RNA-Sequencing Quantification of Hepatic Ontogeny of Phase-I Enzymes in Mice

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ABBREVIATIONS: ADH or Adh: alcohol dehydrogenase; AKR or Akr: aldo-keto reductase; ALDH or Aldh: aldehyde dehydrogenase; AOX or Aox: aldehyde oxidase; CES or Ces: carboxylesterase; DPYD or Dpyd: dihydropyrimidine dehydrogenase; EPHX or Ephx: epoxide hydrolase; FMO or Fmo: flavin monooxygenase; FPKM: fragments per kilobase of exon per million reads mapped; NQO or Nqo: quinone oxidoreductase; P450: cytochrome P450; PON or Pon: paraoxonase; POR or Por: NADPH-cytochrome P450 oxidoreductase; RNA-Seq: RNA sequencing; XDH or Xdh: xanthine oxidoreductase
ABSTRACT:

Phase-I drug metabolizing enzymes catalyze reactions of hydrolysis, reduction, and oxidation of drugs and play a critical role in drug metabolism. However, the functions of most Phase-I enzymes are not mature at birth, which markedly affect drug metabolism in newborns. Therefore, characterization of the expression profiles of Phase-I enzymes and the underlying regulatory mechanisms during liver maturation is needed for better estimation of using drugs in pediatric patients. Mouse is an animal model widely used for studying the mechanisms in the regulation of developmental expression of phase-I genes. Therefore, we applied RNA sequencing to provide a “true quantification” of the mRNA expression of Phase-I genes in mouse liver during development. Liver samples of male C57BL/6 mice at 12 different ages from prenatal to adulthood were used for defining the ontogenic mRNA profiles of Phase-I families, including hydrolysis: carboxylesterase (Ces), paraoxonase (Pon), epoxide hydrolase (Ephx); reduction: aldo-keto reductase (Akr), quinone oxidoreductase (Nqo), dihydropyrimidine dehydrogenase (Dpyd); and oxidation: alcohol dehydrogenase (Adh), aldehyde dehydrogenase (Aldh), flavin monooxygenases (Fmo), molybdenum hydroxylase (Aox and Xdh), cytochrome P450 (P450), and cytochrome P450 oxidoreductase (Por). Two rapidly increasing stages of total Phase-I gene expression after birth reflect functional transition of liver during development. Diverse expression patterns were identified and some large gene families contained the mRNAs of genes that are enriched at different stages of development. In conclusion, this study has revealed the mRNA abundance of Phase-I genes in mouse liver during development and provided a valuable foundation for mechanistic studies in the future.
Introduction

Drug-metabolizing enzymes play a central role in the elimination of drugs from the body. The metabolic reactions of hydrolysis, reduction, and oxidation usually introduce a small functional group to the substrate or convert an existing functional group to a new group that may be further conjugated with a large and bulky water-soluble co-substrate. The reactions associated with hydrolysis, reduction, and oxidation are often called Phase-I reactions, and the reactions associated with conjugation are often referred as Phase-II reactions. Liver is the major organ for Phase-I drug metabolism, but before birth, the liver functions as a hematopoietic organ. A functional transition occurs in liver after birth, and most of the drug-metabolizing enzymes mature during this period. Changes in expression of some Phase-I enzymes during liver maturation in humans have been reported, including P450s (Stevens et al., 2003; Koukouritaki et al., 2004; Stevens et al., 2008; Croom et al., 2009), CES (Yang et al., 2009; Zhu et al., 2009), PON (Cole et al., 2003; Huen et al., 2009), ADH (Smith et al., 1971), and FMO (Cherrington et al., 1998; Koukouritaki et al., 2002; Hines, 2006). The dynamic changes in the ontogenic expression of these genes are thought to be responsible for the substantial pharmacokinetic differences between newborns and adults, and this contributes to differences in therapeutic efficacy and adverse drug reactions in pediatric patients (Kearns et al., 2003; Blake et al., 2005; Hines, 2007; Hines, 2008; Hines, 2013). An in-depth understanding of the regulatory mechanisms of the ontogeny of Phase-I enzymes is needed for safer and more effective drug therapy for pediatric patients.

Several limitations exist in studies of the developmental regulation of Phase-I enzymes with human samples. The first limitation is the ethical and technical issues in recruiting human subjects and obtaining suitable human samples (Rowell and Zlotkin, 1997). Secondly, variations
in human metabolic capacity, which may be caused by genetic or environmental factors, can interfere with studies aimed to reveal the regulatory mechanisms that are only due to age. Furthermore, mechanistic loss-of-function or gain-of-function strategies are not applicable directly in human samples. Animal models are advantageous in overcoming these limitations. In recent years, mice and rats have surpassed other laboratory animals as the experimental models of choice for the study of physiology, metabolism, and disease (Muruganandan and Sinal, 2008; Hrycay and Bandiera, 2009). Advantages of these models include rapid growth, easy maintenance, and the development of genetic manipulation techniques for mechanistic studies with gain-of-function and loss-of-function strategies. Several laboratories, including us, have examined the ontogenic gene expression profiles in mouse or rat liver for some Phase-I genes, including P450s (Hart et al., 2009; Cui et al., 2012b), Ces (Zhu et al., 2009), Akr (Pratt-Hyatt et al., 2013), Adh and Aldh (Smolen et al., 1990; Alnouti and Klaassen, 2008), Pon (Li et al., 1997), and Fmo (Falls et al., 1995; Cherrington et al., 1998; Janmohamed et al., 2004). Developmental expression patterns of some Phase-I genes in mice and rats are similar to those in humans.

