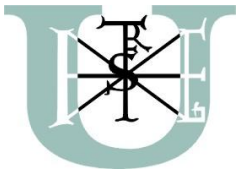

SZENT ISTVÁN UNIVERSITY

Establishment of a biomarker zebrafish line for the detection of estrogenic substances

PhD thesis

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1 Antecedents and objectives

1.1 Antecedents

The hormone system has long been a major focus of interest. Besides the complex molecules of the body a number of environmental substances may also have hormonal effects including estrogenicity. Chemical structure of estrogenic substances is highly diverse, so estrogenicity can hardly be predicted on the basis of structure. In addition, environmental samples might be extremely complex and other compounds of the sample might also affect overall estrogenicity of a substance. The full estrogenic impact of an environmental sample can thus only be revealed by a complex series of *in vitro* and *in vivo* ecotoxicological tests.

Several cell line and yeast (*Saccharomyces cerevisiae*) based screens have been developed recently, however they are usually only able to detect the binding to the estrogen receptor, but do not provide information on alternative routes of hormonal action. Moreover, none of them is able to model the complex physiological processes of the body, or to detect the hormone sensitive life stages, so they often lead to false results.

It is well known that some genes are sensitive to estrogens. The gene product can be detected by molecular biological tools at the protein and *mRNA* level too, but usually involves the sacrifice of the model. As animal protection laws are becoming stricter, threre's an increasing demand for alternative test systems to which the number and suffering of experimental animals can be minimized or animal model systems may be replaced. The development of transgene technologies and the discovery of fluorescent proteins paved the way towards the establishment of biomarker lines. Such lines enable to test eg. the activation of estrogen-sensitive genes *in vivo*.

Among vertebrates the role of fish models in environmental risk assessment is outstanding. Compared to mammalian models fish offer many advantages: as aquatic animals they are able to absorb pollutants through the entire body, they produce a large number of offspring and the generation interval of some species is short. Their endocrine system and physiological processes show a high similarity to other vertebrates, and even mammals so humans as well. Moreover ecotoxicological tests conducted on fish also have economic benefits. Fish play an increasingly

important role in human nutrition, and so the health status of farmed fish as well, which may also be forecasted by fish experiments. In addition, current animal welfare regulations do not consider fish as living animals until the free feeding age, so experiments carried out on them are not regarded as animal experiments.

In fish, several genes proved to be suitable for the detection of estrogenicity. Among these estrogen receptor, aromatase-b, choriogenin-H and vitellogenin (*vtg*) are the most significant. Recently, a number of estrogen biosensor fish lines have been developed from laboratory fish models, such as medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). Transgenic fish models are significant in studying estrogenic actions, but lines established up to the start of my PhD work have not been sensitive enough to detect environmental estrogen concentrations.

The main advantage of zebrafish in terms of creating biosensor lines is that the embryos and larvae are transparent, therefore, the signal of the fluorescent reporter is easy to be monitored *in vivo*, without killing the animal. In addition to the animal welfare aspects it is also a valuable feature as it allows to study the response of the same individual at different times of the treatment. Another important benefit is that recently the entire zebrafish genome sequence has been revealed which facilitates the design of transgene constructs, and a number of techniques have been developed for the transgenesis of the species.

1.2 Objectives

The main objective of my work was to develop a transgenic zebrafish model for the quantitative detection of estrogens and to establish the appropriate experimental protocol. In zebrafish, several estrogen inducible genes are known that would be suitable for the development of such lines. At the onset of the work, only estrogen receptor activation detecting reporter lines were known. The advantage, of vitellogenin transgenic lines, compared to these, is that the expression of the transgene is tissue (liver) -specific, and there is virtually very low or no detectable basic expression in embryos, larvae, juveniles and mature males. Liver is one of the key toxicological target organs, therefore the line, besides being a biomarker, may be suitable for the *in vivo* detection of adverse effects of contaminants on the liver, without sacrificing the animal. As in mature females due to the endogenous estrogen the fluorescent signal is activated under normal conditions too, they enable to study hepatotoxic effects of non-estrogenic compounds. Zebrafish have 8 vtg genes from which vtg-1 shows the strongest expression. Therefore, the promoter region of vtg-1 and a red fluorescent protein, the mCherry was selected for the establishment of the transgenic line.

In addition to the transgenic line, the impact of zearalenone on the development and vitellogenin production of fish was also studied on wild-type zebrafish.

Zearalenone is the only known estrogenic mycotoxin, which is mainly detected in animal feed and cereal foodstuffs, but can also be found in soil and water. Its occurence and effects have been studied extensively on of farmed animals and *in vitro* test systems, however, information is relatively scarce on the biological effects on fish. Besides natural populations the toxin may also pose a threat to economically important fish species (eg carp) through zearalenone contaminated water and cereals so results may also be valuable for fish farms in the future.

