Establishment of multicolor transgenic zebrafish lines for environmental toxicology and pharmacology tests (NNF 78834)

Publications

Results of the project have been published on scientific conferences and a reviewed scientific paper has been submitted:

Poster/Oral presentation on international conference:

Zs. Csenki, A. Zaucker, B. Kovács, Y. Hadzhiev, Á. Hegyi, K.K. Lefler, T. Müller, R. Kovács, B. Urbányi, L. Váradi, F. Müller: ***Intraovarian transplantation of stage I-II follicles results in viable zebrafish embryos***, 6th European Zebrafish Genetics and Development Meeting Rome, Italy, Book of Abstracts 148. p, 2009 (Poster)

Katalin Bakos, Balázs Kovacs, Zsolt Csenki, Róbert Kovács, Dóra Kánaine Sipos, Yavor Hadzhiev, Ferenc Müller, Béla Urbányi: ***Establishment of transgenic zebrafish lines for EDC tests. "The zebrafish model in toxicology and teratology" konferencia***, Karlsruhe, Németország, 2010. szeptember 2-3., 2010 (Poster)

Katalin Bakos, Balázs Kovács, Zsolt Csenki, Róbert Kovács, Dóra Kánaine Sipos, Yavor Hadzhiev, Ferenc Müller, Béla Urbányi: ***Establishment of a new, zebrafish based toxicological test system***, Aquaculture Europe 2010. "Seafarming Tomorrow" konferencia, Porto, Portugália, 2010. október 6.-8., 2010 (Poster)

Bakos K.: ***Ösztrogén hatású szennyezőanyagok kimutatására alkalmas zebradánió alapú tesztrendszer létrehozása.***, Kárpát-medencei Doktoranduszok Nemzetközi Konferenciája (TUDOC), Gödöllő, 2010. május 27., 2010 (international conference in Hungarian) (Oral presentation)

K. Bakos, Zs. Csenki, R. Kovács, S.D. Kánainé, D. Bencsik, Y. Hadzhiev, B. Kovács, F. Müller, B. Urbányi: ***Transgenic zebrafish lines as in vivo EDC screening systems***, 7th European Zebrafish Meeting, Edinburgh, Scotland, 5th-9th July, 2011 (Abstract submitted for poster presentation)

K. Bakos, Zs. Csenki, R. Kovács, S.D. Kánainé, Y. Hadzhiev, B. Kovács, F. Müller, B. Urbányi: ***Establishment of a transgenic zebrafish (Danio rerio) based EDC screening system***, Genomics in Aquaculture International Symposium, GIA 2011, Heraklion, Crete, Greece, 14-17 September 2011 (Abstract submitted for poster presentation)

Poster/Oral presentation on national conference:

Bakos K., Csenki Zs., Kovács R., Kánainé S. D., Müller F., Hadzhiev Y., Kovács B., Urbányi B.: ***Multikolor zebradánió alapú tesztrendszer toxikológiai vizsgálatokhoz***, XXXIV. Halászati Tudományos Tanácskozás, Szarvas, 2010. május 12-13., 2010

Reviewed scientific paper (in Hungarian):

Bakos Katalin, Csenki Zsolt, Kovács Róbert, Kánainé Sipos Dóra, Müller Ferenc, Yavor Hadzhiev, Kovács Balázs, Urbányi Béla: ***Hormonháztartást zavaró anyagok monitorozása szennyezett vizekben transzgenikus zebradánióval***, Halászat, 2011 (submitted)

Reviewed scientific paper (in English):

The article is in progress and will be submitted in December, 2011 at the latest.

Detailed final report

In pharmaceutical, toxicological and basic research, there is ever increasing demand for replacing mammalian models with new test systems that enable the detection of harmful effects. Therefore *in silico* and *in vitro* (cell and tissue cultures) approaches are more and more frequently used. However, these have limited value and are only useful in the early phases of drug discovery and toxicity evaluation, as they cannot replicate the complex dynamic, multi-organ events that occur *in vivo*. Even new technologies, like IdMOC (Integrated Discrete Multiple Organ Culture plate), based on the co-culture of cell lines from different organs are unable to model the continuous, interactive communication of cells and organs perfectly. According to the above and in order to reduce cost and comply with animal experimentation directives, researchers have been looking for new animal models such as the zebrafish (*Danio rerio*) or roundworm (*Caenorrhabditis elegans*) because they offer the biological complexity at the level of organ and entire organism. Although the new models are lower organisms, their genes, receptors, molecular and physiological processes are analogous to those in mammals so to humans as well.

