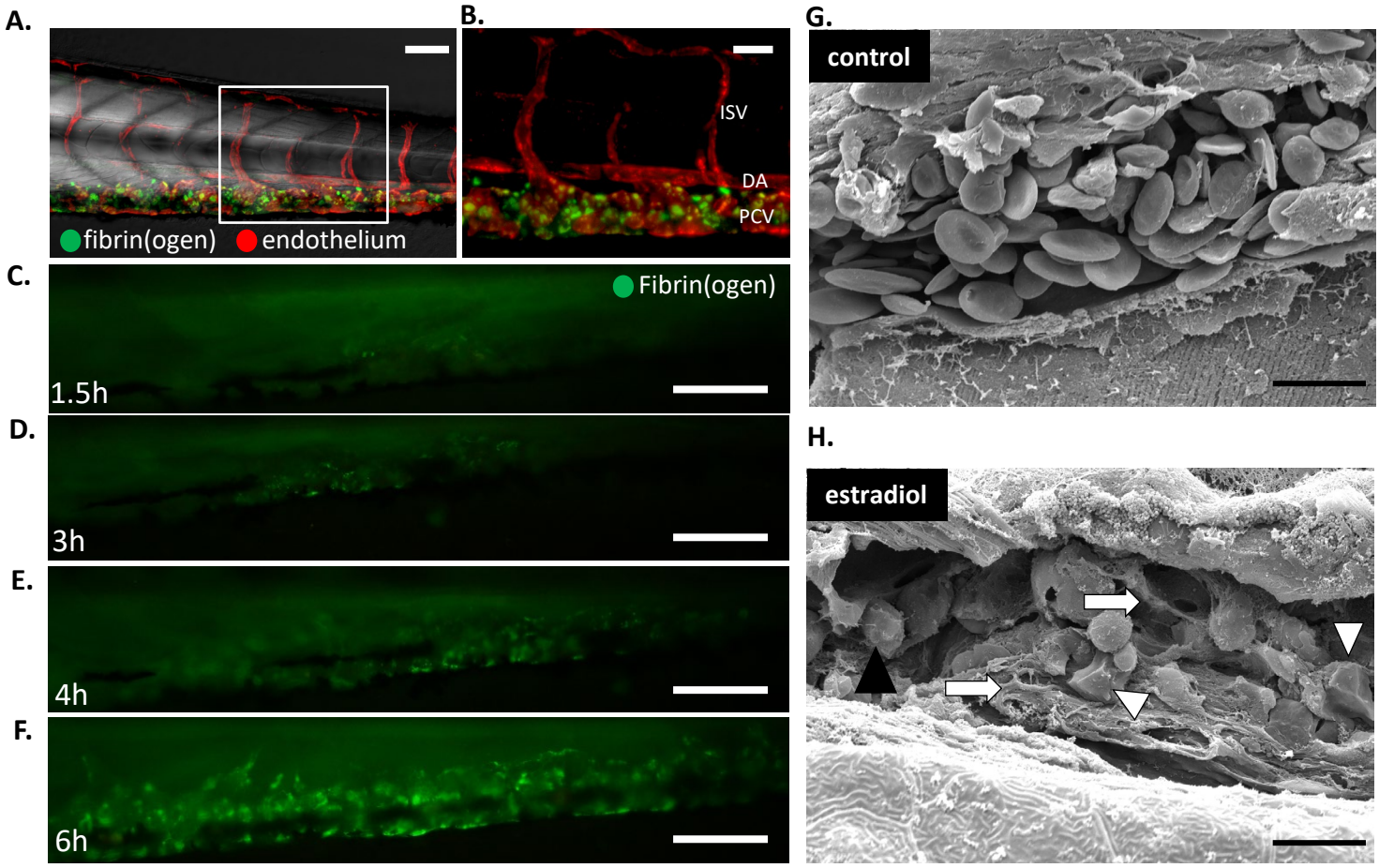

A.
fibrin(ogen)
endothelium
B.
ISV
DA
PCV
C.
Fibrin(ogen)
1.5h
D.
3h
E.
4h
F.
6h
G.
control
H.