2 Materials and methods

2.1 Fish maintenance

All experiments were performed on a laboratory-bred strain (AB) of zebrafish (*Danio rerio).* Prior to treatments fish were maintained in a special zebrafish recirculation system (ZebTEC, Tecniplast) under standard laboratory conditions (at 25.5°C with 14 hour light-10 hour dark cycle). Adults were fed twice a day with complete fish food (zebrafish basic food, Special Diets Services /SDS/) supplemented with freshly hatched live *Artemia nauplii* twice a week. Larvae after the free swimming age were fed three times a day with fish food, supplemented with *Artemia* from 10 dpf (days post fertilization).

Fish were placed in breeding tanks (Tecniplast) in the afternoon the day before the experiment and eggs were spawn synchronously the next morning. One hour later eggs were harvested and normally developing embryos were selected under a microscope.

2.2 Establishment of the vtg1-mCherry transgene construct

The putative transcription start site was identified upstream the vtg-1 coding sequence by PROSCAN 1.7. Then the upstream sequence was screened for elements needed for the estrogen sensitivity of the construct (EREs - Estrogen Response Elements) by DRAGON ERE Finder and ALGEN PROMO MATINSPECTOR softwares, and manual sequence analysis.

Primers were designed, fragments for the gene consturct were amplified and recombination sequences were incorporated to the 5' ends by PCR. 4082 and 3357 bp fragments harbouring the putative promoter region upstream the vtg-1 the transcription start site were amplified from zebrafish genomic DNA, while for the amplification of the fluorescent protein encoding sequence, the pCS2 mCherry plasmid was used.

PCR products containing different *attB* recombination sites (vtg-1 promoter – *attB3*, *attB5*, mCherryt – *attB1*, *attB2*) were cloned into separate plasmids (pDONR221-P1P2 and pDONR221-335) by site-specific recombination (*BP* reaction). Sequences were then cloned into the final vector (pSP17.2BSSPE-R3-R5-RFA-Venus Tol2LR) harbouring *Tol2* arms by *LR* cloning. Plasmids were propagated in XL10 GOLD *Escherichia coli* competent cells and were pre-screened by colony PCR. Incorporation of the desired sequences were checked in purified plasmids

by restriction digestion using *RsaI*, *PstI* + *XbaI* and *HindIII* + *EcoRV* enzymes.

2.3 Establishment of the *vtg1-mCherry* transgenic line

For efficient transposition, the construct was linearized. Transposase mRNA was synthetized from the encoding plasmid by SP6 RNA-polymerase. The construct (25 ng) was co-introduced with the transposase mRNA (25 ng) into the yolk of 1-2 cell stage zebrafish embryos.

Transient expression of the reporter gene was examined in 5 and 10 dpf embryos following 100 ng/L 17-ß-estradiol (E2) under a fluorescent microscope using RFP filter.

Some untreated, unselected injected embryos were raised until fertility and crossed to wild-type zebrafish. The so-called P0 founders, transmitting the gene construct were selected by fluorescent microscopy and mCherry specific PCR of estrogen treated offspring.

Offspring of founders found positive by the PCR were raised and so F1 generation of the *vtg1-mCherry* line was estbalished. F2 was generated by mating F1 individulas with wild-type fish. F2 were then crossed again with wild-type individuals. F4 generation was established by intra- and intergenerational crossing of F2 and F3 “transgene carriers”. The above mentionned microscopic examination was carried out in every generation while prior to the establishment of F4, PCR-based selection was also used.

2.4 Examination of mCherry induction

The start of mCherry induction was studied daily in 500 embryos exposed to 200 ng/L 17-α-ethinyl-estradiol (EE2) from 0 dpf, under a fluorescent microscope at different magnifications.

2.5 Investigation of mCherry inducibility by estrogenic substances

Transmission of the transgene in the F1 and F2 was checked by 100 ng/L EE2 treatment (30-70 embryos/treatment). In F3 and F4 embryos, fluorescent protein induction was tested by several concentrations of other estrogenic compounds too. The exposure was carried out from 3 to 5 dpf in three replicates on 50-100 embryos/concentration.