Zebrafish is a popular, well-studied model organism, previously considered as an excellent genetic model for developmental biology. The focus of research now shows a noticeable shift towards the application of zebrafish as disease model, and test tool in pharmacology and toxicology.

The popularity of zebrafish in toxicological research is mainly due to its small size, high tolerance, short generation interval and cheap and easy husbandry. It produces a large number of eggs in a single spawning so many embryos/larvae can be obtained in a short period of time. Additionally whole genome information and many simple and cheap experimental techniques such as gene expression analysis, transgenesis, gene function manipulation tools are also available for the species. Organ/tissue and cellular phenotypes can easily be observed in the transparent *ex utero* developing embryos and larvae. Manipulation techniques developed for mammals can be used in zebrafish too, but there are fish specific methods as well. Zebrafish are able to detect low toxic agent levels. Importantly, their embryos and larvae up to the free feeding age are not considered as

animals by the Animals (Scientific Procedures) Act 1986 of the UK and the relevant Directive 86/609/EEC Art. 2b of the European Union. Thus, promoting the use of zebrafish embryos/ larvae instead of rodents, and other laboratory animals including adult fish remains a high priority. Somewhat paradoxically, the increasing popularity of zebrafish in medical research also led to the increasing number of laboratories in which zebrafish is used also after the start of the free feeding stage (such as regeneration, liver and cardio toxicity and neurotoxicity, behaviour and cancer).

Using transgenic zebrafish in medical research is not a novel concept, so hundreds or even thousands of transgenic zebrafish lines have been generated in the last decade. However the majority of these are single colour lines and the application of double transgenics has been sporadic while multiple transgenic lines have not yet been reported.

In the framework of the NNF 78834 project our aim was to develop multiple transgenic zebrafish lines based on which new, rapid *in vivo* embryonal test systems could be established. As in case of zebrafish, the chorion and the developing larvae are transparent, transgenic lines expressing fluorescent proteins enable the rapid and easy *in vivo* detection of toxic effects.

If the toxicologically important target organs (liver, blood vessels, blood cells, nervous system) are labelled with different fluorescent proteins, toxic effects on these organs can be detected simultaneously in a single zebrafish line.

For the development of the multiple transgenic lines, first we crossed already available transgenic lines: two lines that produce fluorescent proteins in the neurotic tissues (Neurogenin and Nestin), a line in which the blood cells (GATA-1) and another in which the blood vessels (Fli) are labelled with fluorescent proteins.

Nestin is an intermediate filament protein expressed mostly in dividing nerve cells eg. during the radial growth of the axon. Nestin is only expressed during the early stages of development in the central nervous system, later on it becomes downregulated and replaced by tissue specific intermediate filaments. In adults, nestin expression is reinduced in the ijury of the central nervous system as a sign of regeneration. The transgenic line was developed by a construct in which the expression of the green flurescent protein (GFP) is driven by the nestin promoter. Since in this line, the presence

of GFP can only be detected in developing nervous tissues, it is suitable for analysing the effects of toxic agents on nervous system development.

Neurogenin-1 is a tissue specific key protein in the development of the nervous system. Neurogenin-1 is expressed only in embryonic neural tissues during the development of the neural plate and supports the differentiation of primary neurons. The neurogenin transgenic line expresses red fluorescent protein (RFP) under the control of the neurogenin promoter, enabling the in vivo detection of toxic effects on neural plate development.

GATA-1 (globin transcription factor-1) is a transcription factor essential for the differentiaition of erythroid cells, eosinophyl granulocytes and mastocytes. Playing a key role, this protein is one of the best tools to identify haematopoietic tissues. In the transgenic line blood cells are labelled with a red fluorescent protein (DsRed) making the monitoring of toxic effects possible on these cells and tissues.

Fli-1 (Friend leukemia integration site-1) is a transcription factor present in the blood-forming (haematopoietic) tissues and organs (embryonal ICM, kidney, thymus, heart) and also in endothelial cells (interior surface of blood vessels). The transgenic line produces green fluorescent protein (GFP) in these cells and organs, making the line suitable to monitor toxic effects exerted on the labelled tissues.

