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TOXICOGENOMICS

Exploring the zebrafish embryo as an alternative model for the evaluation of liver toxicity by histopathology and expression profiling

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Abstract The whole zebrafish embryo model (ZFE) has proven its applicability in developmental toxicity testing. Since functional hepatocytes are already present from 36 h post fertilization onwards, whole ZFE have been proposed as an attractive alternative to mammalian in vivo models in hepatotoxicity testing. The goal of the present study is to further underpin the applicability of whole ZFE for hepatotoxicity testing by combining histopathology and next-generation sequencing-based gene expression profiling. To this aim, whole ZFE and adult zebrafish were exposed to a set of hepatotoxic reference compounds. Histopathology revealed compound and life-stage-specific effects indicative of toxic injury in livers of whole ZFE and adult zebrafish. Next-generation sequencing (NGS) was used to compare transcript profiles in pooled individual RNA samples of whole ZFE and livers of adult zebrafish. This revealed that hepatotoxicity-associated expression can be detected beyond the overall transcription noise in the whole embryo. In situ hybridization verified liver specificity of selected highly expressed markers in whole ZFE. Finally, cyclosporine A (CsA) was used as an illustrative case to support applicability of ZFE in hepatotoxicity testing by comparing CsA-induced gene expression between ZFE, in vivo mouse liver and HepaRG cells on the levels of single genes, pathways and transcription factors. While there was no clear overlap on single gene level between the whole ZFE and in vivo mouse liver, strong similarities were observed between whole ZFE and in vivo mouse liver in regulated pathways related to hepatotoxicity, as well as in relevant overrepresented transcription factors. In conclusion, both the use of NGS of pooled RNA extracts analysis combined with histopathology and traditional microarray in single case showed the potential to detect liver-related genes and processes within the transcriptome of a whole zebrafish embryo. This supports the applicability of the whole ZFE model for compound-induced hepatotoxicity screening.

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Introduction

The liver is a critical target for xenobiotic-induced toxicity, and drug-induced liver injury is a major contributor in attrition of drugs in preclinical development. A first factor in hepatic susceptibility is that active hepatic processing leads to high intracellular concentrations of xenobiotics. Secondly, hepatocellular biotransformation of xenobiotics, which normally protects against the direct-acting toxicity through inactivation and facilitation of excretion, may also lead to reactive metabolites that are more toxic than the parent compound, resulting in liver cell injury (Jaeschke et al. 2002).

Xenobiotic-induced hepatotoxicity occurs through different mechanisms associated with distinct histopathological and clinical phenotypes, including cholestasis, steatosis and necrosis, which will be further referred to as nominal phenotypes. These pathological conditions represent the consequences of, respectively, impaired hepatocellular bile excretion, triglyceride accumulation resulting from disruption of fatty acid oxidation and hepatocellular cell death (Jaeschke et al. 2002). Traditionally, histopathology and clinical chemistry are used to detect hepatotoxic properties of chemicals in in vivo rodent studies. Such studies require high numbers of animals, interfere with animal welfare and are associated with high costs and are not always predictive for the human situation. Therefore, alternative test systems are needed to improve predictivity and to contribute to reduction, refinement and replacement of in vivo rodent studies.

Following this necessity, several alternative methods to monitor liver injury have been proposed, including liver slices (Elferink et al. 2008), cultured primary hepatocytes (Kienhuis et al. 2009) and continuous cell lines, particularly the human hepatoma-derived HepG2 line (Schoonen et al. 2005) and the more recently derived human hepatocyte HepaRG line (Guillouzo et al. 2007). However, these in vitro systems each have their limitations related to their reductionistic nature and to loss of functionality compared to the liver in vivo (Boess et al. 2003).

The zebrafish (*Danio rerio*) is a widely used model organism to study human biology and pathology because physiological and molecular functions at the organ and cell level are highly comparable between the zebrafish and humans (Chu and Sadler 2009). Consequently, its value in the area of toxicology testing as well as drug discovery has also been recognized, making it a potentially important alternative for in vivo testing in rodents (Dooley and Zon 2000). The zebrafish has several experimental advantages such as its short life cycle, accessibility and availability, and the power to generate high numbers of embryos easily, which makes the model suitable for high throughput testing. Moreover, the zebrafish genome is completely sequenced, and a wide variety of genetic, molecular and cellular manipulation tools are available (Zon and Peterson 2005).

As a particular refinement, the use of the whole zebrafish embryo (ZFE) is advantageous since the early embryo is considered not to perceive pain or otherwise discomfort. According to legislation (EFSA 2005), whole ZFE can be considered as an alternative model system and it is already applied for studying chemical toxicity using developmental and lethality endpoints (Hermsen et al. 2011; Hill et al. 2005). In contrast to in vitro cell cultures, in vivo cell type and organ interactions are maintained in the whole zebrafish embryo. These aspects may contribute to a better representation of hepatotoxic responses that occur in vivo.

From a hepatotoxicity testing perspective, the liver in the zebrafish embryo is fully functioning with active drug metabolism at 72-hpf (Alderton et al. 2010). A few promising studies showed that zebrafish embryos are suitable to detect human hepatotoxicants. In a study by Jones et al., the whole ZFE model was evaluated through morphological endpoints after exposure to a set of compounds including drugs which were falsely identified by the HepG2 cells. In this design, whole ZFE successfully detected more hepatotoxicants with higher specificity than the HepG2 cells (Jones et al. 2009). In another study, Amali et al. (2006) carried out histopathological, molecular and biochemical analysis in ZFE exposed to a single dose of thioacetamide and showed that the whole ZFE model is suitable to detect steatohepatitis. These descriptive studies certainly indicate the potential of the whole ZFE model for hepatotoxicity testing, but further validation of the model is needed. A potential addition in this perspective is toxicogenomics, as it enables detailed analysis of the underlying mechanisms of cellular responses upon xenobiotics exposure. The most practical way to study hepatic gene expression in ZFE is to use whole ZFE RNA extract for analysis, instead of liver extracts, but this may result in masking of signals or lowering the signal/noise ratio of the regulated genes. Although available, sophisticated methods such as micro-dissection to study hepatic gene expression in small organism hinder the throughput of the system (Voelker et al. 2007).

The main objective of this study was to further substantiate the applicability of whole ZFE as a model for testing hepatotoxicity by combining histopathology with toxicogenomics, by means of next-generation sequencing (NGS)-based gene expression profiling. To this end,

histopathology in the ZFE and adult zebrafish liver was studied after exposure to a set of hepatotoxic reference compounds to confirm hepatocellular pathology and to extrapolate between developmental stages. Subsequently, induction of hepatotoxicity relevant genes was analyzed in a single RNA pool composed from a set of whole ZFE and adult zebrafish liver, individually exposed to one out of a range of reference hepatotoxicants, using NGS. The use of such a single combined pool of RNA is a cost-effective way to obtain robustly expressed transcripts after treatment with compounds of a similar toxicity class (Pronk et al. 2011). The advantage of NGS is that, in contrast to expression arrays, it does not build on sequence-specific probe hybridization and does not suffer from background and cross-hybridization problems. Furthermore, it provides an absolute measure of all transcripts, not just the relative abundance in an array selection, enabling to assess the whole transcriptome ('t Hoen et al. 2008). The ZFE and adult zebrafish liver transcriptomes were then compared to reveal whether hepatotoxicity-associated signals, as present in the overlap between ZFE and adult liver, can be detected in the noise of other tissues that contribute to the whole ZFE RNA. Highly expressed markers in ZFE were confirmed for liver specificity through in situ hybridization. Further analysis of the transcriptome was on regulation of pathways related to liver toxicity. General conclusions on hepatotoxic responses in the ZFE were verified in a dedicated case, that is, cyclosporine A (CsA), where ZFE data were compared with available data from in vivo mouse liver and from the HepaRG cell line, at the levels of single genes, pathways and transcription factors. Pathway and transcription factor-based analyses attractively leveled out differences between models due to species variation, model sensitivity and power.

Altogether, using pooled RNA extracts in NGS analysis combined with histopathology, followed by case-directed traditional microarray, we supportively demonstrate the applicability of the whole ZFE model for toxicogenomics-based compound-induced hepatotoxicity screening.