In F3 only E2, EE2 and zearalenone (ZEA) was tested in 50 and 100 ng/L, 25, 50, 100 ng/L and 50, 100, 250 µg/L concentrations. F4

embryos were exposed to E2 (200; 100; 50; 25; 12,5; 6,25; 0,1 μg/L), EE2 (200; 100; 50; 25; 12,5; 6,25; 3,125 ng/L), ZEA (500; 250; 100; 50; 10; 5; 1; 0,1 μg/L), bisphenol-A (BPA) (20; 10; 8; 6; 5; 2,5; 1; 0,1; 0,01 mg/L), nonylphenol (NP) (300, 150, 75 μg/L) and atrazine (ATR) (10000; 1000; 100; 50; 25; 25; 12,5; 6,25 μg/L). Treatments were carried out in petri-dishes, at 27,5 °C. The effects of solvents used for the stock solutions was tested at the solvent concentration of the highest test concentration.

Prior to microscopic analysis, embryos were anaesthetized in 0,02%-os MS-222 (Tricaine-methane-sulphonate) solution, then larvae were oriented (to the left side) in 0,5% methyl-cellulose. Bright field and fluorescent images were captured from all embryos in 30x magnification (exposure time: 1s, Leica M205 FA, Leica DFC 425C camera, LAS V3.8 software).

Signal in the red range of the RGB (Red, Green, Blue) colour range on the pictures was evaluated by the ImageJ software. An elliptical area of the same size was selected on all images and moved to the area of the liver, then the signal strength and the size of the affected area was determined. From the two values an integrated value (Integrated density) was generated and the induction was evaluated on the basis of these.

The estrogenicity (so-called relative estrogenic potency) of the tested chemicals was determined by comparing the integrated density derived EC_{50} of compounds to the EC_{50} of E2.

2.6 Testing the mCherry induction in males

Adult (3-month-old) males of the F1 generation were exposed to 5, 10, 50 and 100 ng/L E2. Treatments were carried out in two replicates in 3 liter tanks containing 15 individuals each. Induction of the fluorescent signal was checked under a fluorescent microscope. Individulas were anaesthetized in 0,02% MS-222 prior to the examination.

2.7 Vitellogenin „*whole mount" in situ* hibridization

Expression of the endogenous vtg gene was examined in wild-type and transgenic embryos. Wild type embryos (50) were treated from 0 to 5 dpf with 300 ng/L EE2, while transgenics (20) were exposed to 200 ng/L EE2 from 3 to 5 dpf.

In situ hibridization was carried out with a 545 bp probe designed for the *vtg mRNA.* Total RNA of estrogen treated embryos was reverse transcribed (to cDNA) and the probe sequence was amplified by PCR.

Purified PCR product was ligated to pCRII plasmid (Dual Promoter TA cloning Kit, Invitrogen), plasmids were propagated in TOP10 *E. coli* competent cells then colonies were selected by colony PCR. Carrier plasmids were linearized and DIG-labelled antisense RNA probe was generated by *in vitro* transcription (DIG RNA Labeling Kit SP6/T7, Roche). Hybridization was carried out according to the protocol developed for zebrafish embryos by Thisse and Thisse (2008).

2.8 Examination of the effects of zearalenone on ontogeny and vtg induction

Effects of zearalenone was studied on zebrafish embryos and adult males.

Seven sexually mature males were placed in each 3-liter tank containing 1000, 10, 0.1 μg/L ZEA. 0.1 μg/L 17-β-estradiol was used as positive control, and system water as negative control. Treatments were performed under semi-static conditions, in duplicates. During the 21-day period test solution was changed in every 4 days. Fish were fed once a day with fish food (SDS). Zearalenone concentrations were measured by HPLC following immunoaffinity enrichment (Food Analitika Ltd., Békéscsaba, Hungary).

Embryos were placed in 24-well plates (one embryo/well) at 1 hour post fertilization (hpf) containing ZEA test solutions of 1 ng/L, 0.1; 1; 5; 10; 25; 50; 100; 250; 500, 750, 1000, 1250, 1500, 1750, 2000 , 3000, 4000, 5000 μg/L. 0.1 μg/L 17-β-estradiol was used as positive control, and system water as negative control. All treatments were performed in 4 repliactes, using 1 embryo/replicate. Plates were incubated at 27,5 ^{o}C. All larvae were screened daily under a microscope for survival until 120 hpf for lethal endpoints and malformations. Digital images of embryos and larvae in lateral orientation were taken every day at 30x magnification.

To measure vtg induction, a whole body homogenate was prepared individually from adult males in PBS. Homogenates were then divided into two (one for RNA preparation and the other for ELISA).

In case of embryos, pools of 10 120 hpf larvae were collected for RNA isolation and real-time PCR.