In order to develop multiple transgenic lines, first single transgenic, homozygous individuals were crossed. In order to find out the genotype of transgenic individuals, fish were crossed to wild type individuals and developing embryos were examined under a fluorescent microscope, using the appropriate filter. If all larvae are hemizygous and express the fluorescent protein, the transgenic parent was homozygous. But if non-expressing, wild-type larvae can also be seen amongst, the parent was hemizygous. Identified homozygous parents were then crossed to each other and double transgenic individuals, hemizygous for the transgenes were derived. In hemizygous fish, the transgene is only present in one of the chromosome pairs (Figure 1.).

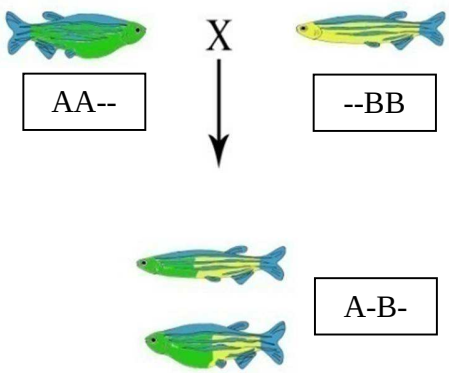

Figure 1. Crossing scheme of single transgenic lines. One of the parents is homozygous for the „A" and the other for the „B" construct. Offspring will get only one chromosome from each parent that harbour the transgene.

Double transgenic, heterozygous offspring were raised until fertility and mated amongst or crossed to other single or double transgenic individuals. The first results in homozygous, hemizygous and wild-type F2 individuals, from which homozygous ones can be identified as described above. In the other case, triple or quadraple transgenic, hemizygous fish can be derived. From these, if crossed *inter se* multiple transgenic, homozygous lines can be established. During the project, homozygous and hemizygous Nestin-Neurogenin (Figure 2.), Nestin-GATA (Figure 3.), GATA-Fli (Figure 4.), Fli-Neurogenin (Figure 5.), Fli-Nestin Neurogenin (Figure 6.), Fli-Nestin-GATA (Figure 7.), and Fli-Nestin-Neurogenin-GATA (Figure 8.) transgenic zebrafish were produced from the available lines (GATA-1, Fli, Nestin and Neurogenin) expressing green and red fluorescent proteins in the blood cells, blood vessels and nervous tissues.

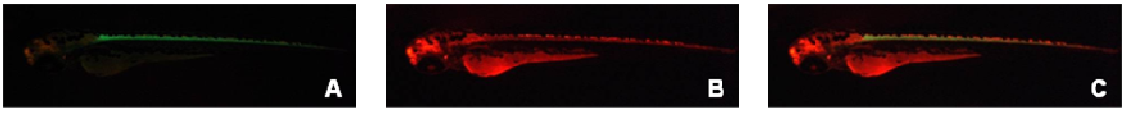

Figure 2. Fluorescent image of a 3 dpf (days post fertilization) Nestin-Neurogenin transgenic zebrafish fry (A: green /GFP/ filter, B: red /mCherry/ filter, C: green /GFP/ + red /mCherry/ filter)

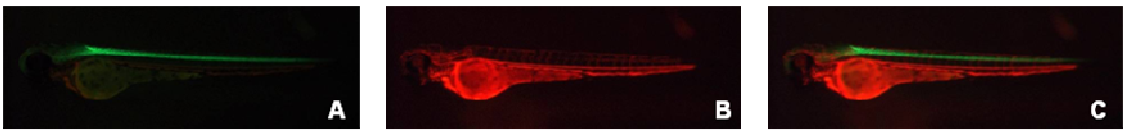

Figure 3. Fluorescent image of a 3 dpf (days post fertilization) Nestin-GATA transgenic zebrafish fry (A: green /GFP/ filter, B: red /mCherry/ filter, C: green /GFP/ + red /mCherry/ filter)

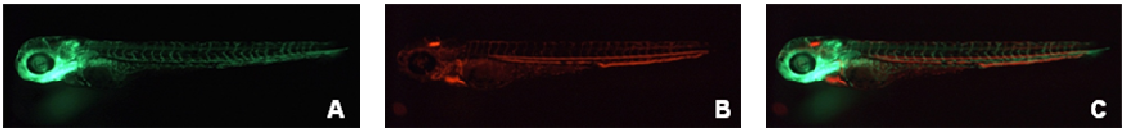

Figure 4. Fluorescent image of a 3 dpf (days post fertilization) GATA-Fli transgenic zebrafish fry (A: green /GFP/ filter, B: red /mCherry/ filter, C: green /GFP/ + red /mCherry/ filter)

