RNA-Sequencing Quantification of Hepatic Ontogeny and Tissue Distribution of mRNAs of Phase II Enzymes in Mice^S

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ABSTRACT

Phase II conjugating enzymes play key roles in the metabolism of xenobiotics. In the present study, RNA sequencing was used to elucidate hepatic ontogeny and tissue distribution of mRNA expression of all major known Phase II enzymes, including enzymes involved in glucuronidation, sulfation, glutathione conjugation, acetylation, methylation, and amino acid conjugation, as well as enzymes for the synthesis of Phase II cosubstrates, in male C57BL/6J mice. Livers from male C57BL/6J mice were collected at 12 ages from prenatal to adulthood. Many of these Phase II enzymes were expressed at much higher levels in adult livers than in perinatal livers, such as *Ugt1a6b*, *-2a3*, *-2b1*, *-2b5*, *-2b36*, *-3a1*, and *-3a2*; *Gsta1*, *-m1*, *-p1*, *-p2*, and *-z1*; *mGst1*; *Nat8*; *Comt*; *Nnmt*; *Baat*; *Ugdh*; and *Gclc*. In contrast, hepatic mRNA expression of a few Phase II

enzymes decreased during postnatal liver development, such as *mGst2*, *mGst3*, *Gclm*, and *Mat2a*. Hepatic expression of certain Phase II enzymes peaked during the adolescent stage, such as *Ugt1a1*, *Sult1a1*, *Sult1c2*, *Sult1d1*, *Sult2as*, *Sult5a1*, *Tpmt*, *Glyat*, *Ugp2*, and *Mat1a*. In adult mice, the total transcripts for Phase II enzymes were comparable in liver, kidney, and small intestine; however, individual Phase II enzymes displayed marked tissue specificity among the three organs. In conclusion, this study unveils for the first time developmental changes in mRNA abundance of all major known Phase II enzymes in mouse liver, as well as their tissue-specific expression in key drug-metabolizing organs. The age- and tissue-specific expression of Phase II enzymes indicate that the detoxification of xenobiotics is highly regulated by age and cell type.

Introduction

Liver, kidney, and intestine are the three major tissues involved in the absorption, distribution, metabolism, and elimination of xenobiotics. Phase II conjugating enzymes, such as sulfotransferase (*Sult*), uridine diphosphate-glucuronosyltransferase (*Ugt*), glutathione *S*-transferases (*Gst*), *N*-acetyltransferase (*Nat*), catechol *O*-methyltransferase (*Comt*), and amino acid (AA) conjugation enzymes, play key roles in catalyzing the conjugation reaction of xenobiotics (Jancova et al., 2010). Phase II conjugates usually have decreased biologic activities. Moreover, products of most of these Phase II conjugation reactions, with the exception of methylation and acetylation, have markedly increased water solubility and are readily excreted from the body. Therefore, Phase II conjugation reactions are generally considered as

major inactivation and detoxification pathways for xenobiotics, although activation of certain xenobiotics by these Phase II enzymes has been reported (Glatt, 2000; Regan et al., 2010). In addition to xenobiotics, Phase II enzymes are also important in the biotransformation of endobiotics, such as steroid hormones, thyroid hormones, bilirubin, and bile acids (Jancova et al., 2010).

Many drug-processing genes undergo marked changes in expression during tissue development and maturation. Children and newborn animals are often more susceptible to the adverse effects of therapeutic drugs and environmental chemicals due to their immature capacity to metabolize and detoxify these chemicals (Blake et al., 2005; Hines, 2008; Funk et al., 2012). Understanding of developmental changes in expression patterns of these Phase II enzymes will help to elucidate the mechanism of ontogenic regulation of gene expression. Additionally, extrahepatic tissues, including intestine and kidney, also play key roles in determining the absorption, distribution, metabolism, and excretion (ADME) of xenobiotics (Krishna and Klotz, 1994). It is essential to understand the tissue-specific expression patterns of Phase II enzymes among the three major tissues in ADME, namely liver, kidney, and

