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Estrogen Inhibits Development of Yolk Veins and Causes Blood Clotting in Transgenic Medaka Fish Overexpressing Estrogen Receptor

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ABSTRACT—We established three transgenic medaka fish lines overexpressing the medaka estrogen receptor under the constitutive medaka β-actin promoter. The transgenic embryos became hypersensitive to estrogens (17β-estradiol and 17α-ethinylestradiol), and failed to develop yolk veins while blood clots formed in the blood island within 3 days after exposure to the estrogens. The embryos developed normally if exposed to estrogen after an early neurula stage, suggesting that the sensitive stage is before neurulation. The developmental defects were recovered by incubation with an anti-estrogen, tamoxifen. These results indicate that activation of estrogen receptor caused the estrogen-induced developmental defects. Our results show that the transgenic embryos can be used to assay the blood clotting activity of estrogenic compounds *in vivo*.

Key words: transgenic medaka fish, blood clotting, yolk vein, estrogen, estrogen receptor

INTRODUCTION

Estrogens play key roles in vertebrate physiology. The vasculature, like the reproductive tissues, bone, liver, and brain, is an important target of estrogen's action. In the ovary and uterus, initiation of blood vessel formation during follicular development, menstrual cycle, and pregnancy is associated with increases in the levels of circulating estradiol, and regression of the newly formed microvessels is accompanied by a fall in estradiol concentrations, suggesting that the angiogenic process is estradiol-regulated (Dubey *et al*., 2000). Multiple lines of evidence suggest that estrogen directly modulates angiogenesis via effects on endothelial cells (surrounding the inner surface of blood vessel) (Losordo and Isner, 2001). On the contrary, recent studies have found that estrogen medication in hormone replacement therapy (HRT) is associated with increased risk of venous thromboembolism (VTE, occlusion of blood vessels with blood clots) (Daly *et al*., 1996; Jick *et al*., 1996). Using contraceptive (containing estrogen) also increases the risk of thrombotic occlusion in young women (Godsland *et al*., 2000). Pregnant women, who suffer from 100 times higher circulating estrogen than usual, are five times more likely to experience VTE than non-pregnant women (Burns, 2000). Although the estimated rate of such event is low, venous thromboembolic disease is a leading cause of maternal death. Despite 40 years of research, the mechanisms behind these adverse effects of estrogen are not understood. There is no convincing evidence that the balance between clotting and fibrinolysis is disturbed. Given the widespread use of oral contraceptive and HRT, it is important to uncover a mechanism by which estrogens act in the blood coagulation pathway.

The medaka fish, *Oryzias latipes* (a Japanese freshwater fish), possesses a unique combination of features that make it particularly well suited for experimental and genetic analysis of vertebrate vascular development. Medaka embryos and fry are optically clear, allowing for direct, non-invasive observation of virtually the entire vascular system. Medaka embryos but not zebrafish embryos, another genetically tractable killifish, develop yolk veins that are easily observable under a dissecting microscope. Furthermore, the small size of embryos permits them to receive enough oxygen by passive diffusion to survive and continue to develop

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in reasonably normal fashion for many days even in the complete absence of blood circulation (Vogel and Weinstein, 2000). This is particularly useful when one examines genetically or experimentally manipulated animals with circulatory defects. Furthermore, methods for generating transgenic (Tg) medaka fish have been established (Tanaka and Kinoshita, 2001). Medaka embryos are also useful for the study on the role of estrogen and estrogen receptor (ER) in blood clotting, because we previously reported that estrogen specifically inhibits the development of yolk veins and stimulates blood clotting in the blood island (Kawahara *et al*., 2000).

To genetically elucidate the possible role of ER in vascular physiology (development of yolk veins and blood clotting) in medaka embryos, we constructed Tg medaka fish overexpressing medaka ER, and examined for the development of blood vessels and blood clotting in the presence of estrogen. The Tg embryos were defective in the development of yolk veins and blood clots formed in response to more than 1,000 times lower concentration of estrogen than that for wild type, indicating that ER is involved in the estrogen-induced vascular defects. Our Tg fish model may be useful for studying molecular mechanisms for ER in the estrogen-induced vascular diseases, because formation of new blood vessels in the adult involves not only angiogenesis (sprouting from preexisting blood vessels) but also postnatal vasculogenesis, in which circulating endothelial progenitor cells are incorporated into newly formed capillaries (Asahara *et al*., 1999; Zhang *et al*., 2002). The Tg fish can also be used to assay the thrombotic activity of estrogenic compounds *in vivo* and to monitor estrogens in the aquatic environment.