Previous studies quantified Phase-I gene expression on the mRNA level by either Northern blot, RT-PCR, microarrays, or multiplex suspension bead arrays, which only provide relative quantification of a given gene and don’t allow a quantitative comparison of genes in different families. With the development of next-generation sequencing technologies, such as RNA sequencing (RNA-Seq), it is possible to define a whole transcriptome with low background noise, no upper limit for quantification, and a high degree of reproducibility for both technical and biological replicates (Mortazavi et al., 2008; Nagalakshmi et al., 2008). More importantly, RNA-Seq quantifies the true abundance of mRNA molecules in biological samples and enables comparison of the expression of all genes (Malone and Oliver, 2011). We have reported RNA-
Seq to reveal ontogenic patterns of P450s (Peng et al., 2012), phase-II enzymes (Lu et al., 2013), transporters (Cui et al., 2012a), and epigenetic modifiers (Lu et al., 2012) in mouse liver during maturation.

In this report, RNA-Seq was used to systematically quantify the mRNA expression of major non-P450 Phase-I genes in mouse liver during postnatal maturation to define the ontogenic profiles of these mRNAs. The groups included enzymes catalyzing reactions in hydrolysis (carboxylesterase, paraoxonase, and epoxide hydrolase), reduction (aldo-keto reductase, quinone oxidoreductase, and dihydropyrimidine dehydrogenase), and oxidation (alcohol dehydrogenase, aldehyde dehydrogenase, flavin monooxygenases, molybdenum hydroxylase, and cytochrome P450 oxidoreductase). The purpose of this study was to generate comprehensive information on the ontogeny of mRNAs of Phase-I genes in livers of mice, which will form the foundation for determining the regulatory mechanisms controlling the various transcription patterns of Phase-I genes during liver maturation.
Materials and Methods

Animals. Eight-week-old C57BL/6 breeding pairs of mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed according to the American Animal Association Laboratory animal care guidelines and were bred under standard conditions in the Laboratory Animal Resources Facility at the University of Kansas Medical Center (KUMC). The use of these mice was approved by the Institute of Laboratory Animal Resources at KUMC. Liver samples (n=3) were collected at the following 12 ages: day -2 (gestational day 17), day 0 (right after birth and before the start of suckling), day 1 (exactly 24 h after birth), and day 3, 5, 10, 15, 20, 25, 30, 45, and 60 (collected at approximately 9:00 AM). These ages represent periods of prenatal (day -2), neonatal (day 0-10), juvenile (day 15-30), and young adult (day 45-60). Due to potential variations caused by the estrous cycle in maturing adult female mice, only male livers were used for this study. Livers were immediately frozen in liquid nitrogen after removal and stored at -80°C.

Total RNA Extraction, Sequencing Library Construction, and RNA-Seq. RNA extraction, library construction, and RNA-Seq were performed as previously described (Peng et al., 2012).

RNA-Seq Data Analysis. After the sequencing images were generated by the sequencing platform, the pixel-level raw data collection, image analysis, and base calling were performed using Illumina’s Real Time Analysis (RTA) software. The output bcl files were converted to qseq files by Illumina BCL Converter 1.7 software and subsequently converted to FASTQ files for downstream analysis. The RNA-Seq reads from the FASTQ files were mapped to the mouse reference genome (NCBI37/mm9) by Tophat 1.2.0. The output files in BAM (binary sequence
alignment) format were analyzed by Cufflinks 1.0.3 to estimate the transcript abundance (Trapnell et al., 2010). The mRNA abundance was expressed as the number of fragments per kilobase of exon per million reads mapped (FPKM).

**Data Visualization and Statistics.** The significance of the observed expression (measured FPKM) of a gene at a given age relative to null expression (zero FPKM) was determined by the drop-in-deviance F test of the fitted FPKM values to a Poisson log linear regression model with a zero intercept that permits extra Poisson variation. The resulting \( p \)-values were adjusted for multiple-hypothesis testing by the Benjamini-Hochberg method (FDR-BH; Benjamini and Hochberg, 1995). Phase-I drug metabolizing enzymes that were significantly expressed (FDR-BH \( \leq 0.05 \)) in at least one of the 12 time-points were selected for analysis. Genes that were significantly differentially expressed between at least two time points during liver development were determined by the drop-in-deviance F test of the fitted FPKM values to a full \( \log [E(FPKM_{ij}|AGE)] = \beta_0 + \beta_j \text{AGE}_{ij} \) where AGE is an indicator variable representing the 12 ages, \( j=1..11 \) and reduced \( \log [E(FPKM_{ij}|AGE)] = \beta_0 \) Poisson-log-linear regression model accounting for extra Poisson variation. The resulting \( p \)-values were adjusted for false discovery as before and those genes with a FDR-BH \( \leq 0.05 \) were considered significantly differentially expressed over time. Two-way hierarchical clustering dendrograms were generated by JMP (version 10; SAS Institute, Inc., Cary, NC) to determine the expression patterns of the Phase-I genes during liver development.
Results