Materials and methods

Materials

Exposure studies were performed with reference compounds known to induce nominal hepatotoxic phenotypes in humans (cholestasis, steatosis and necrosis; Table 1). All test chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) and included acetaminophen or paracetamol (*N*-actyl-para-aminophenol; APAP, CAS no. 103-90-2), paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride, PQ, CAS no. 1910-42-5), thioacetamide($(CH_3$-C(S)NH2); TA, CAS no. 62-55-5), amiodarone hydrochloride (2-butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride; AM, CAS no. 19774-82-4), valproic acid (2-propylpentanoic acid sodium; VPA, CAS no. 1069-66-5), tetracycline (TET, CAS no. 64-75-5), cyclosporine A (CsA, CAS no. 59865-13-3), 17α-ethynylestradiol (17α-Ethynyl-1,3,5(10)-estratriene-3,17β-diol; EE2, CAS no.57-63-6) and chlorpromazine (2-Chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride; CPZ, CAS no. 69-09-0), tricaine methansulfonate (MS-222; CAS no. 886-86-2). Dimethylsulfoxide (DMSO, CAS no. 67-68-5) was ordered from Fisher Scientific. The RNeasy MinElute Cleanup kit (Cat. No. 74204) and the QIAzol Lysis reagent (Cat. No. 79306) were obtained from Qiagen Benelux B.V. (Venlo, the Netherlands). Phase-lock Gel Heavy (Cat. No. 2302870) and the metal micro pestle (P985.1) were purchased from VWR International B.V. (Amsterdam, the Netherlands).

Zebrafish

Wild-type zebrafish (*Danio rerio*) were originally obtained as commercially bred Singapore import (Ruinemans Aquarium BV, Montfoort, the Netherlands), which were maintained and bred in our facilities for more than 5 generations. Adult zebrafish (aZF) used in this study were approximately 14 months of age. For generation of ZFEs, two male and two female aZF were set together 1 day before spawning, after a period of 4-day separation to improve egg production. The breeding tank was constructed to prevent egg predation, and after spawning, a glass siphon was used to collect the eggs. All eggs were checked for fertility and thereafter pooled in a single petri dish containing Dutch Standard Water (DSW, demineralized water supplemented with $NaHCO_3$ (100 mg/l), $KHCO_3$ (20 mg/l), $CaCl_2{\cdot}2H_2O$ (200 mg/l), and $MgSO_4{\cdot}7H_2O$ (180 mg/l) aerated for 24 h at 27 °C) until the start of the exposure.

Exposure conditions

All ZFE exposures started at 3 days post fertilization (3dpf) with embryos which were randomly derived from at least 2 spawning units. Exposures were performed in 48-well plates (BD Biosciences) containing 1 ml of test medium per well. Exposures were performed in an incubator at 26.5 ± 1 °C in a static way and lasted for 48 h. Concentrations for the expression study were defined in a range finding study conducted with ZFE, where, respectively, 6 and 7 exposures were tested for DMSO and water-diluted compounds, with 12 ZFEs per condition, each embryo in a single well. The exposures in the expression study were conducted with three concentrations (Table 1),

Table 1 Reference compounds

Compounds	Nominal phenotype	Test concentrations (μM)[1]	Vehicle control
Chlorpromazin (CPZ)	Cholestasis	3–1–0.3[2]	DMSO[3]
Cyclosporine A (CsA)	Cholestasis	6–2–0.7	DMSO
17α-ethynylestradiol (EE2)	Cholestasis	3.5–1.2–0.4	DMSO
Amiodarone (AM)	Steatosis	10–3.3–1.1	DMSO
Valproic acid (VPA)	Steatosis	600–200–67	DSW[4]
Tetracycline (TET)	Steatosis	200–66.7–22.2	DMSO
Acetaminophen (APAP)	Necrosis	660–220–73.3	DMSO
Paraquat (PQ)	Necrosis	3,000–1,000–330	DSW
Thioacetamide (TA)	Necrosis	10,000–3,333–1,111	DSW

[1] Test concentrations of the reference compounds for transcriptomics and histopathology

[2] aZF were exposed to a different range of CPZ, that is, 1–0.3 μM–0.1 μM

[3] Compounds initially dissolved in DMSO and diluted further in DSW with a final DMSO concentration of 0.2 % v/v. DSW and DMSO 0.2 % v/v were included as vehicle controls

[4] Dutch Standard Water, demineralized water supplemented with $NaHCO_3$ (100 mg/l), $KHCO_3$ (20 mg/l), $CaCl_2{\cdot}2H_2O$ (200 mg/l), and $MgSO_4{\cdot}7H_2O$ (180 mg/l) aerated for 24 h at 27 °C

where the highest concentration was defined as the no-effect concentration in ZFE (no mortality or teratogenicity) in the range finding study. Each concentration was performed in five replicate units, where each unit had 15 embryos (three wells each with five embryos). Each plate was set up to have 6 vehicle control wells. After exposure, the 15 embryos of each replicate unit were sampled in a single tube and snap-frozen in liquid nitrogen.

aZF were transferred to the static test system 3 days prior to testing for acclimatization, and this consisted of full-glass aquaria containing three liters of DSW covered by a glass plate. The water was continuously aerated, and ambient conditions were daily monitored. The temperature was kept at 27 ± 2 °C, pH between 7.4 and 9.0, and oxygen >7.10 mg/l at all times. The light/dark cycle was 14-h light and 10-h dark. Each experimental group consisted of four male zebrafish. Test concentrations and exposure duration were as for ZFE (except CPZ; Table 1). After the exposure, the zebrafish were euthanized with a solution of 100 mg/l MS-222 buffered with sodium bicarbonate. Following decapitation, the fish were slit ventrally from heart to anus and livers were dissected and snap-frozen in liquid nitrogen. ZFE and aZFL samples were stored at −80 °C until RNA extraction.

Experiments with adult zebrafish were approved by the RIVM Animal Experimentation Ethical Committee and carried out in accordance with relevant Dutch national legislation, including the 1997 Dutch Act on Animal Experimentation.

Histopathology

For histopathology, ZFE were sampled from three highest concentrations without mortality or teratogenicity of the concentration range finding study and adult zebrafish livers were dissected from adult fish exposed in the expression study. Whole ZFE and aZFL were fixed in 4 % paraformaldehyde for 24 h, transferred and then stored in 70 % ethanol until use. Samples were first embedded in a specially designed 1 % agarose mold for adequate positioning of the embryos (Tsao-Wu et al. 1998; Sabaliauskas et al. 2006), and then transferred to paraffin, whereafter 4-μm sections were routinely stained with hematoxylin and eosin (H&E) and covered with a glass coverslip. Additional cryosections from AM, VPA and TET were stained with oil-red-O to determine fatty droplet accumulation in ZFE and aZFL. Furthermore, in aZFL, additional sections from CPZ, EE2 and CsA were stained with Fouchet staining to detect bile accumulation. For the aZFL, only one replicate per compound was included. As for the ZFE, approximately 12 replicates were included per compound.

RNA isolation and processing

Total RNA was isolated using the MinElute Clean up kit according to the protocol of de Jong et al. (2010). RNA concentration was measured spectrophotometrically (ND1000; NanoDrop technologies, Wilmington, DE, USA), and RNA integrity was assessed using Bioanalyzer 2100 (Agilent Technologies, Amstelveen, the Netherlands). All samples contained intact total RNA with RNA Integrity Number (RIN) >8. To reduce cost of NGS, RNA extracts of all ZFE of the highest exposure concentration of each compound were combined to a single pool. This is justified because our primary interest was in responsiveness potency of the ZFE, not in effects of single compounds, and pooling of samples has analytical advantages (see Discussion). We added control samples to be able to pick

up any transcripts that are highly expressed in control conditions and downregulated in samples treated with liver toxicants, although these transcripts may be harder to detect because they now have their highest expression level only in a minority of the pooled samples (2 control samples versus 9 hepatotoxicants). A pool contained 500 ng/µl RNA, with equal quantities derived from approximately 45 embryos per compound (Fig. 1). A similar RNA pool was prepared from three adult zebrafish livers per compound at the same concentration.

RNA NGS sequencing

NGS sequencing was performed by BaseClear B.V. (Leiden, the Netherlands) using the Illumina GAII instrument. RNA-Seq libraries were made from 10 µg total RNA for each pool using the Illumina mRNA-Seq Sample Preparation Kit according to manufacturer's instructions (Illumina, Inc., San Diego, USA). In brief, this included purification of the RNA, fragmentation through divalent cations under elevated temperatures, followed by cDNA synthesis. Next, adaptors were ligated, and the product was again purified and then amplified. A quantity of 4 pmol of each library was transferred to a flow cell; there again amplified to produce clusters of fragment copies, which were then paired end sequenced with a read length of 51 nucleotides. Fragments in a cluster were sequenced twice (forward and reverse), ensuring highly accurate alignment of the reads. Paired end sequences can be considered as technical duplicates, to control for reliability of the procedure. The Illumina system is based on fluorometric image analysis, in which base calling and tag counting were performed using the Illumina pipeline. The sequence reads were mapped to the reference genome (Ensemble, Zv8, Release 59, August 2010) using the CLC Genomics Workbench (version 4.0.3., October 28, 2010).