MATERIALS AND METHODS

Fish and embryo culture

We used the d-rR strain of medaka fish, *O. latipes* (Kawahara and Yamashita, 2000). The fish were maintained at 25–26°C under artificial photo-period of 14L:10D, and fed by powdered Tetramin (Tetra). Eggs were collected within 10 h postfertilization (hpf), rinsed with tap water, and immersed in Yamamoto's salt solution (Yamamoto, 1969) with or without test chemicals. At least 50 eggs were used in each experiment. All chemicals were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). The stock solutions were diluted over 1,000-fold with Yamamoto's solution. The solvent was added to the mock-treated eggs as a control. Eggs were incubated under the same condition as above unless otherwise indicated and inspected for blood clotting under a dissecting microscope. Eggs in which blood clots formed were counted. Data are presented as mean±SEM.

Observation of blood vessels

In order to observe the development of blood vessels, eggs were fixed with 4% paraformaldehyde for 3 days and observed under green fluorescence with a filter set in Leica MZ FLIII stereo-fluorescence microscope as described (Kawamura and Yamashita, 2002). The fixed eggs were also dechorionated with forceps and stained with hematoxylin.

Tg medaka fish

The plasmid pOL21 was constructed by ligation (after fill-in reaction) of the 3.5-kb *Sph*I-*Pst*I DNA fragment containing medaka β-actin promoter (Hamada *et al*., 1998) (provided by Dr. Minoru Kimura, Tokai University, Japan) with the *Ase*I- and *Nhe*I-digested plasmid pS65T-C1 (Clontech) containing the GFP-coding sequence and the SV40 poly(A) signal. The medaka ER cDNA cloned in the plasmid pMER (Kawahara *et al*., 2000) was amplified by PCR using the primers (5'-TCGGTGACATGTACCCTGAA-3' and 5'-CTGTGTGCTCAGTCTTGAAG-3') and replaced for the GFP after digestion of pOL21 with *Sal*I, yielding the plasmid pOL22. We microinjected circular forms of both plasmid DNAs into fertilized eggs at the one- or two-cell stage. Fries that hatched from the injected eggs were reared to adulthood. Tg founder fish were selected by pair mating with non-transgenic wild-type fish and by PCR and Southern blot analysis of DNA extracted from caudal fins of individual fish as described (Kinoshita *et al*., 2000). Tg fish are currently maintained as homozygotes (for A-line) and hemizygotes (for A-, C-, and D-lines) by mating with wild-type fish.

PCR, Southern blot analysis, RT-PCR, and *in situ* hybridization

DNA was extracted from posterior half of an individual caudal fin by using an extraction kit (Isohair, Nippongene). PCR was performed by using a PCR kit (Toyobo Co.). Aliquots of the reaction mixture were separated by agarose gel electrophoresis, and the DNA was then transferred to nylon membranes. Blots were hybridized with probes and visualized by using a kit (ECL direct system, Amersham). Extraction of total RNA, RT-PCR analysis, and *in situ* hybridization were performed as described previously (Kawahara *et al*., 2000). The probe for *olvas* mRNA was provided by Dr. Minoru Tanaka (Hokkaido University, Japan). Nucleotide sequences of the primers used are available upon request.