Total Expression and Proportions of Individual Phase-I Families during Liver maturation. Transcript abundances of the 186 mouse Phase-I genes were calculated by Cufflinks and presented as FPKM values in Supplemental Table 1. If the Benjamini-Hochberg adjusted $p$ value of the drop-in-deviance F test (FDR-BH) for gene expression in at least one of the 12 ages was less than 0.05 for a Phase-I gene, then that gene was considered to be expressed in liver during maturation. Table 1 lists the number of genes in each category that are significantly expressed at various developmental stages of prenatal (day -2), neonatal (day 5), adolescence (day 25), and adult (day 60) as well as the total number of genes expressed during liver development (Day -2 to Day 60) in each Phase-I family. Of the 186 mouse Phase-I genes, 136 genes were expressed in liver during maturation, but only about half of them (64) were expressed in prenatal liver. A significant change in expression of Phase-I enzyme genes was observed around birth. The number of the expressed genes increased to 112 in neonatal liver at day 5. The total expression levels of all Phase-I mRNAs represented as cumulative FPKM values increased approximately 15 fold during postnatal liver maturation from day -2 (FPKM = 1,383) to day 60 (21,285) with two surges (Fig. 1A), the first of which with six fold increase from day -2 (1,383) to day 1 (8,736) and the other with 1.8 fold increase from day 10 (10,511) to day 20 (18,482), with each surge followed by a relatively stable value of cumulative FPKM. Fig. 1B shows the composition of the Phase-I families represented as percentages of the total FPKM value for each stage at prenatal (day -2), neonatal (day 5), adolescence (day 25), and adult (day 60). The major Phase-I families expressed in prenatal liver were P450 (35%), Aldh (25%), Akr (21%), and Adh (10%) are. After birth, there is a further increase in the relative proportions of
the mRNAs of the P450s (from 35% prenatal levels to about 60% after birth), and other highly expressed Phase-I families include *Ces*, *Adh*, and *Aldh*.

**Ontogeny of Genes of Phase I Enzymes Involved in Hydrolysis Reactions.**

**Carboxylesterases:** CESs are enzymes that participate in the hydrolysis and trans-esterification of a variety of esters and amides. Five families of *Ces* genes are annotated in the NCBI reference sequence database for mice. mRNA expression of four families of *Ces* (*Ces1-Ces4*) was detected in mouse liver samples. The most abundant family in adult liver was *Ces1*, and it consisted up to 58% of the total liver *Ces* mRNAs at age day 60 (Fig. 2A). Within the *Ces* family 1, *Ces1c* had much higher expression than other members at postnatal ages. The mRNA levels of *Ces1* genes generally increased with age, but *Ces1c* and *Ces1g* showed a peak of expression around day 20 and day 25 respectively (Fig. 2B). The *Ces2* family was moderately expressed, with *Ces2a* and *Ces2e* being the major members. The expression of *Ces2a* gradually increased after birth till day 60, and *Ces2e* reached its adult levels at around day 25 (Fig. 2C). The *Ces3* family was also highly expressed in adult liver. *Ces3a* and *3b* exhibited similar developmental patterns with a rapid increase in expression between day 20 and day 30 (Fig. 2D). *Ces4* family was expressed at a low level in liver, and it did not show significant difference in expression during development. In total, 13 *Ces* genes had significant changes in expression during development. A two-way hierarchical clustering analysis revealed that the mRNAs of these *Ces* were all low in pre- and neonatal ages, but markedly increased to adult levels around 20-30 days after birth (Fig. 2E).

**Paraoxonases:** PON function in hydrolyzing a broad range of organophosphates, organophosphinites, aromatic carboxylic acid esters, cyclic carbonates, and lactones (Parkinson
Mouse livers expressed all three *Pons* with *Pon1* being the most abundant form. Hepatic expression of *Pon1* increased with age to a peak level around day 20 and then modestly decreased thereafter (Fig. 3A). At the prenatal age day -2, *Pon2* mRNA level was higher than *Pon1* and *Pon3*. After birth, the *Pon2* mRNA expression first increased till day 5 then decreased till day 25, and then went up again to reach adult level at day 60. *Pon3* had overall increased expressions after birth with small fluctuations at adolescent ages (Fig. 3B).

**Epoxide Hydrolases**: Ephxs are important in hydrolyzing and detoxifying electrophilic epoxides, which may otherwise cause cellular and genetic toxicity through binding to proteins and nucleic acids. *Ephx1* and *Ephx2* were the microsomal and soluble forms of these enzymes, respectively. *Ephx2* had a higher expression at mRNA level than *Ephx1*, and they exhibited similar ontogenic patterns, with a sharp increase around birth, followed by a slight decrease through at least day 10, and then a gradual increase till adulthood (Fig. 4).

