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Dynamic and differential expression of the gonadal aromatase during the process of sexual differentiation in a novel transgenic *cyp19a1a*-eGFP zebrafish line

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ABSTRACT

In zebrafish, there exists a clear need for new tools to study sex differentiation dynamic and its perturbation by endocrine disrupting chemicals. In this context, we developed and characterized a novel transgenic zebrafish line expressing green fluorescent protein (GFP) under the control of the zebrafish *cyp19a1a* (gonadal aromatase) promoter. In most gonochoristic fish species including zebrafish, *cyp19a1a*, the enzyme responsible for the synthesis of estrogens, has been shown to play a critical role in the processes of reproduction and sexual differentiation. This novel *cyp19a1a*-eGFP transgenic line allowed a deeper characterization of expression and localization of *cyp19a1a* gene in zebrafish gonads both at the adult stage and during development. At the adult stage, GFP expression was higher in ovaries than in testis. We showed a perfect co-expression of GFP and endogenous Cyp19a1a protein in gonads that was mainly localized in the cytoplasm of peri-follicular cells in the ovary and of Leydig and germ cells in the testis. During development, GFP was expressed in all immature gonads of 20 dpf-old zebrafish. Then, GFP expression increased in early differentiated female at 30 and 35 dpf to reach a high GFP intensity in well-differentiated ovaries at 40 dpf. On the contrary, males consistently displayed low GFP expression as compared to female whatever their stage of development, resulting in a clear dimorphic expression between both sexes. Interestingly, fish that undergoes ovary-to-testis transition (35 and 40 dpf) presented GFP levels similar to males or intermediate between females and males. In this transgenic line, our results confirm that *cyp19a1a* is expressed early during development, before the histological differentiation of the gonads, and that the down-regulation of *cyp19a1a* expression is likely responsible for the testicular differentiation. Moreover, we show that although *cyp19a1a* expression exhibits a clear dimorphic expression pattern in gonads during sexual differentiation, its expression persists whatever the sex suggesting that estradiol synthesis is important for gonadal development of both sexes. Monitoring the expression of GFP in control and exposed-fish will help determine the sensitivity of this transgenic line to EDCs and to refine mechanistic based-assays for the study of EDCs. *In fine*, this transgenic zebrafish line will be a useful tool to study physiological processes such as reproduction and sexual differentiation, and their perturbations by EDCs.

Key-Words: gonadal aromatase, transgenic zebrafish, sexual differentiation

1. Introduction

Despite all the interest granted to zebrafish (*Danio rerio*) in the last few decades, the developmental genetic mechanisms controlling sexual fate in this species remain unclear (for review see (Liew and Orban, 2014; Nagabhushana and Mishra, 2016; Wilson et al., 2014)). In natural wild strains of zebrafish, a strong sex determinant located at the end of the long (right) arm of chromosome 4 was recently discovered and this determinant is necessary but not sufficient for female development (Wilson et al., 2014). In contrast, laboratory strains most often lack this sex determination system (Wilson et al., 2014). Analysis of the available literature suggests that sex in those laboratory zebrafish strains would result of a complex interaction between genome (a polygenic sex determination system), primordial germ cells and environmental factors along with epigenetic factors possibly playing a role in mediating these processes (Liew and Orban, 2014; Luzio et al., 2015; Nagabhushana and Mishra, 2016).

During the sexual differentiation process, all male zebrafish develop a “juvenile ovary” which is later degenerated (“ovary-to-testis” transforming gonad) and transformed into a testis (Maack and Segner, 2003; Takahashi, 1977). While the identity of the gene(s) triggering sex determination/differentiation in zebrafish is not known at present, one common conserved factor in teleost sexual differentiation process is the involvement of estrogens-to-androgens ratios and P450 aromatase, the enzyme responsible for estrogens synthesis (for review see (Guiguen et al., 2010)). In this regard, it is well-established that exposure to estrogens during the process of sexual differentiation resulted in a female biased sex-ratios at the adult stage (Baumann et al., 2014b; Brion et al., 2004; Holbech et al., 2006; Maack and Segner, 2004), while androgen and aromatase inhibitor exposures led to male biased sex-ratios (Baumann et al., 2014a; Fenske and Segner, 2004; McAllister and Kime, 2003; Morthorst et al., 2010; Uchida et al., 2004).

In most teleost fish, including zebrafish, the aromatase gene was duplicated giving rise to two different genes named *cyp19a1a* and *cyp19a1b* (Blazquez and Piferrer, 2004; Chang et al., 2005; Chiang et al., 2001a; Chiang et al., 2001b; Dalla Valle et al., 2002a; Dalla Valle et al., 2002b; Kobayashi et al., 2004; Tchoudakova and Callard, 1998). In zebrafish, the *cyp19a1b* gene is mainly expressed in the brain, specifically in radial glial cells, of both male and female zebrafish and is called the “brain aromatase” (Chiang et al., 2001a; Menuet et al., 2005; Pellegrini et al., 2007; Sawyer et al., 2006). The *cyp19a1a* gene is mainly expressed in the gonads, especially the ovary, and is also called the “gonadal aromatase” or “ovarian aromatase” (Chiang et al., 2001a; Sawyer et al., 2006). Current studies do support a decisive role of *cyp19a1a* gene in the phenotypic sex orientation of the zebrafish gonads. For instance, in *vas::egfp* transgenic zebrafish, *cyp19a1a* transcript levels clearly decreased in gonads undergoing subsequent testicular differentiation (Wang and Orban, 2007). Moreover, by producing loss-of-function mutations in zebrafish, Dranow *et al.* (2016) recently confirmed that Cyp19a1a is necessary for female sexual development in this species. Altogether, these studies support a critical role of Cyp19a1a in the ovarian differentiation and in the maintenance of the ovarian condition.

Aromatase expression and/or activities have been shown to be disrupted by environmental endocrine disrupting chemicals (EDCs) (Hinfray et al., 2006b; Sanderson et al., 2002). For instance, several contaminants belonging to diverse class of chemical families can induce or inhibit aromatase in fish leading to biased sex ratios and/or disruption of reproductive outputs (Fenske and Segner, 2004; Kitano et al., 2000; Skolness et al., 2013; Sun et al., 2007; Uchida et al., 2004). In an attempt to further study the spatio-temporal expression and perturbation of aromatase A expression by EDCs, transgenic fish expressing a fluorescent reporter under the control of the *cyp19a1a* promoter would therefore be relevant biological models. In the present study, we developed and characterized a novel transgenic zebrafish line expressing green fluorescent protein (GFP) under the control of the zebrafish *cyp19a1a* promoter. Herein we report the expression and/or localization of *cyp19a1a* gene in adult zebrafish gonads both in control and in E2-exposed fish. Through fluorescence image analysis of GFP, the spatio-temporal expression of *cyp19a1a* was analyzed and semi-quantified along the process of histological sexual differentiation of the zebrafish and we confirmed that the down-regulation of *cyp19a1a* gene expression is likely responsible for the testicular differentiation in zebrafish. The potential use of this novel transgenic zebrafish line for EDCs studies is also discussed.

2. Materials and Methods

2.1. *cyp19a1a*-eGFP transgenic zebrafish line

2.1.1. Extraction of genomic DNA

cyp19a1a promoter was isolated from genomic DNA of AB strain zebrafish. Genomic DNA was retrieved using a proteinase K protocol. Briefly, 30 hatched larvae were euthanized by an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich, Lyon, France) and incubated in 250 μ L solution of Proteinase K, (Merck, 10 mg/mL) overnight at 56°C. The solution was completed with 83.3 μ L of saturated NaCl solution (Sigma, 350 g/L) and centrifuged (18,000 g, 20 minutes). The liquid phase (250 μ L) was retrieved, precipitated using iso-propanol and washed twice in 70 % ethanol. The pellet was resuspended in 30 μ L Tris buffer (Sigma, 10 mM, pH 8) by incubation at 65°C for one hour and subsequently stored at 4°C.

2.1.2. Transgenic vector design

We made use of transgenesis vector designed by AMAGEN as 'all fish species' transgenesis carriers. The sequences and important features of the cloning and transgenesis vectors are provided in Supplemental figures 1 to 4. Briefly, the vectors were designed upon the multiple Gateway® cloning system described in (Roure et al., 2007) and carried 2 expression cassettes. The first cassette is an enhanced Cyan Fluorescent Protein (eCFP) coding sequence under the control of the zebrafish beta-crystallin-b1 promoter (CrystBB1 cassette) which allows to follow the presence of the transgene in zebrafish from 48 hours post-fertilisation (hpf) onward (Emelyanov and Parinov, 2008). The second expression cassette allows to clone a promoter of interest (*cyp19a1a* in the present study) in front of an enhanced GFP (eGFP) reporter coding sequence. The insertion of the promoter is realised with a

Gateway® Cloning kit using the attP3 and attP5 sites of the pEXP-GFP35H vector (Roure et al., 2007). The two cassettes are separated by a NOS terminator in order to reinforce transcriptional arrest after the CrystBB1 cassette (Emelyanov and Parinov, 2008). The transgenesis is realised through Involin (Thermes et al., 2002) using the two I-SceI sites present on each side of the transgene.

2.1.3. PCR amplification of the promoter

The 7.8 kb region upstream of the start codon of *cyp19a1a* was used as promoter to drive GFP expression. The PCR was designed in order to use the initiation codon and the Kozak consensus translation start of *cyp19a1a* fused to the eGFP coding sequence. The promoter was inserted using Gateway® cloning system (Invitrogen) as described in (Roure et al., 2007) into the pDONR35H (Supplemental figure 1). Briefly, a first fragment (8233 bp) was amplified from genomic DNA. We used the primers Cyp19a1a_Fwd and Cyp19a1a_Rev (Supplemental table 1) in a 50 µL PCR mix (1 µL of genomic DNA (14 ng), KAPA Long Range HotStart DNA Polymerase (KAPA Biosystems, 1.25 U), 5x Kapa Long range buffer (1 µL), dNTPs (0.3 mM each), MgCl₂ (1.75 mM) and 0.5 µM of each primer) to run the first PCR: 1x(94°C – 3 sec); 35x(94°C - 25 sec; 49°C - 15 sec; 68°C - 11 min 30 sec); 1x(72°C – 11 min 30 sec). This reaction yielded several products. A second reaction was used to improve specificity and insert the attB3 and attB5 Gateway recombination sites necessary for the cloning. The first PCR was diluted to the 10th and 2 µL were used as template of the nested PCR reaction. The primers Cyp19a1a_B3 and Cyp19a1a_B5 (Supplemental table 1) allowed to insert attB3 and attB5 recombination sites respectively at the 5' and 3' end of the promoter. The PCR conditions were identical to the first round except hybridization temperature, which was lowered to 47°C. This reaction yielded a unique 7897 bp fragment (7839 bp of promoter and 58 bp of linkers). The attB3-attB5 tagged PCR fragment was purified using QIA quick PCR purification kit (Qiagen) according to manufacturer instructions. 50 femtomole of the PCR product and the vector pDONR35H (Supplemental figure 1) were used to carry out the cloning reaction using the BP clonase II kit (Invitrogen) to generate the pENT24-3 (Supplemental figure 2). The entry vector pENT24-3 was then recombined with pDEST35H (Supplemental figure 3) to generate the transgenesis vector pEXP24-3 (Supplemental figure 4). Both the pENT24-3 and the pEXP24-3 were fully sequenced (the sequences are provided in the supplemental figure 3 and 4).

