

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

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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 affects 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. The 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 the 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*), and epoxide hydrolase (*Ephx*); reduction: aldo-keto reductase (*Akr*), quinone oxidoreductase (*Nqo*), and 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 the liver during development. Diverse expression patterns were identified, and some large gene families contained the mRNA of genes that are enriched at different stages of development. Our study reveals the mRNA abundance of phase-I genes in the mouse liver during development and provides 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 cosubstrate. 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. The liver is the major organ for phase-I drug metabolism; however, before birth, the liver functions as a hematopoietic organ. A functional transition occurs in the 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 cytochrome P450 (P450) (Stevens et al., 2003, 2008; Koukouritaki et al., 2004; Croom et al., 2009), carboxylesterase (CES) (Yang et al., 2009; Zhu et al., 2009), paraoxonase (PON) (Cole et al., 2003; Huen et al., 2009), alcohol dehydrogenase (ADH) (Smith et al., 1971), and flavin monooxygenase (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, 2008, 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 ethical and technical issues in recruiting human subjects and obtaining suitable human samples (Rowell and Zlotkin, 1997). Second, variations in human metabolic capacity, which may be caused by genetic or

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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.

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). The 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 ours, have examined the ontogenic gene expression profiles in mouse or rat livers for some phase-I genes, including P450s (Hart et al., 2009; Cui et al., 2012b), *Ces* (Zhu et al., 2009), aldo-keto reductase (*Akr*) (Pratt-Hyatt et al., 2013), *Adh* and aldehyde dehydrogenase (*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). The 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, reverse-transcription polymerase chain reaction, microarrays, or multiplex suspension bead arrays, which only provide relative quantification of a given gene and do not 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 biologic replicates (Mortazavi et al., 2008; Nagalakshmi et al., 2008). More importantly, RNA-Seq quantifies the true abundance of mRNA molecules in biologic samples and enables comparison of the expression of all genes (Malone and Oliver, 2011). We have reported that RNA-Seq can 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 the 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 the 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 dihydro-primidine 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 the 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 hours after birth), and days 3, 5, 10, 15, 20, 25, 30, 45, and 60 (collected at approximately 9:00 AM). These ages represent the periods of prenatal (day -2), neonatal (days 0-10), juvenile (days 15-30), and young adult (days 45-60). Due to potential variations caused by the estrous cycle in maturing adult female

mice, only male livers were used for this study. The 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 elsewhere (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 (Illumina, San Diego, CA). 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 (<http://tophat.cbcb.umd.edu/>). The output files in BAM (binary sequence alignment) format were analyzed by Cufflinks 1.0.3 (<http://cufflinks.cbcb.umd.edu/>) 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 ≤ 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

$$(\log[E(\text{FPKM}_i|\text{AGE})]) = \beta_0 + \beta_j \text{AGE}_{ij},$$

where AGE is an indicator variable representing the 12 ages, $j=1\dots 11$) and reduced

$$(\log[E(\text{FPKM}_i|\text{AGE})]) = \beta_0)$$

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 ≤ 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 the 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 the liver during maturation, but only about half of them (64) were expressed in the 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 the 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 = 1383) to day 60 (21,285) with two surges (Fig. 1A), the first

TABLE 1

Number of expressed genes in each phase I family in mouse livers at specific ages of prenatal (day -2), neonatal (day 5), adolescence (day 25), and adult (day 60), as well as during development (days -2 to 60)

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

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

of which with a 6-fold increase from day -2 (1383) to day 1 (8736) 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. Figure 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 the prenatal liver were P450 (35%), *Aldh* (25%), *Akr* (21%), and *Adh* (10%). 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). 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 National Center for Biotechnology Information (NCBI) reference sequence database for mice. Expression of mRNA of four families of *Ces* (*Ces1-Ces4*) was detected in mouse liver samples. The most abundant family in the adult liver was *Ces1*, which comprised 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 and 2C).

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. 2D).

The *Ces3* family was also highly expressed in adult livers. *Ces3a* and *Ces3b* exhibited similar developmental patterns, with a rapid increase in expression between days 20 and 30 (Fig. 2E). The *Ces4* family was expressed at a low level in the liver, and it did not show a 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 mRNA of these *Ces* were all low in prenatal and neonatal ages, but were markedly increased to adult levels around 20–30 days after birth (Fig. 2F).

Paraoxonases. PON function in hydrolyzing a broad range of organophosphates, organophosphinites, aromatic carboxylic acid esters, cyclic carbonates, and lactones (Parkinson and Ogilvie, 2008). 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 the adult level at day 60. *Pon3* had an overall increased expression 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 five 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 *Akrs* exhibited significant differential expressions across the ages. The cumulative FPKM values of all expressed *Akr* mRNAs increased 2- to 3-fold during postnatal liver development (Fig. 5A).