GeneChip hybridization

For control and CsA, RNA samples were used as produced for the RNA NGS sequencing (Fig. 1). Sample labeling and hybridization to Affymetrix GeneChip zebrafish ST Genome Arrays were performed by ServiceXS B.V. (Leiden, the Netherlands). The Ambion WT Expression kit (#4411974) was used to synthesize labeled sense stranded cDNA starting from 100 ng total RNA. The minimal yield of the cRNA product is 10 µg. The Affymetrix Terminal Labeling Kit (901524) was used to perform the fragmentation and terminal labeling step using 5.5 µg of the ss cDNA. The concentration and the quality of the cRNA and fragmented ss cDNA samples were assessed using the Nanodrop and the BioAnalyzer. A total amount of 2.9 µg (25 ng/µl) fragmented ss cDNA was finally utilized for the hybridization on the Affymetrix Zebrafish ST Array. The Ambion WT Wash and Stain Kit for GeneTitan Hybridization (#901622) was used for the hybridization, washing, staining and scanning of the chips. The entire experimental procedure was carried out according to ServiceXS Standard Operating Procedures (SOPs) which have been validated for use with the Affymetrix kits and GeneChips and Array

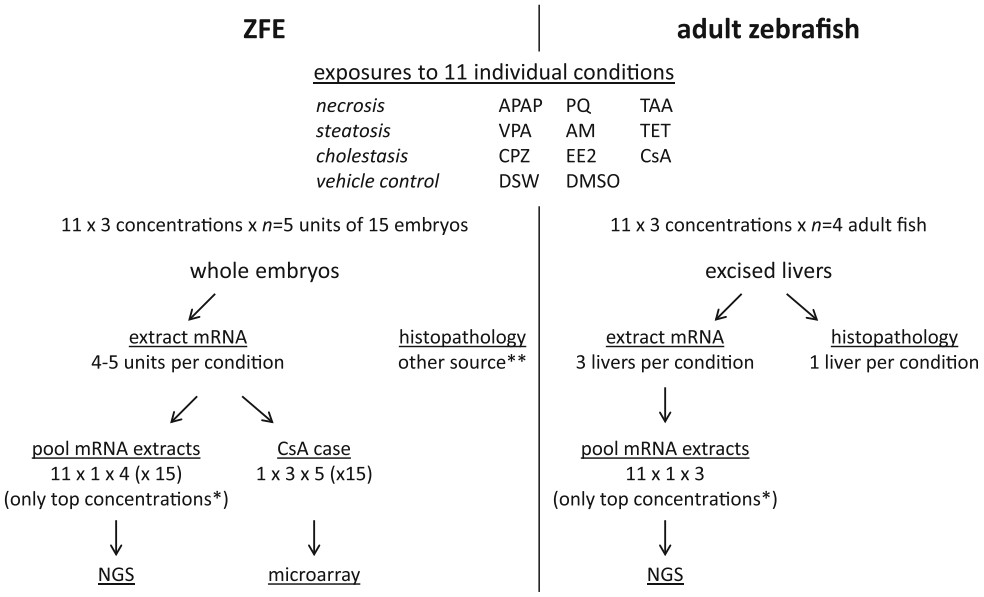

Fig. 1 Study design for next-generation sequencing analysis. Each pool contained 500 ng/µl total RNA, consisting of equal absolute quantities of total RNA from each exposure to the highest concentration of each compound

* The 2 lower concentrations were for contingency in case of toxicity in the top concentration, and could be included in the CsA case study.

** Embryos for histopathology were sampled from the concentration range finding study.

Plates and are completely compatible with the Affymetrix protocols.

The software program Affymetrix GeneChip Command Console (v3.2) was used for fully automated operation of the Affymetrix fluidics stations, which process the washing and staining of the cartridges. After scanning, the array images (DAT files) as well as the correct alignment of the grid were inspected using the program Affymetrix Command Console Viewer software.

Data analysis

In the NGS output file, transcripts were quantified to reads per kilobase of exon model per million mapped reads (RPKM) values, which are expression values corrected for library size and transcript length. These RPKM values were calculated with the CLC Genomics Workbench software (Mortazavi et al. 2008). Previous RNA NGS studies of zebrafish embryos indicated that this technique is extremely sensitive and can even detect gene expression at levels that are hard to detect with other high-density technologies such as micro-arrays (Hegedűs et al. 2009; Ordas et al. 2010; Stockhammer et al. 2010). On the other hand, at extremely low expression levels, exact quantification and reproducibility are often impaired. We therefore used a filter level to exclude unreliable values. Based on previous experience (unpublished results), this filter level was set at an RPKM of 0.12 and only genes with a higher RPKM value were included in the analyses. Statistical analyses were carried out using the R statistical software environment (http://www.r-project.org). To investigate the expression of genes associated with hepatotoxicity, gene lists were made using a text-mining tool (Anni2.1, April, 2010) (Jelier et al. 2008). This tool provides an interface to Medline and retrieves associations for several classes of biomedical concepts. For determining the relevance to the applied search term, every concept is given a concept weight. The used search terms were "hepatotoxicity" and "heart-, eye-, brain development" and were based on human data. As a specificity threshold, the maximum value of the multiple search terms combined should exceed 0.01. Functional overrepresentation analysis was performed using the software program PathVisio2 (2.0.11, February, 2011) (van Iersel et al. 2008). A pathway was considered "overrepresented" when the Z score was greater than two and the minimally required number of changed genes was set at two. Pathways were retrieved from Curated collection of Pathways (Barbazuk et al. 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG), and manually curated collection of Pathways (MC). BLAST was employed to search for homologs of the hypothetical transcripts against the NCBI Non-Redundant database. This procedure was used to assign hypothetical transcripts with a functional annotation, based on protein sequence similarity.

All Affymetrix Cell Intensity Files (*.cel) generated for each GeneChip using the Affymetrix GeneChip Command Console (v3.0) software were normalized using the Robust Multichip Average (RMA) algorithm. For all systems, the data were annotated with a MBNI custom CDF specifically designed for the chips (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/CDF/) (Dai et al. 2005). Mouse liver expression data were obtained from an exposure study of Kienhuis et al. (under review), where the mice were exposed to a dose of 26.6 mg/kg b.w. of CsA for 11 days. Expression data of HepaRG cells exposed for 12 h to 300 μM were obtained from Jennen et al. (2010), EBI ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) with accession number E-MEXP-2458. All gene expression signals were log2-transformed before calculations were performed.

In the CsA case study, comparisons were made on the level of single significantly expressed genes, pathways and enriched motifs for transcription factors. Significantly expressed genes were selected in whole ZFE, in vivo mouse liver, and the in vitro cell line using ANOVA performed over different exposure conditions (concentrations, doses, time-points) with an FDR cut-off of 0.05, 0.1 and 0.01, respectively.

Comparison of regulated pathways between the whole ZFE, mouse in vivo and the cell line was made using the T-Profiler software, which enables a threshold and parameter-free analysis of genome-wide expression patterns (Boorsma et al. 2005). The input for T-Profiler consisted of individual samples of the three models. After pathway calculation, the enrichment score, that is the t-value, was averaged per condition per model. Heatmaps are produced using the GeneMaths XT Software (Version, Applied Maths NV). Transcription factor enrichment analysis was performed using the significant genes in whole ZFE, in vivo mouse liver and the in vitro cell line.

Transcription factor-binding motifs associated with the significant genes were determined using the C3 dataset, which contains transcription factor-binding motifs found 2kB up- or downstream of target genes (Liberzon et al. 2011). For optimal retrieval, the zebrafish and mouse gene IDs were converted to their human homologues by using the homologene dataset of the NCBI. To determine which transcription factor-binding motifs were present significantly more than random in the gene sets, a random permutation test was applied using a cut-off value of $p < 0.01$. As an additional criterium, the transcription factor-binding motifs should be found by at least four genes in the set. To calculate the probability of overlap of the transcription factor-binding motifs between models, the *phyper* package in R was used.

RNA probes

Anti-sense RNA probes were designed using Primer-BLAST from the NCBI website (Rozen and Skaletsky 2000) based on the complete RefSeq sequence of the gene of interest. Primers were ordered by Invitrogen (Life Technologies, Breda, the Netherlands) and are summarized in Table 2. Primers were used for an RT-PCR with the Titan One Tube RT-PCR System (Roche Applied Science). This PCR product was purified with the QIAquick PCR purification kit (Qiagen, Venlo, the Netherlands), and a nested PCR was then conducted to amplify the PCR product. A synthesis step using T3 and T7 polymerase promoters was then performed to generate DIG-labeled RNA probes.