**Ontogeny of Genes of Phase I Enzymes Involved in Reduction Reactions.**

**Aldo-keto reductases**: AKRs are a group of cytosolic enzymes that catalyze the reduction of aldehydes and ketones to primary and secondary alcohols, respectively (Jin and Penning, 2007). Mouse *Akr* genes consist of two families, *Akr1* and *Akr7*, with a total of 16 genes. *Akr1* is the larger family with 5 subfamilies (*Akr1a-e*) and 15 genes. The RNA-Seq data showed 14 out of the 16 mouse *Akr* genes were significantly expressed during liver maturation, and all the expressed *Akr* genes exhibited significant differential expressions across the ages. The cumulative FPKM values of all expressed *Akr* mRNAs increased 2-3 fold during postnatal liver development (Fig. 5A). The composition of *Akr* mRNAs also changed with age, evidenced by alterations in individual *Akr* genes. At day -2, *Akr1a4* was the most abundant member, which accounted for
65% of the total Akr mRNAs. After birth, Akr1a4 mRNA slightly increased, but its proportion in Akr mRNAs decreased (Fig. 5B-C). Akr1b7 was highest during the neonatal stage. It accounted for 28% of the total Akr mRNA at day 1, but was undetectable after day 20. Akr1c6 was one of the major Akr genes in liver. It was lowly expressed at birth, gradually increased to a peak at day 30, and then slightly decreased to adult levels around day 45. The mRNA expression profile of Akr7a5 was similar to the profile of total Akr mRNAs, so the percentage of Akr7a5 mRNA was relatively constant during development (10~15 %) (Fig. 5B-C). Two-way hierarchical clustering analysis of the differentially expressed Akrs revealed two major patterns (Fig. 5D). Unlike Ces genes, which all had low expression at the neonatal stage, some Akr genes displayed neonatal enriched expression (Group 1). In Group 2, Akr genes however showed relatively low expression during the neonatal ages. These genes were a diverse group with some genes enriched at the adolescent stage (e.g. Akr1c20) and some at the adult stage (e.g. Akr1c12).

**Quinone oxidoreductase:** NQO1 and NQO2 perform two-electron reduction of quinones to hydroquinones. The RNA-Seq results demonstrated that the expression of Nqo1 was relatively stable during development, with two small drops around birth and day 25. Nqo2 mRNA was expressed higher than Nqo1 in mouse liver 5 days after birth. Nqo2 showed two periods of increased expression during development; one from day 0 to day 15, and the other from day 30 to day 60 (Fig. 6A).

**Dihydropyrimidine dehydrogenase:** DPYD is located mainly in liver cytosol. It catalyzes the reduction of 5-fluorouracil and related pyrimidines (Parkinson and Ogilvie, 2008). The mRNA expression of Dpyd showed a marked increase at day 1, dropped slightly till day 3, and then went up gradually to adult levels at day 60, with a small plateau between day 20 and day 30 (Fig. 6B). The adult mRNA level of Dpyd was about 30 times higher than newborns.
Ontogeny of Genes of Phase I Enzymes Involved in Oxidation Reactions.

**Alcohol dehydrogenases**: ADHs metabolize a wide spectrum of substrates, including ethanol, retinol, other aliphatic alcohols, hydroxysteroids, and lipid peroxidation products (Duester et al., 1999). *Adh* generally had lower expression at younger ages and reached stable mature levels at day 30 or earlier (Fig. 7A). Five families of *Adh* genes exist in the mouse genome, and each family consists of only one member that is significantly expressed during liver development (Fig. 7B-D). *Adh1* mRNA was the most highly expressed, consisting of over 90% of *Adh* mRNAs in adult liver (Fig. 7B), followed by *Adh5* and then *Adh4* (Fig. 7C). *Adh6* and *Adh7* were minimally expressed in liver (Fig. 7D). *Adh1* and *Adh5* also showed a peak of expression around day 0 or day 1.

**Aldehyde dehydrogenase**: ALDHs are a group of enzymes that catalyze the oxidation and detoxification of aldehydes. There were total 20 *Aldh* genes in mouse genome, and 15 of these genes were significantly expressed at mRNA levels during liver maturation. Of these 15 genes, 14 showed differential expression across the ages. The total mRNA of *Aldh* genes increased almost linearly over fivefold from 2 days before birth to 60 days after birth (Fig. 8A). *Aldh1l1* and *Aldh2* were the major *Aldh* genes expressed at all ages of liver development. *Aldh1b1*, *Aldh4a1* and *Aldh9a1* accounted for a high percentage of total *Aldh* mRNAs at the prenatal and adolescent stages, whereas *Aldh1a1* matured later and became highly expressed only at the adult stage (Fig. 8B). Two-way hierarchical clustering analysis of the differentially expressed *Aldhs* also demonstrated diverse ontogenic patterns of individual *Aldhs*. Similar to *Akr*s, Group 1 *Aldh* genes had enriched expression at the perinatal stage, and genes in Group 2 were expressed higher at the adult stage than at the perinatal stage (Fig. 8C).
Flavin monooxygenases: Like P450s, FMOs are microsomal enzymes that require NADPH and O2. They oxidize a variety of xenobiotics, including the nucleophilic nitrogen, sulfur, and phosphorus heteroatom (Parkinson and Ogilvie, 2008). Five families of Fmos are annotated in the mouse genome. The mRNA expression of Fmo5 increased gradually with age, and was the most abundant in adult males, followed by Fmo1, which showed a rapid increase of expression from birth to day 5, and then slightly increased to a peak at day 25 and decreased to adult expression levels at day 30. The expression of Fmo3 became detectable at 10 days after birth, increased to peak levels at day 25, and then dropped to negligible levels after day 30 in male mice (Fig. 9A). Fmo2 and Fmo4 were very lowly expressed in liver during maturation, and Fmo2 was mainly detected at the adolescent stage from day 10 to day 20 (Fig. 9B).