2.1.4. Generation of transgenic *cyp19a1a*-GFP zebrafish

Transgenic zebrafish were generated by injecting the pEXP24-3 expression vector into one cell stage zebrafish eggs. Briefly, a solution containing pEXP24-3 maxiprep (15 ng/µL final) and I-SceI enzyme (Roche, 1U/µL final) was injected into one cell stage eggs using borosilicate glass microcapillaries (GC100-10, Harvard Apparatus Ltd, UK), a femtojet injector (Eppendorf, Germany), a binocular loupe (Stemi Zv6, Zeiss, Germany) and Narishige (Japan) micromanipulator. In total, 789 eggs were injected in 3 sessions. At 48 hpf, the embryos were sorted according to eCFP expression in the lens under an epifluorescence dissecting microscope (MZFLIII Leica). Embryos which displayed differential expression from one eye to the other, strong mosaicism, weak or absence of CFP/GFP expression were eliminated. Roughly 20% of the injected eggs were selected for rearing (a batch of 77

mild expressing embryos and a batch of 90 embryos displaying strong expression). After a rearing period of 4 months, 12 female and 4 male F0 adult fish were tested for their ability to transmit the transgene to their progeny. Among those, 4 females and 1 male transmitted the transgene to their progeny (Table 1). Progeny from each of these founders were further analysed for expression of GFP in the gonads. Two of the 5 lines did not express GFP at all. Each of the 3 other lines did express GFP according to a *cyp19a1a* compatible pattern albeit at levels varying from one line to the other (data not shown). The progeny of female 4 was selected as the most appropriate to our purpose (highest GFP expression intensity) and all further analysis were carried out using the progeny of this animal. The term *cyp19a1a*-eGFP thereafter refers exclusively to the lineage of this female 4.

Table 1: Transmission of the transgene from the five *cyp19a1a*-eGFP founders from generation 0 (F0) to generation 1 (F1)

ID and sex of the transgenic founders (F0)	Total number of fertilized eggs	Number of transgenic embryos (F1)	% positive progeny
1 / female	339	149	44
2 / female	51	11	22
3 / female	103	26	25
4 / female	383	80	21
5 / male	234	88	38

2.2. Zebrafish maintenance and reproduction

Both transgenic (*cyp19a1a*-eGFP) and wild type (AB strain) zebrafish were raised in our laboratory facility at INERIS (Institut National de l'Environnement Industriel et des Risques, Verneuil-en-Halatte, France). Zebrafish were maintained in 3.5 L aquaria in a recirculation system (Zebtec, Tecniplast, Buguggiate, Italy) on a 14:10 light:dark cycle at a temperature of $27.0 \pm 2.0^\circ\text{C}$. Animal handling and experimentations were in accordance with the EU Directive 2010/63/EU for animal experiments.

Transgenic *cyp19a1a*-eGFP zebrafish were crossed with wild type zebrafish (AB strain) and fertilized eggs were harvested. Eggs were disinfected 5 min in water supplemented with 0.1% of commercial bleach (2.6% of sodium hypochlorite). The GFP positive eggs/larvae were maintained in semi-static conditions until 8 days post fertilization (dpf) and then transferred in 3.5 L aquaria of the Zebtec system (around 25 larvae per liter) to grow.

To obtain the transgene transmission rate in the *cyp19a1a*-eGFP zebrafish line, 6 pairs of breeders each consisting of one transgenic and one wild type zebrafish, were allowed to reproduce. Transmission rate was determined by calculating the ratio of transgenic eggs over wild type eggs for each pair of breeders.

2.3. GFP fluorescence imaging and semi-quantification in the entire gonads of mature zebrafish

Mature *cyp19a1a*-eGFP transgenic zebrafish were euthanized in ice-cold water (Strykowski and Schech, 2015; Wilson JM et al., 2009) before sectioning the spinal cord. The abdominal cavity was open to remove gut, liver, spleen and bile vesicle. Gonads were photographed with a Discovery V8 stereomicroscope equipped with an AxioCam Mrm camera (Carl Zeiss GmbH, Göttingen, Germany). GFP fluorescence in the entire gonads was then photographed and semi-quantified using a fluorescence imaging system IVIS Lumina II (Perkin Elmer, Courtaboeuf, France). All the pictures were taken in the same conditions (zoom, time of exposure, binning, F-Stop, excitation filter and emission filter). GFP fluorescence levels in the entire gonads were expressed as a measure of the photons incidence on the CCD camera (counts) and normalized by the area of the entire gonads (in squared millimeters, mm²). For each sex, 6 individuals were analyzed.

2.4. Zebrafish exposure to estradiol and GFP fluorescence measurement

Mature female *cyp19a1a*-eGFP zebrafish were exposed to 10 nM E2 (Sigma–Aldrich) or to solvent alone (dimethyl sulfoxide (DMSO), 0.00001%; Sigma–Aldrich, Lyon, France) during 7 days. Fish were exposed under semi-static conditions in 4 L tanks with 100% water renewal every day (temperature: 28 ± 1°C; pH: 8.0 ± 0.2; dissolved oxygen: 6.6 ± 0.3 mg/l; conductivity: 275 ± 43 mS/cm). Exposure was performed in one tank per condition, each containing four female fish. At the end of exposure, zebrafish were euthanized and gonads were removed and fixed 48h in 4% paraformaldehyde in saline phosphate buffer (PBS) at 4°C. After rinsing in PBS, samples were successively transferred to 10%, 20% and 30% sucrose-PBS solutions at 4°C and finally embedded in Neg-50 cryo-compound (Microm Microtech, France). Frozen sections of gonads were performed at 12 µm thick. Slides were rinsed in PBS to remove Neg-50 cryo-compound and coverslipped using a fluorescence mounting medium (DAKO, France).

GFP fluorescence was observed directly on cryo-sections using a Zeiss AxioImager Z1 fluorescence microscope equipped with an AxioCam Mrm camera (Carl Zeiss GmbH, Göttingen, Germany). Micrographs were taken using the Axiovision Imaging software. Semi-quantification of the fluorescence of GFP of DMSO- and E2-exposed fish ovaries was performed on micrographs taken with the same magnification (x2.5 objective) and time of exposure for all individuals. For each experimental condition, 4 fish were used and 4 micrographs were analyzed per fish. Fluorescence was measured as integrated density (IntDen, ImageJ software), i.e. the sum of the grey values of all the pixels in the region of interest. The IntDen values were then normalized to the total area of tissue on the micrograph.

2.5. Fluorescence immunohistochemistry

cyp19a1a-eGFP transgenic/wild type AB zebrafish were euthanized and gonads were removed and fixed in modified Davidson's fixative (22% v/v formaldehyde 37-40%; 11.5% v/v glacial

acetic acid; 33% v/v ethyl alcohol 95%; 33.5% v/v distilled water) for 48 hours at room temperature. After fixation, samples were dehydrated in ethanol, cleared in toluene and embedded in paraffin, according to conventional procedures. Zebrafish gonads were sectioned at 5 μ m.

Immunohistochemistry was then carried out using an In situ Pro VSI robotic station (Intavis, Köln, Germany, distributed in France by Proteigene) as previously described in (Hinfray et al., 2013). For GFP localization, a primary antibody produced in goat was used at a dilution of 1:600 (polyclonal goat anti-GFP, Abcam, Paris, France, reference number: ab5450). For Cyp19a1a localization, a primary antibody produced in rabbit was used at a dilution of 1:600 (polyclonal rabbit anti-zfCyp19a1a, AnaSpec, Fremont, USA, distributed in France by Tebu-Bio, reference number: 55474s). For co-localization experiment, slides were incubated with a mix of these two antibodies at a dilution of 1:600. These primary antibodies have been already used successfully in zebrafish (Hinfray et al., 2013; Klein et al., 2011). Slides were then incubated with a donkey anti-goat antibody coupled to AlexaFluor® 488 or a mix of donkey anti-goat coupled to AlexaFluor® 488 and donkey anti-rabbit coupled to AlexaFluor® 594 antibodies (Invitrogen, Praisley, UK) at a dilution of 1:200. Nuclei were revealed with Hoechst 33342. The specificity of the immunoreactivity was controlled by processing adjacent sections without primary antibody or with only one primary antibody and the two secondary antibodies. The specificity of the anti-GFP antibody was controlled by performing immunohistochemistry on wild type zebrafish gonad sections. The specificity of the anti-Cyp19a1a antibody was also controlled by performing immunohistochemistry with the anti-Cyp19a1a antibody pre-incubated with 100 μ g/ml of the synthetic peptide. In these negative controls, no cross-reactivity was seen (Supplemental Figure 5).

GFP fluorescence was observed and photographed using an FV1000 confocal microscope (Olympus, Rungis, France). Acquisition parameters (time of exposure, laser intensity...) were optimized for each micrograph independently.

2.6. Gonadal GFP fluorescence during the process of sexual differentiation

cyp19a1a-eGFP transgenic zebrafish larvae were sampled at 20, 30, 35 and 40 dpf (n = 8 at 20 dpf and 15 at the other sampling time). The larvae were euthanized with an overdose of MS-222. Entire larvae (20 dpf) or larvae without their heads (30, 35, 40 dpf) were fixed 48h in 4% paraformaldehyde in PBS at 4°C. After rinsing in PBS, larvae were successively transferred to 10%, 20% and 30% sucrose-PBS solutions at 4°C, embedded in Neg-50 cryo-compound and sectioned transversally at 12 μ m thick. Slides were rinsed in PBS and coverslipped using the DAKO fluorescence mounting medium.

GFP fluorescence was observed directly on cryo-sections using a Zeiss AxioImager Z1 fluorescence microscope equipped with an AxioCam Mrm camera and combined with an ApoTome (Carl Zeiss GmbH, Göttingen, Germany). Illustration micrographs were taken using the ApoTome system and Axiovision Imaging software. Relative quantification of the fluorescence of GFP of the larvae gonads was performed on micrographs taken without the ApoTome system but with the same magnification (x10 objective) and time of exposure for all individuals whatever the sex and stage of

development. For each larva, 3 to 4 micrographs of the entire gonads were analyzed. GFP fluorescence was measured as integrated density (IntDen, ImageJ software).

2.7. Whole Mount In Situ Hybridization (WMISH) on early stage zebrafish

Wild type AB zebrafish larvae were sampled at 20 dpf, euthanized with an overdose of MS-222 and fixed in 4% paraformaldehyde (24 hours, 4°C). After fixation, larvae were rinsed 3 times (PBS-Tween 20 0.1%), progressively dehydrated in methanol and stored in methanol 100% until WMISH.

RNA probes were produced from PCR product obtained by following amplification of the IRBOP991C1282D clone (Source Bioscience, UK) containing the zebrafish *cyp19a1a* cDNA. Colonies containing plasmid were isolated with 50 µg/ml ampicillin. Plasmids were purified using PureLink® Quick Plasmid Miniprep kit (ThermoFisher Scientific) according to manufacturer's protocol. PCR amplification was carried out on 2 ng of plasmid as follow: 1 cycle at 94°C for 1 min, 35 cycles at (94°C for 40 sec, 46°C for 40 sec and 72°C for 2 min), and 1 cycle at 72°C for 10 min; using the following primers 5'-TAA TAC GAC TCA CTA TAG GG-3' and 5'-CAA TTA ACC CTC ACT AAA GG-3'. Ten nanograms of the resulting PCR product was then used as template for digoxigenin-labeled RNA probes synthesis using DIG RNA labeling mix (Roche, France) and T3 or T7 RNA polymerase (Roche, France) following standard protocols. Labeled RNA probes were then purified using Micro Bio-Spin 30 Columns (BioRad, France) and stored at -80°C until use.

WMISH was carried out using an In Situ Pro (Intavis) robotic station as described in Yano et al. (2011) with minor modifications. Briefly, 20 dpf zebrafish larvae were rehydrated, permeabilized by proteinase K treatment (25 µg/ml, 35 min, room temperature), and post-fixed (4% paraformaldehyde and 0.8% glutaraldehyde, 20 min). Pre-hybridization and hybridization medium contained 50% formamide, 5X SCC, 0.1% Tween 20, 0.005% heparin, 0.1 mg/ml tRNA. Hybridization was carried out at 37°C for 16 hours. After post-hybridization washes, larvae were incubated in blocking buffer for 1 hour (Triton 1%, Tween 20 0.2%, 2% serum/PBS) before addition of the alkaline phosphatase coupled anti-digoxigenin antibody (1:2,000, Roche Diagnostics Corp) for 6 hours. After washes, color reaction was performed in the presence of NBT/BCIP (Roche). After WMISH, larvae were dehydrated and embedded in paraffin according to conventional procedures, and sectioned at 5 µm.

2.8. Statistics

To compare mean fluorescence data (female vs male, DMSO vs E2 and GFP expression in the different sexes and stages of development), a non-parametric Wilcoxon's test was performed, where it was possible, using R_{TM} (R software, R development Core Team). Differences between groups were considered to be significant if $p < 0.05$ (*) and $p < 0.01$ (**).

