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Original Article

Transcriptional profiling of cytochrome P450 genes in the liver of adult zebrafish, *Danio rerio*

Akira Kubota[1], Yusuke K. Kawai[1], Natsumi Yamashita[2], Jae Seung Lee[1], Daisuke Kondoh[3], Shuangyi Zhang[2], Yasunobu Nishi[4], Kazuyuki Suzuki[4], Takio Kitazawa[2] and Hiroki Teraoka[2]

[1]Laboratory of Toxicology, Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada-cho Nishi, Obihiro 080-8555, Hokkaido, Japan
[2]Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyodai-Midorimachi, Ebetsu 069-8501, Hokkaido, Japan
[3]Laboratory of Veterinary Anatomy, Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada-cho Nishi, Obihiro 080-8555, Hokkaido, Japan
[4]Department of Large Animal Clinical Sciences, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyodai-Midorimachi, Ebetsu 069-8501, Hokkaido, Japan

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ABSTRACT — Increasing use of zebrafish in biomedical, toxicological and developmental studies requires explicit knowledge of cytochrome P450 (CYP), given the central role of CYP in oxidative biotransformation of xenobiotics and many regulatory molecules. A full complement of *CYP* genes in zebrafish and their transcript expression during early development have already been examined. Here we established a comprehensive picture of *CYP* gene expression in the adult zebrafish liver using a RNA-seq technique. Transcriptional profiling of a full complement of *CYP* genes revealed that *CYP2AD2*, *CYP3A65*, *CYP1A*, *CYP2P9* and *CYP2Y3* are major *CYP* genes expressed in the adult zebrafish liver in both sexes. Quantitative real-time RT-PCR analysis for selected *CYP* genes further supported our RNA-seq data. There were significant sex differences in the transcript levels for *CYP1A*, *CYP1B1*, *CYP1D1* and *CYP2N13*, with males having higher expression levels than those in females in all cases. A similar feature of gender-specific expression was observed for *CYP2AD2* and *CYP2P9*, suggesting sex-specific regulation of constitutive expression of some *CYP* genes in the adult zebrafish liver. The present study revealed several "orphan" *CYP* genes as dominant isozymes at transcript levels in the adult zebrafish liver, implying crucial roles of these *CYP* genes in liver physiology and drug metabolism. The current results establish a foundation for studies with zebrafish in drug discovery and toxicology.

Key words: Zebrafish, Cytochrome P450, CYP, Liver, Transcript expression

INTRODUCTION

Cytochromes P450 (CYPs) comprise a large and ancient superfamily of genes encoding heme-containing monooxygenase enzymes, with broad significance in biology. Many CYPs have important roles in basic physiological processes. Some CYPs, including CYP1, CYP2 and CYP3, have critical roles primarily in catalyzing oxidative biotransformation of xenobiotics and thus can determine the persistence and actions of many drugs and toxicants. CYP enzymes are responsible for the metabolism of 70-80% of all drugs in clinical use, CYP3A4(+3A5) being the main enzyme involved, followed by CYP2D6, CYP2C9 and CYP1A2 (Zanger and Schwab, 2013). There are obvious associations between the actions of some CYPs and the toxicity of some chemicals either through activation or inactivation of those chemicals. Studies with knockout mice showed that CYPs mediate adverse effects of many environmental chemicals as well as drugs and dietary chemicals (Gonzalez and Kimura, 1999, 2003). Thus, understanding the susceptibility of organisms to effects of drugs and toxicants as well as the

Correspondence: Akira Kubota (E-mail: akubota@obihiro.ac.jp)
Hiroki Teraoka (E-mail: hteraoka@rakuno.ac.jp)

basic physiology is critically dependent on knowledge of CYP functions.