The composition of *Akr* mRNA also changes with age, as evidenced by alterations in individual *Akr* genes. At day -2, *Akr1a4* was the most abundant member, accounting for 65% of the total *Akr* mRNAs. After birth, *Akr1a4* mRNA slightly increased, but its proportion in *Akr* mRNAs decreased (Fig. 5, B and 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 the liver; it was lowly expressed at birth, gradually increased to a peak at day 30, and then slightly decreased to

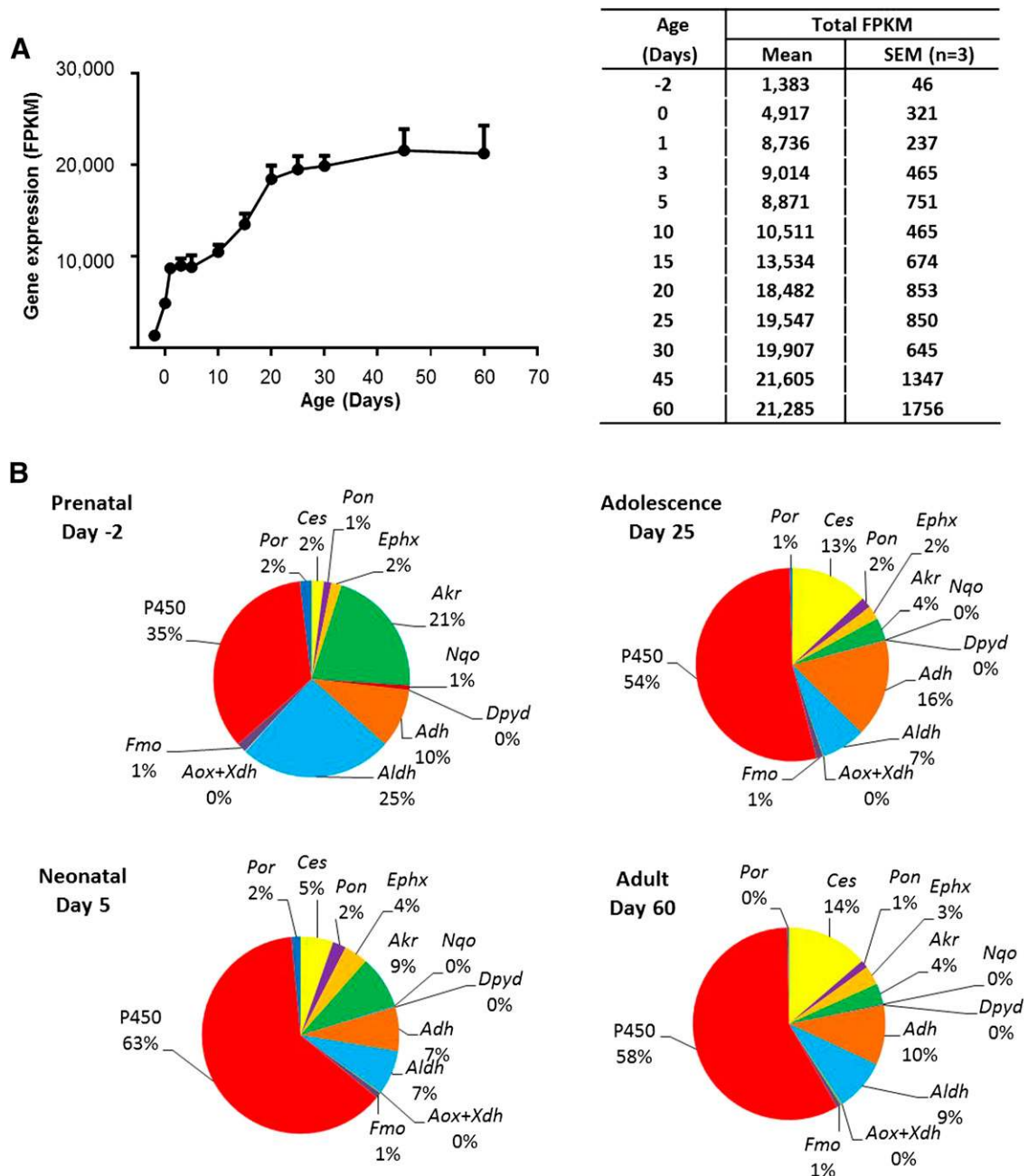


Fig. 1. (A) Total mRNA levels of the 186 mouse phase-I genes in the liver during postnatal maturation. RNA-Seq was done for liver mRNAs of male C57BL/6 mice at 12 ages from 2 days before birth to 60 days after birth. The FPKM values of all 186 phase-I genes at each age were added and plotted to show the developmental pattern of total phase-I mRNAs. Bars represent the mean \pm 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.

adult levels around day 45. The mRNA expression profile of *Akr7a5* was similar to the profile of total *Akr* mRNA, so the percentage of *Akr7a5* mRNA was relatively constant during development (10%~15%) (Fig. 5, B and C).