3. Results

3.1. Transmission of the transgene in the *cyp19a1a*-eGFP transgenic zebrafish line

Adult *cyp19a1a*-eGFP heterozygote transgenic zebrafish (N=3 males and 3 females) were allowed to reproduce with wild type AB-strain zebrafish to determine the transmission rate of the transgene from generation 3 to generation 4. Transgenic larvae were identified by the presence of CFP fluorescence in the eyes. Transmission of the transgene occurred for the 6 transgenic fish and the transmission rate was about 50% for each breeder fish (Table 2) suggesting a unique insertion of the transgene in these transgenic fish. Besides the unique insertion of the transgene, this *cyp19a1a*-eGFP line also displays interesting features such as being a stable transgenic line with the possibility to select transgenic eggs as soon as 48 hpf.

Table 2: Transmission rate of the transgene from generation 3 (F3) to generation 4 (F4) in the *cyp19a1a*-GFP transgenic zebrafish line. 6 transgenic zebrafish were cross-bred with wild type AB zebrafish.

ID and sex of the transgenic fish (F3)	Total number of fertilized eggs	Number of transgenic embryos (F4)	Transmission rate (%)
1 / female	388	196	50.5
2 / female	187	87	46.5
3 / female	216	104	48.1
4 / male	203	93	45.8
5 / male	183	87	47.5
6 / male	123	64	52.0

3.2. GFP expression, localization and regulation in the *cyp19a1a*-eGFP transgenic zebrafish line

The expression of GFP in mature zebrafish gonads was determined by fluorescence imaging and relative quantification (Figure 1). In all male and female fish studied, GFP expression was higher in ovaries than in testis (Figure 1A, B) with a 9-fold higher expression in ovaries compared to testis (Figure 1B).

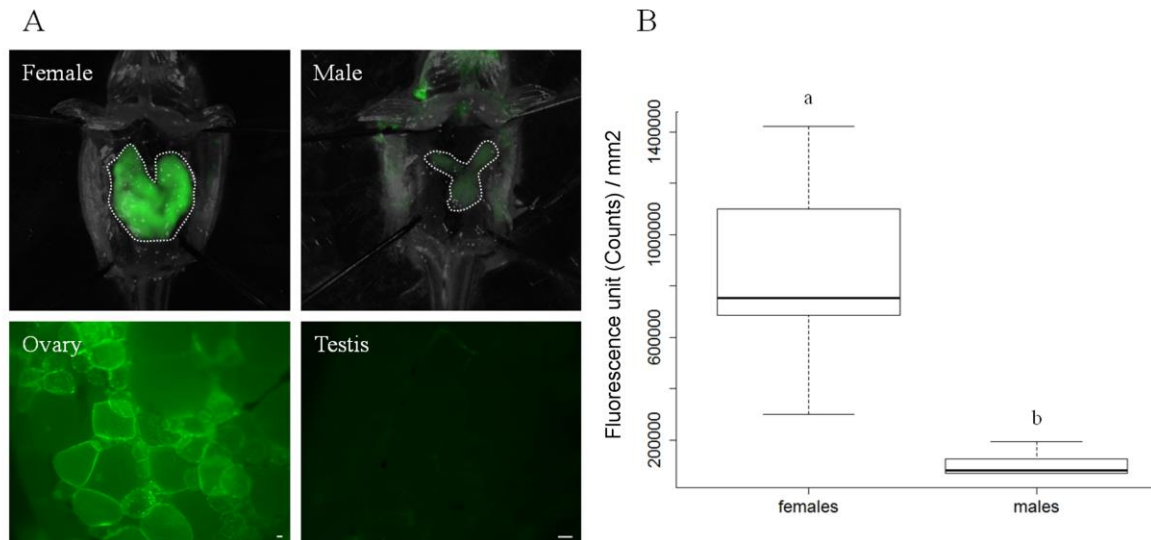


Figure 1: Relative quantification of GFP fluorescence in mature zebrafish gonads of the *cyp19a1a*-GFP transgenic line. (A) Imaging of GFP fluorescence (green) in the entire gonads using a fluorescence imaging system IVIS Lumina II from Perkin Elmer (Top) and a Discovery V8 stereomicroscope from Carl Zeiss (Bottom). Scale bars = 50 μ m (B) Relative quantification of GFP fluorescence in the entire gonads of females (N=6) and males (N=6) with the IVIS Lumina II system. Results are expressed as the median value (bars in the middle of the box), upper quartile (upper edge of the box) and lower quartile (lower edge of the box). Different letters indicate a statistically significant difference between the groups (Wilcoxon's Test, $p < 0.05$).

At the cellular level, in 55 dpf-old zebrafish ovary, GFP was mainly localized to the cytoplasm of peri-follicular cells surrounding previtellogenic oocytes, with a slight fluorescence also in the cytoplasm of oocytes (Figure 2 A,B). In mature ovary, GFP was immunolocalized also in the cytoplasm of peri-follicular cells (theca and granulosa cells) surrounding oocytes from a slight expression around some previtellogenic oocytes to a strong expression around vitellogenic oocytes (Figure 2 C-G). A slight GFP expression was also noticed in oocyte cytoplasm. In mature testis, GFP localized to the cytoplasm of both Leydig cells and germ cells (Figure 2 H).

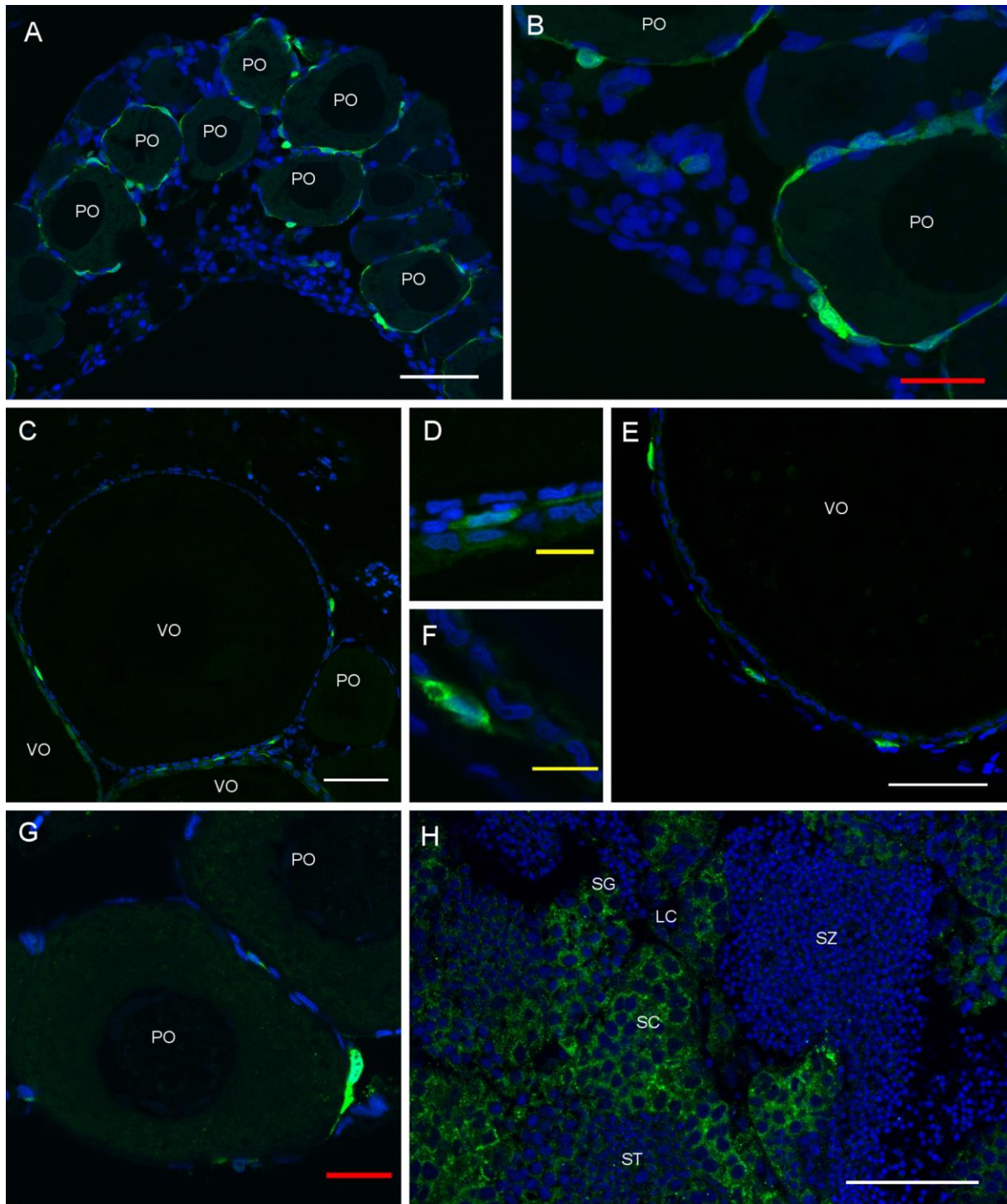


Figure 2: GFP fluorescence localization in zebrafish gonads of the *cyp19a1a*-GFP transgenic line. In 55 days post fertilization (dpf) zebrafish, GFP was localized to the cytoplasm of peri-follicular cells surrounding pre-vitellogenic oocytes (A,B). In mature ovary of adult zebrafish, GFP is mainly immunolocalized in the cytoplasm of peri-follicular cells (granulosa and thecal cells) surrounding vitellogenic oocytes and some pre-vitellogenic oocytes (C-G). (D) Enlargement of peri-follicular cells from micrograph C. (E) Enlargement of peri-follicular cells from micrograph E. In mature testis, GFP localized to the cytoplasm of both Leydig cells and germ cells (H). Imaging was realized with an Olympus FV1000 confocal microscope. Acquisition parameters were optimized for each micrograph independently. Nuclei are in blue. PO: pre-vitellogenic oocyte; VO: vitellogenic oocyte; LC: Leydig cells; SC: spermatocytes; SG: spermatogonia; ST: spermatids; SZ: spermatozoa. White bars: 50 µm; Red bar: 20 µm; Yellow bars: 10 µm.

To assess whether GFP is indeed expressed in Cyp19a1a-expressing cells, sections of *cyp19a1a*-eGFP transgenic fish gonads were immunolabeled both with anti-GFP and anti-Cyp19a1a antibodies. In both mature ovary and testis, a perfect co-localization of GFP and Cyp19a1a proteins was observed (Figure 3).