The zebrafish (*Danio rerio*), mainly in its embryonic and larval stages, has been increasingly used as a model organism for drug discovery, developmental toxicity testing and ecotoxicology, due largely to rapid development coupled with its transparent feature throughout embryonic development, allowing us to examine chemical interference with organogenesis and functions of organs (Stegeman *et al*., 2010; Strähle *et al*., 2011; Nishimura *et al*., 2016). The ease of genetic detection and genetic manipulation makes zebrafish highly suitable for a mechanistic understanding of chemical effects on organisms. A striking example of the use of zebrafish in toxicology is cardiovascular toxicity posed by dioxins and related compounds; the cardiovascular system of developing zebrafish is particularly susceptible to these chemicals. An initial step of dioxin-induced cardiovascular toxicity in zebrafish embryos occurring as early as 50 to 72 hr post fertilization (hpf) involves activation of aryl hydrocarbon receptor 2 (Ahr2), and the toxicity was explained, at least partially, by increasing vascular permeability (Dong *et al*., 2004) as well as structural and functional changes in the heart (Antkiewicz *et al*., 2005). Molecular mechanistic investigations further revealed the involvement of prostanoid signaling in dioxin-induced cardiovascular toxicity in zebrafish embryos (Teraoka *et al*., 2009, 2014; Nijoukubo *et al*., 2016). Some CYPs, including CYP1A, CYP1C1, CYP1C2 and CYP5A, have also been suggested to be involved in dioxin-induced cardiovascular toxicity in zebrafish embryos (Teraoka *et al*., 2003, 2009; Kubota *et al*., 2011). Another example is thalidomide-induced teratogenicity; zebrafish embryos appear to be sensitive to this chemical, which causes pectoral fin malformations within 48 to 72 hpf, being similar to the limb malformations seen in thalidomide embryopathy in humans (Ito *et al*., 2010). An enantiomer selectivity of thalidomide teratogenicity was also indicated in zebrafish (Mori *et al*., 2018).

The liver is one of the largest organs in the abdominal cavity and is crucial to homeostasis and protecting individuals from xenobiotics. Although the structural organization of the zebrafish liver is different from that of the livers of rodents and humans, with particular emphasis on the lack of a typical lobular arrangement in the zebrafish liver, drug-metabolizing properties of the liver are generally similar in zebrafish, rodents and humans (reviewed in Vliegenthart *et al*., 2014). In zebrafish, formation of the hepatic primordium begins at 28 hpf (Tao and Peng, 2009), hepatic organogenesis is completed at 72 hpf (Isogai *et al*., 2001), and the liver is fully functional at 120 hpf, after which zebrafish become capable of independent feeding. Thus, most of the developmental toxicology studies using zebrafish embryos have been conducted with the liver not being fully functional. This is, in one aspect, advantageous because there is no need to take hepatic metabolism into consideration and the effect of a parent chemical can be solely assessed by using zebrafish embryos. On the other hand, many drugs and environmental contaminants require metabolic activation in which hepatic CYPs play important roles to produce an ultimate toxic metabolite. Therefore, studies on CYP involvement in chemical toxicities are particularly important in this premier non-mammalian model species, though there have been few such studies. Little is known about basal levels of CYP expression in the liver. Understanding basal levels of CYP expression in the liver will establish a foundation for studies with zebrafish in areas of drug discovery and toxicology.

Various drug-metabolizing CYPs that are chemically induced in the adult zebrafish liver have been identified (Bresolin *et al*., 2005; Jönsson *et al*., 2007; Kubota *et al*., 2013; Poon *et al*., 2017). In a broader point of view, stress-responsive signaling pathways have been shown to be significantly enriched in the adult liver compared with those in the whole larvae following exposure to hepatotoxicants (Poon *et al*., 2017). Thus, knowing the expression, regulation and function of CYPs in the adult zebrafish liver might be beneficial for evaluating stress responses and the roles of CYPs in detoxification and metabolic activation of xenobiotics, which may also lead to a substantial contribution to toxicological studies with larval zebrafish.

The objective of this study was to unveil transcriptional profiling of a full complement of *CYP* genes in the adult zebrafish liver. For this purpose, liver samples from male and female fish were subject to RNA-seq analysis using the Illumina NextSeq 500 platform. Transcript levels of selected *CYP* genes were further confirmed by real time RT-PCR to examine sex-related and individual differences in expression levels.

MATERIALS AND METHODS

Fish husbandry and liver collection

Zebrafish (*Danio rerio*) of the RIKEN wild-type strain (10 months old) were used in the present study. Male and female zebrafish (approximately 50 fish each) were separately reared in 60 cm glass tanks (GEX, Osaka, Japan) filled with water (approximately 60 L) which was prepared in an independent filtration system equipped with a cartridge filter (Advantec Toyo, Tokyo, Japan) and an

activated carbon filter (Advantec Toyo). Rearing water was maintained at 28.5°C under a cycle of 14 hr light and 10 hr dark and was partially refreshed every three days after being transferred from RIKEN CBS (Saitama, Japan). Water quality was regularly monitored and kept at a pH of 7.0-8.0, NH_4^+ of < 0.2 mg/L and NO_2^- of < 0.1 mg/L. Twelve fish were sampled randomly from the two tanks containing males and females separately. The sampled fish were weighed (0.52 ± 0.054 g for males and 0.62 ± 0.083 g for females) and dissected for livers (n = 6 each) according to a previously described method (Gupta and Mullins, 2010) using 0.2% ethyl 3-aminobenzoate methanesulfonate (MS-222) (Sigma-Aldrich, Tokyo, Japan) for anesthesia and subsequent immersion in ice water and decapitation for euthanasia. Each liver was flash frozen in liquid nitrogen and stored at -80°C until total RNA isolation. The experimental procedures were approved by the Animal Experimental Committee of Obihiro University of Agriculture and Veterinary Medicine (Notification No. 28-27).