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ABBREVIATIONS: AA, amino acid; ADME, absorption, distribution, metabolism, and excretion; *As3mt*, arsenic (+3 oxidation state) methyltransferase; *Baat*, bile acid-CoA:amino acid *N*-acyltransferase; *Bal*, bile acid-CoA ligase; bp, basepair; BPA, bisphenol A; *Ccb1*, cysteine conjugate- β -lyase 1; *Comt*, catechol *O*-methyltransferase; FPKM, fragments per kilobase of exon per million reads mapped; *Gclc*, glutamate-cysteine ligase catalytic subunit; *Gclm*, glutamate-cysteine ligase modifier subunit; *Ggt1*, γ -glutamyltransferase 1; GH, growth hormone; *Glyat*, glycine-*N*-acyltransferase; GSH, glutathione; *Gst*, glutathione *S*-transferase; IPA, Ingenuity Pathways Analysis; IPKB, Ingenuity Pathway Knowledge Base; *Mat1a*, methionine adenosyltransferase 1a; *Nat*, *N*-acetyltransferase; *Nnmt*, nicotinamide *N*-methyltransferase; *Papss1*, 3'-phosphoadenosine 5'-phosphosulfate synthase 1; PCR, polymerase chain reaction; RNA-seq, RNA sequencing; *Sult*, sulfotransferase; *Tpmt*, thiopurine *S*-methyltransferase; *Ugdh*, UDP-glucose 6-dehydrogenase; *Ugp2*, UDP-glucose pyrophosphorylase 2; *Ugt*, uridine diphosphate-glucuronosyltransferase.

small intestine. Therefore, the purpose of the present study was to elucidate ontogenic changes in mRNA expression of all major known Phase II enzymes during liver development and the distribution of mRNA expression of these Phase II enzymes in adult tissues of liver, kidney, and small intestine.

There is already some information about the ontogenic expression patterns and tissue distribution of certain Phase II enzymes in mice, such as *Sult* (Alnouti and Klaassen, 2006) and *Gst* enzymes (Knight et al., 2007; Cui et al., 2010). However, hepatic ontogenic changes in the mRNA expression of *Ugt*, *Nat*, methyltransferase, and AA-conjugating enzymes remain unknown. Moreover, conventional mRNA-profiling methods cannot compare the real abundance of transcripts between various genes. In contrast, RNA sequencing (RNA-seq) provides a “true-quantification” of transcript counts (Malone and Oliver, 2011). Therefore, the purpose of this study was to use RNA-seq technology for the first time to generate comprehensive information on the ontogenic mRNA expression of all major known Phase II enzymes in liver, and the distribution of mRNAs of these genes in adult liver, kidney, and small intestine using mouse as a model in a quantitative mode. This information will provide a foundation for understanding the relative importance of various Phase II enzymes and their subfamily members in xenobiotic metabolism in liver at different developmental stages, as well as in the three key tissues of ADME, namely liver, kidney, and small intestine, in adulthood.

Materials and Methods

Animals. Eight-week-old C57BL/6J breeding pairs of mice were purchased from the Jackson Laboratory (Bar Harbor, ME). They were housed according to the American Animal Association Laboratory Animal Care guidelines, and were bred under standard conditions at the University of Kansas Medical Center. Livers from offspring were collected at the following 12 ages: days –2 (gestational day 17.5), 0 (right after birth and before the start of suckling), 1, 3, 5, 10, 15, 20, 25, 30, 45, and 60. Due to potential variations caused by the estrous cycle in maturing adult female mice, only male livers were used for this study ($n = 3$ per age, randomly selected from multiple litters). For the study of tissue distribution, tissues of liver, kidney, and small intestine were collected from adult male mice at 60 days of age ($n = 2$ per tissue). Tissues were frozen immediately in liquid nitrogen and stored at -80°C before use.

Total RNA Preparation. Total RNA from liver, kidney, and small intestine was isolated using RNeasy Lysis reagent (Qiagen, Crawfordsville, IN) per the manufacturer's protocol. RNA concentrations were quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at a wavelength of 260 nm. Integrity of the total RNA samples was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA), and the samples with RNA integrity values of >7.0 were used for the following experiments.

cDNA Library Preparation and RNA-Seq of Liver Ontogeny Samples. The cDNA libraries from all total RNA samples of livers of 12 ages of mice were prepared using an Illumina TruSeq RNA sample prep kit (Illumina, San Diego, CA). Three micrograms of total RNA was used per the RNA input recommendations of the manufacturer's protocol. The mRNAs were selected from the total RNAs by purifying the poly(A)-containing molecules using poly-T primers. The RNA fragmentation, first- and second-strand cDNA syntheses, end repair, adaptor ligation, and polymerase chain reaction (PCR) amplification were performed per the manufacturer's protocol. Fragments of the cDNA library ranged from 220 to 500 basepair (bp), with an average size of 280 bp (including 100-bp adapter sequences). The quality of cDNA libraries was validated using an Agilent 2100 Bioanalyzer before sequencing by the Genome Sequencing Facility at the University of Kansas Medical Center. Briefly, the cDNA libraries were clustered onto a TruSeq paired-end flow cell and sequenced (2×100) using a TruSeq 200-cycle SBS kit (Illumina). For the initial run, a PhiX control (Illumina) was loaded on each flow cell as well as universal human reference RNA on 1 of the 16 lanes of the Illumina HiSeq 2000 sequencer, and sequenced in parallel with other samples to ensure the data generated for each run were accurately calibrated during the image analysis and