estradiol

Figure 5

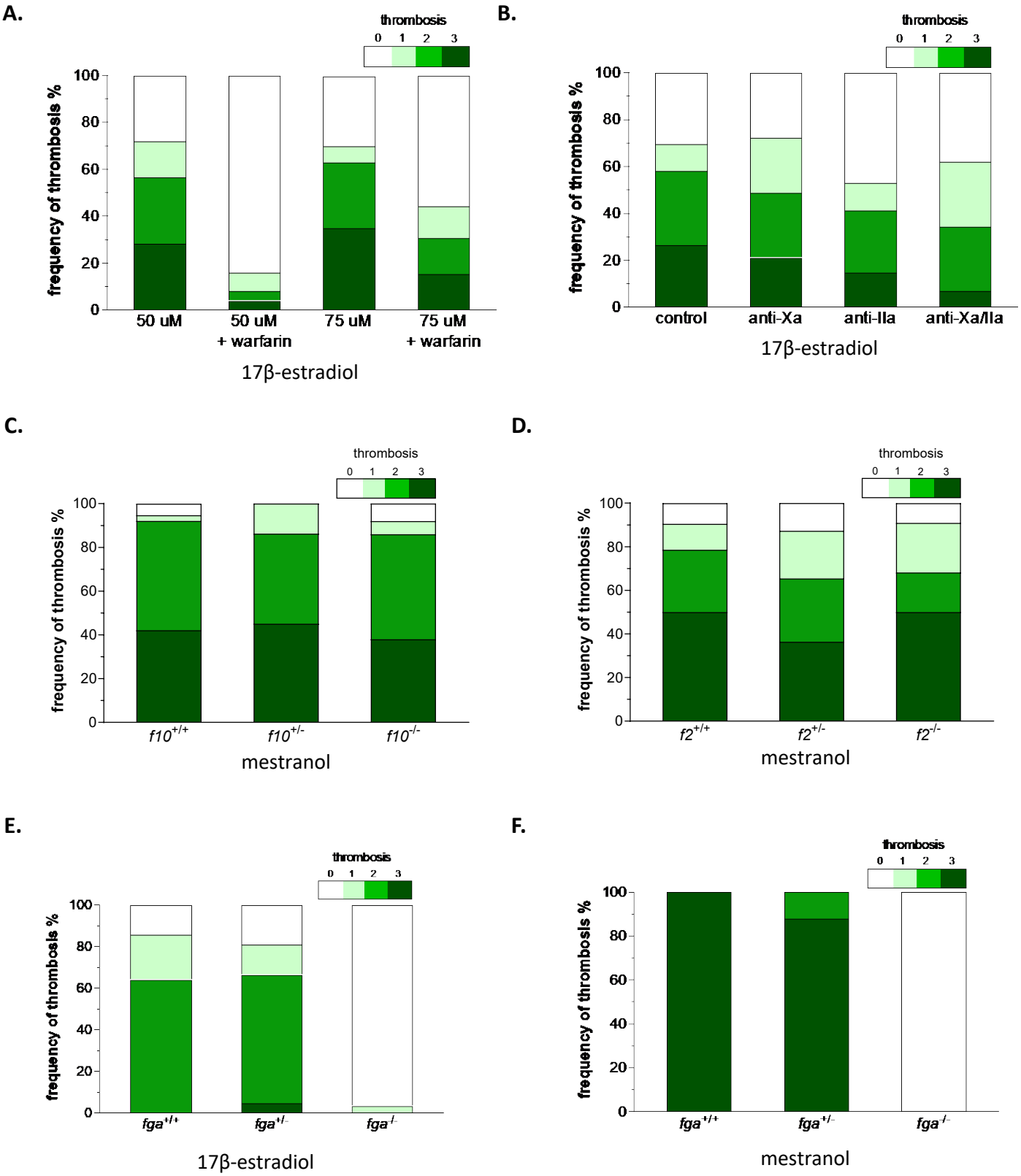
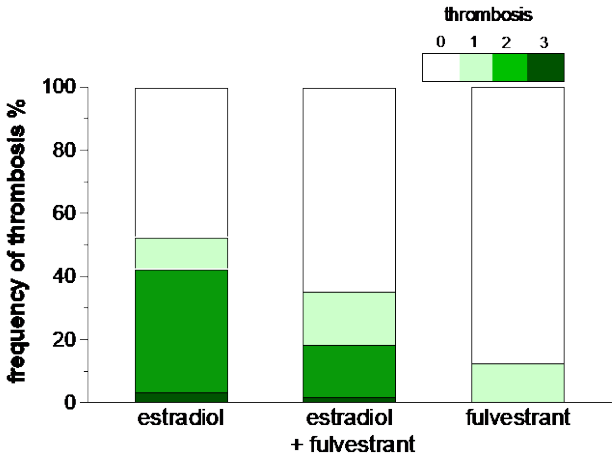
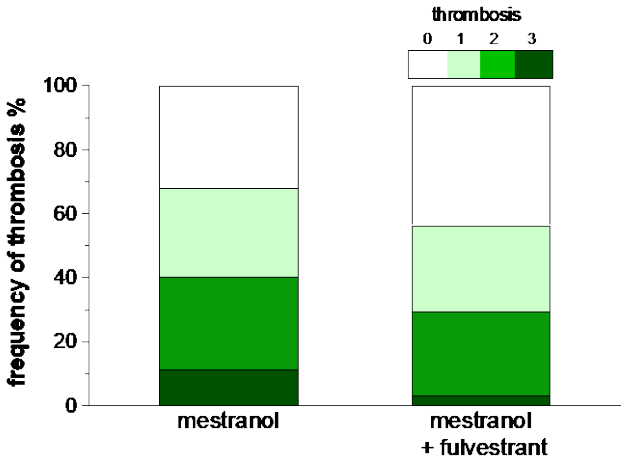