Molybdenum hydroxylases: Mammalian molybdenum hydroxylases require FAD and molybdenum cofactor for their catalytic activity. There are two major molybdenum hydroxylases participating in the metabolism of xenobiotics: aldehyde oxidases (AOX) and xanthine oxidoreductase (XDH) (Parkinson and Ogilvie, 2008). Four Aox genes are annotated in the mouse genome and two of them were expressed during liver maturation. Aox3 was the major Aox in mouse liver. Its mRNA level increased over 10 fold from day -2 to day 3, remained stable from day 3 to day 25, and then went up about 5 fold to adult level at day 60. Aox1 was expressed at lower level than Aox3, and also showed an increase of expression after day 25 (Fig.10A). Xdh mRNA expression gradually increased about 10 fold from day -2 to day 20, and then remained relatively stable (Fig. 10B).

NADPH-cytochrome P450 oxidoreductase: POR transfers electrons from NADPH to cytochrome P450s for their catalytic function. The expression of Por mRNA rose sharply around birth, and decreased to about half the level of day 1 by day 5. From day 5 to day 20, Por
expression was relatively stable. Then at day 25, it decreased again to about half the level of day 20 and remained at that level till maturity (Fig. 11).
Discussion

The current study provided a comprehensive quantitative analysis of the developmental expression of major non-P450 Phase-I enzymes on mRNA levels in mouse liver by RNA-Seq, including enzymes involved in hydrolysis, reduction, and oxidation. Compared with other commonly used methods for mRNA quantification, such as microarray, branched DNA, and real-time PCR, which detect mRNAs by probe hybridization and rely on hybridization specificity and efficiency, RNA-seq directly counts sequence reads of the nucleotide molecules in biological samples, thus it is able to quantify the mRNA expression with minimal bias. The expression of a gene transcript is represented by FPKM, which normalized sequencing depths between different samples and sizes between various genes, allowing direct comparison of mRNA abundance among various transcripts on a genome-wide scale.

From perinatal through neonatal to adult liver, the total FPKM values of Phase-I mRNAs increase 15 fold with two rapidly increasing stages (Fig. 1A), which may reflect functional transition of liver from a hematopoietic organ to a metabolic organ during liver maturation. The first surge occurs from 2 days before birth to 1 day after birth. During that period of time, the total FPKM values increase 8 fold and the composition of each Phase-I family also changes dramatically. P450s are the most changed Phase-I enzymes during this perinatal surge, indicating an urgent need for liver to deal with exposure to xenobiotics immediately after birth. The second surge occurs from day 10 to day 20 with a rapid increase in abundance of Phase-I mRNAs. This period is also the most rapidly growing stage of postnatal liver maturation. After day 25 (adolescence), the total FPKM levels and composition of the Phase-I enzymes are consistent till day 60 (adult). These data indicate that during the developmental period from neonatal to adolescence, hepatic expression of many Phase-I enzyme genes change dramatically.
Expectedly, the ability to metabolize xenobiotics including drugs by Phase-I enzymes should also be changed dramatically in this period of time. Determination of the mechanisms in regulation of the Phase-I gene expression during this developmental period will provide insight for understanding drug metabolism in pediatric patients.

The ontogenic patterns of many Phase-I genes we found in this study are consistent with the previous findings in mice and humans by other researchers. For example, the high expression of carboxylesterase 1 (Ces1) in our results was consistent with the literature that Ces1 was the major expressed carboxylesterase gene in mouse liver (Holmes et al., 2010). Another study demonstrated that the expression of mouse Ces1 and Ces2 in the liver were markedly lower in newborns than in adults and increased gradually to levels of adult animals in 2 to 4 weeks (Zhu et al., 2009). Our data confirmed these, and provided more details on changes with age and more information about individual members of each gene family (Fig. 2). Adult humans also express higher levels of CES1 and CES2 than children and fetus (Yang et al., 2009). As a former study suggested that the CES genes from mouse and human had evolutionally conserved transcriptional regulatory mechanisms (Hosokawa et al., 2007), these ontogeny data in mice are expected to provide important resources for interpreting the developmental regulation of CESs in humans.

A study showed that lower levels of PON1 enzyme persisted in young children till at least 7 years of age (Huen et al., 2009), which was similar to the ontogenic pattern of Pon1 mRNA expression in mice. Another group found that the plasma PON1 activity in mice reached a plateau in 3 weeks after birth (Li et al., 1997), which is similar to our data of Pon1 mRNA expression in mouse liver (Fig. 3). A transgenic mouse strain that lacks endogenous Pon1, but with the human PON1 gene, exhibited a similar developmental pattern of expression as wild-type
mice, indicating conserved developmental regulatory elements between mouse and human PON1 (Cole et al., 2003), making our data helpful for mechanistic studies.