In situ hybridization

Whole mount in situ hybridization was performed following a protocol adapted from Thisse et al. (Thisse and Thisse 2008). ZFEs were collected at 5-dpf and fixed overnight in 4 % paraformaldehyde. After fixation, ZFEs were washed 2 times in 1× PBS, bleached in 3 % H_2O_2/0.5 % KOH solution to remove pigment and washed in 1× PBS. Following bleaching, the ZFEs were dehydrated in a graded methanol/PBS series and stored in 100 % methanol at −20 °C. Before in situ hybridization, ZFEs were rehydrated with 50/50 PBS/MeOH for 5 min and washed 4 times for 5 min with PTW (1× PBS, 0.1 % Tween20) followed by 40-min incubation in 5 µg/ml proteinase K in PTW at 37 °C. Embryos were fixated again in 4 % paraformaldehyde, followed by 5 times 5-min wash in PTW. Until here, all the washing steps were performed in 24-well plates using small baskets made from 15-ml tubes with a nylon mesh melted to its opened bottom end. After this, the embryos were transferred to transparent 4-ml screw cap vials and prehybridized in hybridization buffer (50 % deionized formamide; 5× SSC; 500 µg/ml tRNA; 50 µg/ml heparin; 0.1 % Tween20; pH 6.0 with 1 M citric acid) for 3 h at 70 °C. The buffer was then replaced by fresh hybridization buffer (pre-heated for 5–10 min at 70 °C) containing digoxigenin-labelled RNA probe and incubated overnight at 70 °C. After hybridization, the embryos were washed twice at 70 °C for 20 min with, respectively, 50 % hybridization buffer (without tRNA and heparin)/50 % 2× SSC; and 2× SSC. Then, the ZFE were washed twice with 0.2× SSC for 60 min, followed by 5-min washes at room temperature with 50 % PBT (PTW with 2 % sheep serum and 0.2 % BSA)/50 % 0.2× SSC and finally 100 % PBT. The embryos were incubated at room temperature while shaking with PBT for 3 h, followed by incubation overnight at 4 °C with anti-DIG antibody solution diluted at 1/2,000 with PBT under gentle agitation. The embryos were subsequently washed 6 times 15 min each with PBT at room temperature. Embryos were transferred to a 24-well plate and washed 4 times for 20 min with staining buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1 % Tween 20, 5 mM levamisole). The first of these washing steps was done with staining buffer without $MgCl_2$. Embryos were stained with 20 µl/ml NBT/BCIP staining buffer until an optimal signal was obtained. The staining reaction was stopped by three

Table 2 Primer sequences for in situ hybridization

Primers	Strand	PCR	Sequences
FABP10	Forward	1st PCR	AGCGGGACGTGGCAGGTTTAC
FABP10	Reverse	1st PCR	CCTCCGACTGTCAGCGTCTCCAC
FABP10	Forward	Nested PCR	CTCAGAGCCATCTCTCTGCCAGA
FABP10	Reverse	Nested PCR	CCTGGATGTGGGAGAATCGGTCA
PPARγ	Forward	1st PCR	GACGTTTGGCTGGCCCGTGG
PPARγ	Reverse	1st PCR	CAGGAACAGCGCCATGTCGCA
PPARγ[a]	Forward	Nested PCR	GAAGATCCGTCTTCATCCTCAC
PPARγ[a]	Reverse	Nested PCR	GATCTGTCCGTAGGAGATCAGG
Zgc:193613	Forward	1st PCR	TGGGCACAGGAATGGCCCGT
Zgc:193613	Reverse	1st PCR	TGGACACACAGCTGTCAGATTGGT
Zgc:193613	Forward	Nested PCR	TCAAGAGGCTTGTCAATGCTTGGG
Zgc:193613	Reverse	Nested PCR	TTCCCTGGGGCAGTACGGTGT
Wu:fj16a03	Forward	1st PCR	TTCCAACTTGCTGAACATCCGTGAA
Wu:fj16a03	Reverse	1st PCR	ATGTTGCTGCATTGCTGTCGGAT
Wu:fj16a03	Forward	Nested PCR	TGTTCCTGCTGCTGCCTGTCTG
Wu:fj16a03	Reverse	Nested PCR	AGCATTTCCAGCCTTTCTTCTCGC

[a] Primer set from literature (Flynn et al. 2009)

washes in 0.5 ml PTW, followed by overnight fixation in 4 % paraformaldehyde at 4 °C. Embryos were subsequently washed 3 times in 0.5 ml PTW and transferred to 100 % glycerol, placed on a rocker and agitated gently overnight at room temperature in the dark, mounted in 100 % glycerol and observed and photographed microscopically.

Results

Hepatotoxicant-specific liver pathology in zebrafish embryo and adult zebrafish liver

Histopathological examination in the whole ZFE and aZFL was conducted after 48 h of exposure to the model compounds. In H&E staining, hepatocytes of the control adult zebrafish showed a similar morphology as mammalian hepatocytes (Fig. 2a, b). Although the liver as a whole did not show the obvious lobular structure which is present in mammals, substructures such as bile canaliculi could be discerned (Fig. 2b, arrow). Hepatocytes in the control embryos showed a more open cytoplasm, although with varying degrees, indicative of varying levels of glycogen contents (Fig. 3a, b). After 48 h of exposure, cholestasis was a remarkably frequent observation in aZFL, with a high incidence in nominal cholestatic compounds (CPZ, EE2, CsA), but also with most of the non-cholestatic compounds (Table 3). Cholestasis appeared as intracellular and intra-canalicular brown pigment accumulation in H&E staining (Fig. 2c–e), and these subtypes were confirmed by Fouchet staining (Fig. 2g, h, compared with control in 2f). In addition, with TAA, the nucFouchet staining was confined tolei (Fig. 2i). Cholestasis was not observed in ZFE. Lipid vacuoles as a mark of steatosis were not observed in aZFL, but well discernible in ZFE with two of the nominal steatotic compounds and one nominal cholestatic compound (Fig. 3c–e). However, oil-red-O staining also revealed lipid droplets in aZFL, with nominal steatotic compounds (illustrated for TET in Fig. 2k, compare with control in Fig. 2j). Necrosis did not show in either aZFL or ZFE, that is, not in a zonal pattern as can be observed in mammal liver. On the other hand, marks of cell death, particularly chromatin condensation and cytoplasmic eosinophilia were observed in both aZFL (Fig. 2l) and ZFE with some compounds, although without much consistency between life stages and clearly more compound than class specific. Additional observations were chromophobic and eosinophilic vacuolization, which occurred in aZFL and ZFE (Figs. 2m, n, 3f–h), mainly coinciding with nominal cholestatic and necrotic compounds (Table 3); and cytoplasm basophilia, in aZFL only observed with EE2 (Fig. 2o) and in ZFE with CPZ (Fig. 3c).

Overall assessment of all exposed animals indicated that simple histopathological effects were mainly observed with nominal steatotic compounds and that complex histopathology effects were present in most nominal cholestatic and necrotic compounds (Table 3). Furthermore, serial sections of the whole ZFE revealed additional histopathology in the intestinal epithelium, which showed vacuolization after exposure to CPZ, EE2 and APAP (Table 3). Histopathological observations are summarized in Table 3, without considering the xenobiotic concentration conditions.

Next-generation sequencing of the whole zebrafish embryo and adult zebrafish liver transcriptomes

Next-generation RNA sequencing was used to compare the transcriptomes of aZFL and whole ZFE. A total of 21914 transcripts were sequenced from ZFE (Fig. 4a, blue and yellow area) and 16,459 transcripts from the aZFL (Fig. 4a, blue and green area). There were 15,801 overlapping transcripts (Fig. 4a, blue area). These three areas can also be distinguished when comparing relative expression (RPKM values) between ZFE and aZFL (Fig. 4b). Here, the gray diagonal (Fig. 4b) separates transcripts that are more highly expressed in the whole ZFE (above) from those that are more highly expressed in the aZFL (below).

PathVisio2 was used to identify overrepresented biological pathways and processes from the uniquely expressed transcripts in ZFE (6,113 transcripts, Fig. 4a, b, yellow area) and aZFL (658 transcripts, Fig. 4a, b, green area). ZFE thus showed overrepresentation in developmental processes, signaling pathways and in other pathways not obviously related to the liver. In aZFL, over-represented pathways were mainly found in the immune and intracellular processes (Table 4). It should be noted that due to nature of the experimental setup and the pooling of all individual samples, regulation of neither adaptive or toxicity pathways nor of transcription factors can be attributed to any individual of our hepatoxicants.

Hepatotoxicity-associated gene expression in the exposed zebrafish transcriptomes

Next, to discover whether transcripts related to either human hepatotoxicity or development were expressed in exposed whole ZFE or aZFL, we performed text mining using an automated search strategy (Supplementary Table 1 for hepatotoxicity, supplementary Table 2 for development).