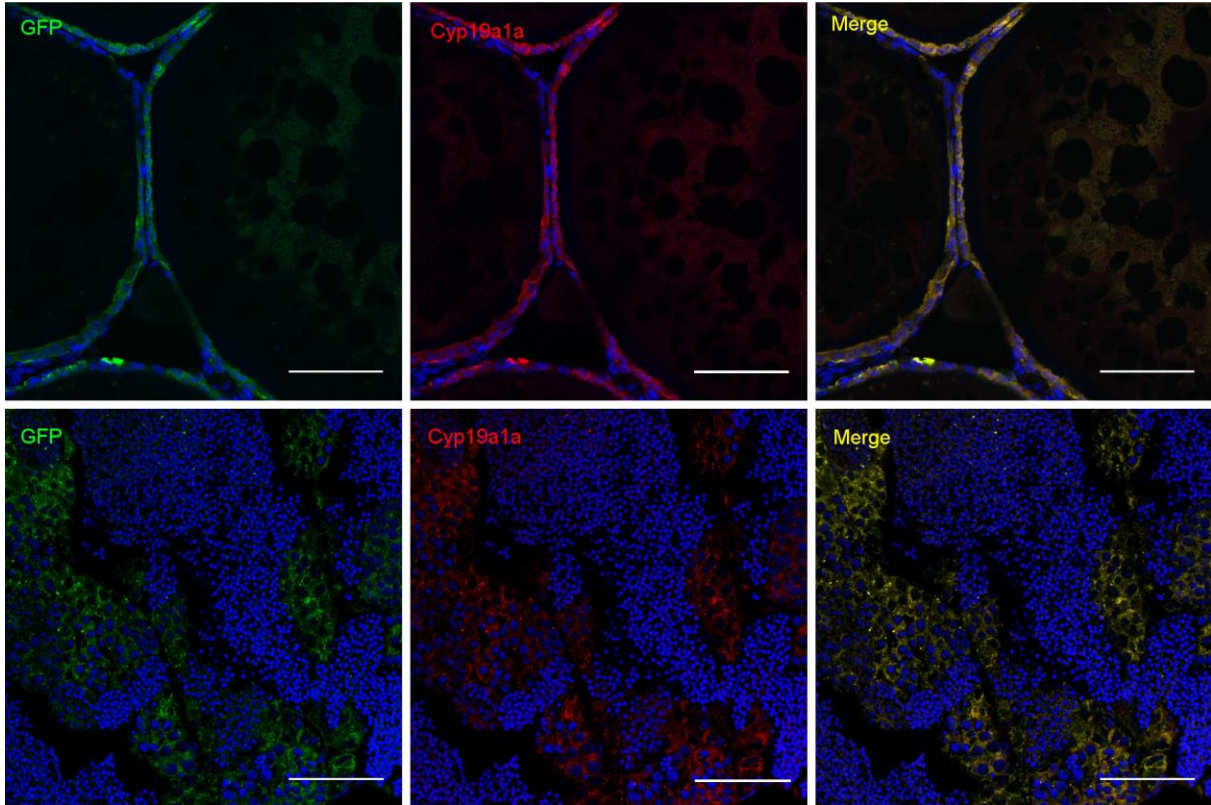


Figure 3: Co-localization of GFP and Cyp19a1a in mature zebrafish gonads of the *cyp19a1a*-GFP transgenic line. GFP (green) and Cyp19a1a (Red) proteins were immunolocalized both in ovaries (Top) and testis (Bottom). The merge of the two is showed in yellow. Nuclei are in blue. Imaging was realized with an Olympus confocal microscope FV1000. Acquisition parameters were optimized for each sex independently. White bars: 50 μ m.

In a previous study, we showed that exposure to 10nM of E2 for 7 days inhibited *cyp19a1a* gene expression in wild type zebrafish ovary (Hinfray et al., 2006a). In the present study, GFP fluorescence in adult *cyp19a1a*-eGFP transgenic zebrafish ovaries was also inhibited by exposure to E2 (10nM, 7 days) (Figure 4), indicating that GFP expression regulation by E2 in transgenic zebrafish is similar to that of *cyp19a1a* gene in wild type zebrafish.

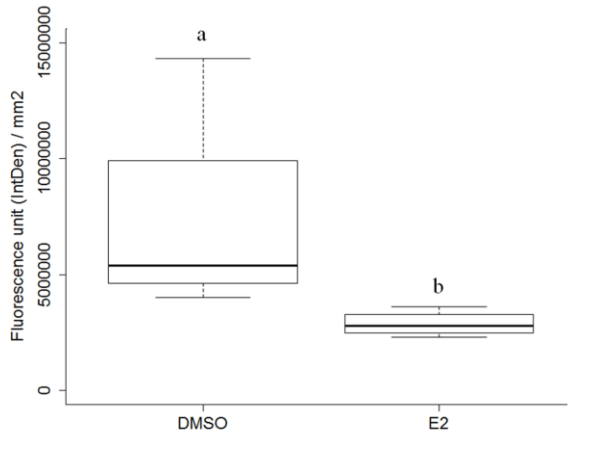


Figure 4: Relative quantification of GFP fluorescence in mature *cyp19a1a*-GFP transgenic zebrafish ovaries after a 7-days exposure to 10 nM of Estradiol (E2) or solvent (DMSO). Results are expressed as the median value (bars in the middle of the box), upper quartile (upper edge of the box) and lower quartile (lower edge of the box). For each individual (n=4 individuals / condition), four micrographs of ovarian sections were analyzed. Different letters indicate a statistically significant difference between the groups (Wilcoxon's Test, $p < 0.05$).

3.2. Spatio-temporal expression of GFP in *cyp19a1a*-eGFP transgenic zebrafish gonads during development

The spatio-temporal expression of *cyp19a1a* gene was studied during the process of sexual differentiation using GFP fluorescence imaging and semi-quantification in gonad sections of transgenic zebrafish between 20 and 40 dpf (Figure 5, 6 and 7). A slight expression of GFP was observed in all immature gonads of 20 dpf-old zebrafish and tends to increase in 30 and 35 dpf-old zebrafish immature gonads (Figure 5). In immature gonads of 20 dpf *cyp19a1a*-eGFP transgenic zebrafish, GFP and *Cyp19a1a* expression was localized in the cytoplasm of all cell types (Figure 6 A, B). At the same stage of development, *cyp19a1a* mRNA was also localized in the cytoplasm of all cell types of wild type zebrafish gonads (Figure 6 C). In females (from 30 dpf), GFP expression was higher than in immature gonads and its expression increased to reach high GFP intensity in well-differentiated ovaries of 40 dpf-old zebrafish (Figure 5). At this stage, GFP was mainly observed in the cytoplasm of peri-follicular cells but a slight fluorescence was also noticed in the cytoplasm of oocytes (Figure 7). On the contrary, males consistently displayed low GFP expression as compared to females whatever their stage of development, resulting in a clear dimorphic expression between both sexes (Figure 5). At 40 dpf, GFP was localized in the cytoplasm of several isolated cells distributed in the entire testis (Figure 7). Interestingly, gonads of fish that undergo ovary-to-testis transition (35 and 40 dpf) presented GFP levels similar to males or intermediate between females and males (Figure 5). In this ovary-to-testis transition gonads, GFP is expressed in cells at the periphery of the gonad while no GFP was found in the peri-follicular cells surrounding remaining oocytes (Figure 7).

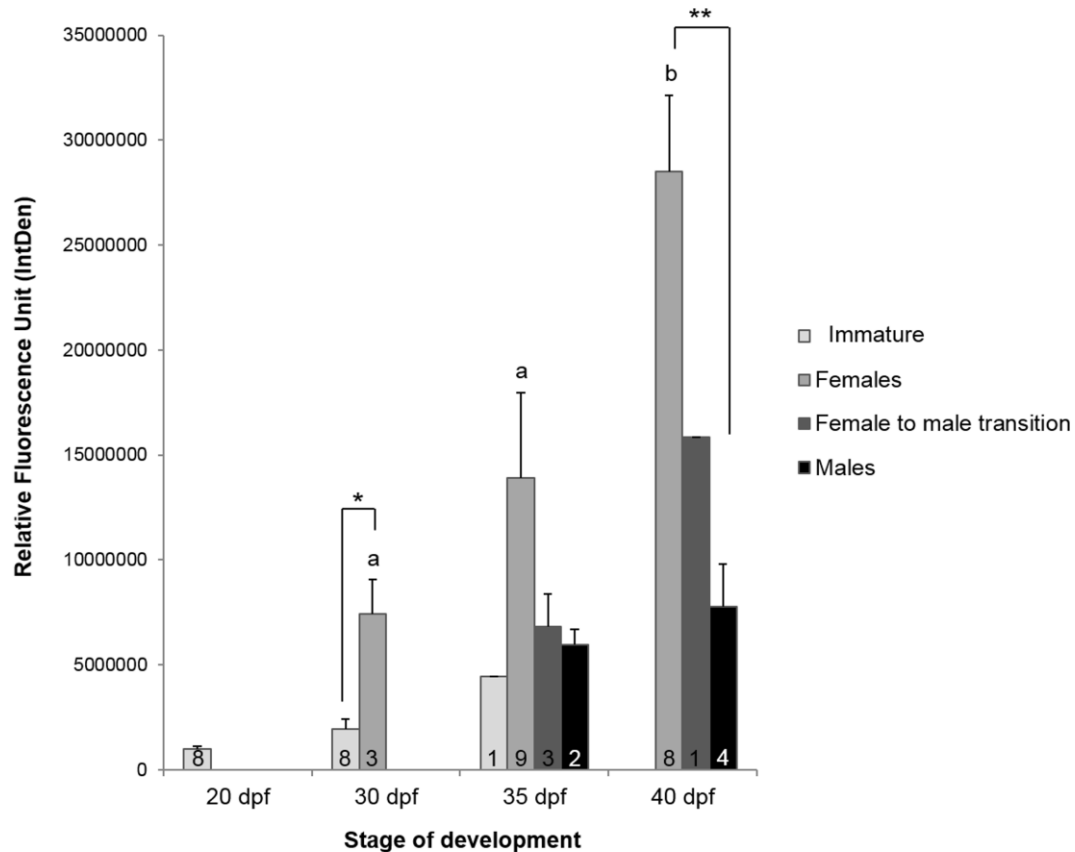


Figure 5: Relative quantification of GFP fluorescence in gonads of *cyp19a1a*-GFP transgenic zebrafish during development. Results are expressed as mean \pm S.E.M. Number of fish analyzed is indicated in the bars of the histogram and 3-4 sections were analyzed for each fish. Different letters indicate statistically significant differences across development for one sex (Wilcoxon's test, $p < 0.05$). * and ** indicate statistically significant differences between sexes within one stage of development (Wilcoxon's test, * $p < 0.05$, ** $p < 0.01$). dpf: days post fertilization.

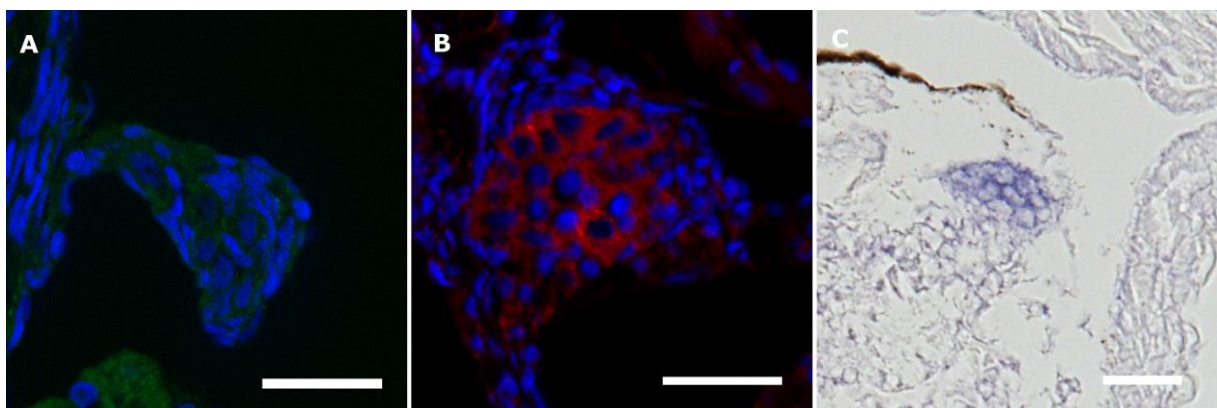


Figure 6: Localization of GFP and *cyp19a1a* protein and mRNA in 20 dpf gonads of zebrafish. A: Imaging of GFP fluorescence (green) was realized directly on cryo-sections of *cyp19a1a*-GFP transgenic zebrafish gonads. B: Cyp19a1a (Red) proteins were immunolocalized using the anti-Cyp19a1a antibody in *cyp19a1a*-GFP transgenic zebrafish gonads. C: *cyp19a1a* mRNA was localized by whole mount *in situ* hybridization in wild type zebrafish gonads. Fluorescence imaging was realized using a Zeiss AxioImager Z1 fluorescence microscope equipped with a MRm5 camera combined with an ApoTome. Nuclei are in blue. Acquisition parameters were optimized for each micrograph independently. Transmitted light imaging was performed using a Nikon 90i microscope equipped with a Nikon DS Ri1 camera. dpf: days post fertilization. Scale bars: 20 μ m.