The ELISA plate (Biosense) was read with Gene5 microplate reader (BioTek) at 492nm. For the evaluation, a dilution series of purified zebrafish Vitellogenin was used as standard. The concentration of the VTG in each sample was calculated with non-specific binding correction and regression analysis, performed by log-log transformation of the data according the manufacturer's recommendations.

vtg induction at the transcription level was measured by a duplex real-time PCR. ß-actin was used as a housekeeping gene. PCR reactions were carried out in a StepOne Plus real-time PCR equipment by using TaqMan probes. 3 dilutions (1x, 4x, 16x) were prepared from all samples and all dilutions were examined in 3 replicates. Ct values were determined and data were analyzed with Relative Expression Software Tool (REST).

2.9 Dose-response curves, statistical analysis

In case of ZEA, one-way analysis of variance (ANOVA) was used to test the effect of ZEA concentrations on the relative abundance of development defects, compared to the control. As variances accross groups were not equal, comparison of exposure groups was carried out by Dunnett's T3 post hoc test. On the basis of these no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were determined. Dose-response curves and EC_{50} values were calculated by Probit analysis (Minitab 16.1.1) and all graphs were plotted by Graphpad Prism 4.0.

In the course of integrated density evaluation, outliers were removed by the ROUT method and dose-response analysis was carried out following the log transformation and normalization of results. Results of ELISA vs. quantitative real-time PCR and larvae vs. adults were compared with Pearson's correlation, following normalization (presented as percentages). Sigmoidal dose-response curves were generated by non-linear regression and plotted with Graphpad Prism 4.0. EC_{10} and EC_{90} values were calculated according to the recommendations of GraphPad ($EC_F= (F/100-F)^{1/H}$ x EC_{50}) (F=effective concentration %, H=slope of the dose-response curve).

Induction results of ELISA and real-time PCR were compared with Pearson correlation following the normalization of results. Homogeneity of variance was tested with Levene test, prior to normalization.

3 Results

3.1 Establishment of an estrogen sensitive biomarker zebrafish line

The putative transcription start site of vtg-1 was successfully identified. On the amplified promoter sequences (4082 bp and 3357 bp) 15 and 10 ERE sites were identified with ALGEN PROMO software, 5 half ERE sites with MATINSPECTOR and one full sequence with DRAGON ERE Finder on both fragments. From sequences described in the literature 10 half ERE sites were found on the shorter and 11 on the longer promoter fragment. Elements identified by the different methods overlapped, and on the basis of these 17 ERE regions were located on the shorter and 19 on the longer sequence. Recombination cloning of the longer promoter region to the plasmid of the Gateway cloning system failed. However the shorter fragment harboured most of the identified EREs, it was likely that the construct would be functional with the shorter fragment too.

Vectors harbouring the vtg1 promoter and the mCherry coding region have successfully been generated by *BP* cloning from which fragments have been introduced to the vector with *Tol2* arms by *LR* reaction. Plasmids including both sequences became full transgene constructs, while some plasmids only harboured the fluorescent protein gene or the promoter region.

The transgene construct was microinjected to the yolk of zebrafish embryos. Following estrogen induction, the fluorescent signal appeared in 20% of the injected embryos. In addition to the fluorescence in the liver, the majority of embryos expressed strong transient fluorescent signal in the yolk, while in some embryos (<1%) weak expression was observed in the eye and the kidney.

Some injected embryos were raised without treatment until maturity, and the P0 generation of the transgenic line was successfully established.

To establish further generations, individuals transmitting the transgene needed to be identified. Therefore a construct-specific PCR reaction was developed, and 11 founders (4 milters and 7 spawners) were selected. Results of estrogen induction confirmed the PCR and proved the incorporation of the construct.

Fluorescent signal was detected in the liver of 0-20% of F1 embryos. Fluorescent protein expression in the kidney and eye region

disappeared, while in response to estrogen it was still detected in the yolk, suggesting that the signal is a result of transgene activity.

For the sake of maintaining a manageable number of fish, one founder (20 ♂) was selected on the basis of estrogen sensitivity. F2 generation was originated from 3 male and 3 female F1 offspring of this male. In F2, transgene integration was stable, and presumably all individuals had only a single copy of the transgene. F3 was established by crossing F2 individuals to wild-type fish. On the basis of estrogen induction, 11 males and 6 females were found to be sufficient for producing future generations.