Transcripts specific for hepatotoxicity were evenly distributed in both pooled ZFE and aZFL samples and mainly located in the set of overlapping genes (Fig. 4b, red squares, $n = 127$, and explained in supplementary Table 3). Furthermore, transcripts related to development were

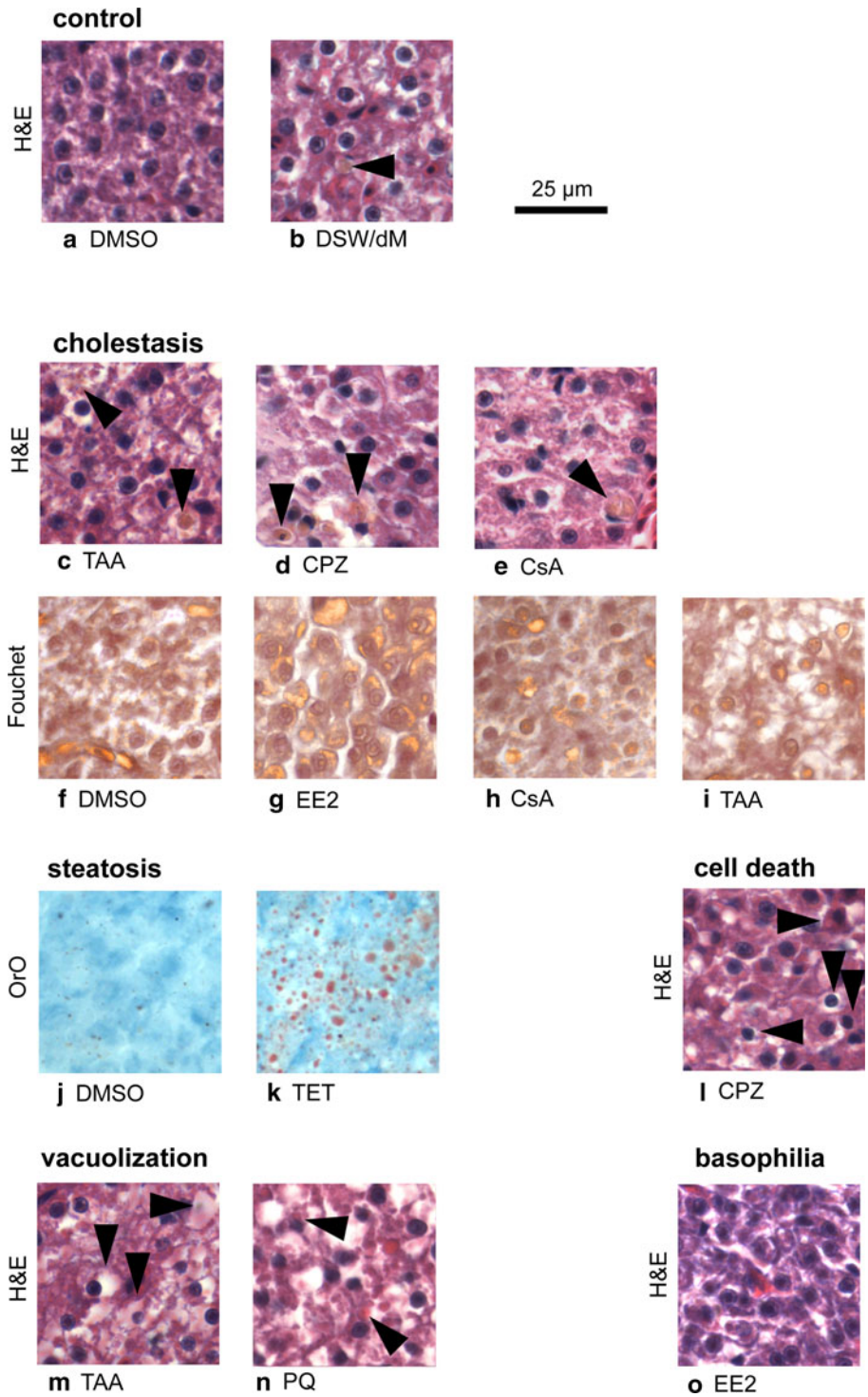

Fig. 2 Hepatotoxicant-specific liver pathology in adult zebrafish. Microphotographs are illustrations of various observations recorded in Table 3. Reference histology in control (**a**); *arrowhead* indicates bile canaliculus (**b**). Cholestasis is shown in H&E (**c**–**e**) and Fouchet staining (**f**–**i**). *Arrowheads* in **c**, **d** intracellular cholestasis, in **e**, intracanalicular cholestasis. Corresponding intracellular and intracanalicular cholestasis with Fouchet staining are shown in **g** and **h**, respectively; additional intranuclear cholestasis in **i**. Steatosis is shown with Oil-red-O staining in **k**, absence of OrO is shown in **j**. Cell death is indicated by chromatin condensation and eosinophilic cytoplasm (*arrowheads* in **l**). *Arrowheads* in **m**, chromophobic vacuolization, *arrowheads* in **n**, eosinophilic cytoplasm inclusions; **o**, basophilic cytoplasm. Compound abbreviations are explained in Table 1, except for dM (d-Mannitol, negative control). *Size bar* refers to all microphotographs

predominantly present in the whole ZFE (Fig. 4b, green diamonds, $n = 324$, and explained in supplementary Table 4). Several hepatotoxicity-associated genes (red squares) were uniquely present in ZFE, namely *aanat1*, *cyp1b1*, *fabp10a*, *hgfa*, *npy*, *otc*, *pomca* and *si:dkey-22d17.3*. In aZFL, the uniquely expressed hepatotoxicity-related gene was *faslg* and the uniquely expressed genes for development were *amh* and *bmp10*.

Validation of liver-specific gene expression by in situ hybridization

Genes which by text mining were associated with hepatotoxicity and also were highly expressed in both whole ZFE and aZFL were selected to verify liver specificity of the expression using in situ hybridization in whole ZFE. This set included three known genes, that is, fatty acid-binding

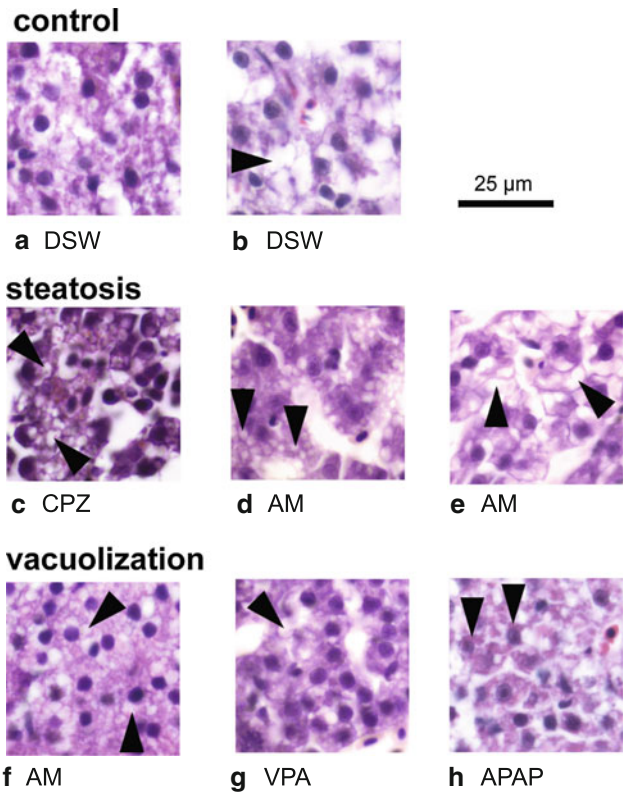

Fig. 3 Hepatotoxicant-specific liver pathology in whole zebrafish embryo. Microphotographs are illustrations of various observations recorded in Table 3. Reference histology in controls (**a**, **b**); *arrowhead* indicates irregular unstained area indicative of glycogen storage. Lipid vacuoles of varying sizes (*arrowheads*) as observed in H&E are illustrated in **c**–**e** (small in **c**–**d**, larger in **e**). *Arrowheads* in **f** and **g** indicated chromophobic vacuolization, and eosinophilic inclusions in **h**

protein 10a (*fabp10a*), peroxisome proliferator-activated receptor gamma (*pparγ*), and apolipoprotein A2 (*apoa2*) and one unannotated hypothetical gene, *wu:fj16a03*. All four genes thus showed expression in the liver region, whereas *pparγ* and *wu:fj16a03* showed additional staining in the brain and gut (Fig. 5). For *fabp10a*, *pparγ* and *apoa2,* the sense probes were negative, while for *wu:fj16a03,* the sense probe stained the same areas as the antisense probe, cautioning for conclusiveness of the *wu:fj16a03* antisense signal. Still, the in situ hybridization confirmed the liver specificity as concluded from NGS in at least three of the four analyzed expression markers.