Two-way hierarchical clustering analysis of the differentially expressed *Akr*s 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 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 livers 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,

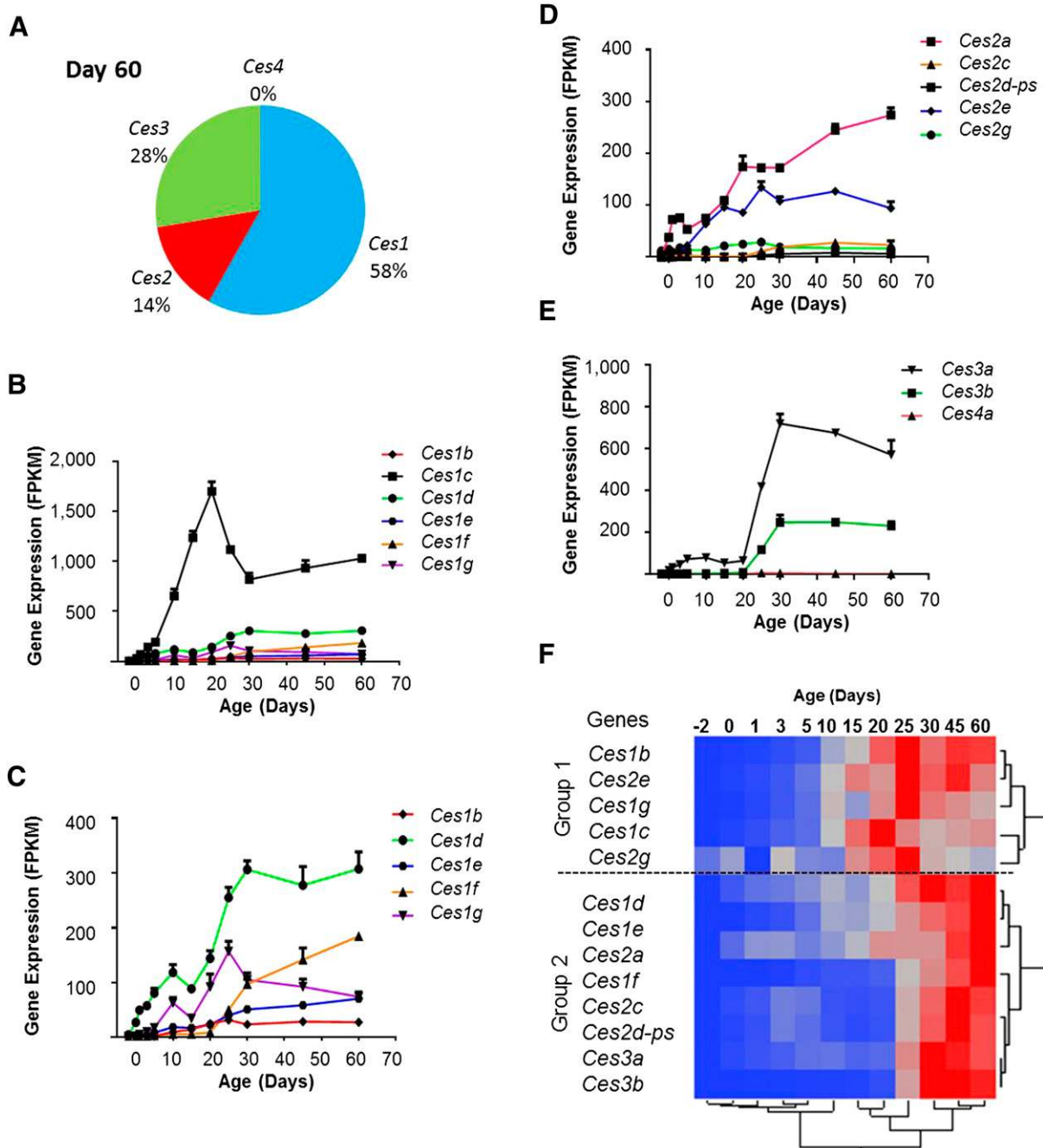


Fig. 2. Expression of *Ces* mRNAs during liver development in male C57BL/6 mice. (A) mRNA proportion of each *Ces* family at the age of day 60. (B) Expression profiles of *Ces1* gene family. (C) Expression profiles of *Ces1* gene family without *Ces1c*. (D) Expression profiles of *Ces2* gene family. (E) Expression profiles of *Ces3* and *Ces4* gene families. Data are expressed as mean FPKM and S.E.M. 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.

and then went up gradually to adult levels at day 60, with a small plateau between days 20 and 30 (Fig. 6B). The adult mRNA level of *Dpyd* was about 30 times higher than in 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). *Adhs* 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. 7, B–D). *Adh1* mRNA was the most highly expressed, and was over 90% of all *Adh* mRNAs expressed in the adult liver (Fig. 7B), followed by *Adh5* and then *Adh4* (Fig. 7C). *Adh6* and *Adh7* were minimally expressed in the liver (Fig. 7D). *Adh1* and *Adh5* also showed a peak of expression around day 0 or day 1.