Subsequently, transgenic parent pairs from which presumably the most sensitive, stable biomarker line(s) could be derived were selected. Initially, only F2 individuals were crossed in various combinations. Later F1 fish were also involved. Estrogen inducible fluorescent signal still appeared in the yolk of embryos in these generations too, their number varied from 5-100%. Therefore in the course of selection besides the fluorescent activity in the liver, signal of the yolk was also taken into account. On the basis of these, 3 pairs were selected: 203♂ x 202♀, 216♂ x 208♀ and 204♂ x 104♀. Although fluorescence in the yolk was relatively strong in case of the first pair, offspring proved to be highly senstitive to estrogen. Offspring of the other two pairs showed no unspecific fluorescent activity. Embryos from the same parents were raised and crossed *inter se* to create three F4 "sublines". Fish were hemizygous for the transgene until F3, as one of their parents was a wild-type fish. In F3 and F4, homozygous individuals were also present.

3.2 Examination of the expression of the fluorescent signal at different developmental stages

Initially embryos were treated with estrogen from 0 to 5 dpf. However liver development in zebrafish only starts at 6 hpf, and the functional organ is formed at 50 hpf. Results of this work proved that vitellogenin production begins in later stages. The fluorescent signal was induced from 2-3 dpf and was strong from 3 dpf. The size of the fluorescent area increased gradually along with liver development and reached the maximum at 5 dpf in the experimental period. Therefore the start of the exposure period was modified to 3 dpf.

3.3 Concentration dependency of the fluorescent signal

Vtg is normally expressed only in mature females, while in males the level is lowish or undetectable. As in larvae, vtg production can be triggered by estrogens in males too, and the effect can be enhanced in a concentration-dependent manner. The gene consruct works similarly. In the liver of untreated females mCherry production is so high that the signal can be observed through the pigmented skin, while in males the fluorescent signal is undetectable even after autopsy. Inducibility of the transgene was proved by E2 treatment of mature milters. mCherry production was detected after 4 days of exposure in the lowest concentration (5 ng/L) too.

Concentration dependency of the fluorescent signal in embryos was first examined in F3. Due to inhomogeneity (presumably different integration sites), results showed a large variation, so significant signal intensity differences were only observed in case of EE2, but the concentration-dependent response was assumed for all three materials (E2, EE2, ZEA). There was no fluorescent signal in the solvent control in either case.

Sensitivity of offspring of the three parent pairs selected for establishing the transgenic line was compared on the basis of EE2 exposure. Significant difference was only detected in 100 and 200 ng/L concentrations. The highest level of induction was observed in case of the 204 ♂x 104 ♀ pair, but the other two sublines also proved to be suitable for the detection of estrogenic effects.

Dose-dependency of the fluorescent response was studied in the F4 generation of the most sensitive 204 ♂ x 104 ♀ subline. The lowest effective concentration of mCherry expression was 0.1 mg/L for E2, 6.25 ng/L for EE2, 0,1 µg/L for ZEA and 1.25 mg/L for BPA. In case NP a very small area of the liver showed fluorescent protein expression in only a few embryos. ATR in this developmental stage had no effect on vtg levels.

On the basis of the fluorescent protein induction of the *vtg:*mCherry transgenic line BPA proved to be approx. 5000x weaker, ZEA was nearly equivalentl, while EE2 was an approx. 78x stronger estrogenic substance compared to natural estrogen.

3.4 *vtg in situ* hybridisation

Regardless of the estrogenic substance or its concentration it was found that the liver of some embryos showed weaker while others

stronger signal in all generations, and the same was true for the area concerned. In addition, the size of the area and strength of the signal showed no correlation.

Vitellogenin *in situ* hybridization showed that in case of both wild-type and transgenic embryos the gene was not induced in all individuals and there was a significant difference in sensitivity between embryos. In addition, *vtg* producing embryos also showed differences. Some embryos produced high level of *vtg* in the whole liver, while in others expression was weaker or involved smaller areas.

In situ hybridization images of transgenic embryos was compared to fluorescent images captured before fixation. Results clearly showed that the endogenous *vtg* production functioned similarly to the fluorescent protein in embryos showing weaker fluorescent signal or smaller area of expression.

Although 5 dpf zebrafish embryos had right liver lobes, the production of vtg at this stage was only observed in the left lobe.

3.5 Studies of an estrogenic mycotoxin

Studies on zearalenone started with the embryotoxicity test. Concentrations bellow 50 μg/L had no adverse effects during the exposure period. Severity of the adverse effects in higher concentrations varied depending on the duration of the treatment or the concentration used. Embryos hatched normally (to 3 dpf) bellow 1000 μg/L. Those that did not hatch in higher concentrations remained unhatched throughout the whole experiment.

Dose response curves for lethality were plotted for every 24 hours on the basis of the number of dead embryos, from which LC_{10} and LC_{50} values were calculated.

In addition to the general phenotypic changes of toxicity, two ZEA or estrogenic substance specific abnormalities were observed. Normally at the base of the prospective caudal fin there is a gap in the melanophore streak. In 72 hpf embryos exposed to 500 μg/L ZEA and above, this gap was missing and the caudal fin was thin and symmetrical.