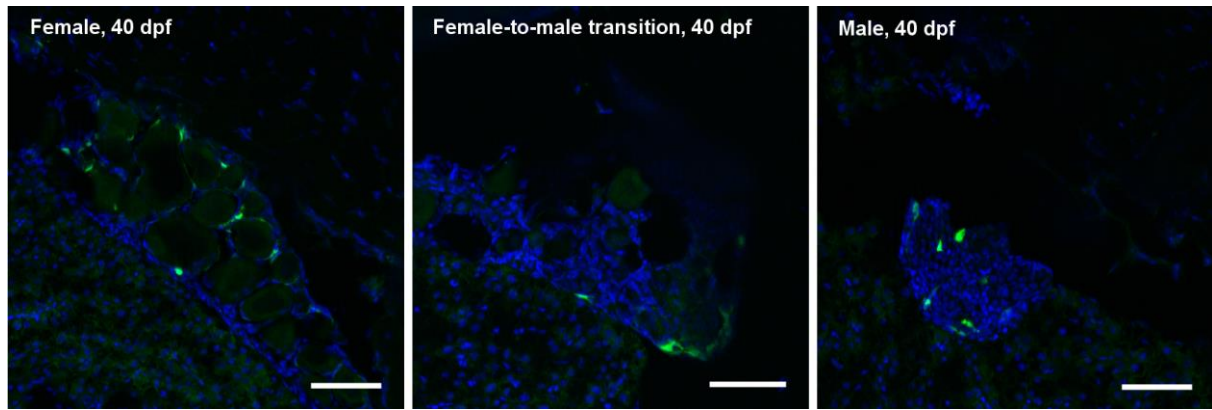


Figure 7: Localization of GFP fluorescence in 40 dpf gonads of *cyp19a1a*-GFP transgenic zebrafish. Imaging of GFP fluorescence (green) was realized directly on cryo-sections using a Zeiss Axiomager Z1 fluorescence microscope equipped with an MRm5 camera combined with an ApoTome. Acquisition parameters were optimized for each micrograph independently. Nuclei are in blue. dpf: days post fertilization. Scale bars: 50 μ m.

4. Discussion

4.1. The *cyp19a1a* gene is expressed both in female and male gonads

In the present study, we established a novel and useful transgenic line of zebrafish expressing GFP under the control of the zebrafish *cyp19a1a* gene promoter. In these transgenic animals, GFP was expressed in both male and female gonads with a 9-fold higher fluorescence level in ovaries, which is consistent with the crucial production of estrogens in females with important roles in oocyte growth and hepatic vitellogenin synthesis (Nagahama, 1994). This dimorphic GFP expression is in agreement with the higher expression of *cyp19a1a* gene (Fenske and Segner, 2004; Sawyer et al., 2006; Trant et al., 2001) and Cyp19a1a protein (Caulier et al., 2015) measured in wild type zebrafish ovaries compared to testis as well as with the higher circulating concentrations of estradiol in female zebrafish (Deng et al., 2010; Ji et al., 2013; Liu et al., 2013; Liu et al., 2012; Ma et al., 2012; Yu et al., 2014).

In ovaries, GFP was detected in the cytoplasm of peri-follicular cells (thecal and granulosa cells) surrounding both previtellogenic and vitellogenic oocytes with a stronger expression around vitellogenic oocytes. This pattern of expression is similar to the wild type pattern of *cyp19a1a* gene and its protein in zebrafish ovaries (Caulier et al., 2015; Goto-Kazeto et al., 2004; Rodriguez-Mari et al., 2005; Wang and Orban, 2007) and to another recently developed *cyp19a1a*-EGFP transgenic zebrafish (Dranow et al., 2016). A slight GFP fluorescence was also measured in the cytoplasm of oocytes. In other fish species such as medaka, rainbow trout, air-breathing catfish or gilthead seabream, *cyp19a1a* gene or its protein product have already been detected in the germinal compartment, i.e. the ooplasm (Gohin et al., 2011; Park et al., 2008; Raghuv eer et al., 2011; Zapater et al., 2012), and proved to be functional in rainbow trout late vitellogenic oocytes (Gohin et al., 2011).

In zebrafish, however, due to the presence of Cyp19a1b protein in the ooplasm (Caulier et al., 2015), the specific functionality of Cyp19a1a protein remains difficult to demonstrate in this compartment.

In testis, both Leydig cells and germ cells expressed GFP. In wild type zebrafish testis, while *cyp19a1a* gene expression has been measured, no mRNA localization was achieved (Hinfray et al., 2006a; Rodriguez-Mari et al., 2005; Sawyer et al., 2006; Wang and Orban, 2007). Nevertheless, this GFP localization to Leydig and germ cells is consistent with our previous studies showing the same pattern of localization of Cyp19a1a protein in wild type zebrafish testis (Caulier et al., 2015; Hinfray et al., 2013). In other teleosts, *cyp19a1a* gene and/or protein have been detected in both Leydig cells (Guiguen et al., 2010; Kobayashi et al., 2004; Kotula-Balak et al., 2008; Vinas and Piferrer, 2008; von Schalburg et al., 2013) and germ cells (Kotula-Balak et al., 2008; Vinas and Piferrer, 2008; von Schalburg et al., 2013) of the testis. All these data argue for a role of estrogens in fish testis that might be linked to the involvement of estradiol in spermatogonial stem cell renewal in fish (Miura et al., 1999; Miura et al., 2003).

Altogether, expression of GFP in the *cyp19a1a*-eGFP transgenic zebrafish is consistent with the expression of endogenous *cyp19a1a* gene expression in the gonads of wild type zebrafish. The presence of the CrystBB1-CFP reporter allows for early selection of transgenic eggs and does not seem to disturb the expression of the *cyp19a1a* promoter. These combine to make of this line a useful and powerful tool to monitor *cyp19a1a* expression and determine its precise role in different physiological processes.

4.2. Down-regulation of gonadal *cyp19a1a* expression is likely responsible for testicular differentiation

In the present study, the *cyp19a1a*-eGFP transgenic zebrafish was used to analyze the spatio-temporal expression of the *cyp19a1a* gene in individual gonads during the sexual differentiation process. At the early stages (from 20 dpf to 35 dpf), a slight GFP fluorescence was detected in all cell types of all immature gonads. At 20 dpf, *cyp19a1a* mRNA and protein were also localized in the cytoplasm of all cell types of zebrafish gonads. This early expression of the *cyp19a1a* gene has already been evidenced in wild type zebrafish trunks by RT-PCR (Fenske and Segner, 2004; Wang and Orban, 2007) and individual gonads by *in situ hybridization* (Rodriguez-Mari et al., 2005), and also in gonads of the TgBAC(*cyp19a1a*:EGFP)^{uc44} zebrafish of 13 dpf (Dranow et al., 2016). However, contrary to the localization of *cyp19a1a* mRNA by *in situ hybridization* which has been shown to be restricted to some regions of the immature gonads (Rodriguez-Mari et al., 2005), a wider distribution of GFP and Cyp19a1a protein within the *cyp19a1a*-eGFP gonads and of *cyp19a1a* mRNA within the wild type gonads is reported herein. The rationale behind this discrepancy is not known at present and deserves further study.

In *cyp19a1a*-eGFP female ovaries, GFP was mainly localized to the cytoplasm of peri-follicular cells which is consistent with the localization of *cyp19a1a* mRNA in wild type zebrafish ovaries (Rodriguez-Mari et al., 2005; Wang and Orban, 2007). In ovaries, GFP fluorescence was higher than in immature gonads and increased during ovarian development in relation with the key role of

cyp19a1a in ovarian differentiation in fish (Guiguen et al., 2010). In gonads of *cyp19a1a*-eGFP fish that undergo ovary-to-testis transition, GFP fluorescence levels were intermediate between females and males or similar to males. This pattern of gender expression of GFP is similar to the pattern of *cyp19a1a* gene expression in wild type zebrafish trunks/gonads (Rodriguez-Mari et al., 2005; Wang and Orban, 2007). The results of the present study suggest that *cyp19a1a* expression is necessary for the maintenance of the ovarian fate and that the down-regulation of *cyp19a1a* expression is likely responsible for testicular differentiation. Estrogens depletion may be one of the reasons leading to the degeneration of ovary during gonadal transformation in males as previously suggested by Wang and collaborators (2007). Recent studies in zebrafish but also tilapia and common carp showed that exposure of adult fish to aromatase inhibitors could lead to the transdifferentiation of fully differentiated ovaries into testis (Ogawa et al., 2008; Sun et al., 2014; Takatsu et al., 2013). Moreover, in tilapia, this transdifferentiation could be rescued by exogenous estradiol supplementation (Sun et al., 2014), confirming the importance of estrogens in ovarian maintenance.

Estrogens in fish are produced by two different aromatase proteins, i.e. Cyp19a1a and Cyp19a1b. While some results suggested a bimodal expression of *cyp19a1b* gene in the entire zebrafish larvae during the sexual differentiation process (Trant et al., 2001), more detailed studies established that *cyp19a1b* gene and protein are not expressed in a sexually dimorphic manner in the zebrafish larvae heads/brains during gonadal differentiation (Kallivretaki et al., 2007; Vosges et al., 2010). Moreover, a recent study showed no defects in the sexual differentiation process of *cyp19a1b* loss-of-function mutant zebrafish while *cyp19a1a* loss-of-function mutant zebrafish all developed as males (Dranow et al., 2016). Current studies thus do not support a decisive role of Cyp19a1b in the phenotypic sex orientation of the zebrafish gonads. Altogether, these observations rather support earlier studies (Fenske and Segner, 2004; McAllister and Kime, 2003; Uchida et al., 2004; Wang and Orban, 2007) that favor the hypothesis of Cyp19a1a being the rate-limiting step in female sexual development.

Regarding males, GFP expression was detectable in the present study in male gonads of the *cyp19a1a*-eGFP transgenic fish and this persisted during male development contrary to the absence of mRNA detection by in situ hybridization (Rodriguez-Mari et al., 2005). The presence of GFP in testis is relevant regarding the known *cyp19a1a* gene expression in male gonads both at the adult stage (Hinfray et al., 2006a; Sawyer et al., 2006) and during development (Wang and Orban, 2007). Estrogen receptors have also been localized in the testis of many fish including zebrafish (Bouma and Nagler, 2001; Chandrasekar et al., 2010; Menuet et al., 2002; Vinas and Piferrer, 2008; Wu et al., 2001). Altogether, these data suggest a role of estrogens in testis development, steroid production and spermatogenesis. Indeed, evidences indicate that spermatogonial stem cell renewal is regulated by estradiol (Miura et al., 1999; Miura et al., 2003).

4.3. *cyp19a1a*-eGFP transgenic zebrafish as a reliable model to investigate reproductive functions and their perturbations by EDCs

During the recent years, an increasing interest has emerged as regards *in vivo* transgenic fish models to assess environmental hazard of chemicals notably EDCs. Several transgenic fish models have been developed to study the effects of chemicals acting on the endocrine system through nuclear receptor pathways such as estrogen receptors (Gorelick and Halpern, 2011; Green et al., 2016; Legler et al., 2002) or glucocorticoid receptor (Weger et al., 2013) and disrupting the tissue-specific expression of hormone-regulated genes in the brain (Brion et al., 2012) or in the liver (Kurauchi et al., 2005; Sébillot et al., 2014; Spirhanzlova et al., 2016). Mechanism-based *in vivo* fish bioassays that use transgenic fish were recently developed and have been shown to be very useful tools to screen EDCs alone or in combination (Brion et al., 2012; Cano-Nicolau et al., 2016; Gorelick and Halpern, 2011; Green et al., 2016; Hinfray et al., 2016), some of them being under validation at the OECD level (i.e. EASZY assay that uses *cyp19a1b*-GFP zebrafish embryos). The usefulness of the newly developed *cyp19a1a*-eGFP line for studies on environmental effects of EDCs need to be further investigated. However, we demonstrated that this model has favorable and interesting features such as being a stable transgenic line, possessing only one transgene insertion with the possibility of inheritance of the transgene from both males and females making this line easier to maintain. Further, in adult *cyp19a1a*-eGFP transgenic zebrafish ovaries, pharmacological treatment with E2 resulted in inhibition of GFP fluorescence in a similar manner to *cyp19a1a* gene expression in wild type zebrafish ovaries (Hinfray et al., 2006a). Furthermore, the clear involvement of “ovarian” aromatase in the processes of reproduction and sexual differentiation in fish makes this transgenic zebrafish line a powerful model for studies onto these physiological processes and their perturbations by EDCs. Currently, at OECD, several test guidelines for screening and testing of endocrine active substances have been adapted in fish to detect compounds acting as aromatase inhibitors (Test Guideline 229/230) and affecting the sex development of the gonads and/or the sex-ratio (Test Guideline 234). The use of *cyp19a1a*-eGFP line for these assays should allow the relative quantification of GFP in gonads through image analysis thus providing additional mechanistic insight on aromatase that are currently lacking in OECD test guidelines. To do so, it would however be advisable to (i) evaluate the sensitivity of the *cyp19a1a*-eGFP zebrafish line to estrogens and also to other EDCs such as aromatase modulating compounds to refine actual mechanistic based assays, and (ii) improve the *in vivo* measurement of GFP in developing and adult fish in a non-invasive way to assess the time-dependent effect of chemicals on GFP without increasing the number of fish. This could be notably achieved by cross-breeding our transgenic fish with the casper mutant zebrafish that do not develop pigments (White et al., 2008). Altogether, the time- and concentration-dependent measurement of GFP in live transgenic *cyp19a1a*-eGFP together with other “classical” endpoints (e.g., vitellogenin concentrations, gonadal histology) would usefully bring complementary comprehensive data for the refinement of Adverse Outcome Pathways (AOPs) and the development of quantitative AOPs thereby increasing the relevance of such model and bioassays for advanced assessment of EDCs in fish.