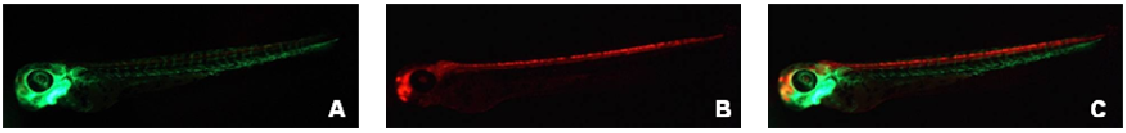

Figure 5. Fluorescent image of a 3 dpf (days post fertilization) Fli-Neurogenin transgenic zebrafish fry (A: green /GFP/ filter, B: red /mCherry/ filter, C: green /GFP/ + red /mCherry/ filter)

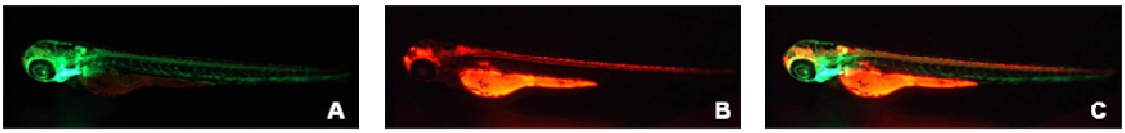

Figure 6. Fluorescent image of a 3 dpf (days post fertilization) Fli-Nestin-Neurogenin transgenic zebrafish fry (A: green /GFP/ filter, B: red /mCherry/ filter, C: green /GFP/ + red /mCherry/ filter)

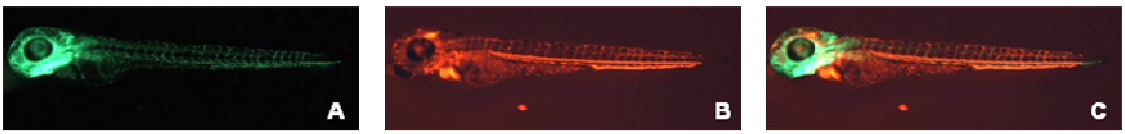

Figure 7. Fluorescent image of a 3 dpf (days post fertilization) Fli-Nestin-GATA transgenic zebrafish fry (A: green /GFP/ filter, B: red /mCherry/ filter, C: green /GFP/ + red /mCherry/ filter)

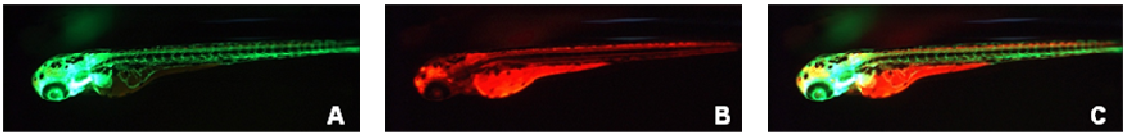

Figure 8. Fluorescent image of a 3 dpf (days post fertilization) Fli-Nestin-Neurogenin-GATA transgenic zebrafish fry (A: green /GFP/ filter, B: red /mCherry/ filter, C: green /GFP/ + red /mCherry/ filter)

As liver is also one of the main target organs of toxicity, and at the beginning of the project, no lines expressing fluorescent protein in the liver existed, a new transgenic line needed to be generated.

In order to establish the new line, first we needed to specify a gene of a protein produced only in the liver and a gene of a fluorescent protein (as a visible marker.)

From liver specific genes, vitellogenins are among the most suitable genes for building the construct for transgenesis. Vitellogenins in the absence of inducers are normally synthetised only in females, so as vitellogenins are inducible by estrogenic substances, besides following liver development (and detecting abnormalities as effects of toxic agents) the male individuals from the liver transgenic line derived this way could also be used for screening Endocrine Disrupting Chemicals (EDC) e.g. in pesticides, herbicides, drugs, wastewaters. Zebrafish has 7 vitellogenin genes from which vitellogenin-1 (vtg-1) has the strongest expression. Therefore the promoter of vitellogenin-1 was chosen for establishing the new transgenic line. If the vitellogenin promoter is followed by the gene of a fluorescent protein in the construct, the promoter will guide the expression of this protein. For this purpose, a red (mCherry) and a blue fluorescent protein (EBFP) was chosen (as visible markers of vitellogenin synthesis in the liver).