RESULTS

Construction of the chimeric gene and Tg madaka fish

We constructed the plasmid (pOL22) carrying a chi-

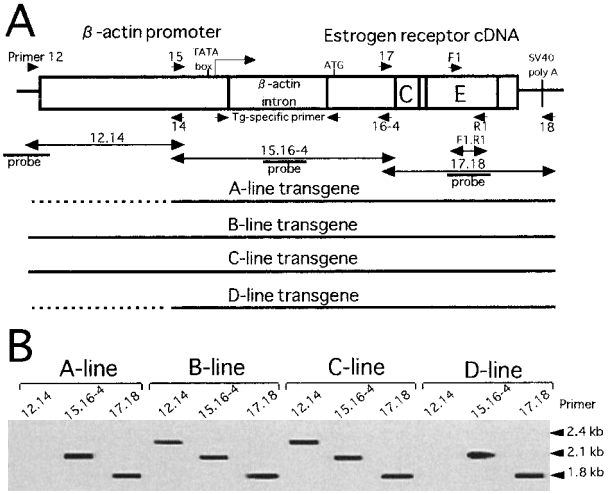


Fig. 1. (**A**) Illustration of gene construct used to transform medaka fish. DNA- and estrogen-binding domains of ER are described as C and E, respectively. Primer sets and the probes used in PCR, Southern blots, and RT-PCR are also indicated. (**B**) PCR and Southern blot analysis of Tg fish. DNA was extracted from individual fins of "A"- to "D"-line Tg fish. PCR was performed using each of the primer sets indicated. Amplified DNA was processed for Southern blotting using each of the probes indicated. Representative data are shown with molecular sizes of the amplified bands. Note that 5'-half of the β-actin promoter is lost from "A"- and "D"-line transgenes.

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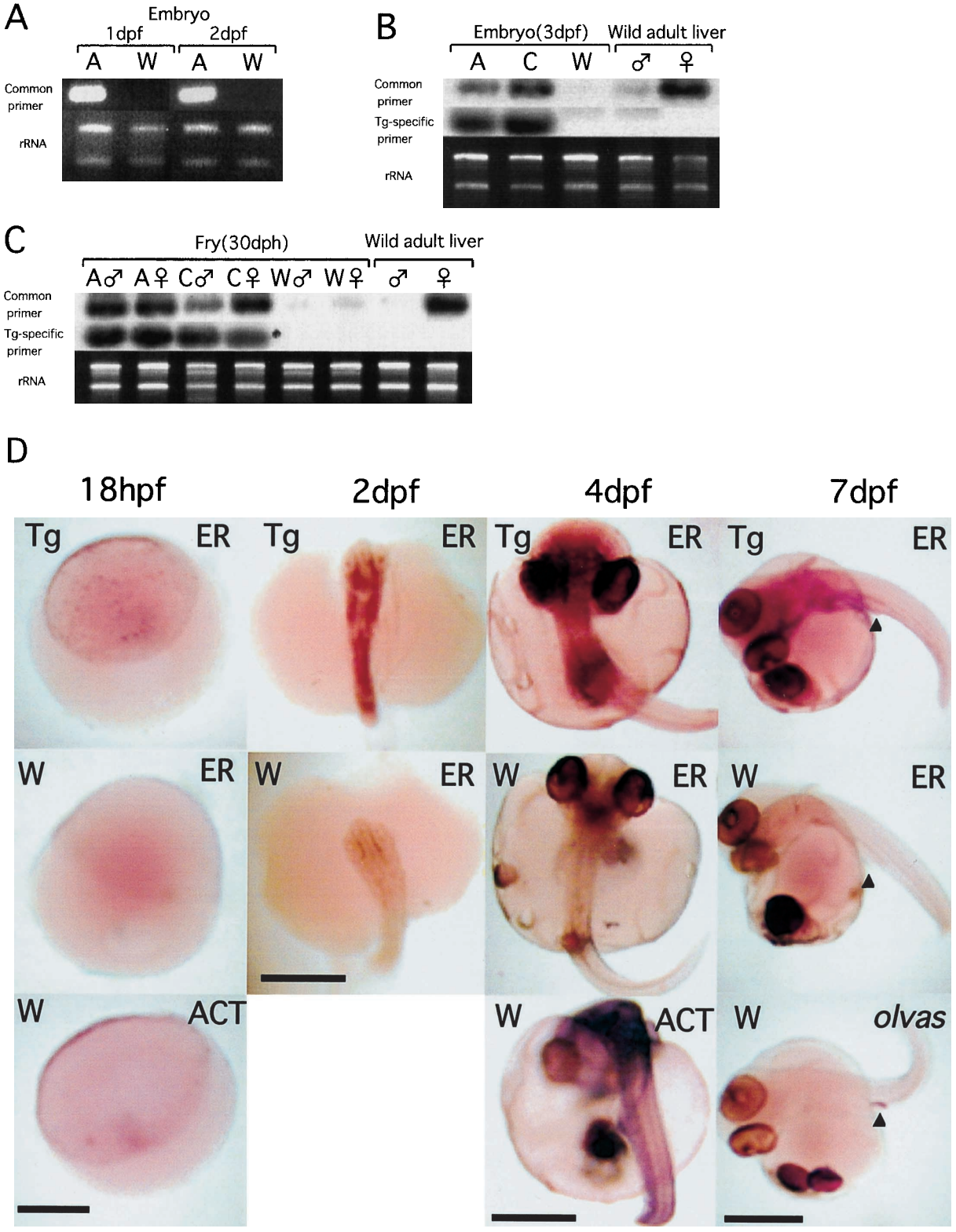