5. Conclusions

In the present study, we successfully established a stable transgenic zebrafish line expressing GFP under the control of zebrafish *cyp19a1a* gene promoter. In the *cyp19a1a*-eGFP zebrafish line, GFP is expressed, localized and down-regulated by estradiol in the same way as *cyp19a1a* gene in wild type zebrafish showing that GFP can be used reliably to monitor *cyp19a1a* expression. In the *cyp19a1a*-eGFP transgenic line, our results confirm that *cyp19a1a* is expressed early during development, before the histological differentiation of the gonads, and that the down-regulation of *cyp19a1a* expression is likely responsible for testicular differentiation. Moreover, we show that although *cyp19a1a* expression exhibits a clear dimorphic expression pattern in gonads during sexual differentiation, its expression persists whatever the sex suggesting that estradiol synthesis is important for gonadal development including for testis where the expression was low but significant. In the end, monitoring the expression of GFP in control and exposed-fish will help determine the sensitivity of this transgenic line to EDCs and its relevance to refine mechanistic based-assays for the study of EDCs. *In fine*, this transgenic zebrafish line is a useful tool to study physiological processes such as reproduction and sexual differentiation, and their potential perturbations by EDCs.

Declaration of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SUPPLEMENTARY MATERIALS

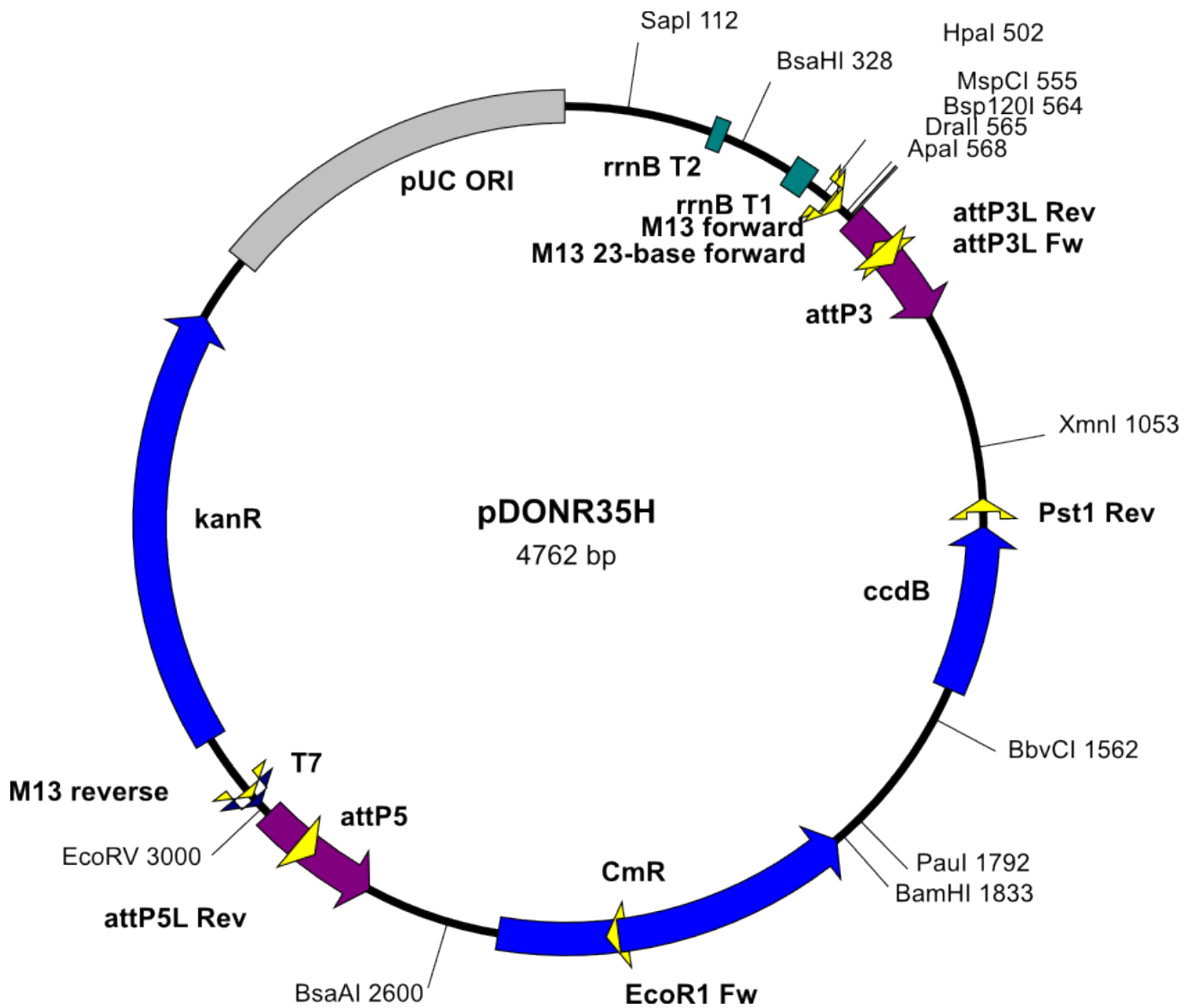
Supplemental Table 1: Sequence of the primers used to clone and verify the promoter of Cyp19a1a. The underlined sequences in Cyp19a1a_B3 and Cyp19a1a_B5 primers correspond respectively to the attB3 and attB5 sites inserted during the second round of nested PCR. Those recombination sites are necessary for the cloning by Gateway(R) recombination.

Used for	Primer name	Sequence
First round of nested PCR	Cyp19a1a_Fwd	5'-AAGGGCATCTGCTGCGTAAA
	Cyp19a1a_Rev	5'-GTGGAATGAAGCCGGTGCCT
Second round of nested PCR	Cyp19a1a_B3	5'- <u>GGGGACAAGTTTGTATAATAAAGTAGGCTCCTTTCTGATCAGGAGCAGA</u>
	Cyp19a1a_B5	5'- <u>GGGGACCACTTTGTATACAAAAGTTGGGT</u> CCTGCCATAAGAACGGATTA
Sequencing primers	24-3-a_rev	5'-GGTGGAGGTTTGTTCATT
	24-3-b_fwd	5'-CGAGACAAATCCAGAAAAG
	24-3-c_rev	5'-TCTCCCTTTTAAACAATACGC
	24-3-d_fwd	5'-TACAGTATGCAAGATAAGCC
	24-3-e_rev	5'-TATTACCCGATGTACCACA
	24-3-f_fwd	5'-AGCTTTTGTGCTCTCTGT
	24-3-g_rev	5'-TACAACGAACTCACCAAG
	24-3-h_fwd	5'-TCCGCCCTGTGTATTTCT
	24-3-i_rev	5'-TATGCTGTGAAGTGGACC
	24-3-j_fwd	5'-TGGAGGACAAAGGGGAAA
	24-3-k_rev	5'-GATATTAGACATCTGCGGC
24-3-l_fwd	5'-GTGAGCAGCTTGTGAATG	

Supplemental figure 1

Name pDONR35H
 Type Donor Vector
 Size 4762 bp
 Origine Roure et al, (2007) PLoS ONE 2(9): e916.

Selectable makers Kanamycin – chloramphenicol
 Bacterial strain DB3.1(TM) or any [gyrA462]
 Comments listed as 'pDONR-221-P3/P5' in Roure et al.



The elements found are detailed below

name : rrnB T2 terminator of transcription
type : Terminator of transcription
subtype : E. coli
origin : ND
start : 268
end : 295
complementary (yes/no) : no
comments : Stops transcription in procaryotes

name : rrnB T1 terminator of transcription
type : Terminator of transcription
subtype : E. coli
origin : ND
start : 427
end : 470
complementary (yes/no) : no
comments : Stops transcription in procaryotes

name : bacteriophage M13 -40 forward primer
type : Binding site
subtype : Primer
origin : bacteriophage M13
start : 517
end : 533
complementary (yes/no) : no
comments : Sequencing and PCR primer

name : bacteriophage M13 23-base forward primer
type : Binding site
subtype : Primer
origin : bacteriophage M13
start : 522
end : 544
complementary (yes/no) : no
comments : Sequencing and PCR primer

name : attP3
type : Recombinaison binding site
subtype : Gateway
origin : ND
start : 570
end : 801
complementary (yes/no) : no
comments : None

name : attP3L Rev
type : Binding site
subtype : Primer
origin : ND
start : 631
end : 671
complementary (yes/no) : yes
comments : primer from PLOS One paper of A. roure

name : attP3L Fw
type : Binding site
subtype : Primer
origin : ND
start : 645
end : 683
complementary (yes/no) : no
comments : primer from PLOS One paper of A. roure

name : PstI Rev
type : Binding site
subtype : Primer
origin : ND
start : 1146
end : 1171
complementary (yes/no) : yes
comments : primer from PLOS One paper of A. roure

name : ccdB gene
type : Selectable marker
subtype : Metabolism
origin : ND
start : 1197
end : 1502
complementary (yes/no) : yes
comments : toxic in E. coli which do not carry the mutation gyrA462

name : chloramphenicol acetyl transferase
type : Selectable marker
subtype : Antibiotic
origin : ND
start : 1847
end : 2506
complementary (yes/no) : yes
comments : None

name : EcoRI Fw
type : Binding site
subtype : Primer
origin : ND
start : 2286
end : 2309
complementary (yes/no) : no
comments : primer from PLOS One paper of A. roure

name : attP5
type : Recombinaison binding site
subtype : Gateway
origin : ND
start : 2754
end : 2985
complementary (yes/no) : yes
comments : None

name : attP5L Rev
type : Binding site
subtype : Primer
origin : ND
start : 2872
end : 2910
complementary (yes/no) : yes
comments : primer from PLOS One paper of A. roure

name : bacteriophage T7 promoter
type : Promoter
subtype : Procaryotic
origin : bacteriophage T7
start : 3006
end : 3022
complementary (yes/no) : yes
comments : promoter for in vitro transcription.

name : bacteriophage M13 reverse primer
type : Binding site
subtype : Primer
origin : bacteriophage M13
start : 3027
end : 3043
complementary (yes/no) : yes
comments : Sequencing and PCR primer

name : kanamycin resistance gene
type : Selectable marker
subtype : Antibiotic
origin : ND
start : 3156
end : 3965
complementary (yes/no) : no
comments : Genetically engineered. Allows resistance to kanamycin in
procaryotes

name : E. coli pBR322 origin of replication
type : Origin of replication
subtype : E. coli
origin : E. coli
start : 4086
end : 4759
complementary (yes/no) : no
comments : Necessary for DNA replication in E. coli

name : pUC origin of replication
type : Origin of replication
subtype : E. coli
origin : ND
start : 4089
end : 4732
complementary (yes/no) : no
comments : exclusion group : pMB1/ColE1