Total RNA extraction

Total RNA was extracted by QIAzol Lysis Reagent (Qiagen, Venlo, Netherlands) in MagNA Lyser Green Beads (Roche, Basel, Switzerland) using Fast Prep 24 Instrument ver. 4 (MP Biomedicals, Santa Ana, CA, USA). Total RNA was quantified by a Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Tokyo, Japan), and RNA integrity was assessed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Tokyo, Japan). The RNA integrity number (RIN) of all 12 liver samples was above 8.0, and the samples were thus used for further experiments.

High-throughput sequence analysis

A single RNA sample per sex, which consisted of RNA pooled from 4 fish, was prepared for high-throughput sequence analysis. Libraries were prepared from 1,000 ng pooled total RNA (250 ng each) and amplified by PCR with 12 cycles using a KAPA Stranded mRNA-Seq Kit (Kapa Biosystems, Wilmington, MA, USA) with a FastGene Adapter Kit (Nippon Genetics, Tokyo, Japan) following the manufacturer's protocol. The libraries were quantified by a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and qualified by an Agilent 2100 Bioanalyzer using a High Sensitivity DNA Kit (Agilent Technologies). The prepared libraries were sequenced on an Illumina NextSeq 500 platform (Illumina, San Diego, CA, USA) with 76-bp paired-end reads. All steps for the high-throughput sequence analysis were conducted at the Bioengineering Lab. Co., Ltd. (Kanagawa, Japan).

Processing RNA-seq data

Poor-quality reads (score below 20) were removed from the raw paired-end sequenced files using Sickle ver. 1.33, followed by removal of short length reads (shorter than 30 bases). The filtered reads were mapped into the reference genome (*Danio rerio* GRCz11) obtained from the NCBI assembly database (http://www.ncbi.nlm.nih.gov/assembly/GCF_000002035.6) using Hisat2 ver. 2.1.0 (Kim *et al*., 2015). After the mapping step, mapping files (.SAM files) were converted into binary alignment files (.BAM file) using Samtools ver. 1.5 (Li *et al*., 2009).

Count of reads on CYP isoforms

The number of reads mapped in the reference genome was counted by featureCounts ver. 1.5.3 (Liao *et al*., 2014). Reads overlapping with more than one gene were not counted in this analysis. The count of reads of each gene was converted to Reads Per Kilobase per Million (RPKM) (Mortazavi *et al*., 2008). In each of the *CYP* isoforms, mapping data were visualized by Integrative Genomics Viewer (IGV) ver. 2.4 (Robinson *et al*., 2011; Thorvaldsdóttir *et al*., 2013) and it was confirmed that there was no differential expression of transcript variants. Therefore, the RPKM were averaged in each *CYP* gene. In cases in which there was a difference between the reference genome assembly and alternate loci scaffolds (ALT_REF_LOCI) for representations of variant sequences, the RPKM with a higher value was chosen. The sequence data used in the present study are available at NCBI with the accession number DRA007652.

Real-time RT-PCR

Another portion of total RNA was DNase treated by the TURBO DNA-free kit (Thermo Fisher Scientific). cDNA was synthesized using the Transcriptor First Strand cDNA synthesis kit (Roche) according to the manufacturer's protocols. Quantitative real-time PCR was performed using Thunderbird qPCR Mix (Toyobo, Osaka, Japan) in a LightCycler 480 System II (Nippon Genetics), according to the manufacturer's instructions. Real-time PCR primers used for selected *CYP* genes are listed in Supplemental Table S1. In each sample, the genes were analyzed in duplicate with the following protocol: 95°C for 1 min and 95°C for 15 sec/60°C for 1 min (40 cycles). Melt curve analysis was performed at the end of each PCR run to ensure that a single product was amplified. Specificity of the qPCR primer pairs was confirmed by direct sequencing of PCR products.