Fig. 2. RT-PCR and *in situ* hybridization analysis of Tg fish. (**A, B**, and **C**) RNA was extracted from 1- and 2-dpf embryos (**A**), 3-dpf embryos (**B**), and 30-dph fries (**C**) spawned by pair mating between "A"- and "C"-line Tg and wild-type fish (denoted by A and C, respectively), and from age-matched wild-type embryos and fries (W) (n=30 each). Adult female and male livers of wild-type fish were extracted as controls. RT-PCR proceeded using each of the primer sets that can detect the E domain-coding message (common primers F1 and R1; Fig. 1) and the transgene-specific joining region between the β-actin promoter and ER cDNA (Fig. 1). After electrophoresis of amplified DNA, gels were stained by ethidium bromide (**A**) or processed for Southern blotting (**B** and **C**). Ethidium bromide-stained rRNAs are also shown. (**D**) Whole-mount *in situ* hybridization. Signals for ER, β-actin (ACT), and *olvas* messages were examined using antisense probes in "A"-line Tg and wild-type (W) embryos at the indicated times in the development. Arrowheads indicate the gonadal region. Scale bar is 500 μm.

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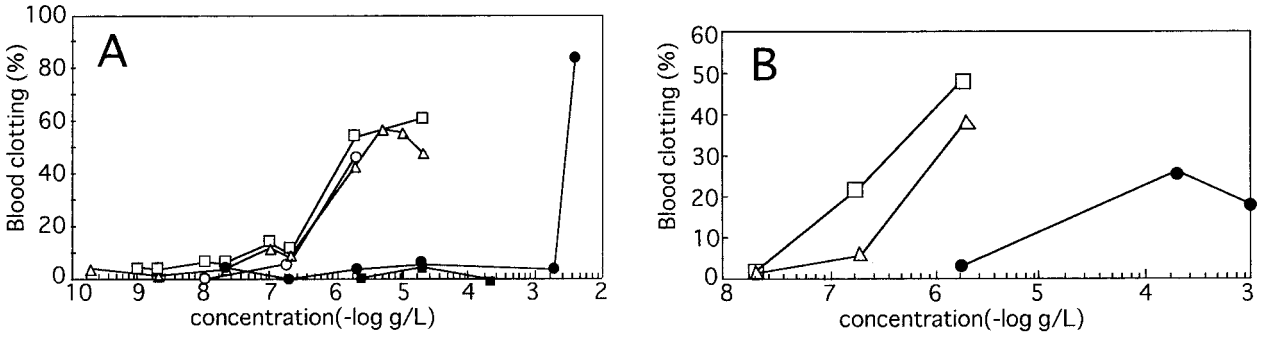


Fig. 3. Dose-response of blood clotting after exposure to estrogens. (**A** and **B**) Embryos were collected from mating between wild-type fish (closed circle) and from mating between the "A" (triangle)-, "B" (closed square)-, "C" (open square)-, or "D" (open circle)-line Tg males and wild-type females. Embryos (n>50 each) were incubated in the Yamamoto's solution containing E2 (**A**) or EE (**B**) at the indicated concentrations for 3 days, then blood clotting was evaluated.