Instead of using BAC clones, the highly efficient transposon-based Gateway cloning system was used for the establishment of the transgenic line. BAC clones are large DNA sequences (100-300 kbp) difficult to work with and the efficiency of transgenesis is low (<5%). The Gateway vector is much smaller (3-8 kbp), easier to work with and provides much higher transgenesis efficiency (>80%). The Gateway system is fast and dynamic.

The Gateway system is a two-step system in which cloning is based on site-specific recombination making cloning much more efficient than the traditional endonuclease based method. First the genes of interest (in this case the vitellogenin promoter and the fluorescent protein gene) should be amplified by a special PCR using primers that have the attachment sites needed for the recombination on their ends. We looked up the genomic sequence of the vtg-1 gene in the databases (NCBI, Ensembl) and

analysed the region upstream the presumed transcription start site. A 4200 bp, then as the first construct did not work, a 3400 bp fragment (harbouring the transcription start site and the upstream regulatory sequences as well) was amplified from zebrafish genomic DNA. The fluorescent protein gene was amplified in the same way, using the encoding PCS2 plasmid (Figure 9.).

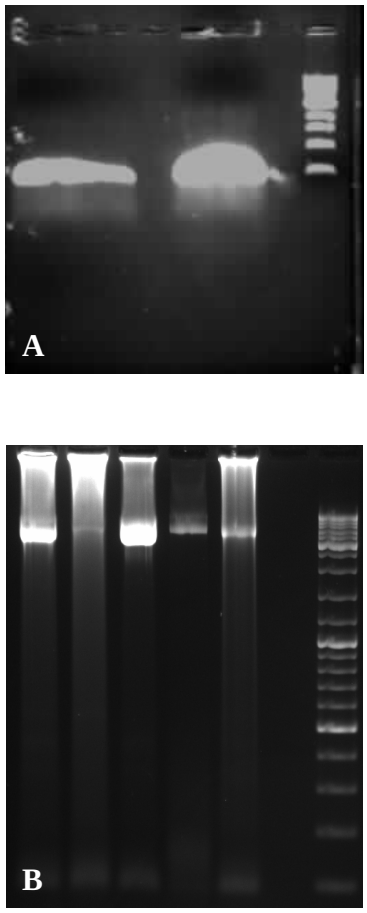

Figure 9. Gel electrophoretic images of the amplified fluorescent protein (mCherry) gene (A) and the vitellogenin-1 promoter (B)

Fragments were cleaned up from the agarose gel and their sequence was analysed. PCR products were then cloned separately to circular plasmid DNA vectors called donor vectors, and finally to the Gateway itself (Figure 10.).