Calculations and statistics

The $E^{-\Delta Ct}$ method was used to compare the expres-

sion levels of different *CYP*s in the liver of zebrafish (Schmittgen and Livak, 2008). The efficiency (E) of the PCR reactions for each gene was calculated using standard curves generated by serially diluting PCR products amplified by the quantitative PCR primers. In the present study, eukaryotic translation elongation factor 1α1, like 1 (*eef1a1l1*) was used as a reference gene based on its low degree of expression variability across tissue types, during development and in chemical treatment experiments (Tang *et al*., 2007; McCurley and Callard, 2008). Relative mRNA expression of each target gene was normalized to that of *eef1a1l1* ($E^{-\Delta Ct}$; where ΔCt = [Ct(target gene) − Ct(*eef1a1l1*)]). In the figure showing qPCR results, the whiskers show the data range, while the box extends from the 25th to 75th percentiles. Plots are also shown for clarifying the individual differences in transcript levels. Significance of difference in the expression levels between females and males was determined by the Wilcoxon rank sum test adjusted by the Benjamini-Hochberg (BH) method (Benjamini and Hochberg, 1995) for false discovery rate (FDR) in R ver. 3.4.4 (R Core Team, 2018). The significant level was set at 0.05. Spearman's rank correlation analysis for each possible gene pair in the transcript expression data was also performed in R ver. 3.4.4 (R Core Team, 2018). The FDR was calculated using the BH method and the significant level was set at 0.05. All graphical representations were made by ggplot2, ggbeeswarm and GGally packages (Wickham, 2016; Clarke and Sherrill-Mix, 2017; Schloerke *et al*., 2018).

RESULTS

Transcript profiling of CYP genes in the adult zebrafish liver

Transcriptional profiling identified a diverse array of *CYP* genes that are expressed in the adult zebrafish liver (Supplemental Table S2). The ranking orders of the expression levels that accounted for > 5% of total *CYP* transcripts were as follows (Fig. 1): *CYP3A65* (13.4%) > *CYP2AD2* (12.2%) > *CYP2K18* (11.4%) > *CYP27B1* (5.7%) > *CYP2Y3* (5.2%) > *CYP2P9* (5.1%) > *CYP2R1* (5.0%) > *CYP1A* (5.0%) for females and *CYP2AD2* (17.2%) > *CYP1A* (14.5%) > *CYP2K22* (7.1%) > *CYP3A65* (6.5%) > *CYP2P9* (6.4%) > *CYP51* (6.1%) > *CYP2Y3* (5.3%) for males. Hepatic expression of *CYP2K22* was highly specific for males, while *CYP2K18* transcript expression appears to be high in females in the RNA-seq data.

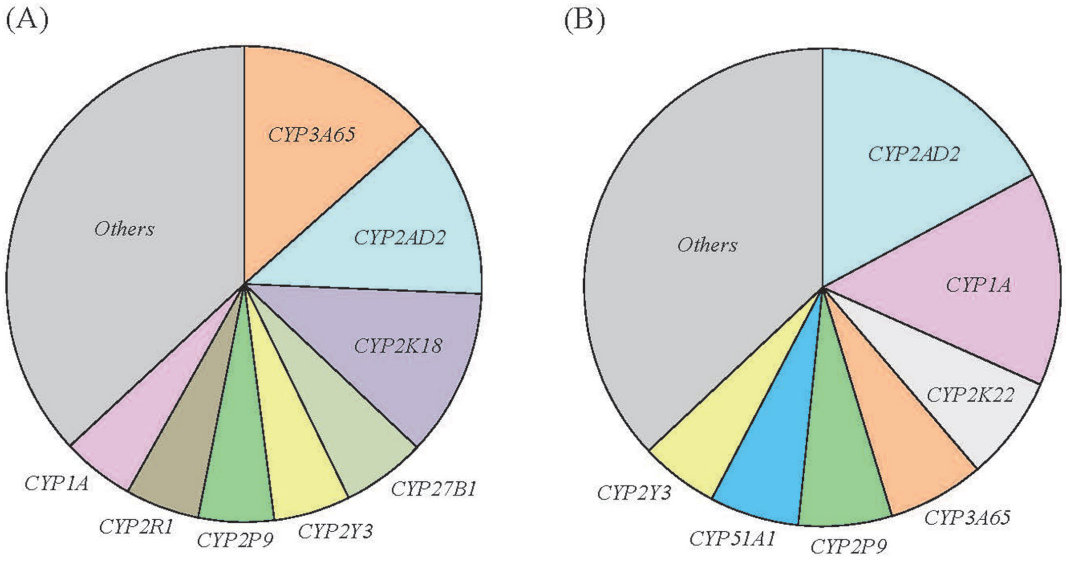

Fig. 1. Transcript profiling of a full suite of *CYP* genes in females (A) and males (B) of adult zebrafish. *CYP* genes with a contribution of > 5% to total *CYP* transcripts are highlighted with the isozyme names, while *CYP* genes with a contribution of < 5% are denoted as "others". Same genes in females and males share the same color in the pie chart. A single RNA sample per sex, which consisted of RNA pooled from 4 fish, was prepared for high-throughput sequence analysis. Libraries prepared from 1,000 ng pooled total RNA (250 ng each) and amplified by PCR with 12 cycles were sequenced on an Illumina NextSeq 500 platform with 76-bp paired-end reads. See Materials and Methods for detailed description of the analysis of the RNA-seq data.