A.
thrombosis
0 1 2 3
frequency of thrombosis %
100
80
60
40
20
0
50 uM
50 uM + warfarin
75 uM
75 uM + warfarin
17β-estradiol
B.
thrombosis
0 1 2 3
frequency of thrombosis %
control
anti-Xa
anti-IIa
anti-Xa/IIa
17β-estradiol
C.
thrombosis
0 1 2 3
frequency of thrombosis %
f10+/+
f10+/-
f10-/-
mestranol
D.
thrombosis
0 1 2 3
frequency of thrombosis %
f2+/+
f2+/-
f2-/-
mestranol
E.
thrombosis
0 1 2 3
frequency of thrombosis %
fga+/+
fga+/-
fga-/-
17β-estradiol
F.
thrombosis
0 1 2 3
frequency of thrombosis %
fga+/+
fga+/-
fga-/-
mestranol

Figure 6

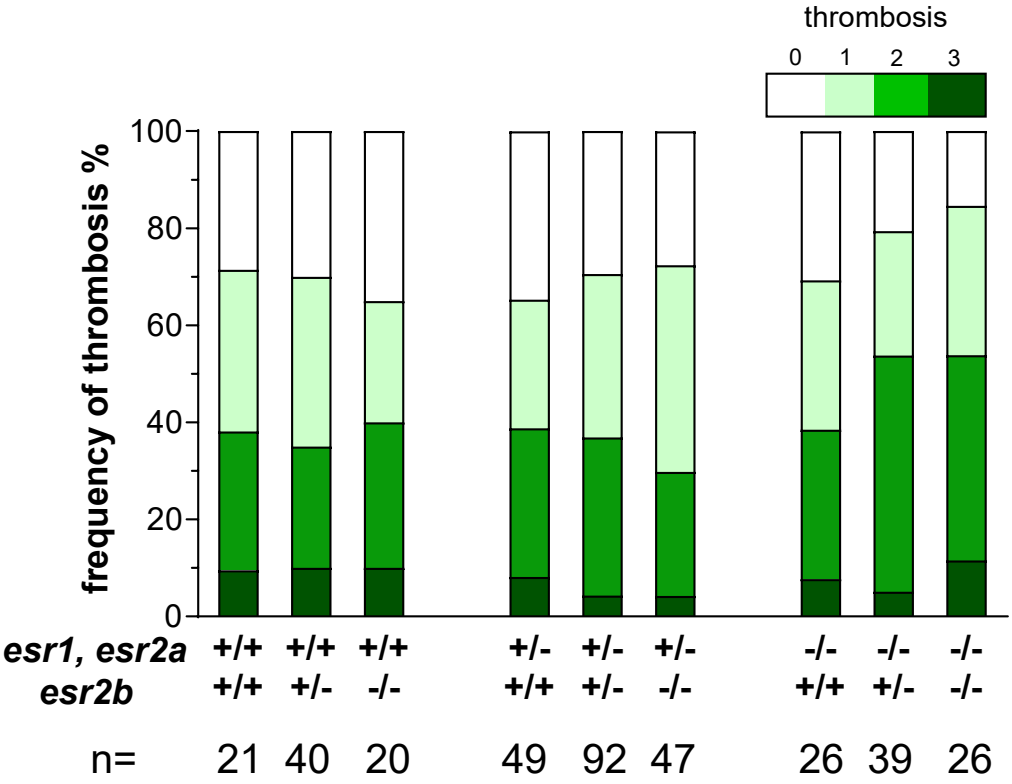
A.



B.



C.



D.

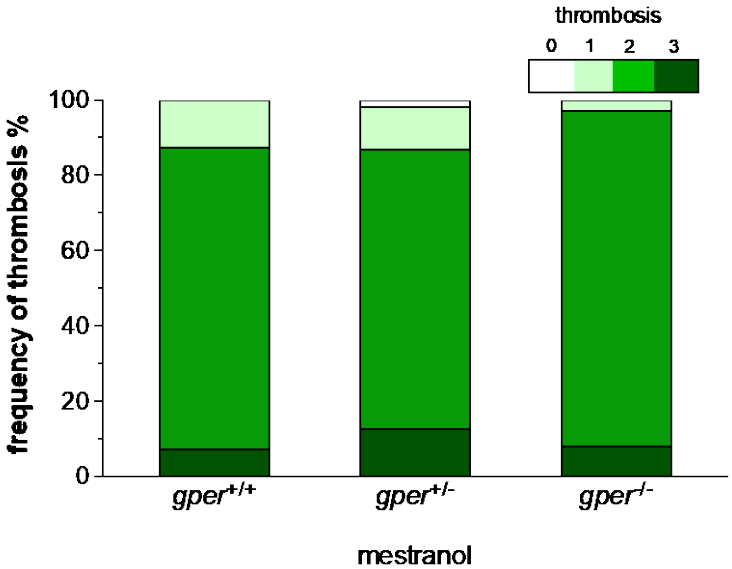
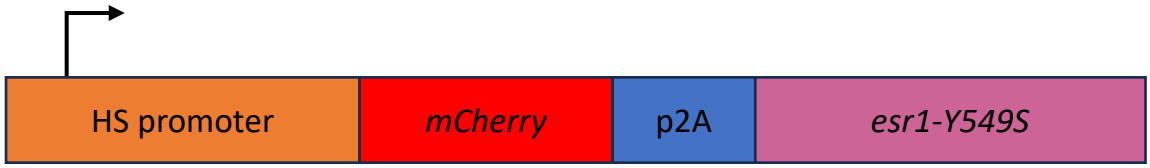
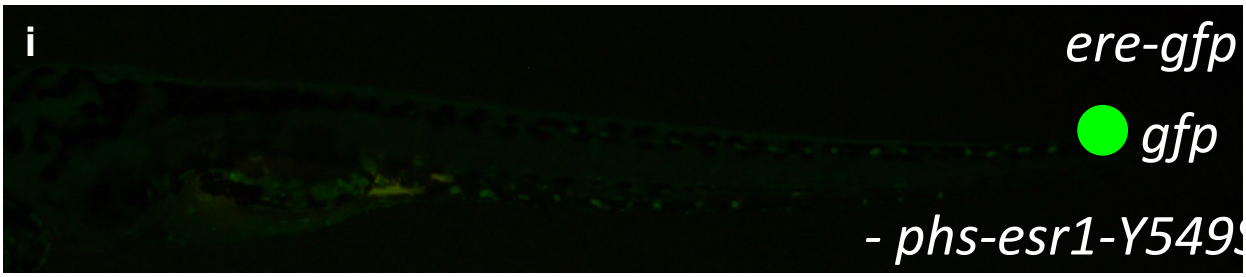