data analysis. In addition, the PhiX was spiked into each cDNA sample at $\sim 1\%$ as an internal quality control.

RNA-Seq Data Analysis. After the sequencing platform generated the sequencing images, the pixel-level raw data collection, image analysis, and base calling were performed by the RTA (Real Time Analysis) software on a Dell PC attached to the HiSeq 2000 sequencer. The data were streamed off to the analysis server as the run was progressing. The BCL Converter converted the BCL (base calling) files to qseq files, and the qseq files were 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), and the splice junctions were identified by TopHat. The output files in BAM (binary sequence alignment) format were analyzed by Cufflinks to estimate the transcript abundance. The transcript structure predictions of Cufflinks were compared with Ensembl GTF version 65 by Cuffcompare. RNA-seq generated an average of 175 million reads per sample, and $>80\%$ of the reads were mapped to the mouse reference genome (NCBI37/mm9) by TopHat (unpublished data). The mRNA abundance of genes was estimated by Cufflinks. The mRNA abundance was expressed in FPKM (fragments per kilobase of exon per million reads mapped), which normalizes sequencing depths between different samples and sizes between different genes, allowing direct comparison of expression levels among different transcripts on a genome-wide scale.

cDNA Library Preparation and RNA-Seq of Tissue Distribution Samples. The preparation of cDNA libraries of adult male liver, kidney, and small intestine and sequencing of these cDNA libraries were conducted by Beijing Genomic Institute (Shenzhen, People's Republic of China). Beads with oligo(dT) were used to isolate poly(A) mRNA from total RNA. Fragmentation buffer was added for breaking mRNA into short fragments. Taking these short fragments (200–700 nucleotides) as templates, random hexamer primers were used to synthesize the first-strand cDNA. The second-strand cDNA was synthesized using buffer, dNTPs, RNase H, and DNA polymerase I, respectively. Short fragments were purified with QiaQuick PCR extraction kit (Qiagen, Germantown, MD) and resolved with elution buffer for end repair and adding poly(A). After that, the short fragments were connected with sequencing adaptors. For amplification with PCR, suitable fragments were selected as templates with respect to the result of agarose gel electrophoresis. The resultant cDNA libraries were sequenced using Illumina HiSeq 2000. Images generated by sequencers were converted by base calling into nucleotide sequences, which were called raw reads and were stored in FASTQ format. After removal of poor-quality reads containing adaptors, unknown, or low-quality bases from the raw reads, the resultant clean reads were mapped to the mouse reference genome (NCBI37/mm9) and the splice junctions were identified by TopHat. The FPKM method was used to calculate mRNA abundance, which normalizes sequencing depths between different samples and sizes between different genes. For the study of tissue distribution, the mRNA abundance was expressed as ratio to value of liver, with that of liver set as 1.0.

Validation of RNA-Seq Data with Real-Time PCR. The total RNAs of male mouse livers at eight ages, days –2, 1, 5, 10, 20, 25, 45, and 60 ($n = 3$ per age), were reverse-transcribed into cDNAs using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The cDNAs were used for real-time PCR quantification of mRNA expression of *mGst3* and *Nat8* using the iTaq Universal SYBR Green Supermix and a MyiQ2 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). No single commonly used housekeeping gene had consistent mRNA expression levels throughout liver development (RNA-seq data, not shown). In contrast, the average expression levels of two housekeeping genes, namely *Gapdh* and *Ncu-g1* (an integral membrane protein of the lysosome), were found to be consistent throughout liver development (Lu et al., 2012). Thus, amounts of mRNAs were calculated using the comparative computed tomography method, which determines the amount of target normalized to the geometric mean of *Gapdh* and *Ncu-g1*. Real-time PCR primers for *mGst3* and *Nat8* were: *mGst3_for*, AGATGGCTGCTCTCTAAGG; *mGst3_rev*, ATATGCCCGTCTCTCAGATCT; *Nat8_for*, GGACTACAAACAGGTCGTGGA; *Nat8_rev*, GCATACAACAGCCAGGAACCA.