Quantitative real-time PCR of CYP1-3 genes

Six liver samples per sex, including the same 4 samples as those used for the high-throughput transcriptional profiling as indicated above and 2 additional samples, were further tested for individual differences in the *CYP* transcript levels. To this end, the expression levels of all *CYP1* and *CYP3* genes and selected *CYP2* genes that were shown to be highly expressed in the liver in the high-throughput analysis were measured by quantitative real-time PCR (Fig. 2). *CYP* gene expression levels measured by the two methods basically showed a good correlation (Supplemental Fig. S1). The differences observed can be at least in part explained by different sets of samples; for example, the *CYP3C2/3C3* expression levels were low in both sexes in the high-throughput analysis, whereas substantial levels of transcripts were detected in the male liver. Two male liver samples that had greater transcript levels were examined only by qPCR and not included in the pooled liver samples from 4 fish in the high-throughput analysis. Significant sex differences (FDR < 0.05) in the gene expression were observed for *CYP1A*, *CYP1B1*, *CYP1D1* and *CYP2N13*, although the expression level of *CYP1B1* was quite low in both sexes. Expression levels of *CYP2AD2* and *CYP2P9* also showed tendency to be higher in males than in females.

Correlations of transcript levels among CYP genes

Pairwise correlation was determined for each possible gene pair in the transcript expression data to predict whether a shared regulatory mechanism of basal expression exists in the adult liver (Supplemental

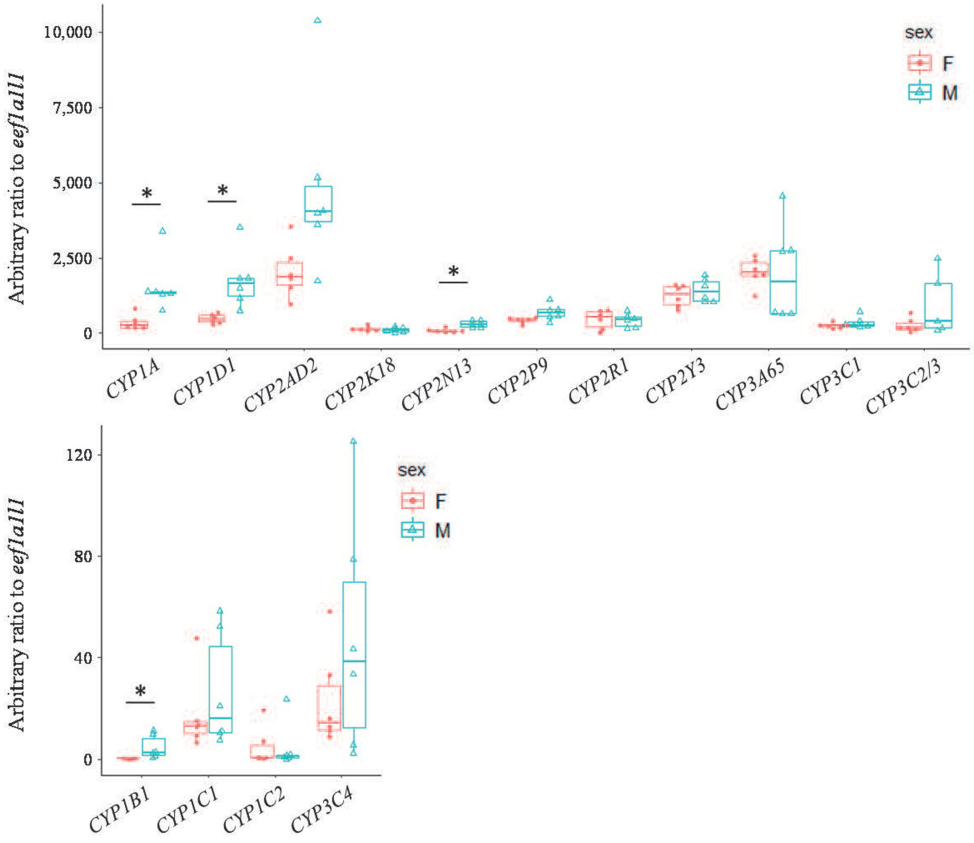

Fig. 2. Comparison of transcript levels of selected *CYP* genes in females and males of adult zebrafish. The expression data were shown in two separate figures, one containing genes with higher transcript levels (upper) and the other containing genes with lower levels (lower). A median value of 40 was set as a threshold. The whiskers show the data range, while the box extends from the 25th to 75th percentiles. Plots are also shown for clarifying the individual differences in transcript levels. Statistical differences in transcript levels between females and males were determined by the Wilcoxon rank sum test adjusted by the BH method and are shown by an asterisk (*< 0.05). N = 6.