Quinones are highly reactive molecules. They can undergo one-electron reduction, commonly catalyzed by NADPH-cytochrome P450 reductase, and generate semiquinone radicals, which are reactive metabolites themselves and may cause oxidative stress by redox cycling. The resultant reactive oxygen species can lead to DNA damage, lipid peroxidation, membrane damage, cytotoxicity, and neoplasia. NQO1 and NQO2 compete for the above reaction and catalyze the two-electron reductive metabolism of quinones to produce stable hydroquinones, which are removed by glucuronidation or sulfonation (Long and Jaiswal, 2000; Parkinson and Ogilvie, 2008). Quinones are ubiquitous in nature and human exposure to quinones occurs through diet, airborne pollutants, and drugs (Monks and Jones, 2002). Therefore, quinone oxidoreductase has a highly important role in developmental toxicology. The present study is the first demonstration of the developmental expression of these genes. NQO1 and NQO2 are two closely related flavoproteins. Although they have overlapping substrate specificities, significant differences exist in relative affinities for various substrates (Das et al., 2006). Our results also revealed the differential expression of Nqo1 and Nqo2 mRNAs during development (Fig. 6A). The changes of expression during liver maturation may have strong toxicity impact in children. Further studies are needed to address the ontogeny of NQO enzymes and their significance in human health.

Although FMO3 is the most highly expressed FMO family member in adult human liver, it demonstrated gender-specific expression in mice, and was not detectable in male liver of mice (Falls et al., 1995). Our data only examined mRNA expression in male animals and was consistent with this result. Fmo3 was expressed equally in male and female mice, even at 4
weeks of age (Cherrington et al., 1998), and then after puberty, the gender difference appeared due to sex steroids (Falls et al., 1997). We also showed the detailed time window when the shutdown of Fmo3 happened, which was between day 25 and day 30 (Fig. 9A). Fmol in mice showed increased expression after birth, unlike human, in which Fmol was most abundant in fetal liver and absent after birth (Hines, 2006). Interestingly, Fmo3 was not expressed immediately after birth in mice, and appeared after 10 days of age, which is similar in humans (Hines and McCarver, 2002). Thus, neonatal mice may serve as a model to study the mechanism of the delayed onset of FMO3 expression in humans.

AOX and XDH are important enzymes that catalyze the oxidation of electron-deficient carbon atoms, often found in nitrogen heterocycles, such as purines and pyrimidines. These typically complement oxidations by cytochrome P450s, which catalyze the oxidation of carbon atoms with a high electron density. A broad range of xenobiotics are substrates for molybdozymes, including immunosuppressive drugs like 6-mercaptopurine, antiviral drugs like 6-deoxyacyclovir, and antidepressant citalopram. They also perform important physiological functions by metabolizing biogenic amines and catecholamines, and may be related to neuron disease (Bendotti et al., 1997). The final electron acceptor of AOX and XDH is oxygen, so the reactions can generate reactive oxygen species and lead to oxidative stress and lipid peroxidation (Parkinson and Ogilvie, 2008). Although having critical roles in biotransformation, the expression and regulation of molybdozymes are largely understudied, especially compared to other drug metabolizing enzymes. Our first report on the developmental expression pattern of these genes will facilitate drug metabolism and toxicity studies related to molybdozymes.

POR is the only electron donor for all microsomal P450s, and alteration in POR activity can affect P450-catalyzed drug oxidation (Hart et al., 2008). Our previous study had revealed the
ontogenic mRNA expression of all P450s in mouse liver, and a large number of P450 genes had increased expression after birth (Peng et al., 2012). Here we showed hepatic mRNA expression of *Por*, which could influence P450 activities, actually decreased during postnatal development (Fig. 11). Thus, the level of *Por* needs to be taken into considerations when we study the developmental enzyme activity of P450s.

Protein levels and enzyme activities of Phase-I genes were not determined in this study due to extensive workload and technical limitations. Specific antibodies, substrates, and inhibitors for many individual mouse Phase-I enzymes are not available. Yet compared with the few studies that measured protein expression or enzyme activities of certain Phase-I genes in mouse liver during development, such as CES (Zhu et al., 2009), PON (Cole et al., 2003), EPHX (Rouet et al., 1984), and FMO (Cherrington et al., 1998), our mRNA expression patterns were fairly indicative of the protein or enzyme activity levels. But whether the mRNA expression can be extrapolated to the protein expression and enzymatic activities of the Phase-I enzymes remains to be validated. For future studies, it may be informative to apply certain pediatric drugs to the mice of different developmental stages and to check the pharmacokinetics of these drugs to see if the metabolism patterns can be predicted by the current gene expression patterns. Technological breakthroughs in proteomics and metabolomics are essential to the study of ontogeny of Phase-I enzymes on the protein contents and metabolite levels in the future.

In summary, the present study has provided the first knowledge regarding the true quantification of the mRNA ontogenic patterns of all major known non-P450 Phase-I enzymes during mouse liver development. Such knowledge will serve as a foundation for further understanding the regulation of gene expression and physiological function of these enzymes in
liver during development and aid in a better understanding of the kinetics of xenobiotic metabolism during perinatal and postnatal maturation.
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Authorship Contributions

Participated in research design: Zhong, Klaassen, Lu, Gunewardena, Peng, Cui, and Yoo.