Case study cyclosporine A

As a next step, we performed a comparative study using the widely studied hepatotoxicant cyclosporine A (CsA). Here, CsA-regulated gene sets, pathways and transcription factors in ZFE were compared with existing data from studies of CsA-treated in vivo mouse livers and HepaRG cells. Statistical analysis of Affymetrix microarray data

Table 3 Histopathological observations in ZFE and aZFL

	Embryo liver								Adult liver								Illustration in Figs.
	Cholestasis			Steatosis			Necrosis		Cholestasis			Steatosis			Necrosis		
	CPZ*	CsA	EE2*	AM	VPA	TET	APAP*	PQ	TAA	CPZ	CsA	EE2	AM	VPA	TET	APAP	PQ	TAA	
n	7	3	12	6	6	5	10	5	8	3	3	3	3	3	3		3	3	
Cholestasis (H&E)	–	–	–	–	–	–	–	–	–	2	1	3	–	–	–	–	1	3	2c–e
Cholestasis(Fouchet)											cn	cp						in	2f–i
Lipid vacuoles (H&E)	6	–	–	6	6	–	–	–	–	–	–	–	3	3	3	–	–	–	3c–e
Oil-red-O (cryosections)				6	6	5							3	3	3				2j, k
Chromatin condensation	2	–	11	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	2l
Chromophobic vacuolization	–	1	8	–	–	–	–	–	–	–	3	–	–	–	–	–	–	3	2m, 3f, g
Eosinophilic vacuolization	4	2	–	–	–	–	–	3	5	1	2	3	–	–	–	–	3	–	2n, 3h
Cytoplasmic basophilia	4	–	–	–	–	5	7	–	–	–	–	3	–	–	–	–	–	–	2o

Observations are qualitative, that is, without considering severity or intensity of effects, and without considering concentration dependency of effects. *Cn* canalicular, *cp* cytoplasmatic, *in* intranuclear. * CPZ, EE2 and APAP showed remarkable vacuolization in the intestinal epithelium. Empty cells indicate not tested. –Indicate no observation

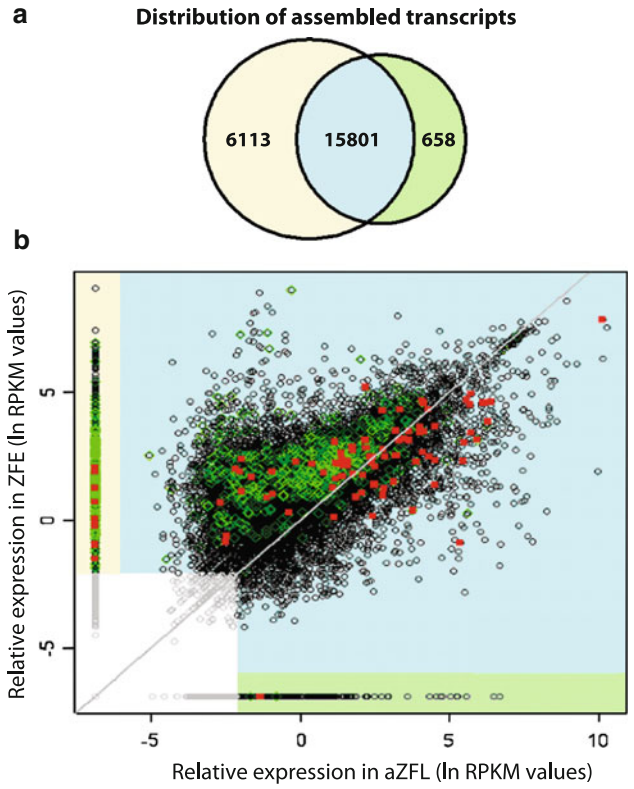

Fig. 4 Expression comparison of hepatotoxicity-associated genes and development-specific genes for the aZFL and ZFE. **a** Venn diagram showing the number of expressed transcripts per experimental pool and the overlap of these expressed transcripts after exposure to a set of model hepatotoxicants. **b** *Black circles* represent genes that were neither related to hepatotoxicity nor involved in development of heart, eye and brain based on the results of the text mining. *Gray circles* represent genes that were not included in the analysis due to their RPKM expression below 0.12. *Green diamonds* indicate genes associated with development, *red squares* indicate genes associated with hepatotoxicity. *Colored areas* indicate model specificity of transcripts: *green area* only expressed in adult zebrafish liver (658), *yellow area* only expressed in whole zebrafish embryo (6,113), *blue area* expressed in both model systems (15,801). *White shaded area* indicate transcripts not involved in analysis (color figure online)

revealed 72 significantly regulated liver-associated genes for the whole ZFE model. In comparison, 115 significantly regulated genes were observed in the in vivo mouse liver; and 262 significantly regulated genes in the HepaRG cell line. On single gene level, there was no overlap observed between the significant CsA-induced transcripts of the in vivo mouse liver and whole ZFE, which can be understood from the low number of significant transcripts in each model. For the human HepaRG cell line, 3 transcripts overlapped with the in vivo mouse liver. Pathway analysis using all genes is therefore a more informative approach, and when aligning all regulated pathways in the three models in a cluster analysis, this showed good comparability between whole ZFE and mouse liver in vivo (Fig. 6). These two models had 15 of the 26 regulated pathways regulated in the same direction, whereas the HepaRG cell line showed a deviating regulation of the these 15 pathways, and only 5 pathways regulated in the same direction as compared to mouse. Concordance between mouse liver and the cell line was similar to that between ZFE and the cell line. While most corresponding pathways between whole ZFE and mouse in vivo included hepatotoxicity-related pathways (Fig. 6, italics), the pathways that showed overlap between whole the ZFE and the liver cells included cell cycle-related pathways, which is related to the active proliferation in these two models. Importantly, cholesterol biosynthesis was downregulated in all models.

The high concordance in hepatotoxicity pathways between ZFE and mouse liver is indicative for similar regulation at the level of transcription factors. To verify this, we started out with only those genes which were significantly regulated and liver specific in the ZFE, that is, present in the overlapping genes in the NGS comparison between whole ZFE and aZFL (Fig. 4, blue area). Transcription enrichment analysis thus resulted in 45 enriched transcription factor-binding motifs (Fig. 7). In mouse liver, using the whole set of significantly regulated genes, 58 enriched transcription factors were found, and 19 enriched transcription factors in the HepaRG cell line. The overlap between the whole ZFE and in vivo mouse liver was significant (p value < 0.001, hypergeometric test) and showed eight overlapping transcription factor-binding motifs, which were *Runx2*, *Ets2*, *Atf1*, *Mef2*, microRNA 137, microRNA 181, microRNA 182, microRNA 527. The overlap between the cell line HepaRG and in vivo mouse liver was 5 transcription factor-binding motifs, which were *Meis 1*, *Usf2*, microRNA 145, microRNA 181 and *Ddit3*. The overlap between ZFE, in vivo mouse liver and HepaRG cells was one transcription factor-binding motif, which was microRNA 181.

Discussion

The zebrafish is a powerful vertebrate model for human biology and disease, and zebrafish liver resembles the mammalian liver on the morphological and functional level (Hibiya et al. 1982). In ZFE, hepatocytes are present from 36-hpf, and at 72-hpf, the liver is fully functioning, including functional activity of the cytochrome P450 system, which is important for metabolizing xenobiotics (Alderton et al. 2010). Therefore, hepatic responses can be expected after exposure to hepatotoxicants in ZFE. In this paper, we investigated the applicability of the ZFE as an alternative model system for hepatotoxicity testing. To this aim, we compared hepatotoxic effects induced by a set of reference compounds (Table 1), reflected by histopathology and gene expression profiling, in the whole ZFE and adult zebrafish liver.

Table 4 Biological pathways and processes in the subset of uniquely expressed transcripts in ZFE and aZFL

Biological system	Pathway	ZFE	aZFL
Development	Neural crest development	10.8	–
	Canonical wnt—zebrafish	6.9	–
	Non-canonical wnt pathway	5.0	–
	Wnt signaling pathway	2.6	–
	endochondral ossification	2.0	–
	Hedgehog signaling pathway	3.9	2.0
	Melanogenesis	4.8	–
	BMP signaling pathway	2.8	–
Muscle systems	Vascular smooth muscle contraction	2.0	–
	Calcium regulation in the cardiac cell	6.4	–
	Striated muscle contraction	3.1	–
	Cardiac muscle contraction	4.7	–
Metabolism	Glycosphingolipid biosynthesis—ganglio series	2.1	–
	Taurine and hypotaurine metabolism	2.3	–
	Biogenic amine synthesis	3.8	–
	Nitrogen metabolism	–	5.3
	Alanine, Aspart0061te and glutamate metabolism	–	3.7
Signal transduction, signaling molecules and interaction	Nodal signaling pathway	3.7	–
	Peptide GPCRs	3.8	–
	GPCRs, class C Metabotropic glutamate, pheromone	2.4	–
	Monoamine GPCRs	2.3	–
	MAPK signaling pathway	3.8	–
	ERK1–ERK2 MAPK cascade	2.8	–
	FGF signaling pathway	5.0	–
	Myometrial relaxation and contraction pathways	3.3	–
	Calcium signaling pathway	8.2	2.1
	Neuroactive ligand–receptor interaction	14.8	2.9
	GnRH signaling pathway (signaling)	2.2	–
	Proteasome	–	2.4
Intracellular processes	ECM–receptor interaction	3.1	–
	Cell Adhesion Molecules (CAMs)	3.6	–
Immune	Phagosome	–	1.8
	Prostaglandin signaling	2.7	–
	NOD pathway	–	2.6
	Cytokine–cytokine receptor interaction	–	3.0
Other	ACE inhibitor pathway	2.3	–
	Phototransduction	5.8	–
	SIDS susceptibility pathways	4.5	–
	Oocyte meiosis	–	2.9
	Ovarian infertility genes	–	5.3