Fig. S2). Spearman's rank correlation test adjusted by the BH method showed significant positive correlations between *CYP1A* and *CYP1D1* and between *CYP3C2/3* and *CYP3C4* in males. On the other hand, a significant negative correlation was observed between *CYP1B1* and *CYP1C1* in females.

DISCUSSION

The use of zebrafish as a non-mammalian vertebrate model is of growing importance in the fields of embryology, developmental biology, pharmacology and toxicology. Increasing use of zebrafish in those research areas demands knowledge of *CYP* gene expression, function and regulation in this species. The present study clarified the expression profiles of a full suite of *CYP* genes in the adult zebrafish liver using a RNA-seq method, successfully providing a "pie chart" of *CYP* transcript levels in this species. The expression profiles obtained from RNA-seq and qPCR showed close similarity, which is consistent with previous reports (Chandramohan *et al*., 2013; Everaert *et al*., 2017). However, a substantial difference in the expression levels measured by RNA-seq and qPCR was observed for *CYP1D1* (both sexes) and *CYP2K18* (female only). This could be attributed to differences in the experimental conditions between RNA-seq and qPCR in the present study, including selection steps of mRNA (oligo dT for RNA-seq vs. combination of oligo dT and random hexamer for qPCR). Major *CYP* genes that are expressed in both sexes include *CYP2AD2*, *CYP3A65*, *CYP1A*, *CYP2P9* and *CYP2Y3* in the order of expression levels in the RNA-seq data, together with *CYP1D1* for which the expression level was high in the qPCR data, indicating possible physiological and toxicological roles of these genes in the liver.

Transcript levels of *CYP2AD2* were high in both sexes, being most abundant in males and second highest in females, and were somewhat greater in males than in females. In an earlier microarray study in which *CYP* transcript levels were examined during early development from 3 to 48 hpf (Goldstone *et al*., 2010), *CYP2AD2* transcript levels were shown to be relatively high at 3 hpf followed by a decreasing trend at least until 48 hpf. The zebrafish genome includes three *CYP2AD* genes, *CYP2AD2*, *CYP2AD3* and *CYP2AD6*, in a cluster containing 11 *CYP2* genes tandemly arrayed on the chromosome 20 (i.e., 6 *CYP2P*s, 3 *CYP2AD*s, *CYP2N13*, *CYP2V1*), and genes in this cluster share synteny with human *CYP2J2* (Goldstone *et al*., 2010). *CYP2P9*, another *CYP2* gene within this cluster was also highly expressed in male and female zebrafish. Human CYP2J2 is an enzyme involved in the epoxidation of endogenous arachidonic acid in the cardiovascular system (Fleming, 2001, 2008). Some of the fish CYP2 enzymes in this cluster, including killifish CYP2P3, CYP2N1 and CYP2N2, have a catalytic function toward arachidonic acid hydroxylation, with particular similarity between CYP2P3 and human CYP2J2 (Oleksiak *et al*., 2000, 2003). The possibility that zebrafish CYP2ADs have the potential to metabolize arachidonic acid resulting in its homeostasis in the liver warrants further investigation. It is noteworthy that genes in the *CYP2AD* subfamily have not been identified in the current genome assembly of stickleback or Japanese medaka, but duplicated *CYP2AD* genes, named *CYP2AD12*s, have been identified in mangrove killifish (*Kryptolebias marmoratus*), and it was shown that they were induced by octylphenol but not by bisphenol A, nonylphenol or benzo[a]pyrene (B[a]P) (Puthumana *et al*., 2017). Neither the regulation nor function of genes in this subfamily is known at present.