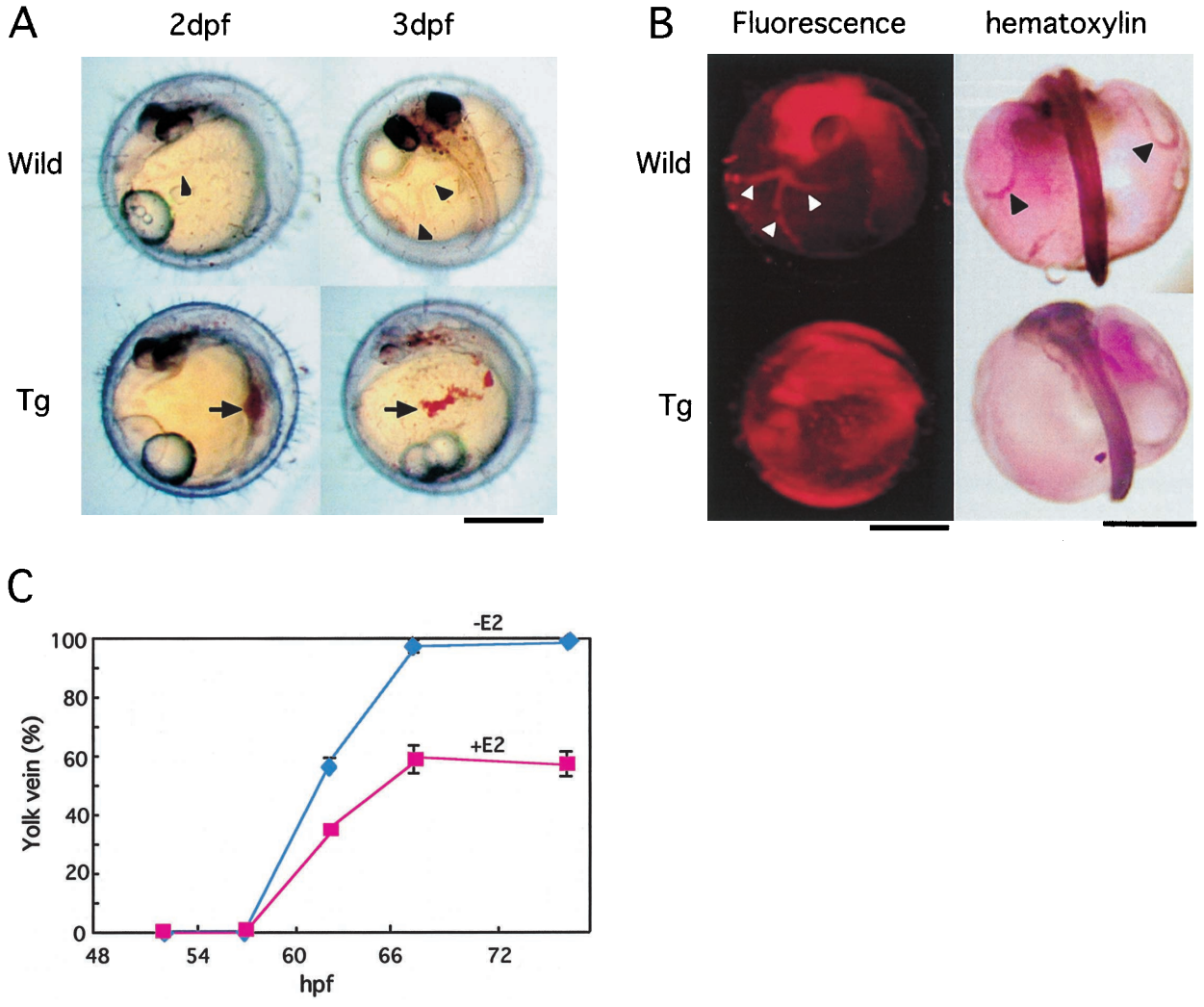


Fig. 4. Blood clotting and developmental defects in yolk veins. (**A**) The "C"-line Tg and wild-type embryos were incubated in the presence of E2 (2 μg/l) for 2 and 3 days and photographed. Blood clots formed at the blood island in the Tg embryos at 2 dpf, then dispersed on the yolk surface. Blood clots and yolk veins are indicated by arrows and arrowheads, respectively. Scale bar is 500 μm. (**B**) The "C"-line Tg and wild-type embryos were examined for yolk veins with fluorescence and hematoxylin after exposure to E2 (2 μg/l) for 3 days. Yolk veins are indicated by arrowheads. Scale bar is 500 μm. (**C**) The 10-hpf embryos from mating between the "C"-line Tg and wild-type fish were incubated in the presence or absence of E2 (2 μg/l). At the indicated times, embryos (>50 each) were taken, fixed, and examined under fluorescence. The embryos that develop yolk veins were counted.