In 48 hpf embryos from 500 μg/L and above a dorsal curvature of the body axis accompanied by abnormal heart and eye development was detected. The degree of curvature was clearly dose dependent from 72 hpf. These phenotypes, according to our previous findings, are typical for estrogenic substances at 5 dpf.

As an estrogenic substance, ZEA induced *vtg* expression in a concentration dependent manner. The induction was measured in mature

zebrafish milters by ELISA, and in larvae and milters by quantitative real-time PCR.

In the adult stage ZEA induced concentration dependent VTG induction in all exposure concentrations, however, significant difference compared to the control was only detected in the highest concentration (1000 μg/L). At the *mRNA* level, significant *vtg* induction was observed in the positive control and in 10 and 1000 μg/L ZEA concentrations.

Induction rates detected by ELISA and real-time PCR were compared and showed high level of statistically verifiable correlation (R^2= 0,9789, p= 0,0037). Hence, a pRT PCR can be used as alternative transcriptional method to protein analysis in measuring estrogenicity response to ZEA exposure.

In embryos, all concentrations except for the lowest (0.001 μg/L) were able to trigger the response of the biomarker as compared to the control. Statistical analysis revealed significant differences between the control and treatment groups above 5 μg/L. The highest relative mRNA level was measured in 250 μg/L, then the rate of induction started to decline. Based on the results of the embryo test 335.1 g/L ZEA concentration is required for 10% lethality. In concentrations higher than LC_{10}, cells of the embryos, such as liver cells are usually damaged, which probably caused the decline of VTG induction, so general toxicology standards suggest that molecular testing should be performed only at concentrations below LC_{10}. Data showed high degree of variation, which may be associated with the individual differences observed by *vtg in situ* hybridization.

A dose response curve was determined on the basis of results obtained for concentrations under LC_{10}.. The median effective concentration was 3.247 μg/L. The rate of induction observed in the positive control was shown to be between the response to 100 and 250 μg/L ZEA.

4 New scientific results

1. I established the first estrogen-sensitive zebrafish line in Europe suitable for the detection of vitellogenin induction, which carries the largest natural promoter region and is the most sensitive vitellogenin transgenic fish line.

2. I developed the recommended test protocol of the newly created transgenic line. Accoringly, treatment of embryos is sufficient to begin at 3 days post fertilization.

3. The *vtg1:*mCherry line enables the detection of estrogenic substances at environmental concentrations. Embryos are able to detect the etrogenicity of 100 ng/L 17-ß-estradiol and equivalents, while adult males are much more sensitive.

4. *In situ* hybridization showed that vitellogenin is only produced in the left hepatic lobe of 5 dpf zebrafish embryos. There is high sensitivity difference between embryos, but fluorescent protein expression of the *vtg1:*mCherry line is similar to the endogenous vitellogenin.

5. In the course of zearalenone exposure I found that ZEA triggers two typical developmental disorders in zebrafish embryos: the gap in the melanophore streak is missing at the base of the caudal fin, and a developmental phenotype reminiscent to the "*heart-and-soul*" mutants characterized by the dorsal curvature of the body axis is formed.

6. I found that zearalenone exposure induces vitellogenin expression in zebrafish embryos and adults as well. In adults induction rate at the protein and mRNA level correlate well, despite the difference in stability. Therefore quantitative real-time PCR could be a good substitute to ELISA measurements in screening the estrogenic potency of chemicals.

5 Conclusions and recommendations

5.1 Conclusions of the vtg1:mCherry transgenic line

The *vtg1:*mCherry line could give better estimation on the real estrogenicity of compounds, as it carries the longest natural promoter region among transgenics and lacks artificial ERE sequences. The incorporated 3357 bp promoter region carries altogether 26 ERE sequences, while on the 1700 bp promoter sequence of a previously developed vtg transgenic zebrafish line (*ere-zvtg1:gfp*) only 12 natural ERE sequences were found. Regions overlap, so the *vtg1:*mCherry construct harbours 17, while the *ere-zvtg1:gfp* line carries 8 ERE regions. The DRAGON ERE Finder software could identify a full, previously undescribed ERE sequence on the promoter of the *vtg1:*mCherry transgene construct, while in the *ere-zvtg1:gfp* construct, no EREs were found.

In vtg transgenic lines expression of the reporter is tissue-specific and restricted to the liver. The promoter is inactive in the lack of estrogenic effect, so these lines give a clear yes or no answer and a pretty good concentration-dependent response. Altough the response of the aromatase transgenic line (*cyp19a1b-GFP*) is tissue (brain) specific too, the transgene shows relatively high basic expression without estrogen induction, making it difficult to evaluate the results.