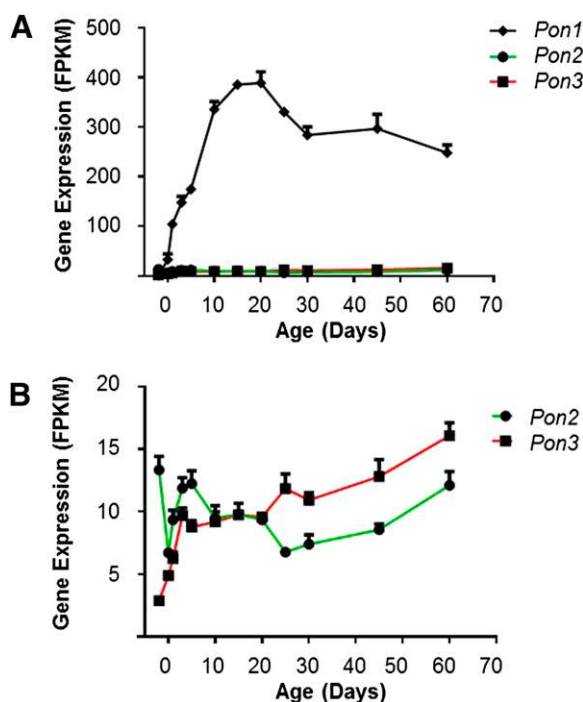


Fig. 3. Expression of *Pon* during liver development. (A) All three *Pon* ontogenic mRNA expression patterns. (B) *Pon1* was removed to enlarge *Pon2* and *Pon3* expression patterns. Data are expressed as mean FPKM and S.E.M. of three individual animals.

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 5-fold from 2 days before birth to 60 days after birth (Fig. 8A). *Aldh11l* 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* has demonstrated diverse ontogenic patterns for the individual *Aldhs*. Similar to *Akrs*, 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).

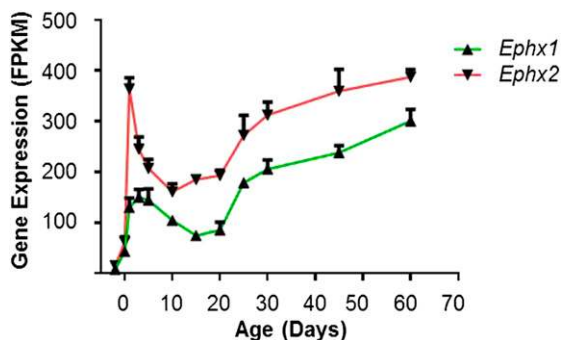


Fig. 4. Expression patterns of *Ephx1* and *Ephx2* during liver development.

Flavin Monooxygenases. Like P450s, FMOs are microsomal enzymes that require NADPH and O_2 . 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* increases gradually with age; it 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 the liver during maturation, and *Fmo2* was mainly detected at the adolescent stage from days 10 to 20 (Fig. 9B).

Molybdenum Hydroxylases. Mammalian molybdenum hydroxylases require flavin adenine dinucleotide (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 livers. 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 a 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 days -2 to 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 days 5 to 20, *Por* expression was relatively stable. Then at day 25, it decreased again to about half the level of day 20, and it remained at that level till maturity (Fig. 11).

Discussion

The current study provides a comprehensive quantitative analysis of the developmental expression of major non-P450 phase-I enzymes on mRNA levels in mouse livers 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 polymerase chain reaction, which detect mRNAs by probe hybridization and rely on hybridization specificity and efficiency, RNA-seq directly counts sequence reads of the nucleotide molecules in biologic samples. Thus, it can quantify the mRNA expression with minimal bias. The expression of a gene transcript is represented by FPKM, which normalizes 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 livers, the total FPKM values of phase-I mRNAs increase 15-fold with two rapidly increasing stages (Fig. 1A), which may reflect the functional transition of the 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 the liver to deal with exposure to xenobiotics immediately after birth. The second surge