Analysis is based on 6,113 unique transcripts in ZFE and 658 unique transcripts in aZFL (Fig. 4). –Pathway is not overrepresented

Pathways were "overrepresented" when the *Z* score was >2 and the minimally required number of changed genes ≥2

Histopathology indicated that the adult zebrafish liver is particularly sensitive for the development of cholestasis, after exposure with both typical and non-typical cholestatic compounds (Table 3). Cholestasis could not be detected in zebrafish embryos. This is probably due to underdevelopment of bile production mechanisms, in line with the observation that genes involved in the formation of bile ducts are first expressed at 48-hpf (Tao and Peng 2009) and that the onset of fully operational bile production in the embryo is only from 5-dpf onwards (Chu and Sadler 2009). On the other hand, comparable para-cholestatic events were present at both life stages reflected by vacuolization observed in both ZFE and aZF and induced by nominal cholestatic compounds, although vacuolization was a rather generalized observation in ZFE. Vacuolization might therefore be a non-specific response in ZFE hepatocytes,

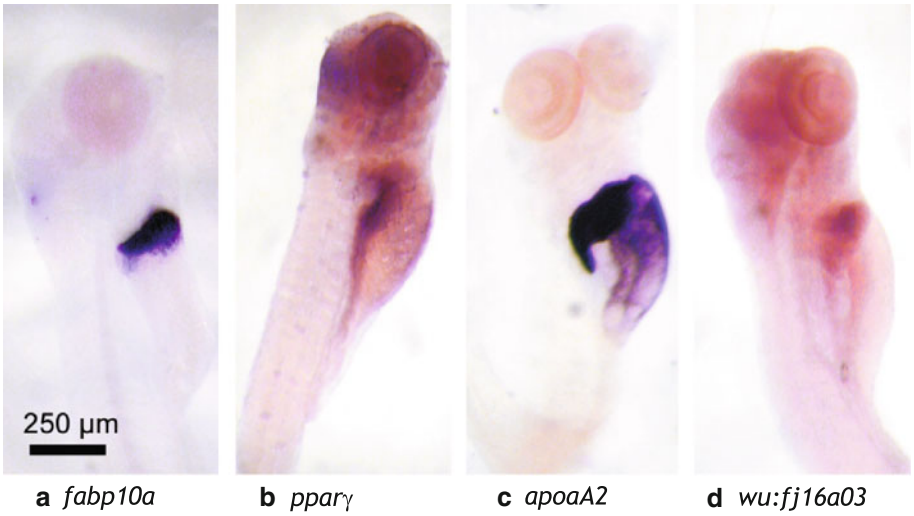

Fig. 5 Validation of liver-specific gene expression by in situ hybridization. Representative in situ hybridization microphotographs for *fabp10a*, *pparγ*, *apoa2* and *wu:fj16a03* mRNAs in 5-dpf ZFE. mRNA staining is *dark blue*. *fabp10a* shows intensely in the liver, and an additional small area in the hindbrain, *pparγ* and *wu:fj16a03* are observed in the liver, gut, and brain, and *apoa2* intensely in liver with additional staining in the yolk sac (color figure online)

for example, resulting from induction of metabolic activity or as an inhibiting effect on the mitochondrial energy production (Strmac and Braunbeck 1999). The steatotic compounds (AM, VPA and TET) induced an effect that was consistent with the nominal phenotype in both zebrafish life stages, that is, lipid accumulation as observed with oil-red-O staining, but lipid accumulation was also present with CPZ in ZFE. All three necrotic compounds (TAA, PQ and APAP) induced hepatocellular vacuoles at both life stages, with the exception of APAP in aZFL, but no apparent apoptosis or necrosis.

There were also additional, mixed effects. Taken together, the nominal phenotypes of the tested compound classes were not simply reproduced in aZFL and ZFE. The final morphology of hepatotoxic effects was related to life stage-dependent capability of hepatocytes, and hepatotoxic responses could lead to mixed histopathology. Still, all of the tested hepatotoxicants do induce specific histopathological effects in the liver, which could be interpreted as a different expression of similar mechanisms of hepatotoxicity compared to humans. Since the design of our study aimed at qualitative assessment of histopathological effects, the information on relation to exposure concentration is limited.

The overall aim of the NGS analysis was to confirm that transcripts associated with hepatotoxicity are expressed in the whole zebrafish embryo (by comparison with adult zebrafish liver) and that hepatotoxicity-specific signals are detectable over the noise of other tissues. To achieve this aim, NGS provides multiple advantages over mRNA microarrays. In contrast to microarrays, NGS does not rely on the probe design and probe selection, thus enabling detection of non-predefined transcripts, including diverse splicing variants of a single gene. The high expenses that come with NGS in a traditional toxicogenomics study setup could in our case be avoided by the use of pooled samples. This was justified because this would sufficiently reveal the liver-specific response capability of the ZFE. Working with pools even has a specific advantage, because it eliminates the blur of non- or low-responding individuals and of non- or less-active compounds (Pronk et al. 2011). On the other hand, good transcript counts depend on a sufficient number of response-evoking compounds, and information on individual compounds is lost. This was, however, not considered as a weakness, because compound-specific activity was not the focus here. Nevertheless, hepatotoxicity-associated gene expression could have been too low to support general conclusions on the applicability of the ZFE model with a specific transcriptional response in only a limited number of compounds.

Bioinformatics-based text mining showed that hepatotoxicity-associated transcripts are detectable in ZFE as well as aZFL and that they were evenly distributed between the two models (Fig. 4, red squares). This indicates that similar processes are active in whole ZFE and aZFL, in spite of the immature hepatocyte morphology in ZFE, and in spite of a different histopathology between the two models. Apparently, initial cellular responses do overlap as reflect in similar gene expression changes, but the downstream biological outcomes differ, depending on the developmental stage of the organism.

Besides a major overlap in the NGS transcripts, unique transcripts were found for both ZFE and aZFL conditions. These transcripts were analyzed for underlying pathways and processes. Unique transcripts in ZFE were mostly related to developmental processes, which is an expected result considering the developmental stage of the embryo. The specific transcripts in aZFL were predominantly involved in immune response pathways. Such responses are not likely to happen in ZFE in view of the immaturity of the immune function at that stage (Reynaud et al. 2008).