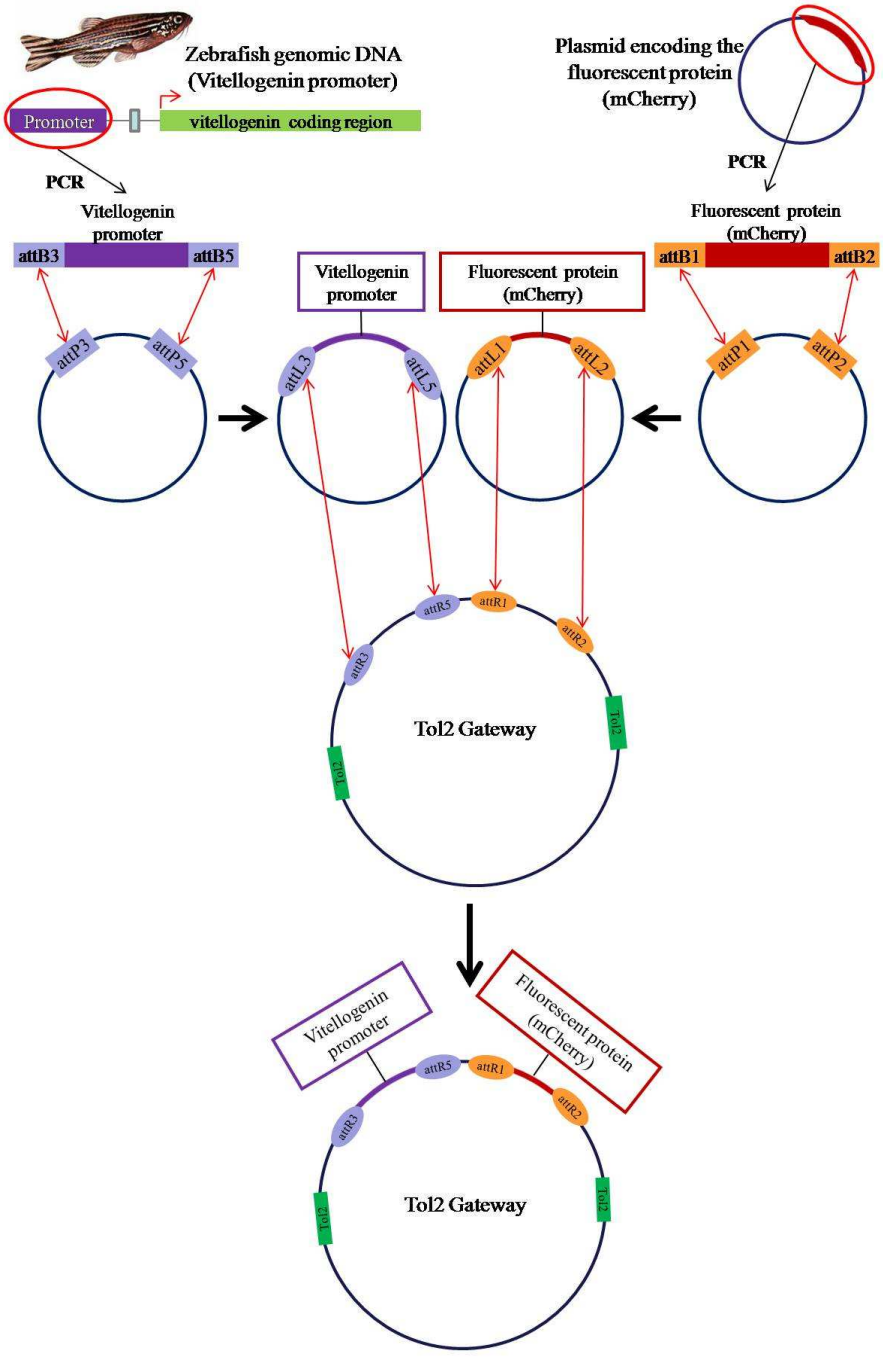

Figure 10. Establishment of the liver-specific construct by using the Gateway cloning system

When the construct is ready (the Gateway is holding the promoter and the fluorescent protein as well) it was linearized by restriction digestion. As the gene and promoter of interest are built between Tol2 transposon arms, to achieve the best transgene

integration efficiency, the mRNA of the transposase enzyme is also needed. Transposase mRNA was synthetized from the encoding PCS2 plamid by using an *in vitro* transcription kit.

The construct (along with the mRNA) was injected to 1-2 cell wild-type and transgenic zebrafish embryos (Figure 11., 12., 13.), resulting single, double or multiple transgenic individuals.

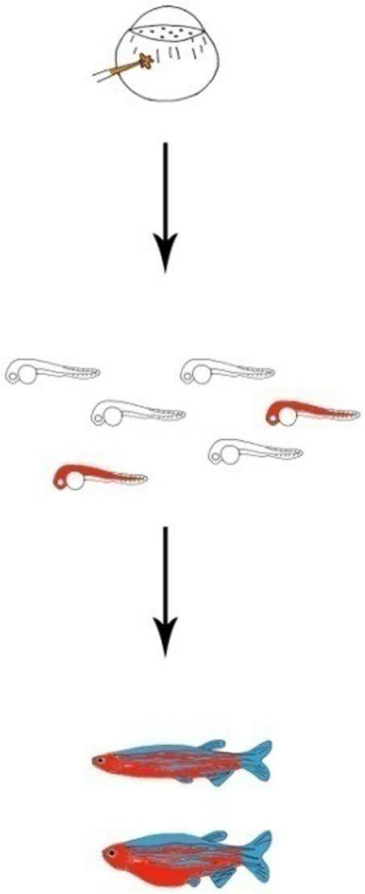

Figure 11. Microinjection of the liver-specific construct to the yolk of 1-2 cell zebrafish embryos

Embryos were raised on 27 ^{o}C, in 100 mm petri dishes containing zebrafish embryo medium. Transgene expression was induced by 100 ng/l estrogen (E2). As the liver in zebrafish only starts to develop on the 3rd-4th day post fertilization (3-4dpf), P0 individuals were treated with estrogen only after reaching this age and examined under

the fluorescent microscope at 10 dpf (Figure 12., 13., 14.). Liver-specific fluorescent protein expression was detected in 12 % of the injected wild-type and 32 % of the transgenic embryos. Transient, non-specific expression was detected in the eye, kidney and in the yolk. Liver specific expression was only detectable in embryos treated with estrogen (E2), while the expression in the yolk was much lower in non-induced embryos. These results correspond to those of other transposon based constructs.