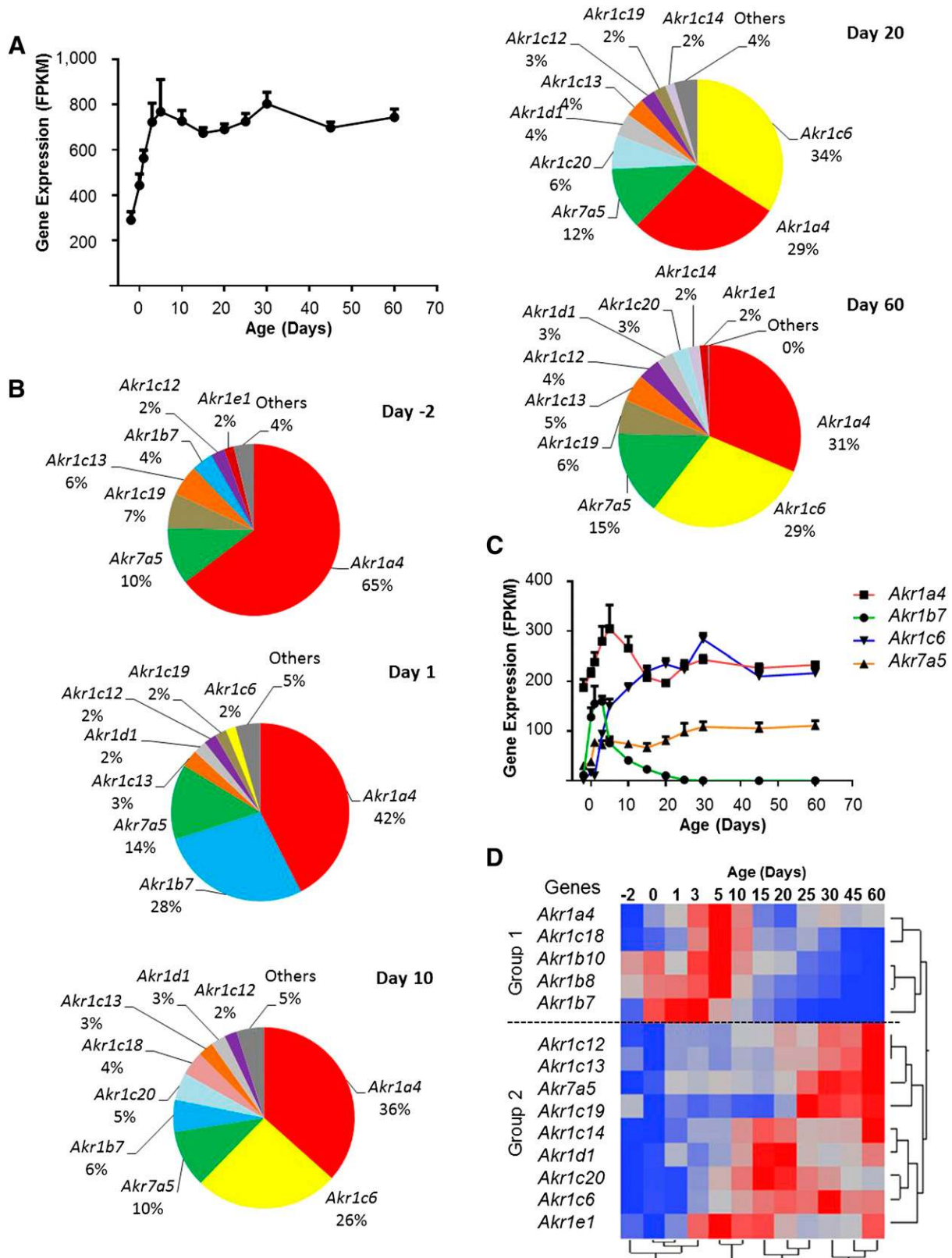


Fig. 5. (A) Total mRNA profile of *Akr* genes in the liver during development. The FPKM values of the 14 significantly expressed *Akr* genes are summed and plotted to show the developmental pattern of total *Akr* mRNAs. (B) Individual *Akr* mRNAs (shown as percentages of total *Akr* mRNAs) at 2 days 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 four highly expressed *Akr*s (>10% in B) in the 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.

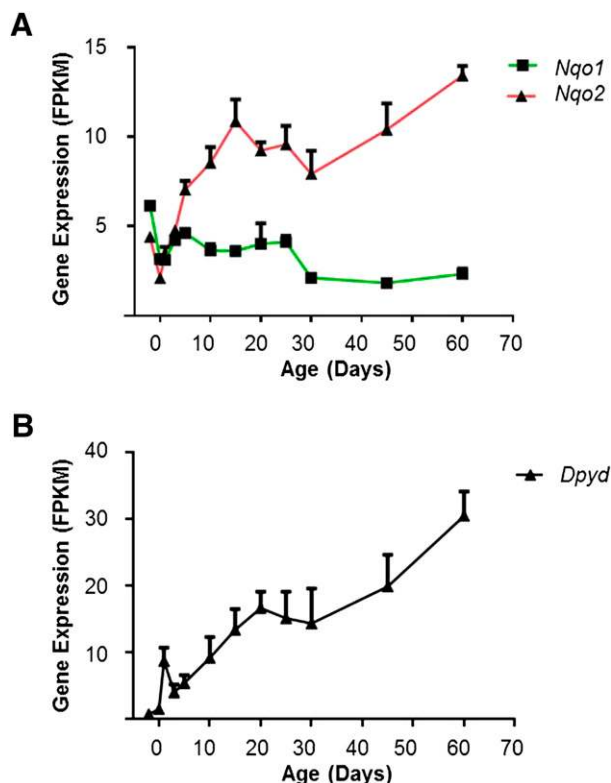


Fig. 6. (A) mRNA expression profile of *Nqo* gene in the liver during development. (B) mRNA expression profile of *Dpyd* in the liver during development.