Statistics. Data are presented as mean $\pm$ S.E. Differences between various groups were determined using analysis of variance followed by post hoc test, with significance set at $P \leq 0.05$. In the study of liver ontogeny, the FPKM values were $\log_2$ -transformed to achieve normal distribution prior to analysis of variance. The statistics for mRNA expression of Phase II

enzymes during liver development (RNA-seq data) are shown in Supplemental Table 1.

Hierarchical clustering of Phase II conjugating enzymes was performed using Matlab (R2012b; The MathWorks Inc., Natick, MA). The data were standardized to have a zero mean and unit variance at a gene level before clustering. The Ward's method was used for the linkage function applied on a Euclidean distance matrix of pairwise distances of gene expression.

Gene set enrichment analysis was carried out using Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA). IPA identifies significant networks, functions, canonical pathways, and associated transcription factors in a set of genes based on information gathered in the Ingenuity Pathway Knowledge Base (IPKB). The IPKB is an extensive repository of information on genes and gene products that interact with each other. The significance (*P* value) of the identified networks, functions, and canonical pathways was calculated using the right-tailed Fisher's exact test. This test measures the significance of the overlap between the input genes and the genes in a particular category in the IPKB, reflecting the statistical significance of the network, function, or canonical pathway with respect to the input genes and the reference genes (all genes in the IPKB identified with the particular network, function, or canonical pathway). The analysis was performed on 85 Phase II genes. The selection criteria for an expressed gene were expression in at least one day measured by the presence of an FPKM value of ≥ 1.0 . Input genes for "gene set enrichment" were perinatal Phase II genes (13 mapped and analyzed in IPA out of 13 in cluster), adolescent Phase II genes (14 mapped and analyzed in IPA out of 18 in cluster), and adult Phase II genes (46 mapped and analyzed in IPA out of 54 in cluster).

Results

The total FPKM values of all mRNA transcripts determined by RNA-seq were very similar among livers from different ages of mice

and/or among the three adult mouse tissues of liver, kidney, and small intestine, which is consistent with the equal loading of total RNAs from these samples in RNA-seq. The RNA-seq data on ontogenic mRNA expression in livers of C57BL/6J mice were highly quantitative, which had been validated in our previous studies (Cui et al., 2012; Lu et al., 2012; Peng et al., 2012). The raw data of FPKM values of RNA-seq data presented in this study are listed in Supplemental Tables 2 and 3.

Total Expression and Proportions of Individual Phase II Enzymes during Liver Development in Male C57BL/6J Mice. mRNA expression of most Phase II enzymes had marked ontogenic changes during liver development (Fig. 1). Total transcripts of *Ugt* enzymes in liver were low before birth, increased gradually after birth, and reached the highest levels of ~ 24 -fold prenatal levels 60 days after birth (Fig. 1A). Conversely, total transcripts of *Sult* enzymes in liver were low before birth, increased rapidly after birth, peaked at 20 days postbirth, and gradually fell back to ~ 5 -fold prenatal levels 60 days after birth (Fig. 1B). In contrast to the low prenatal expression of *Ugt* and *Sult* enzymes, total transcripts of *Gst* enzymes in liver were already high before birth, further increased gradually after birth, and plateaued at ~ 4 -fold prenatal levels 30 days after birth (Fig. 1C). Total transcripts of *Nat* enzymes (*Nat1*, *-2*, and *-8*) in liver were low before birth, slightly increased during postnatal development, started to surge after day 25 (weaning), and reached the highest levels at adulthood (day 60) (Fig. 1D). Compared with *Nat* enzymes, total transcripts of methyltransferases in liver were more abundant, and the postnatal surge started at the earlier time of day 20 (before weaning), reached high levels at day 30, and only increased slightly thereafter (Fig. 1E). Hepatic total transcripts of enzymes involved in AA conjugation of

Fig. 1. Hepatic ontogeny of total transcripts of Phase II enzymes in male C57BL/6J mice. Livers from C57BL/6J mice of ages -2 to 60 days were used for RNA-seq quantification of (A) *Ugt*, (B) *Sult*, (C) *Gst*, (D) *Nat*, (E) methyltransferase, and (F) AA conjugation enzymes. The y-axis represents mRNAs expressed as FPKM. *n* = 3, mean $\pm$ S.E. **P* < 0.05 vs. day -2 .

xenobiotics were low before birth, rapidly increased during perinatal stages, and reached adult levels after weaning (day 25) (Fig. 1F).