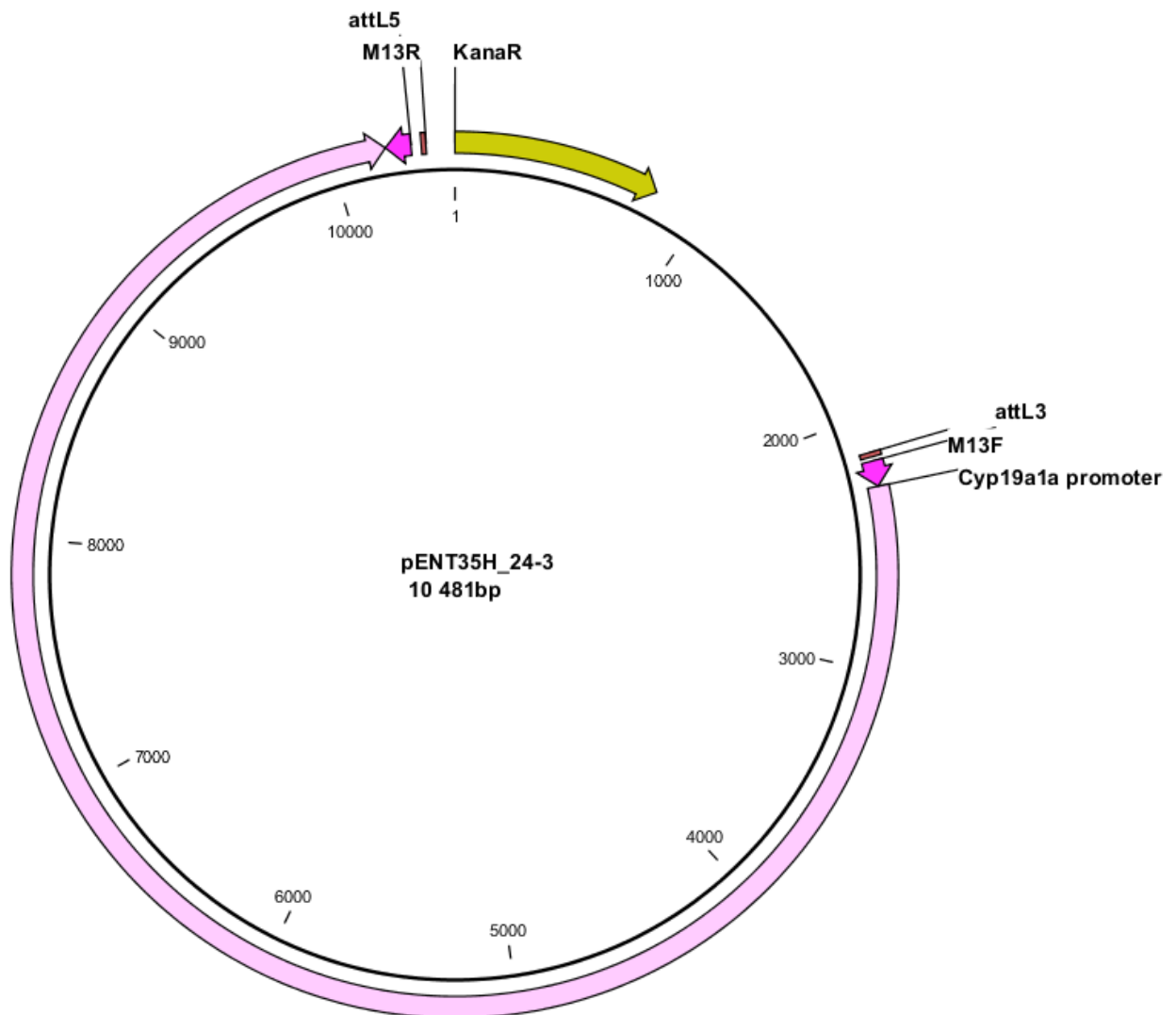
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Supplemental figure 2

Name pENT35H(24-3)
Type Entry Clone
Size 10 481 bp
Origine BP cloning : pDONR35H x PCR (primers Cyp19a1a_B3 and Cyp19a1a_B5)

Selectable makers Kanamycin
Bacterial strain DH5alpha
Comments



pENT35H_24-3 map

Features

name : kanamycin resistance gene
type : Selectable marker
subtype : Antibiotic
origin : ND
start : 1
end : 810
complementary : no
comments : Genetically engineered. Allows
resistance to
kanamycin in procaryotes

name : bacteriophage M13 forward primer
type : Binding site
subtype : Primer
origin : bacteriophage M13
start : 2143
end : 2160
complementary : no
comments : Sequencing and PCR primer

name : attL3
type : Recombination site
subtype : Gateway
origin : ND
start : 2177
end : 2276
complementary : no
comments : none

name : cyp19a1a promoter
type : Promoter
subtype : Insert
origin : Danio rerio
start : 2277
end : 10212
complementary : no
comments : none

name : attL5
type : recombination site
subtype : Gateway
origin : ND
start : 10213
end : 10311
complementary : yes
comments : none

name : bacteriophage M13 reverse primer
type : Binding site
subtype : Primer
origin : bacteriophage M13
start : 10352
end : 10369
complementary : yes
comments : Sequencing and PCR primer

Fasta sequence

>pENT35H_24-3

```
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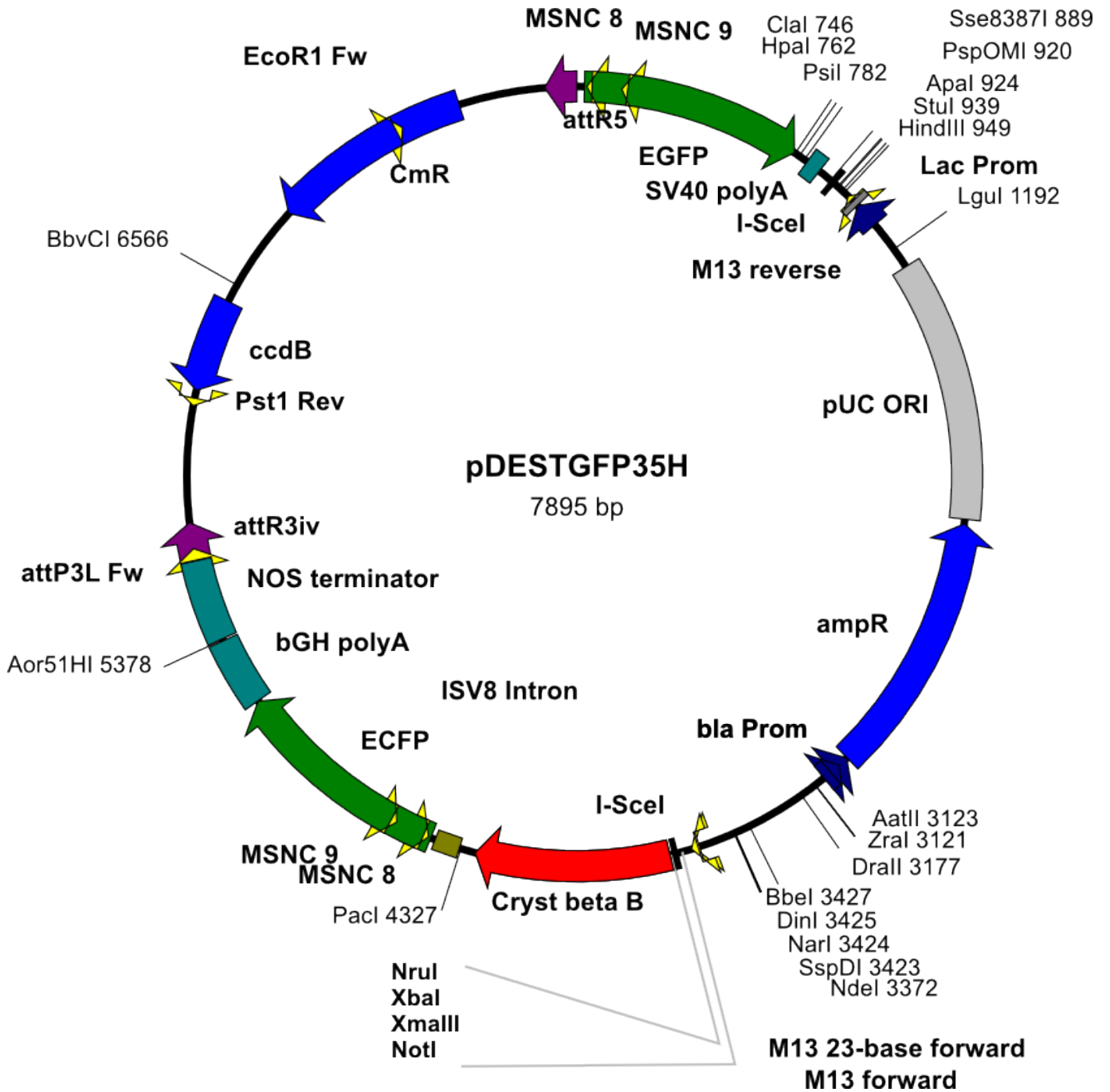
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Supplemental figure 3

Name pDESTGFP35H
 Type Destination Vector
 Size 7895 bp
 Origine Genescript + pDONR35H regeneration

Selectable makers Ampicillin – chloramphenicol
 Bacterial strain DB3.1(TM) or any [gyrA462]
 Comments



The elements found are detailed below

name : Aequorea victoria Enhanced Green Fluorescent Protein
type : Open Reading Frame
subtype : NA
origin : Aequorea victoria
start : 24
end : 743
complementary (yes/no) : no
comments : Genetically Engineered. also known as GFP-mut1.
Excitation maximum = 488 nm; emission maximum = 507 nm.

name : RP-GFP-1
type : Binding site
subtype : Primer
origin : ND
start : 32
end : 49
complementary (yes/no) : yes
comments : Tm=65.5. Orientation : Antisense. Target : cDNA of the EGFP.

name : RP-GFP-2
type : Binding site
subtype : Primer
origin : ND
start : 145
end : 164
complementary (yes/no) : yes
comments : Tm=63.9. Orientation : Antisense. Target : cDNA of the EGFP.

name : SV40 early mRNA polyadenylation signal
type : Terminator of transcription
subtype : polyA
origin : SV40
start : 793
end : 843
complementary (yes/no) : no
comments : None

name : S. cerevisiae I-SceI
type : Recombinasion binding site
subtype : Meganuclease
origin : S. cerevisiae
start : 891
end : 908
complementary (yes/no) : yes
comments : None

name : bacteriophage M13 reverse primer
type : Binding site
subtype : Primer
origin : bacteriophage M13
start : 966
end : 982
complementary (yes/no) : yes
comments : Sequencing and PCR primer

name : Lactose operator
type : Operator

subtype : Transcription
origin : ND
start : 988
end : 1008
complementary (yes/no) : yes
comments : None

name : Lactose promoter
type : Promoter
subtype : Procaryotic
origin : ND
start : 1009
end : 1092
complementary (yes/no) : yes
comments : drive the transcription in procaryotic cells

name : ColE1 origin of replication
type : Origin of replication
subtype : E. coli
origin : ColE1
start : 1260
end : 2112
complementary (yes/no) : yes
comments : Origin of replication of the plasmid ColE1

name : E. coli pBR322 origin of replication
type : Origin of replication
subtype : E. coli
origin : E. coli
start : 1309
end : 1982
complementary (yes/no) : yes
comments : Necessary for DNA replication in E. coli

name : pUC origin of replication
type : Origin of replication
subtype : E. coli
origin : ND
start : 1336
end : 1979
complementary (yes/no) : yes
comments : exclusion group : pMB1/ColE1

name : beta-lactamase
type : Selectable marker
subtype : Antibiotic
origin : ND
start : 2127
end : 2987
complementary (yes/no) : yes
comments : None

name : beta-lactamase promoter
type : Promoter
subtype : Procaryotic
origin : ND
start : 2988
end : 3086
complementary (yes/no) : yes
comments : drive the transcription in procaryotic cells