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 change 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 the mouse liver (Holmes et al., 2010). Another study demonstrated that the expression of mouse *Ces1* and *Ces2* in the liver was 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 observations and provides 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 fetuses (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.

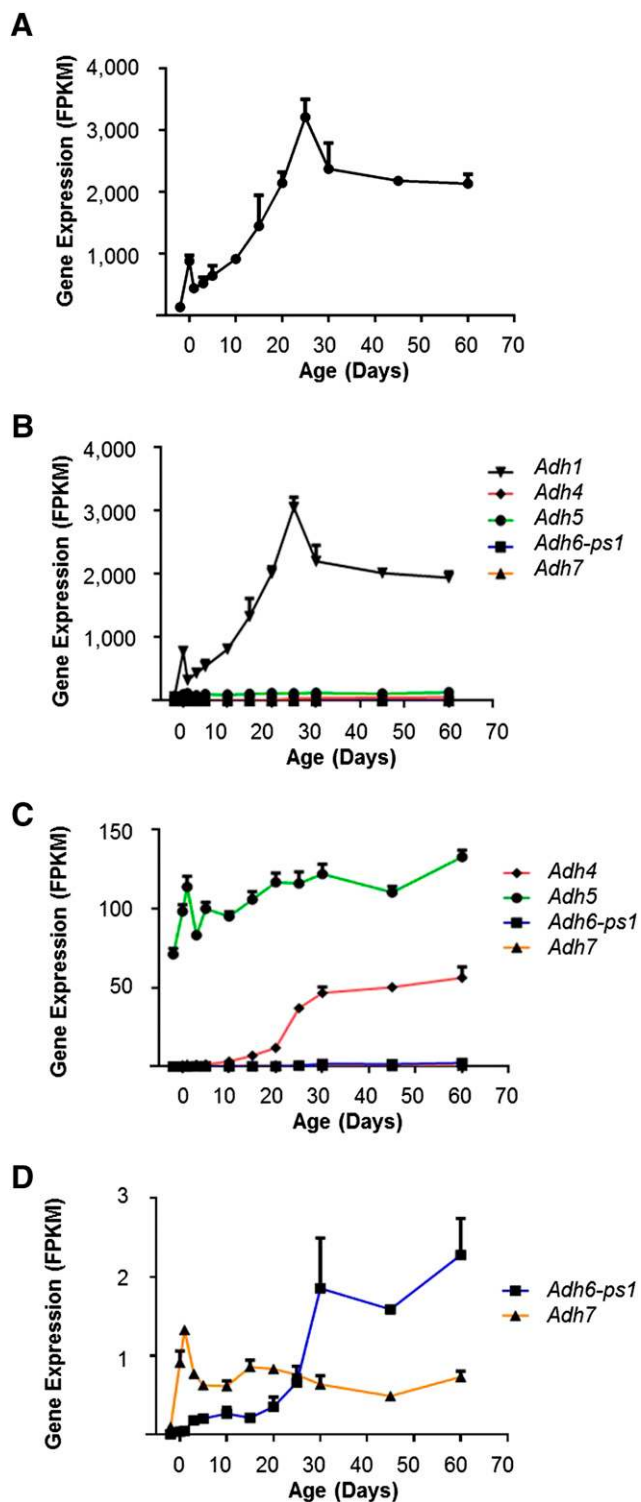


Fig. 7. (A) Total mRNA profile of all five *Adh* genes during liver development. (B) Ontogenic mRNA expression patterns of individual *Adh* gene. (C) *Adh1* removed to enlarge *Adh4* and *Adh5* expression patterns. (D) *Adh4* and *Adh5* removed to enlarge *Adh6-ps1* and *Adh7* expression patterns.