Hierarchical clustering revealed three distinct patterns of expression of Phase II conjugating enzymes in liver with age (Fig. 2). Of the 87 enzymes involved in Phase II conjugation, 84 genes were significantly expressed during different stages of liver development. The first cluster consisting of 13 genes was maximally expressed between prenatal day 2 (day -2) and postnatal day 5, and thus was categorized as perinatal-predominant Phase II conjugating enzymes. The second cluster of 18 genes, whose expression was maximal between day 5 and day 25, was categorized as adolescent-predominant Phase II conjugating enzymes. The third cluster of 53 genes, with maximum expression between day 25 and day 60, was identified as adult-predominant Phase II conjugating enzymes. It was observed that genes in different groups of Phase II conjugating enzymes were very selectively distributed among these three clusters (χ^2 test of independence; Yates' *P* value, 0.0032). For example, of the perinatal-predominant genes, 62% were equally distributed at 4 genes (31%) each between *Gst* and cosubstrate-synthesizing enzymes. A majority of the adolescent-predominant genes were *Sult* genes (11 genes, 61%). Adult-predominant genes were mainly *Gst* (19 genes, 35%) and *Ugt* genes (18 genes, 33%).

Among the main biologic functions significantly associated with the perinatal-predominant Phase II conjugating enzymes are metabolism of peptide, metabolism of glutathione (GSH), synthesis of GSH, synthesis of *S*-adenosylmethionine, and cholestasis. Genes associated with adolescent-predominant Phase II conjugating enzymes show significant function in sulfation of raloxifene, hormone, dopamine, 2-hydroxyestradiol, and β -estradiol; metabolism of estrogen; and steroid metabolism. The significant biologic functions ascribed to the adult-predominant Phase II conjugating enzymes include metabolism of xenobiotics, conjugation of GSH, glucuronidation of hormone, glucuronidation of estrogen, metabolism of GSH, glucuronidation of coumarin, conjugation of lipid, metabolism of peptide, glucuronidation of alcohol, etc.

Hepatic Ontogeny of *Ugt* mRNAs in Male C57BL/6J Mice.

Glucuronidation is critical for the metabolism and excretion of endogenous compounds and xenobiotics. The vast majority of *Ugt* family members displayed the adult-predominant expression pattern, with low expression before birth and gradual postnatal increase to the highest in adulthood (Fig. 3). A prominent change was a marked postnatal surge in hepatic expression of *Ugt1a1*, which peaked during the adolescent stage and then decreased moderately at adulthood (Fig. 3A). In contrast, other *Ugt1a* subfamily members, including *Ugt1a5*, *Ugt1a6a*, *Ugt1a6b*, and *Ugt1a9*, all displayed the adult-predominant expression pattern (Fig. 3A). Among the *Ugt2a* subfamily, *Ugt2a3*, which catalyzes the glucuronidation of simple polycyclic aromatic hydrocarbons (Bushey et al., 2013), had an adult-predominant expression pattern (Fig. 3A), whereas *Ugt2a1* and *Ugt2a2* were undetectable throughout liver development (unpublished data). All seven *Ugt2b* subfamily members, namely *Ugt2b1*, *-2b5*, *-2b34*, *-2b35*, *-2b36*, *-2b37*, and *-2b38*, had adult-predominant expression patterns (Fig. 3A). *UGT3A1* is a UDP-*N*-acetylglucosaminyltransferase with broad substrate activity toward ursodeoxycholic acid, 17α -estradiol, 17β -estradiol, and the prototypic substrates of the *UGT1* and *UGT2* forms, 4-nitrophenol and 1-naphthol (Mackenzie et al., 2008). In contrast, *UGT3A2* uses UDP-glucose and UDP-xylose but not UDP-*N*-acetylglucosamine to glycosidate a broad range of substrates, including 4-methylumbelliferone, 1-hydroxypyrene, bioflavones, and estrogens (MacKenzie et al., 2011). Hepatic mRNA expression of *Ugt3a1* and *Ugt3a2* was very low before birth, increased gradually during postnatal development, and reached the highest levels at adulthood (Fig. 3A).