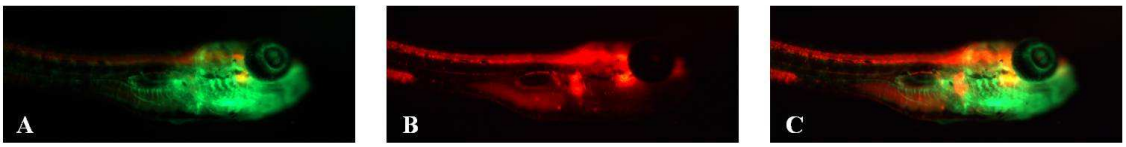

Figure 12. Fluorescent image of 10 dpf, injected Fli-Neurogenin transgenic fry following estrogenic treatment (100 ng/l E2). (A: green /GFP/ filter, B: red /mCherry/ filter, C: green /GFP/ + red /mCherry/ filter)

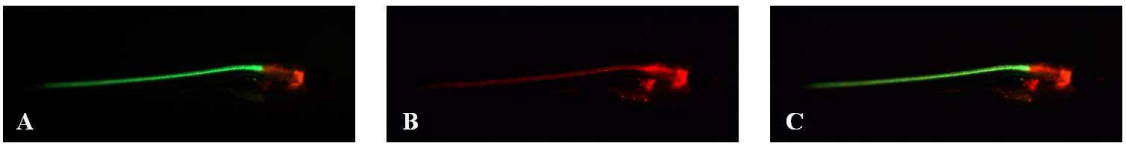

Figure 13. Fluorescent image of 10 dpf, injected Nestin-Neurogenin transgenic fry following estrogenic treatment (100 ng/l E2). (A: green /GFP/ filter, B: red /mCherry/ filter, C: green /GFP/ + red /mCherry/ filter)

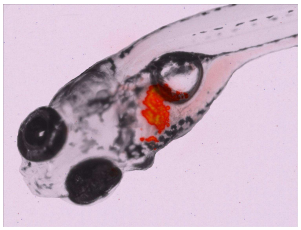

Figure 14. Fluorescent image of 10 dpf, injected wild-type fry following estrogenic treatment (100 ng/l E2).

The injected P0 individuals are mosaic for the transgene integration, so only the genome of certain cells carry the transgene. Therefore individuals that harbour the transgene in their gametes and so transmitting the construct needed to be identified. P0 individuals were crossed to wild-type partners and following estrogenic treatment, fluorescent protein expression was observed under the fluorescent microscope. From the examined 38 wild-type males, 7, while from the injected 12 GATA-Fli males, 2 transmitted the transgene. In case of females, offspring of 8 inherited the transgene from

the examined 50. According to these, 18% of the males and 16% of the females had “transgene carrying” gametes, and about 40% of their offspring produced fluorescent signal (mCherry) in the liver. Our results correspond to the expected and to the results of other similar experiments.

Transgene transmission to subsequent generations was also confirmed at the molecular level. Therefore a PCR reaction, specific for the mCherry was developed, as the other main component of the construct, the vitellogenin promoter may not only be introduced by the construct but is naturally present in the genome. The PCR amplifies a 158 bp fragment of the mCherry and is also suitable for the construct copy number determination. In the reactions we used mCherry as positive control and the genomic DNA of non-injected, wild-type zebrafish as negative control. DNA was prepared from embryos by the phenol-chloroform method, and PCR reactions were performed. Results confirmed that individuals selected on the basis of microscopic analysis could indeed transmit the transgene.

In order to develop the F1 generation of liver transgenic lines, P0 individuals were crossed to wild-type or homozygous transgenic partners. As the transgene in different P0 individuals integrated to different chromosomal locations, they should be handled separately and should not be intercrossed. As in this generation, gonads are mosaic too, not all gametes and so not all offspring will carry the construct. As a cost effective, space saving solution, we raised the offspring for 1 month and selected the transgenic ones on the basis of PCR. Identification would also be possible by estrogenic induction, but the treatment is risky as not all individuals survive, delaying the generation of stable transgenic lines. As F1 individuals are presumably not mosaic, the presence of the transgene can be detected by the mCherry specific PCR (described above) from the tail fin DNA of hatchlings. The tail fin of one month old zebrafish is tiny, so a modified phenol-chloroform based DNA extraction method needed to be elaborated. PCR reactions were performed on the individually isolated DNA samples (Figure 15.).