Conducted experiments: Peng and Cui.

Contributed new reagents or analytic tools: n/a.

Performed data analysis: Peng, Cui, Gunewardena, Yoo, and Zhong.

Wrote or contributed to the writing of the manuscript: Zhong, Klaassen, Lu, Peng, Gunewardena, Yoo, and Cui.
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Footnotes

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Table 1. Number of expressed genes in each Phase-I family in mouse liver at a specific age of prenatal (day -2), Neonatal (day 5), adolescence (day 25), and adult (day 60), as well as during development (Day -2 to Day 60)

<table>
<thead>
<tr>
<th>Gene Family</th>
<th>Prenatal Day -2</th>
<th>Neonatal Day 5</th>
<th>Adolescence Day 25</th>
<th>Adult Day 60</th>
<th>Liver Day -2 to Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydrolysis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ces (18)</td>
<td>4</td>
<td>11</td>
<td>13</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Pon (3)</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Ephx (4)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Reduction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akr (16)</td>
<td>9</td>
<td>14</td>
<td>12</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Nqo (2)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Dpyd (1)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Oxidation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adh (6)</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Aldh (20)</td>
<td>13</td>
<td>15</td>
<td>14</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Aox+Xdh (5)</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Fmo (7)</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>P450 (103)</td>
<td>26</td>
<td>55</td>
<td>59</td>
<td>50</td>
<td>71</td>
</tr>
<tr>
<td>Por (1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total (186)</strong></td>
<td>64</td>
<td>112</td>
<td>114</td>
<td>101</td>
<td>136</td>
</tr>
</tbody>
</table>

Numbers in the brackets represent total number of genes in the family.
Legends

FIG. 1. A. Total mRNA levels of the 186 mouse Phase-I genes in liver during postnatal maturation. RNA-Seq was done for liver mRNAs of male C57BL/6 mice at 12 ages from 2 day before birth to 60 days after birth. The FPKM values of all 186 Phase-I genes at each age were added and plotted to show the developmental pattern of total Phase-I mRNAs. Bars represented the mean ± S.E.M. of three individual animals. B. Percentages of FPKM values of each Phase-I family in prenatal (day -2), neonatal (day 5), adolescence (day 25), and adult (day 60) livers.

FIG. 2. Expression of *Ces* mRNAs during liver development in male C57BL/6 mice: A, mRNA proportion of each *Ces* family at age of day 60. B, expression profiles of *Ces1* gene family; C, expression profiles of *Ces1* gene family without *Ces1c*; D, expression profiles of *Ces2* gene family; E, expression profiles of *Ces3* and *Ces4* gene families. Data are expressed as mean FPKM and SEM of three individual animals. F, hierarchical clustering of expression profiles for 13 differentially expressed *Ces* genes. The two trees describe the relationship between different gene expression profiles (right tree) and various ages (bottom tree). The dendrogram scale represents the correlation distances. Average FPKM values of three replicates per age are given by colored squares: red, relatively high expression; blue, relatively low expression. The dashed line categorizes the expression profiles into two major groups.

FIG. 3. Expression of *Pon* during liver development: A, all three *Pon* ontogenic mRNA expression patterns; B, *Pon1* was removed to enlarge *Pon2* and *Pon3* expression patterns. Data are expressed as mean FPKM and SEM of three individual animals.

FIG. 4. Expression patterns of *Ephx1* and *Ephx2* during liver development.
FIG. 5. A, total mRNA profile of Akr genes in liver during development. The FPKM values of the 14 significantly expressed Akr genes are summed and plotted to show the developmental pattern of total Akr mRNAs. B, individual Akr mRNAs (shown as percentages of total Akr mRNAs) at 2 days before birth and 1, 10, 20, and 60 days after birth. Each gene is presented in a unique color for all ages. Only genes with mRNAs expressed at more than 1% at each age are listed, and the rest are grouped as “Others”. C, expression profile of the 4 highly expressed Akr (∼10% in B) in liver during development. D, hierarchical clustering of expression profiles for the 14 differentially expressed Akr genes. The dendrogram scale represents the correlation distances. Average FPKM values of three replicates per age are given by colored squares: red, relatively high expression; blue, relatively low expression. The dashed line categorizes the expression profiles into two major groups.


FIG. 7. A, total mRNA profile of all five Adh genes during liver development. B, ontogenic mRNA expression patterns of individual Adh gene; C, Adh1 is removed to enlarge Adh4 and Adh5 expression patterns; D, Adh4 and Adh5 are removed to enlarge Adh6-ps1 and Adh7 expression patterns.