According to animal welfare issues tests should be performed in early developmental stages. Estrogen sensitivity of zebrafish is gradually increasing with the development of gonads, and the „receptor composition" also changes. In the liver of embryos, only ß2 receptor is present and cells are unable to produce vitellogenin. The liver of adult males express all three estrogen receptors (α, ß1, ß2) therefore are much more sensitive and probably able to detect some substances that are undetectable with larvae and embryos.

The fluorescent signal in *vtg1:*mCherry embryos become visible at 3 dpf. This is in accordance with previous findings that the liver of the embryo is able to produce vtg from 2-3 dpf. The lower the estrogen concentration is the later the gene product is expressed.

The *vtg1:*mCherry line is probably more sensitive than *ere-zvtg1:gfp* as it is able to detect E2 at very low concentrations (5 ng/l) after 4 days of exposure. Moreover, the line is likely to detect lower concentrations too, as the *ere-zvtg1:gfp* line only gave a response to the lowest concentration after 7 days of exposure.

Estrogenic substance concentrations detectable by *vtg1*:mCherry embryos are similar to that of *zvtg1:gfp* , except for ZEA, however in the latter case exposure was carried out from 7 to 20 dpf, when the number of estrogen receptors is higher than at 5 dpf. EC_{50} levels calculated from dose-response curves of the aromatase biosensor line are lower than the EC_{50} values of the new transgenic line. Treatments were carried out from 0 to 5 dpf. The difference is probably due to the different senitivity of the genes and the different number and type of estrogen receptors expressed in the two areas.

NP triggered response only in a few emryos of the *vtg1*:mCherry line, and the expression was restricted to a very small area in the liver. The *zvtg1:gfp* line did not respond to NP, while the aromatase transgenic line could detect the estrogenicity of a mixture of 4-nonyl-phenols with an EC_{50} of 406 nM. On the basis of my studies, ATR had no effect on the vtg levels at 5 dpf. ATR does not directly act through estrogen receptors, but induces the aromatse enzyme. As an effect estrogen is formed from testosterone, so vtg might be induced indirectly. Testosteron, as other sex hormones is normally not expressed in embryos, so ATR might trigger fluorescent protein induction in adult males.

According to „relative estrogenic potencies" EE2 proved to be 78x stronger than E2, ZEA was equivalent while the estrogenic potency of BPA was only about 5% compared to the reference. On the basis of literature EE2 seems to be about 16,48x more estrogenic than E2. The most striking result was the similar estrogenicity of E2 and ZEA. According to data described so far the estrogenic effect of ZEA compared to E2 varies in a very large range, and is 5,4-1439x weaker than E2. Presumably the affinity of ZEA to embryonic receptors is higher than in later stages.

In the *vtg1:*mCherry line the fluorescent signal is still not expressed in all 5 dpf embryos even in the 4th generation. The number of fluorescent embryos is substance and dose dependent. The same was observed in the *ere-zvtg1:gfp* and the *3pERE-TATA-Gal4ff* transgenic zebrafish lines. *Vtg in situ* hybridization showed that embryos producing weaker fluorescent signal expressed *vtg* at a lower lever too, in the same area. This phenomenon might be due to other factors affecting estrogen receptors. If these factors are genetic, the most sensitive genotypes can easily be selected and individual differences can be reduced.

Results of my PhD work suggest that sensitivity of the *vtg1:*mCherry line is in the range of environmentally relevant concentrations (detected in waters or effluents), so the line with the developed test protocol could be a sufficient tool in assessing the

estrogenicity of environmental samples. The new transgenic line in the future in addition to environmental toxicology tests may help in determining the onset of estrogen synthesis during sexual development and in investigating the molecular background of estrogenic effects and vitellogenin induction by morpholino injection or gene knockout. Females could also be subjects of examining androgens where the effect could be evaluated on the basis of the decrease in the strength of the fluorescent signal. Moreover, adverse effects of toxic substances on the liver may also be studied *in vivo*.

Conclusions of the zearalenone studies

Embryotoxicity tests revealed that LC_{10} and LC_{50} for ZEA exposure were about 10-1000x higher than environmental concentrations. Besides general phenotypic changes associated with toxicity, two ZEA or estrogenic substance specific abnormalities were observed. Dorsal curvature of the body axis was observed previously in response to other estrogenic substances too. The phenotype is reminiscent to the so-called *heart and soul (has)* mutants. The mutation affects the Par3/Par6/aPKC complex that can indirectly be associated with the curvature of the body axis. The same phenotype could be induced by genistein, which is an effective kinase inhibitor so I suggest that, the mycoestrogen ZEA might also act as a kinase inhibitor.