CYP2Y3, another orphan *CYP2* gene that is a single gene in this subfamily, contributed greatly to the total *CYP* transcript levels in the adult zebrafish liver. An earlier study on developmental expression showed that *CYP2Y3* transcript levels had a peak at 12 hpf during early development (Goldstone *et al*., 2010). *CYP2Y3* shares synteny with a cluster of human *CYP2A6*, *CYP2A13*, *CYP2B6*, *CYP2F1* and *CYP2S1* genes (Goldstone *et al*., 2010). The human genes at this locus code for drug-metabolizing enzymes that are under the regulation of constitutive androstane receptor, pregnane X receptor (PXR) and/or AHR. Despite a possible relevance to drug metabolism, this gene has not been focused on in biomedical and toxicological studies in this model species. The available data showed that either knocking CYP2Y3 down or treatment with antioxidants was able to block ethanol-induced steatosis in the liver of larval zebrafish, implying that this enzyme has important roles in ethanol metabolism to highly reactive acetaldehyde, which is involved in the generation of reactive oxygen species (Tsedensodnom *et al*., 2013). The zebrafish CYP2Y3 gene has 43% amino acid identity to human CYP2E1, a major enzyme, together with alcohol dehydrogenase, which is responsible for ethanol-induced liver steatosis in humans. A few studies in which responses of fish *CYP2Y* genes following exposure to chemicals were examined showed that B[a]P induced *CYP2Y3* transcripts in a dose-dependent manner in the liver of the Chinese rare minnow (*Gobiocypris rarus*) (Yuan *et al*., 2013), whereas nonylphenol and bisphenol A both suppressed *CYP2Y3* expression in the liver of the juvenile Atlantic cod (*Gadus morhua*) (Olsvik *et al*., 2009). Regulation and the drug-

metabolizing property of this enzyme await further investigation.

Other *CYP* genes that had high transcript levels in the liver include *CYP3A65* and *CYP1A*. Zebrafish have five *CYP3* genes, i.e., *CYP3A65* and 4 *CYP3C*s (Goldstone *et al*., 2010). CYP3A65 is 54% identical to human CYP3A4. Regulation of zebrafish CYP3A65 appears to be somewhat different from that of mammalian CYP3As. Constitutive expression of *CYP3A65* in the liver and intestine of larval zebrafish has been identified (Tseng *et al*., 2005) and shown to be under regulation of Pxr and Ahr2 (Chang *et al*., 2013). It is inducible not only by the Pxr agonist pregnenolone (Kubota *et al*., 2015) but also by a typical Ahr agonist, 3,3',4,4',5-pentachlorobiphenyl (PCB126) (Kubota *et al*., 2015), the most potent of the dioxin-like PCBs, and TCDD (Chang *et al*., 2013). Pregnenolone induction of *CYP3A65* was inhibited by knocking Pxr down (Kubota *et al*., 2015), while PCB126 and TCDD induction of this gene was blocked by Ahr2 knockdown (Chang *et al*., 2013; Kubota *et al*., 2015), further demonstrating the presence of Pxr-CYP3A65 signaling and Ahr2-CYP3A65 signaling, depending on the type of agonist. Expression of *CYP3A65* was also induced by an activator of rodent PXR, pregnenolone 16α-carbonitrile (PCN), in the adult zebrafish liver (Bresolin *et al*., 2005). Concerning the catalytic function, 17β-estradiol (E_2) has been tested for metabolism by zebrafish CYP3A65 heterologously expressed in bacteria (Scornaienchi *et al*., 2010a). The results of that study showed concordance in the relative formation of 2-hydroxyestradiol and 4-hydroxyestradiol between CYP3A65 and human CYP3A4, though a huge difference (> 100-fold) in the catalytic rates between the two enzymes was observed. Currently, the roles of CYP3A65 in xenobiotic metabolism are not known.

Zebrafish have five *CYP1* genes including *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2* and *CYP1D1*. The liver is the primary organ for *CYP1A* transcript expression in adult zebrafish (Jönsson *et al*., 2007). Exposure of zebrafish adults to PCB126 caused induction of *CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2* in the liver and other organs in the abdominal cavity (Jönsson *et al*., 2007) but not induction of *CYP1D1* (Goldstone *et al*., 2009). Induction of these four *CYP1* genes by Ahr agonists was also found in larval zebrafish, with the vasculature being the primary tissues of induction (Andreasen *et al*., 2002; Dong *et al*., 2002; Kubota *et al*., 2011; Bugiak and Weber, 2009). Pregnenolone also up-regulated *CYP1A* expression in zebrafish embryos, ostensibly through Pxr signaling (Kubota *et al*., 2015), yet whether *CYP1A* in adult tissues is inducible by this compound or other Pxr agonists is not known. The metabolic capacity of zebrafish CYP1s heterologously expressed in yeast (Miranda *et al*., 2006; Stegeman *et al*., 2015) and bacteria (Scornaienchi *et al*., 2010a, 2010b) has been studied with endogenous and xenobiotic substrates, such as E_2, B[a]P and some alkoxyresorufins. In general, zebrafish CYP1A produced metabolites with regioselectivity similar to that of human CYP1A1, with some difference in the rates of metabolism by different expression systems (i.e., yeast vs bacteria) (Scornaienchi *et al*., 2010b). The overall enzymatic activities of CYP1D1 seem to be lower than those of other CYP1s (Scornaienchi *et al*., 2010b; Stegeman *et al*., 2015). Biological effects of the potent Ahr agonist 6-formylindolo[3,2-b]carbazole (FICZ) *in vivo* in zebrafish embryos were found to be enhanced by loss of CYP1A function in an Ahr2-dependent manner, indicating a crucial role of a functioning CYP1A/Ahr2 feedback loop in the regulation of Ahr signaling by a potential physiological ligand *in vivo* (Wincent *et al*., 2016). Thus, higher transcript levels of *CYP1A* expression in the adult liver might indicate its physiological roles in addition to roles in xenobiotic metabolism.