Figure 7

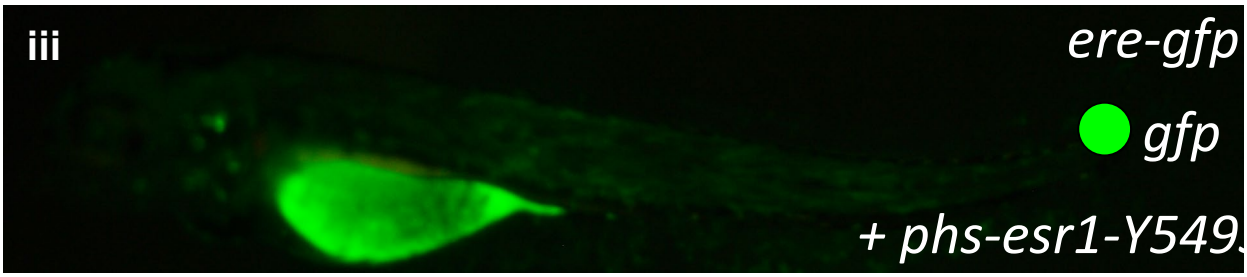
A.

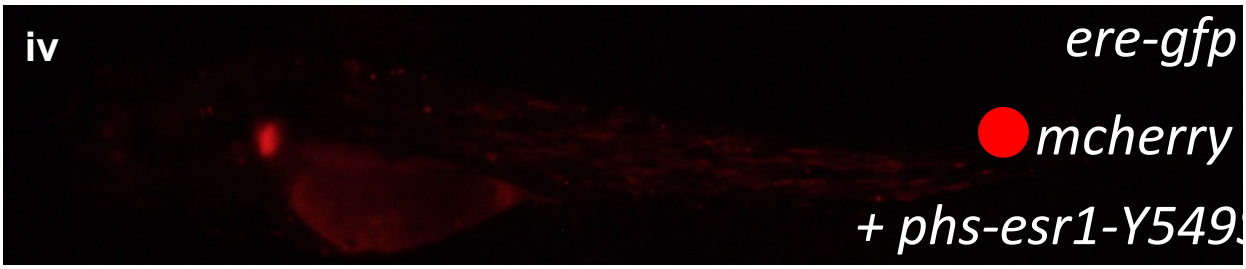

HS promoter
mCherry
p2A
esr1-Y549S

B.


i
ere-gfp
gfp
- phs-esr1-Y549

ii
ere-gfp
mcherry
- phs-esr1-Y549


iii
ere-gfp
gfp
+ phs-esr1-Y549


iv
ere-gfp
mcherry
+ phs-esr1-Y549