```

name          : beta-lactamase promoter
  type        : Promoter
  subtype     : Procaryotic
  origin      : ND
  start       : 3022
  end         : 3057
  complementary (yes/no) : yes
  comments    : drive the transcription in procaryotic cells

name          : bacteriophage M13 -40 forward primer
  type        : Binding site
  subtype     : Primer
  origin      : bacteriophage M13
  start       : 3546
  end         : 3562
  complementary (yes/no) : no
  comments    : Sequencing and PCR primer

name          : bacteriophage M13 23-base forward primer
  type        : Binding site
  subtype     : Primer
  origin      : bacteriophage M13
  start       : 3551
  end         : 3573
  complementary (yes/no) : no
  comments    : Sequencing and PCR primer

name          : Multiple Cloning Site
  type        : MCS
  subtype     : NA
  origin      : ND
  start       : 3604
  end         : 3639
  complementary (yes/no) : no
  comments    : The sites are :
                3604 : NruI
                3613 : XbaI
                3639 : XmaIII, NotI

name          : S. cerevisiae I-SceI
  type        : Recombinaison binding site
  subtype     : Meganuclease
  origin      : S. cerevisiae
  start       : 3619
  end         : 3636
  complementary (yes/no) : no
  comments    : None

name          : Danio rerio Crystallin Beta B promoter
  type        : Promoter
  subtype     : Vertebrate
  origin      : Danio rerio
  start       : 3645
  end         : 4279
  complementary (yes/no) : no
  comments    : zebrafish Crystallin beta B promoter from zebrafish
  genomic DNA using Forward primer: CbBFA: AGAGCTCGAGCAATTGGTGCTATCATCATGACAAAGAG and
  Reverse primer: CbBR: AGAGAGTTTAAACTACAGCTGTGCTGGGAAGGCTG

name          : synthetic IVS8 intron

```

type : Intron
subtype : NA
origin : synthetic
start : 4332
end : 4415
complementary (yes/no) : no
comments : Derived from pGene/V5-HisA(Invitrogen)

name : Aequorea victoria Enhanced Cyan Fluorescent Protein
type : Open Reading Frame
subtype : NA
origin : Aequorea victoria
start : 4430
end : 5149
complementary (yes/no) : no
comments : Genetically engineered. Cyan derivative of the classical GFP. Humanized. excitation max = 433 nm (+ minor peak = 453 nm); emission max = 475 nm (+ minor peak = 501 nm). Sequence origin : Parinov & Emylanov

name : RP-GFP-1
type : Binding site
subtype : Primer
origin : ND
start : 4438
end : 4455
complementary (yes/no) : yes
comments : Tm=65.5. Orientation : Antisense. Target : cDNA of the EGFP.

name : RP-GFP-2
type : Binding site
subtype : Primer
origin : ND
start : 4551
end : 4570
complementary (yes/no) : yes
comments : Tm=63.9. Orientation : Antisense. Target : cDNA of the EGFP.

name : Bovine growth hormone polyadenylation signal
type : Terminator of transcription
subtype : polyA
origin : Bovine
start : 5150
end : 5374
complementary (yes/no) : no
comments : None

name : Agrobacterium tumefaciens nopaline synthase terminator of transcription
type : Terminator of transcription
subtype : Bacterial
origin : Agrobacterium tumefaciens
start : 5381
end : 5647
complementary (yes/no) : no
comments : None

name : attR3iv
type : Recombinasion binding site
subtype : Gateway

origin : ND
start : 5649
end : 5772
complementary (yes/no) : no
comments : None

name : attP3L Fw
type : Binding site
subtype : Primer
origin : ND
start : 5649
end : 5687
complementary (yes/no) : no
comments : primer from PLOS One paper of A. roure

name : Pst1 Rev
type : Binding site
subtype : Primer
origin : ND
start : 6150
end : 6175
complementary (yes/no) : yes
comments : primer from PLOS One paper of A. roure

name : ccdB gene
type : Selectable marker
subtype : Metabolism
origin : ND
start : 6201
end : 6506
complementary (yes/no) : yes
comments : toxic in E. coli which do not carry the mutation gyrA462

name : chloramphenicol acetyl transferase
type : Selectable marker
subtype : Antibiotic
origin : ND
start : 6851
end : 7510
complementary (yes/no) : yes
comments : None

name : EcoR1 Fw
type : Binding site
subtype : Primer
origin : ND
start : 7290
end : 7313
complementary (yes/no) : no
comments : primer from PLOS One paper of A. roure

name : attR5
type : Recombinaison binding site
subtype : Gateway
origin : ND
start : 7791
end : 19
complementary (yes/no) : yes
comments : None

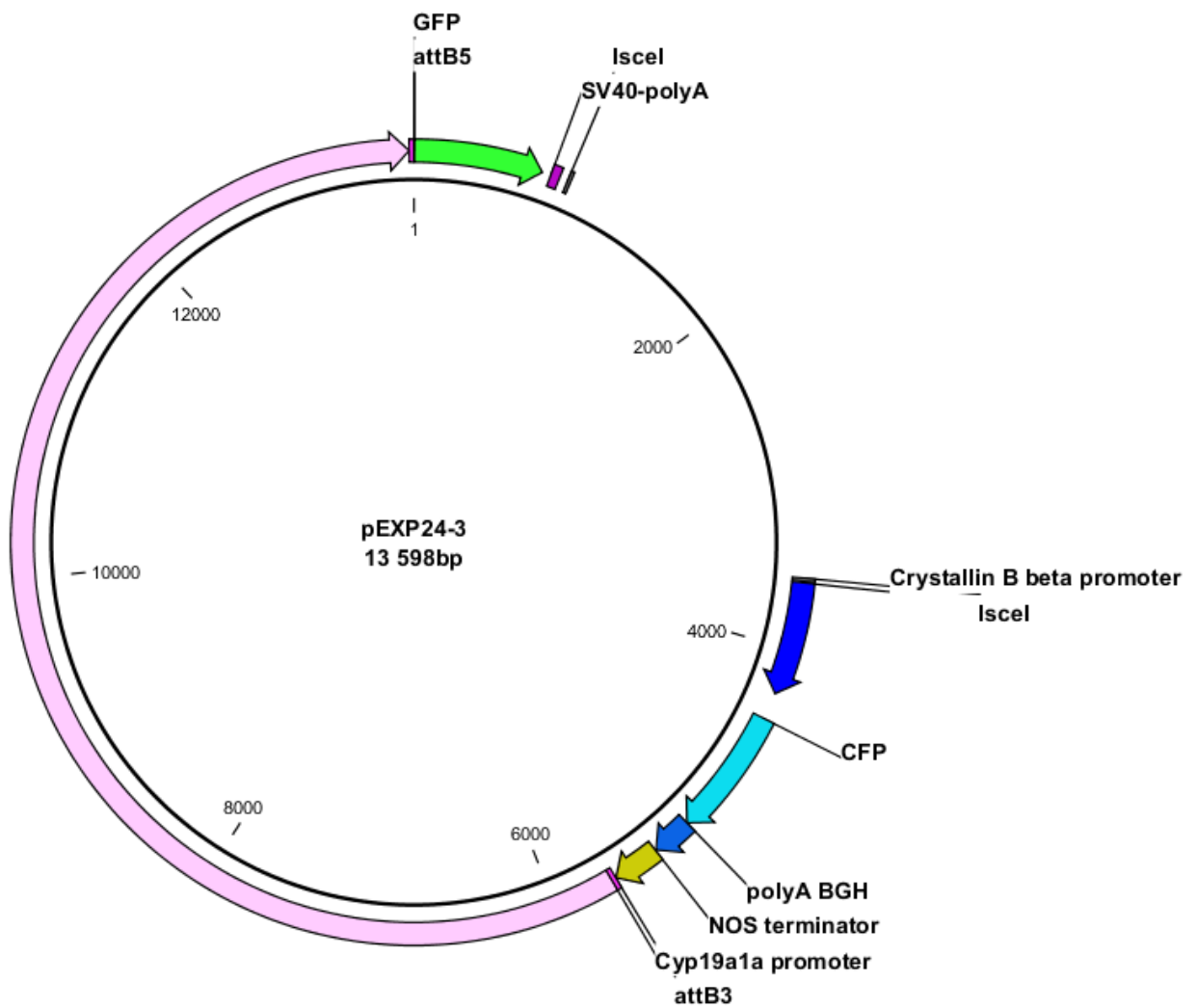
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Supplemental figure 4

Name pEXP35H(24-3)
 Type Expression Vector
 Size 13 598 bp
 Origine LR cloning : pENTR35H (24-3) x pDESTGFP35H

Selectable makers Ampicillin
 Bacterial strain Top10
 Comments Zebrafish selectable marker : expression of CFP in the lens from 48 hours onward



pEXP35H 24-3 map

Features

name : Enhanced Green Fluorescent Protein
type : Open Reading Frame
subtype : NA
origin : Aequorea victoria
start : 4
end : 723
complementary : no
comments : genetically Engineered. also known as GFP-mut1. Excitation maximum = 488 nm; emission maximum = 507 nm.

name : polyA SV40
type : terminator of transcription
subtype : polyadenylation signal
origin : SV40 virus
start : 773
end : 823
complementary : no
comments : none

name : IsceI
type : cleavage site
subtype : meganuclease
origin : S. cerevisiae
start : 871
end : 888
complementary : yes
comments : none

name : IsceI
type : cleavage site
subtype : meganuclease
origin : S. cerevisiae
start : 3599
end : 3616
complementary : no
comments : none

name : beta crystallin promoter
type : promoter
subtype : NA
origin : Danio rerio
start : 3625
end : 4259
complementary : no
comments : zebrafish Crystallin beta B promoter from zebrafish genomic DNA using Forward primer: CbBFA: AGAGCTCGAGCAATTGGTGCTATCATCATGACAAAGAG and Reverse primer: CbBR: AGAGAGTTTAAACTACAGCTGTGCTGGGAAGGCTG

name : Cyan Fluorescent Protein
type : Open Reading Frame
subtype : NA
origin : Aequorea victoria
start : 4410
end : 5129
complementary : no

comments : genetically engineered. Cyan derivative of
the classical GFP.
excitation max = 433 nm (+ minor peak = 453 nm);
emission max = 475 nm (+ minor peak = 501 nm).
Sequence origin : Parinov & Emylanov

name : NOS (nopaline synthetase) terminator
type : terminator of transcription
subtype : NA
origin : Agrobacterium tumefaciens
start : 5361
end : 5626
complementary : no
comments : none

name : attB3
type : recombination site
subtype : Gateway
origin : ND
start : 5628
end : 5652
complementary : no
comments : none

name : cyp19ala promoter
type : promoter
subtype : insert
origin : Danio rerio
start : 5653
end : 13573
complementary : no
comments : none

name : attB5
type : recombination site
subtype : Gateway
origin : ND
start : 13574
end : 13598
complementary : yes
comments : none

Fasta sequence

```
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ACTTTTGTATACAAAGTGGT

Supplemental figure 5: Specificity controls of anti-GFP and anti-Cyp19a1a antibodies to zebrafish gonads. GFP (green, A-D) protein was immunolocalized both in testis (Left) and ovary (Right) of mature zebrafish. A: testis of a cyp19a1a-GFP transgenic zebrafish showing a labeling in Leydig and germ cells. B: ovary of a cyp19a1a-GFP transgenic zebrafish showing labeling in peri-follicular cells. C: testis of a wild type zebrafish showing a very slight green fluorescence background. D: ovary of a wild type zebrafish showing no labeling. Cyp19a1a (E-H) protein was immunolocalized in testis (Left) and ovary (Right) of zebrafish. Cyp19a1a specific labeling is detected in the Leydig and germ cells of the testis (E) and in the peri-follicular cells of the oocytes (F). When this anti-Cyp19a1a antibody is preincubated with the synthetic peptide (100 µg/ml), no labelling is observed in the testis (G) and only a slight unspecific labeling is observed in ooplasm of oocytes (H). Without any primary antibody (secondary antibody: AlexaFluor® 594), no labeling was observed neither in the testis (I) nor in the ovary (J). Nuclei are in blue. Imaging was realized with a Zeiss AxioImager Z1 fluorescence microscope equipped with an MRm5 camera combined with an ApoTome. Acquisition parameters were the same for all GFP photomicrographs and for all Cyp19a1a photomicrographs. Scale bars: 20 µm.