FIG. 8. A, total mRNA profile of Aldh genes in liver during development. The FPKM values of the 15 significantly expressed Aldh genes are summed and plotted to show the developmental pattern of total Aldh mRNAs. B, individual Aldh mRNAs (shown as percentages of total Aldh mRNAs) at 2 days before birth and 1, 10, 20, and 60 days after birth. Each gene is presented in a unique color for all ages. Only genes with mRNAs expressed at more than 1% at each age are
listed, and the rest are grouped as “Others”. C, hierarchical clustering of expression profiles for the 14 differentially expressed Aldh genes. The dendrogram scale represents the correlation distances. Average FPKM values of three replicates per age are given by colored squares: red, relatively high expression; blue, relatively low expression. The dashed line categorizes the expression profiles into two major groups.

FIG. 9. Expression of Fmo genes during liver development: A, all five Fmo ontogenic mRNA expression patterns; B, Fmo1,3,5 are removed to enlarge Fmo2 and Fmo4 expression patterns.

FIG. 10. Expression of Molybdenum hydroxylases during liver development: A, mRNA ontogenic patterns of aldehyde oxidase Aox1 and Aox3; B, mRNA ontogenic patterns of Xdh.

FIG. 11. mRNA expression pattern of Por during liver development.
FIG. 2.

A

Day 60

Ces4
0%

Ces3
28%

Ces1
58%

Ces2
14%

B

Gene Expression (FPKM)

[Ces1b, Ces1c, Ces1d, Ces1e, Ces1f, Ces1g]

Gene Expression (FPKM)

[Ces3a, Ces3b, Ces3c, Ces3d, Ces3e, Ces3f, Ces3g]

C

Gene Expression (FPKM)

[Ces1b, Ces1d, Ces1e, Ces1f, Ces1g]

Gene Expression (FPKM)

[Ces2a, Ces2c, Ces2d-ps, Ces2e, Ces2g]

D

Gene Expression (FPKM)

[Ces2a, Ces2c, Ces2d-ps, Ces2e, Ces2g]

E

Gene Expression (FPKM)

[Ces3a, Ces3b, Ces3c, Ces3d, Ces3e, Ces3f, Ces3g]

F

Gene Expression (FPKM)

[Ces1b, Ces1c, Ces1d, Ces1e, Ces1f, Ces1g, Ces2a, Ces2c, Ces2d-ps, Ces2e, Ces2g, Ces3a, Ces3b, Ces3c, Ces3d, Ces3e, Ces3f, Ces3g]
FIG. 3.
FIG. 4.
FIG. 5.

A

Gene Expression (FPKM)

Age (Days)

0 10 20 30 40 50 60 70

800 600 400 200 0

B

Day -2

Akr1c12 2%  Akr1c19 7%
Akr1c13 4%  Akr7a5 10%
Akr1e1 65%

Day 1

Akr1c12 2%  Akr1c19 3%
Akr1c13 2%  Akr1c17 28%
Akr1c6 42%

Day 10

Akr1d1 3%  Akr1c12 2%
Akr1c13 3%  Akr7a5 10%
Akr1c6 26%

Day 20

Akr1c19 2%
Akr1c13 4%
Akr1e1 6%
Akr7a5 12%
Akr1c12 3%
Akr1c14 29%
Akr1c6 34%

Day 60

Akr1c14 2%
Akr1c20 2%
Akr1c12 4%
Akr1c13 5%
Akr1c19 6%
Akr1a4 15%
Akr1c6 29%

C

Gene Expression (FPKM)

Age (Days)

0 10 20 30 40 50 60 70

Akr1a4 65%
Akr1b7 28%
Akr1c6 14%
Akr7a5 10%

D

Genes

Age (Days)

-2 0 1 3 5 10 15 20 25 30 45 60

Group 1

Akr1a4 100%
Akr1c18 90%
Akr1c13 85%
Akr1c12 80%
Akr1c20 70%
Akr1b7 60%

Group 2

Akr1b10 50%
Akr1b8 40%
Akr1b1 30%
Akr7a5 20%
Akr1c19 10%
Akr1c14 0%
FIG. 6.

A

Gene Expression (FPKM)

Age (Days)

Nqo1

Nqo2

B

Gene Expression (FPKM)

Age (Days)

Dpyd
FIG. 7.

A

Gene Expression (FPKM) vs. Age (Days)

B

Gene Expression (FPKM) vs. Age (Days) for different genes.

C

Gene Expression (FPKM) vs. Age (Days) for Adh4, Adh5, Adh6-ps1, and Adh7.

D

Gene Expression (FPKM) vs. Age (Days) for Adh6-ps1 and Adh7.
FIG. 8.

A

Gene Expression (FPKM) vs Age (Days)

Day 20

Day 60

B

Day -2

Day 1

Day 10

C

Age (Days)

Genes

Aldh18a1
Aldh3a1
Aldh11b1
Aldh1a1
Aldh1a7
Aldh2
Aldh7a1
Aldh11l
Aldh5a1
Aldh6a1
Aldh4a1
Aldh8a1
Aldh3a2
Aldh9a1

Group 1

Group 2
FIG. 9.

A

Gene Expression (FPKM)

Age (Days)

Fmo1
Fmo2
Fmo3
Fmo4
Fmo5

B

Gene Expression (FPKM)

Age (Days)

Fmo2
Fmo4
FIG. 10.

(A) Gene Expression (FPKM) vs. Age (Days) for Aox3 and Aox1.

(B) Gene Expression (FPKM) vs. Age (Days) for Xdh.
FIG 11.