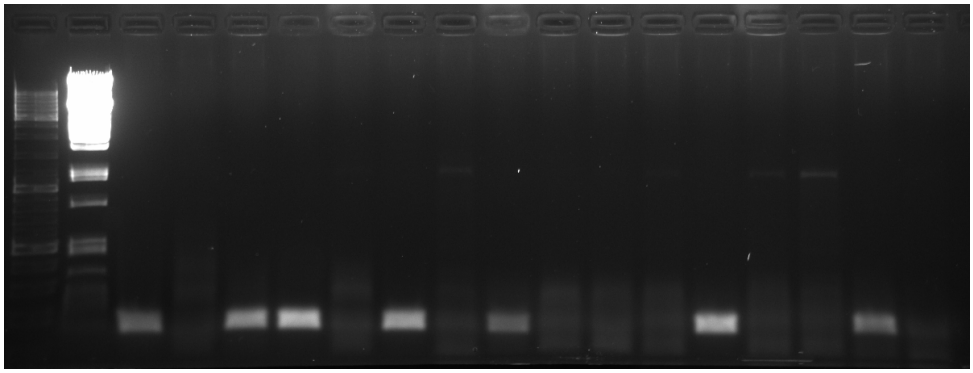

Figure 15. PCR-based identification of 1 month old, transgenic F1 fry (from the left: 1.-2. molecular weight markers, 3. positive control, 4. negative control, 5.-18. PCR products of individual DNA samples).

Hatchlings found to be positive on the basis of the mCherry specific PCR were raised up and up to now the F2 generation of 3 founders (P0) was established. F2 fry are being raised.

Copy number and integration site of the transgene has not been yet determined. Offspring of founders and F1 are regularly bred (every week) and the resulting larvae are subjected to estrogenic treatment. As both the integration site and the copy number may affect the intensity of the induced fluorescent signal, we decided to identify those F1 and F2 individuals that show the highest inducibility prior to the determination of other characteristics at the molecular level. Based on these, further on lines carrying the transgene in more copies could also be generated.

In the project one of our aims was to develop the liver-specific transgene construct with the gene of an other fluorescent protein, the EBFP (Enhanced Blue Fluorescent Protein) too. The excitation and emission spectrum of this protein does not overlap with the other fluorescent proteins present in the multiple transgenic line thus by injecting the construct, multiple transgenic lines expressing proteins of three different colours could be generated. We attempted to develop the construct twice, but the fluorescent protein expression in both cases was so weak, the signal was nearly invisible. So we started to build the construct by using a new blue fluorescent protein, the CFP (Cyan Fluorescent Protein). Although the spectrum of this overlap with that of the green fluorescent protein, the signal it produces will surely be much more intensive.

In summary, we developed a new transgene construct that drives inducible fluorescent protein expression in the liver. This construct was injected to 1-2 cell wild-type and transgenic zebrafish embryos and the F1 and F2 generation of 3 founders was established. The determination of the transgene integration site and copy number is in progress, however, in order to save cost, it will be performed only on individuals showing the highest fluorescent signal inducibility as a response to estrogenic treatment. In the framework of this project, double transgenic, homozygous F2 individuals and hemizygous, multiple transgenic fish were produced from the available single transgenic lines in which the blood cells, the blood vessels or the nervous tissues are fluorescently labelled. We found out, that the EBFP is not a suitable reporter for developing such transgenic lines, as the signal it produces is too weak to be detectable. So a new blue fluorescent protein (CFP) was selected for building a new, blue construct. Purchasing the encoding plasmid also caused a delay.

Further on, the newly developed transgenic lines would enable the *in vivo* identification of toxic effects exerted on the fluorescently labelled tissues and organs and to determine the estrogenic effect of new agents or complex environmental samples. The lines could also be useful when characterizing pharmaceuticals, during the risk assessment of pollutants released to the environment, natural waters and fish ponds or sludge, and treated wastewater and effluents. EDC levels exceeding a certain estrogen (E2) equivalent could also be evaluated with the help of the lines. Results on these transgenic fish could complete previous analytical tests, enabling more precise toxicity evaluation, and could also be a perfect tool in the risk assessment of environmental pollutants and for the determination of toxicity levels of agents.