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meric gene with the coding sequence of the medaka ER cDNA fused to the medaka β-actin promoter and the SV40 poly(A) signal sequence (Fig. 1A). We also constructed the control plasmid (pOL21), for monitoring successful injection, in which the ER sequence is replaced with a GFP reporter gene. A total of 550 one- or two-cell-stage fertilized eggs were injected with a mixture of the two uncut plasmid DNAs (pOL21 and pOL22). Of these, half developed normally and expressed fluorescence when observed 1 day after microinjection. Fourty-seven individuals grew to adulthood and were examined for the presence of the transgene in DNA preparations extracted from posterior half of caudal fins by PCR and Southern blot analysis (Fig. 1B). We obtained 8 transgene-positive fish. These were pair mated with non-transgenic fish, and individual progenies were examined for the transgene. We obtained 4 independent F1 Tg fish named "A" to "D". The "B" and "C" fish had a complete sequence of the designed transgene, but the "A" and "D" fish lacked 5'-half of the β-actin promoter (Fig. 1B) that is not essential for expression (Liu *et al*., 1990). These Tg fish were amplified by pair mating with non-Tg fish and used for further analysis. In each pairing, approximately half of the progenies were transgene positive, indicating that the transgene had been stably integrated in one of the homologous chromosomes. We obtained homozygotes by mating among "A"-line hemizygotes, but could not from "C"-line.

Expression of the transgene

To analyze the steady-state level of the transgene message, total RNA was extracted from mixture of approximately 1:1 Tg and non-Tg embryos (1- to 3-day postfertilization, dpf) and fries (30-day post-hatching, dph) that had been spawned by pair mating between a Tg male and a non-Tg female. RT-PCR analysis was performed using two primer sets to detect messages corresponding to either a coding region of ER cDNA (common to wild-type ER and transgene messages) or a transgene-specific junction region between β-actin promoter and ER cDNA. Both primer sets yielded abundant signals from RNA samples of the mixed embryos and fries in the "A"- and "C"-lines, but signals from RNA samples from wild-type and "B"-line embryos and fries were undetectable (Figs. 2A to C, data not shown). Levels of transgene expression were slightly lower than the ER-message level in wild-type female liver. These results indicated overexpression of the transgene except in the "B"-line and confirmed the previous basal level of wild-type ER gene expression during embryonic and fry development (Kawahara *et al*., 2000).

Spatial pattern of expressions of the transgene and β-actin and *olvas* genes (as controls) was analyzed by *in situ* hybridization to whole mounts of Tg and wild-type embryos, using antisense probes for detection of specific expression (Fig. 2D) and sense probes as controls (data not shown). The specific signal for the ER message was detected in whole body of the Tg embryos in a similar manner as the expression pattern of β-actin message in the wild type, suggesting that the expression of the transgene is governed by the β-actin promoter. The ER signals were detected throughout the embryonic development, and at the tail region including the gonad (marked by arrowheads), where the signal for *olvas* mRNA was detected (Shinomiya *et al*., 2000; Tanaka *et al*., 2001). Specific signals for ER were undetectable in the wild type.

Blood clotting and vascular defects in the Tg embryos

For blood clotting, wild-type embryos and mixture of approximately 1:1 Tg and wild-type embryos were collected 10 hr after fertilization and immersed in a saline solution with or without each of estrogens, 17β-estradiol (E2; a natural estrogen) and 17α-ethinylestradiol (EE; a synthetic estrogen used as a contraceptive pill). Wild-type embryos developed normally in the presence of E2 up to 1.0 mg/l and of EE at 2 μg/l. However, large body of blood clots formed at the blood island (a place where blood cells generate at the developmental stage 23) after 3-day incubation at 4 mg/l concentration of E2 (Fig. 3A) or at 200 μg/l of EE (Fig. 3B). The embryos could not develop blood vessels or hatch as described previously (Kawahara *et al*., 2000). In contrast, blood clots formed in the Tg embryos of lines "A" , "C", and "D", but not "B" (consistent with no expression of the transgene in the "B"-line Tg embryos) as early as 3 days after the start of exposure to extremely lower concentrations of E2 (Fig. 3A) and EE (Fig. 3B). Blood clots formed in most Tg embryos at more than 2 μg/l concentration of E2 or EE (although % embryos with blood clots was nearly 50), because approximately half of the embryos used are Tg. The blood clots formed first at the blood island and were, then, dispersed on the yolk surface (Fig. 4A). The Tg embryos, in which blood clots formed, failed to develop yolk veins 3 days after exposure to estrogen, while wild-type embryos developed them at that time (Fig. 4B). Kinetics of yolk vein formation was examined after incubating the 10-