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

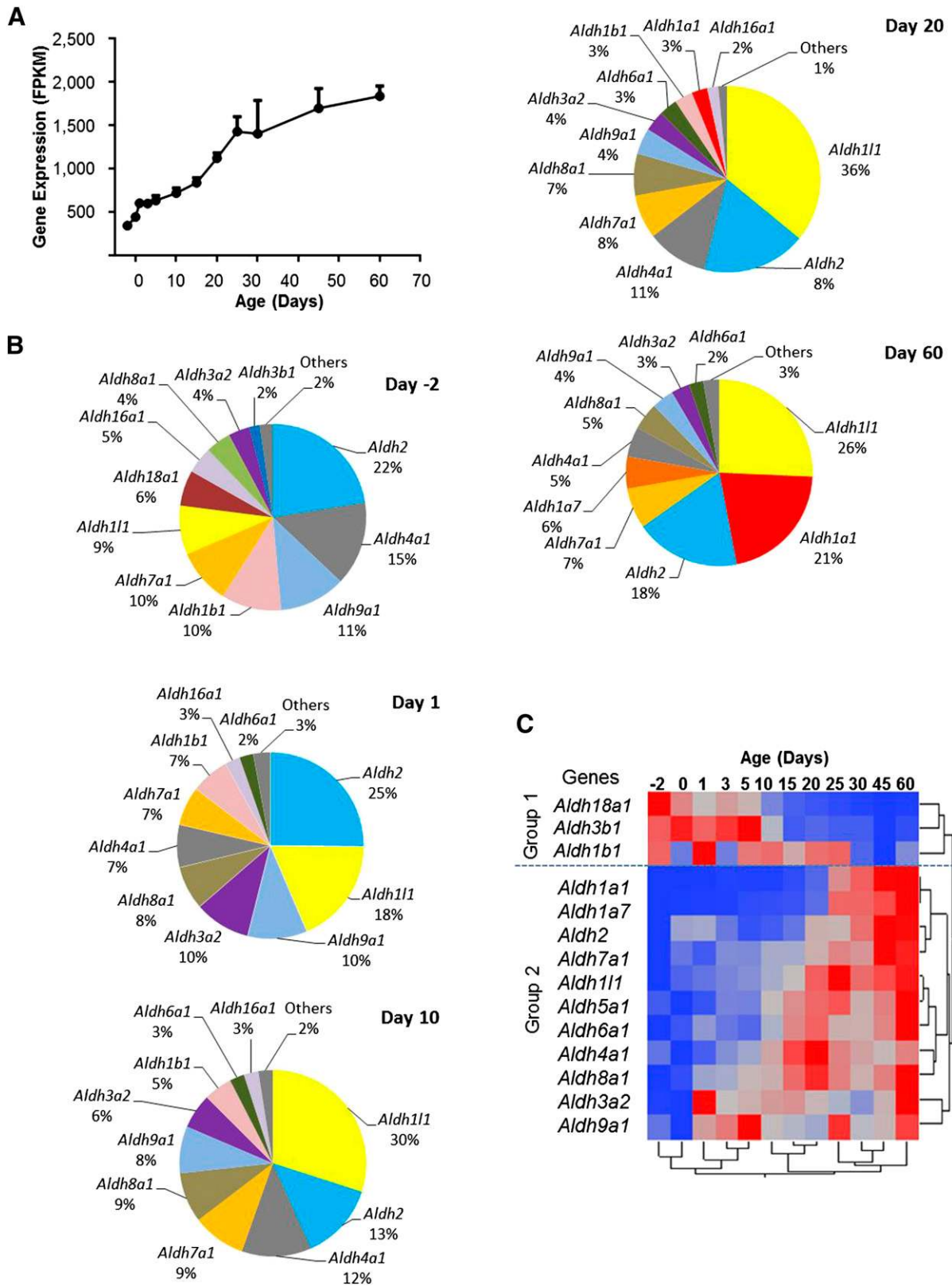


Fig. 8. (A) Total mRNA profile of *Aldh* genes in the liver during development. The FPKM values of the 15 significantly expressed *Aldh* genes are summed and plotted to show the developmental pattern of total *Aldh* mRNAs. (B) Individual *Aldh* mRNAs (shown as percentages of total *Aldh* mRNAs) at 2 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 mRNA 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.

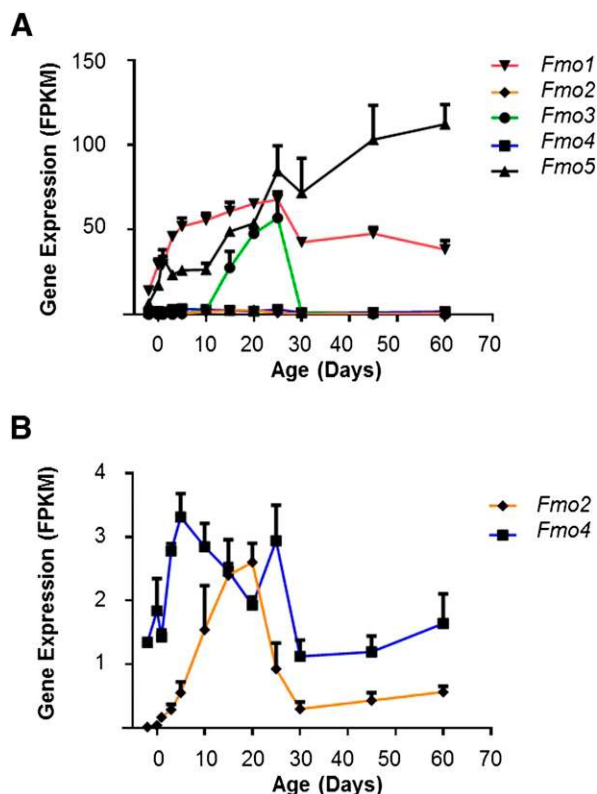


Fig. 9. Expression of *Fmo* genes during liver development. (A) All five *Fmo* ontogenic mRNA expression patterns. (B) *Fmo1*, 3, 5 are removed to enlarge *Fmo2* and *Fmo4* expression patterns.