Normally at the base of the prospective caudal fin there is a gap in the melanophore streak. In embryos exposed to 500 μg/L ZEA and above, this gap was missing and the caudal fin was thin and symmetrical. The phenomenon has been observed in Shh (sonic hedgehog) loss-of-function mutants, and recently in response to methyl-mercury (MeHg) exposure. MeHg might induce the phenotype through the upregulation of tissue remodelling metalloproteases. MeHg also proved to be an endocrine disruptor, so its mode of action underlying these specific phenotypes might be the same as for ZEA.

Estrogenic potency of ZEA was examined through vtg induction. In adult males even the lowest concentration (0,1 μg/L) used could trigger vtg production, and in embryos the same concentration proved to be the lowest effective concentration. ZEA levels in wastewaters are in the same range. EC_{50} for vtg induction in embryos was 100 times higher than environmental concentrations.

In males, results of vtg protein and mRNA induction correlated well, which is strange regarding the higher stability of proteins. The half-life of the VTG protein in zebrafish is 2,4 days, however the stability of

mRNA has not been described yet. In the African clawed frog (*Xenopus laevis*) exogenous estrogens may stabilize *vtg* mRNA, which might also be true for zebrafish. During the comparison it was not the amount of gene products that was compared, but the rates of induction, which could also account for the high similarity.

In males and embryos *vtg* mRNA levels also show high correlation. The comparison is not exact due to the small number of concentrations tested on males and to the different exposure protocols. However males were treated with ZEA for 21 days, according to previous observations it is likely that *vtg* levels (depending on the concentration) reached the maximum to 5 dpf and have not changed subsequently. Results of the transgenic experiments also confirmed this hypothesis.

In contrast to literal data, results of the biomarker line described in the thesis suggest that adult fish may be sensitive to lower ZEA concentrations too. According to their results, 0,1 μg/L ZEA does not induce vtg production. Previous studies shown that zebrafish larvae in the early developmental stages were more sensitive to some estrogenic substances (e.g. E2 and NP), however sensitivity of young larvae and adults is approximately equal. Sensitivity of fish regarding vtg induction raised along with development. According to the results of ZEA studies described in the thesis embryos really proved to be more sensitive to higher concentrations, which was much more pronounced in the survival and the developmental abnormalities than in *vtg* induction. In low concentrations and in respect of *vtg* production, adults seemed to be more sensitive.

According to environmental concentrations detected in waters, ZEA in itself does not seem to carry a potential hazard, however in a mixture with other similar agents might significantly contribute to the overall estrogenicity. ZEA contamination in animal feed is sometimes very high. On the basis of previously described biological effects and results presented herein, monitoring of toxins in feed should be of crucial importance for fish farms too.

Suggestions

- Currently 3 sublines (204 x 104 ♂ ♀, 208 ♂ ♀ x 212 and 203 x 202 ♂ ♀) of the presented transgenic zebrafish line are maintained in our facility. Sensitivity of these sublines is different. Determination of the integration site and copy number of the construct could explane the differences between the sublines. By crossing the sublines of different integration sites, new, more sensitive sublines could be generated.

- In some embryos of the biosensor line, apart from the liver, the fluorescent protein was expressed in the yolk too in response to estrogens. It would be beneficial to raise embryos that only produce liver-specific signal, and then by the crossing of these a new subline could be established. If embryos would be selected on the basis of fluorescence following exposure to low concentrations of estrogen, sensitivity of the new subline could probably exceed the sensitivity of existing sublines.

- Adults of the *vtg1*-mCherry line are presumably more sensitive to environmental estrogens and tolerate wider concentration ranges than larvae. However experimentation on adult zebrafish is regarded as an animal experiment, still I would suggest a more detailed examination of adult fish in the near future.

- The established zebrafish line in the future could be a sufficient alternative test system in the examination of estrogenic substances. I would suggest to test other estrogenic substances and estrogenic mixtures too on the biomarker line.

- *Vitellogenin* whole mount *in situ* hibridization revealed that embryos of the homogenous, laboratory zebrafish strain are also differentially sensitive to estrogens. This can be due to the different developmental stages of the liver, that could be revealed by further *in situ* hibridization.

- On the basis of the results on the estrogenic mycotoxin (zearalenone), several new experiments could be planned. It would be beneficial to disclose the molecular background of ZEA-specific developmental disorders, or to monitor the effects of toxin contaminated feed on the fingerlings of marketable fish species (e.g. carp).

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