CYP2K22 was found to be highly specific for males in the RNA-seq data. Zebrafish *CYP2K22* was shown to be greatly induced by 17α-methyldihydrotestosterone in the female liver (Hoffmann *et al*., 2008) and by androgenic compounds in embryos (Fetter *et al*., 2015). Thus, constitutive expression of *CYP2K22* in the male liver and the androgen-induced expression in the female liver both suggest potential roles of this gene in androgen metabolism and homeostasis in this species. *CYP2K18* transcript expression appears to be high in the female liver on the basis of the RNA-seq data. Apart from gender specificity, a recent genome-wide microarray study by Poon and colleagues (2017) showed that *CYP2K18* in larval zebrafish was induced by some pharmaceuticals including amiodarone, diclofenac and flutamide, which are known as drug-induced liver injury drugs. Thus, CYP2K18 may have key roles in drug metabolism.

There were significant sex-related differences in the transcript levels for *CYP1A*, *CYP1B1*, *CYP1D1 and CYP2N13*, with males having higher expression levels than females in all cases. A similar trend of transcript expression (i.e., male > female) was seen for *CYP2AD2* and *CYP2P9*. These results suggest the sex-specific regulation of basal expression of these 6 *CYP* genes in the adult zebrafish liver.

A significant positive correlation between *CYP1A* and *CYP1D1* in the expression data in males suggests a shared mechanism of constitutive expression in the liver of adult male zebrafish. Basal expression of *CYP1D1* in zebrafish

embryos is unlikely under regulation of AHR2, as knockdown of AHR2 failed to suppress *CYP1D1* expression (Goldstone *et al*., 2009). Role of AHR2 and other AHR isoforms in the basal expression of *CYP1D1* in the adult zebrafish liver remains to be elucidated. A positive correlation between *CYP3C2/3* and *CYP3C4* in males also suggests that the basal expression of these genes is regulated via a shared mechanism in the liver of adult male zebrafish. It should be noted that expression levels of *CYP* genes in females are in many cases less variable as compared to those in males, which may explain no significant correlation in females under the condition of the basal expression. Regulatory mechanisms should also be examined in treated conditions using typical agonists for nuclear/cytosolic receptors.

PXR and AHR2 appear to have a redundant role in regulation of the basal expression of *CYP3A65* (Chang *et al*., 2013; Kubota *et al*., 2015) and *CYP1A* (Kubota *et al*., 2015) in zebrafish embryos. However, no significant correlation between *CYP3A65* and *CYP1A* in the adult zebrafish liver was observed in the current study. It could be that there may be growth stage and tissue type specific mechanisms to maintain constitutive expression of *CYP3A65* and *CYP1A* in which AHR2 or PXR is not involved.

The present study revealed several "orphan" *CYP* genes, in addition to *CYP3A65* and *CYP1A*, as dominant isozymes at transcript levels in the liver of adult zebrafish, implying crucial roles of these *CYP* genes in liver physiology as well as potential metabolism of drugs and environmental contaminants. Studies are underway to examine the regulation and function of these orphan and drug-metabolizing CYPs, particularly CYP2AD2 and CYP2Y3, which will be important to determine the roles of these CYPs in liver physiology and toxicology in this premier non-mammalian vertebrate model. From another point of view, caution should be exercised for possible strain differences in the abundance of CYP isozymes in the liver, considering the strain differences reported for morphological, genetic, physiological and behavioral statuses both in the adult and larval stages (van den Bos *et al*., 2017 and references therein). In particular, both TL and AB strains should be subject to hepatic CYP profiling due to their extensive use in biomedical and toxicological studies.

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