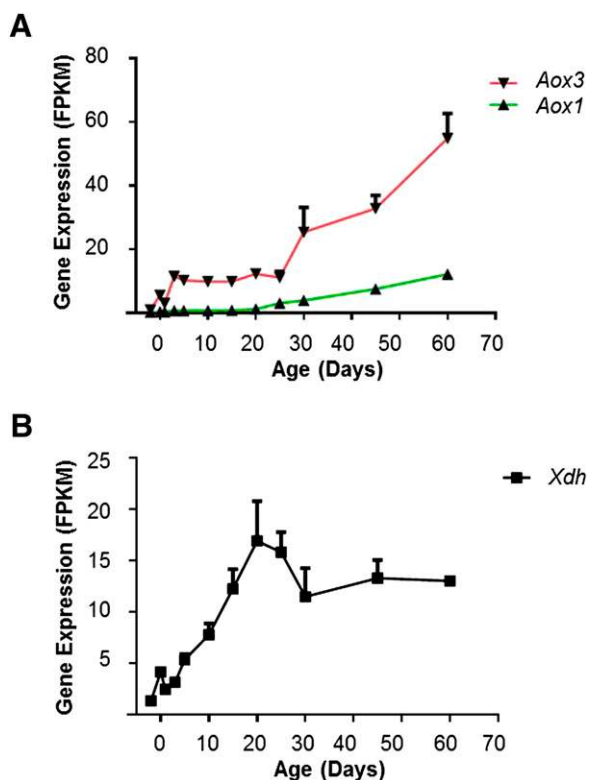


Fig. 10. Expression of molybdenum hydroxylases during liver development. (A) mRNA ontogenic patterns of aldehyde oxidase *Aox1* and *Aox3*. (B) mRNA ontogenic patterns of *Xdh*.

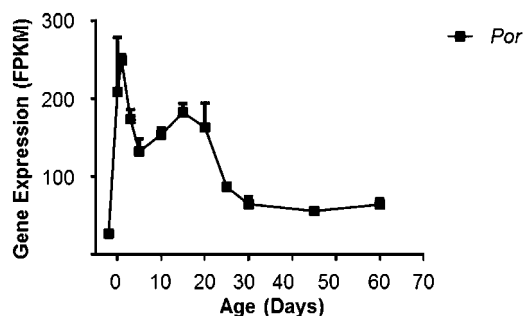


Fig. 11. mRNA expression pattern of *Por* during liver development.

similar to our data of *Pon1* mRNA expression in mouse livers (Fig. 3). A transgenic mouse strain that lacks endogenous *Pon1* but has 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. Our 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 a 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 the adult human liver, it demonstrated gender-specific expression in mice and was not detectable in the male livers of mice (Falls et al., 1995). Our data only examined mRNA expression in male animals and are consistent with this result. *Fmo3* was expressed equally in male and female mice, even at 4 weeks of age (Cherrington et al., 1998); after puberty, the gender difference appeared due to sex steroids (Falls et al., 1997). We also show the detailed time window when the shutdown of *Fmo3* happened, which was between days 25 and 30 (Fig. 9A). *Fmo1* in mice showed increased expression after birth, unlike humans in whom *Fmo1* is most abundant in fetal livers and absent after birth (Hines, 2006). Interestingly, *Fmo3* was not expressed immediately after birth in mice; it 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, which are 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 molybdoenzymes, including immunosuppressive drugs such as 6-mercaptopurine, antiviral drugs such as 6-deoxyacyclovir, and the antidepressant citalopram. They also perform important physiologic functions by metabolizing biogenic amines and catecholamines, and they 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 they have critical roles in biotransformation, the expression and regulation of molybdoenzymes are largely understudied, especially compared with other drug metabolizing enzymes. Our first report on the developmental expression pattern of these genes will facilitate drug metabolism and toxicity studies related to molybdoenzymes.

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 revealed that the ontogenic mRNA expression of all P450s in the mouse liver and a large number of P450 genes had increased expression after birth (Peng et al., 2012). Here, we show hepatic mRNA expression of *Por*, which could influence P450 activities, actually decrease during postnatal development (Fig. 11). Thus, the level of *Por* needs to be taken into consideration when we study the developmental enzyme activity of P450s.

Protein levels and enzyme activities of phase-I genes were not determined in this study because of the 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 have measured protein expression or enzyme activities of certain phase-I genes in the 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 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, our study provides 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. This knowledge will serve as a foundation for further understanding the regulation of gene expression and physiologic function of these enzymes in the 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, Yoo.

Conducted experiments: Peng, Cui.

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

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

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