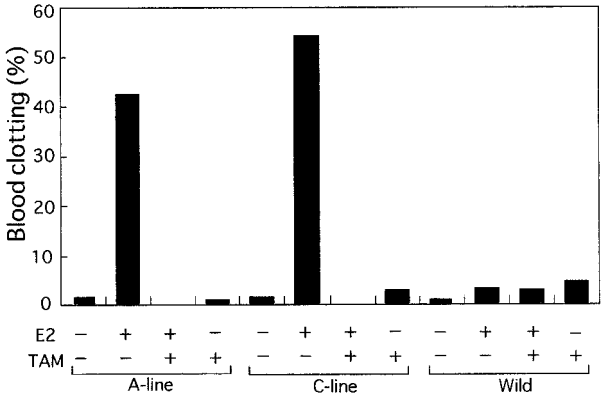


Fig. 5. Tamoxifen inhibits the estrogen-induced blood clotting. Embryos were collected from mating between wild-type fish and from mating between the "A"- or "C"-line Tg males and wild-type females. Embryos (n>50 each) were incubated for 3 days in the presence or absence of E2 (2 μg/l) and TAM (2 mg/l) as indicated, then blood clotting was evaluated.

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hpf embryos from mating between "C"-line Tg and wild-type fish in the presence or absence of E2. Population of the embryos developing yolk veins in the presence of E2 was approximately half of that in the absence of E2 throughout the experiment, indicating that formation of yolk veins is inhibited by E2 in the Tg embryos (Fig. 4C). Blood clots formed in less than 5% of the embryos incubated in mock solution (n ≧100 for each Tg embryos). Co-incubation with an anti-estrogen, tamoxifen (TAM), abolished the estrogen-induced blood clotting (Fig. 5) and rescued the developmental defect in yolk veins. These results indicate that overexpression of ER greatly enhances the thrombotic response to estrogens and that activation of ER is involved in both the estrogen-induced blood clotting and developmental defects in the vasculature.

We examined the sensitive period to estrogen in the development of the Tg embryos by incubating them in E2 after the time interval starting from 10 hpf (Fig. 6A). The embryos of 25 hpf (at the early neurula stage) or later developmental times no longer responded to E2, while the embryos before 22 hpf were susceptible to estrogen (Fig. 6B). These results indicate that the sensitive period is before the early neurula stage.