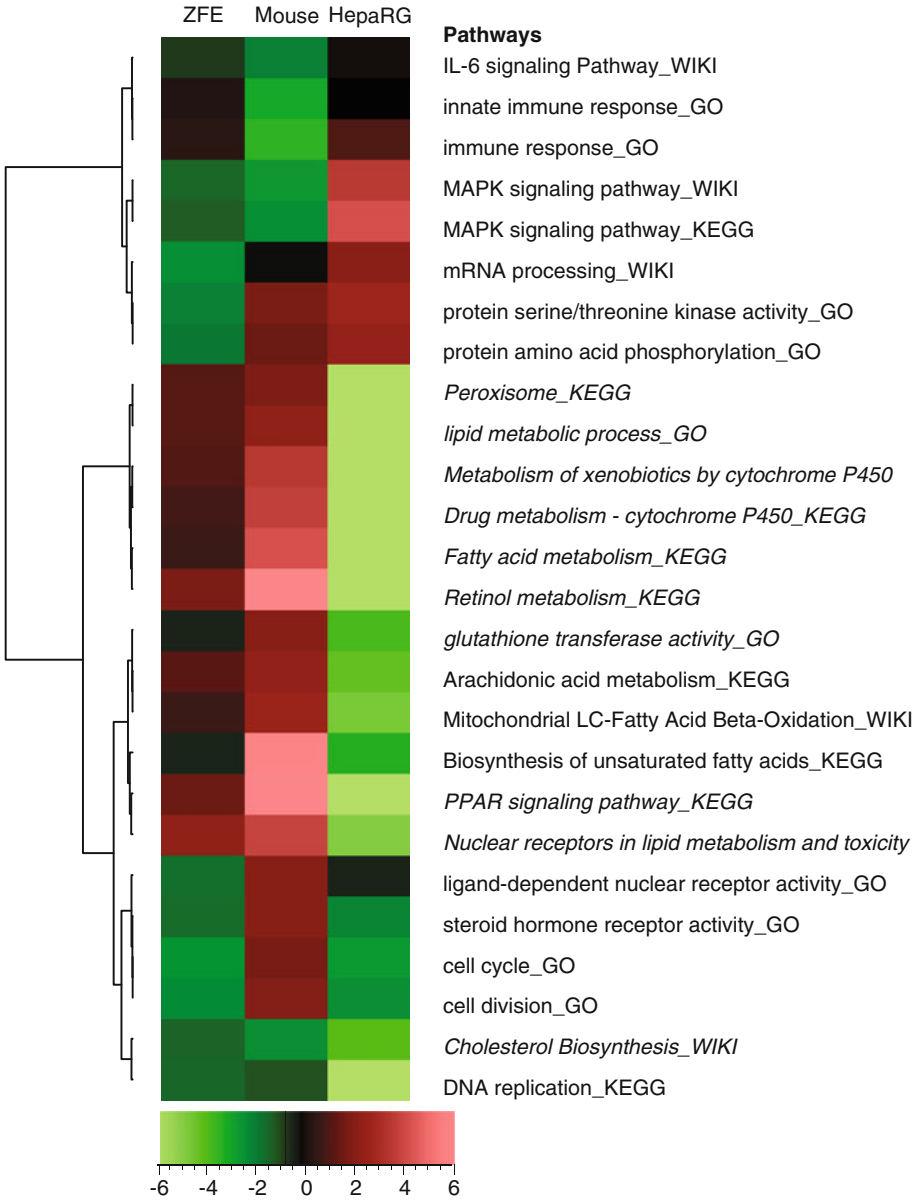

Fig. 6 Heatmap of pathway responses upon cyclosporine A exposure. Pathways regulated in mouse liver in vivo, whole zebrafish embryo and the cell line HepaRG. The cluster of pathways that are upregulated in both whole ZFE and mouse liver in vivo is clearly enriched for hepatotoxicity-related pathways (indicated in *italics*). The *color scale* indicates the *t*-value, with downregulation in shades of *green* and upregulation in shades of *red* (color figure online)

The gene expression showed that some hepatotoxicity-associated genes were only present in whole ZFE. These genes were arylalkylamine N-acetyltransferase 1 (*aanat1*), cytochrome P450, family 1, subfamily B, polypeptide 1 (*cyp1b1*), fatty acid-binding protein 1a (*fabp1a*), neuropeptide Y (*npy*), ornithine carbamoyltransferase (*otc*) and carbamoyl-phosphate synthase 1, mitochondrial (*cps1*, former *si:dkey-225d17.3*) and are highly expressed in other tissues than liver (Bradford et al. 2011). The absence of these gene in aZFL is either because gene expression is below the RPKM cut-off value or transcripts are in ZFE expressed in other tissues than the liver. The ability to identify these off-target gene expressions can be interpreted as an advantage of the ZFE model as it may still contribute to the hepatotoxic response. *Cyp1b1*, for example, is an enzyme for biotransformation of compounds and is highly expressed in the gills of zebrafish (Jonsson et al. 2007). Like the gut in mammals, the gill represents the first-pass organ in fish and its cross-talk with the liver is important for biotransformation of compounds (Ryu et al. 2004). Such interaction between organs is important for assessment of hepatotoxicity and an advantage of whole ZFE model. Moreover, the ZFE model allows for identification of toxic responses in organs outside the liver, shown in the affected intestinal epithelium after exposure to CPZ, EE2 and APAP (Table 3). One hepatotoxicity-related gene was uniquely expressed in the aZFL, *faslg*, which is known to be present only in the adult stages

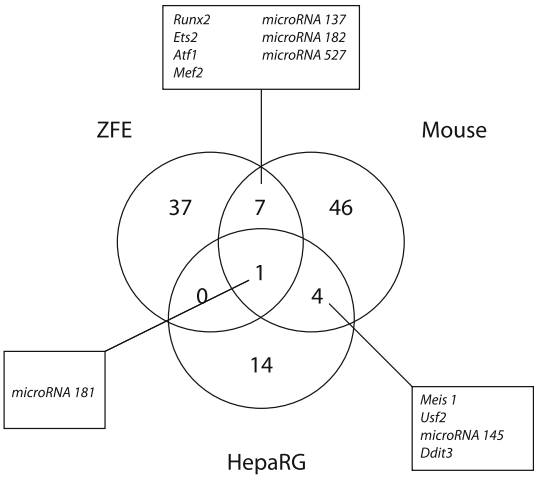

Fig. 7 Venn diagram of the enriched transcription factor motifs enriched after CsA exposure. Venn diagram showing the number of enriched transcription factor motifs in the in vivo mouse liver, whole ZFE model and the two cell lines exposed to CsA. Enrichment of transcription factors is based on the significant genes per system after exposure to CsA using an ANOVA. The overlap of transcription factors between the models is tested for significance with an hypergeometric test

(Bradford et al. 2011). The major conclusion from the comparison ZFE-aZF liver is that using whole ZFE RNA extract allows for detection of important transcripts for hepatotoxicity, either in the liver or in other tissues.

Moreover, none of the important hepatotoxicity pathways as expressed in aZF livers were missed in whole ZFE exposed to reference hepatotoxicants, providing a promising perspective for use of the whole ZFE model for (toxicogenomics-based) hepatotoxicity testing.

The liver expression of key hepatotoxicity-related genes identified in the whole ZFE was confirmed by in situ hybridization. Liver expression of *fapb10a*, *pparγ* and *apoa2* was also observed by others (Chu and Sadler 2009; Sharma et al. 2006; Wang et al. 2011). Neither function nor site specificity of the highly expressed hypothetical gene *wu:fj16a03* has been described. The closest observation is that BLAST analysis indicates that this gene has a strong sequence similarity to the toxin-1 gene, which is found in rainbow trout (*Oncorhynchus mykiss*). In trout, expression of this particular gene is found in the liver, spleen and brain, which is similar to our observations in whole zebrafish embryos (Bradford et al. 2011). Some off-liver expression sites were detected for some of the genes, for example, the *wu:fj16a03* transcript is rather prominent in the head region and in the gut.

To further validate the applicability of the ZFE as a suitable alternative for hepatotoxicity testing, pathways regulated by CsA, which is known to induce cholestasis in humans (Lee 2003), were compared between our ZFE model, a mouse model (Kienhuis et al. under review) and one in vitro model, the HepaRG cell lines (Jennen et al. 2010). Although CsA doses/concentrations and exposure duration differed between models, they were all selected to optimally induce hepatotoxicity, reflected by clinical chemistry (mouse in vivo, (Kienhuis et al. under review), histopathology (whole ZFE) and cytotoxicity (in vitro cell line, (Jennen et al. 2010) and therefore allowed for comparison. Pathway and transcription factor-based analyses attractively level out differences between models due to species variation, and model sensitivity and power, which can hamper model comparison based on single gene level. Transcription factors are key players in the regulation of a wide variety of cellular processes in health and disease. They play an important role in the development of toxic responses, and transcription factor analysis is therefore an attractive way to interpret gene expression changes (Glahn et al. 2008; Zellmer et al. 2010). On single gene level, no overlap was observed in affected significantly different genes between the whole ZFE and in vivo mouse liver. Nevertheless, pathway analysis showed that there is an overlap in regulation of the affected pathways. It appeared that the cluster of pathways showing the same direction of regulation of gene expression was highly enriched for pathways relevant for liver toxicity. The one pathway that was downregulated in all model systems, the cholesterol biosynthesis pathway, corresponds with the inhibiting effect of CsA on cholesterol 7a-hydroxylase, which is the rate-limiting step in cholesterol conversion to bile acids (Vaziri et al. 2000). In addition, a significant overlap in transcription factors was found between whole ZFE and in vivo mouse liver. These transcription factors (*Runx2*, *Ets2*, *Atf1* and *Mef2*) regulate immune processes, which can be understood from the immunosuppressant function of CsA. For the three microRNAs, not enough information is available to link them to the CsA effects. The overlapping transcription factors between hepaRG cells and in vivo exposed mouse liver are mainly involved in wide range of processes which can be linked to DNA binding (*Ddit3*, *Usf2)* and development (*Meis1*). The absence of immune cells in the HepaRG model can explain the absence of immune-function-related transcription factors.

Overall, these results indicate that there is more similarity of responses between the ZFE and in vivo mouse liver after CsA exposure than between HepaRG cells and in vivo mouse liver, particularly on the level of pathways and transcription factors. This supports the notion that the whole ZFE is a better proxy for the traditional in vivo model than the human cell line in vitro model.

In conclusion, we confirmed that the ZFE is a promising alternative model for hepatotoxicity testing, as indicated by hepatotoxicant-induced liver histopathology and by induction of hepatotoxicity-associated gene expression. NGS appeared a powerful tool allowing sensitive and

specific quantitative comparison of transcripts between ZFE and aZFL. While due to high costs, we had to apply NGS on pooled samples from multiple experiments, comparison of gene expression from a single hepatotoxicant experiment between ZFE, in vivo mouse liver, and HepaRG cells further supported the potential of ZFE in hepatotoxicity testing.

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