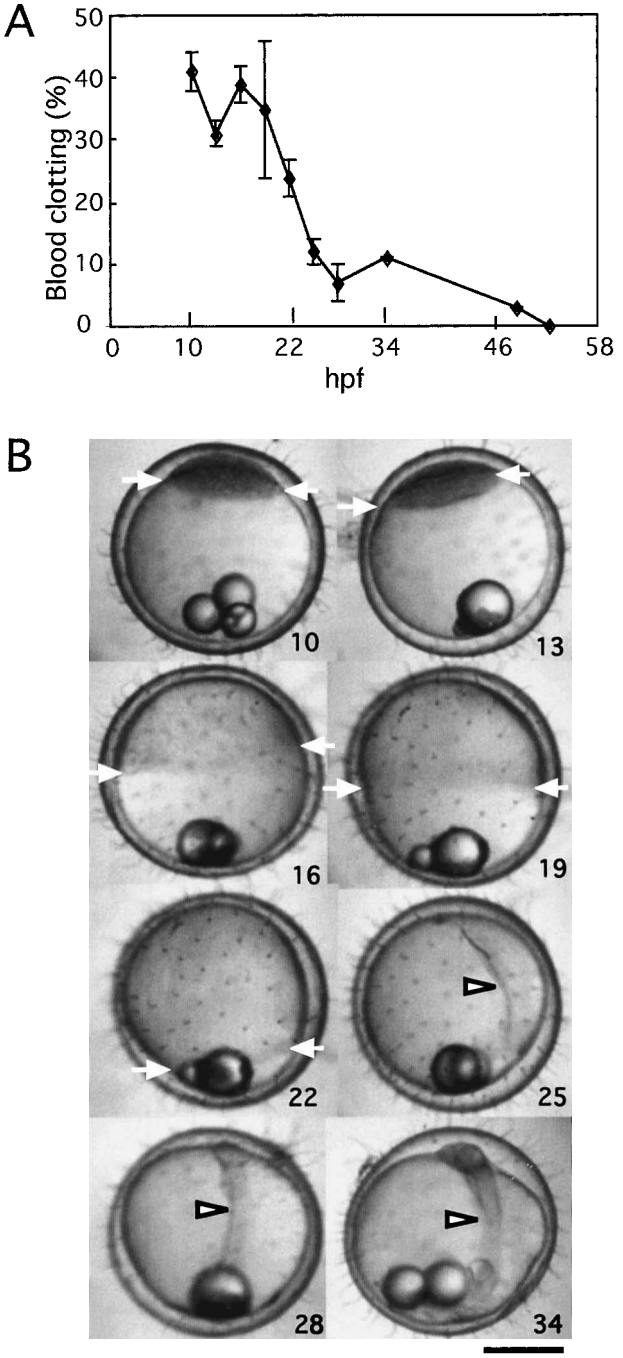


Fig. 6. The sensitive period to estrogen in the development of Tg embryos. (**A**) The 10-hpf embryos (n>50 each) from mating between the "C"-line Tg and wild-type fish were incubated in saline, added with E2 at the final concentration of 2 μg/l at the indicated hpf, followed by incubation, then blood clotting was evaluated at 3 dpf. (**B**) Photographs of the embryos in saline at the indicated hpf. Arrows indicate the developing edge of the blastoderm. Arrowheads indicate the embryonic body. Scale bar is 500 μm.

DISCUSSION

We have created, for the first time, aquatic animals hypersensitive to estrogens (E2 and EE) by overexpressing ER in the medaka fish. Our Tg embryos fail to develop yolk veins and blood clots form within 3 days after exposure to the lowest estrogen concentration (2 μg/l). We therefore propose the use of our Tg embryos to specifically monitor the estrogenic activity in chemicals, foods, and the sewage effluent to be discharged to the aquatic environment, which has been shown to contain E2 and EE (Desbrow *et al.*, 1998). Blood clotting is a novel, useful biomarker for estrogens, because the current bioassay based on the male-to-female gonadal sex reversal or the intersex gonad takes 2–3 months (Patino, 1997; Kawahara and Yamashita, 2000).

The thrombotic progression is considered to be dependent on several risk factors such as aging, cancer, pregnancy and surgery. Several studies have also reported that current use of oral contraceptives (OC) in young women and HRT in postmenopausal women increase risk of VTE and cerebral and myocardial infarction (Daly *et al.*, 1996; Jick *et al.*, 1996; Godsland *et al.*, 2000). Given the widespread use of OC and HRT, it is important to uncover a mechanism by which estrogens act in the blood clotting pathway. Here we overexpressed ER in the medaka fish embryos and produced the estrogen-dependent thrombosis. Our results indicate a key role of ER in the estrogen action. The embryos respond to estrogen before the neurula stage and fail to develop blood vessels at the later stage, suggesting that precursor cells to yolk veins can be damaged by estrogen. Blood clotting may be caused by the embryonic vascular damage, because platelet adhesion to subendothelial collagens and activation by components of the extracellular matrix are crucial for blood clotting (Nieswandt *et al.*, 2001). These situations may be applicable to VTE, because, firstly, circulating endothelial progenitor cells are involved in the

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formation of blood vessels after birth (Losordo and Isner, 2001), and secondly, recent studies in human and animals suggest a breakdown in vessel wall structure and an increase in endothelial permeability in response to estrogen and xeno-estrogen (Godsland *et al*., 2000; Dubey *et al*., 2000). Given the conservation of ER among vertebrates, the estrogen-induced thrombosis may occur by a conserved mechanism between fish and human. We will now be able to study underlying pathological mechanisms in a short-term experiment using the transparent transgenic fish embryos that enable us to detect a blood clot easily and continuously. Accordingly, our transgenic fish is also recommended as a tester in the screening of anti-thrombotic agents or anti-estrogens.

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