On day -2 (2 days before birth), *Ugt2b34* was the most abundant *Ugt* isoform, followed by *Ugt2b35*, *-2b36*, *-1a6b*, and *-2b5*; *Ugt1a1*

only accounted for a very small percentage of total *Ugts* (Fig. 3B). On day 1 (neonatal stage), due to a marked surge in *Ugt1a1* expression, *Ugt1a1* became the most abundant *Ugt* isoform, followed by *Ugt1a6b*, *-2b34*, *-2b35*, and *-2b5*. On day 25 (adolescent stage), due to marked increases in the expression of *Ugt2b* family members, *Ugt2b36* was the most abundant *Ugt* isoform, followed by *Ugt1a1*, *-2b5*, and *-1a6b*. On day 60 (adulthood), *Ugt2b* family members, namely *Ugt2b5*, *-2b36*, and *-2b1*, were the most abundant *Ugt* isoforms; however, many other *Ugt1a*, *-2a*, and *-3a* family members were also expressed at appreciable levels in adult liver (Fig. 3B). *Ugt8a*, a UDP-galactose:ceramide galactosyltransferase, was not expressed at any time during liver development (unpublished data).

Hepatic Ontogeny of *Sult* mRNAs in Male C57BL/6J Mice.

Sult family members had diverse ontogenic expression patterns during liver development (Fig. 4). *Sult1a1*, *-1d1*, *-1e1*, *-2a1* to *-2a6*, and *-5a1* all displayed adolescent-predominant expression patterns (Fig. 4A). Hepatic mRNA expression of *Sult1c2* increased rapidly after birth, peaking at 5 days after birth, and then sharply decreased to low levels at 30 days of age. In contrast, hepatic expression of *Sult1b1*, which sulfonates dopamine and thyroid hormones (Saeki et al., 1998), reached the highest levels 15 days after birth and fluctuated thereafter (Fig. 4A). *Sult3a1*, *-4a1*, and *-6b1* transcripts remained undetectable throughout liver development in male mice (unpublished data).

Before birth (day -2), *Sult1a1*, which sulfonates phenolic compounds, including steroid hormones, catecholamines, and phenolic drugs (Hempel et al., 2007), was the predominant *Sult* isoform in liver, accounting for >80% of total *Sult* transcripts (Fig. 4B). On day 1, there were marked increases in the expression of steroid/bile acid sulfotransferase *Sult2a* (Huang et al., 2010) subfamily members, which account for >50% of total *Sult* transcripts in liver. A similar distribution in *Sult* isoforms was observed on day 25, but with a shrinking share for the *Sult1* family members. On day 60, due to the silencing of most *Sult2a* subfamily genes, *Sult1a1* resumed as the predominant *Sult* isoform in liver, accounting for >80% of total *Sult* transcripts, followed by *Sult5a1*, *-1d1*, and *-1b1* (Fig. 4B).

Hepatic Ontogeny of *Gst* mRNAs in Male C57BL/6J Mice.

Various *Gst* subfamily members had distinct ontogenic expression patterns during liver development (Fig. 5). Members of the *Gsta* subfamily all had adult-predominant expression patterns, with *Gsta3* being the predominant *Gsta* subfamily member, followed by *Gsta4* (Fig. 5A). *Gsta3* is essential for the protection of mice against DNA damage and hepatotoxicity induced by aflatoxin B1 (Ilic et al., 2010). Among the *Gstm* subfamily, *Gstm1* was the predominant isoform throughout liver development (Fig. 5A). *Gstm1* is essential for the protection of mice against methemoglobinemia induced by 1,2-dichloro-4-nitrobenzene through GSH conjugation (Arakawa et al., 2010). *Gstm1*, *-m4*, and *-m6* had adult-predominant, *Gstm2* and *-m3* had adolescent-predominant, and *Gstm5* had fetal-predominant expression patterns, whereas *Gstm7* had no significant ontogenic changes (Fig. 5A). Among the microsomal *Gst* (*mGst*) subfamily, there was an ontogenic switch during liver development: *mGst3* was the predominant *mGst* in fetal liver, whereas *mGst1* was the predominant *mGst* in adult liver, due to marked postnatal downregulation of *mGst3* but an increase of *mGst1* (Fig. 5A). Similar to *mGst3*, hepatic *mGst2* transcripts were markedly decreased during postnatal liver development. The most abundant *Gstt* subfamily was *Gstt1*, an enzyme essential for GSH conjugation of 1,2-epoxy-3-(*p*-nitrophenoxy)propane, dichloromethane, and the alkylating drug 1,3-bis(2-chloroethyl)-1-nitrosourea (Fujimoto et al., 2007). *Gstt1* and *Gstt3* had the adolescent-predominant expression pattern, whereas *Gstt2* mRNA expression surged 1 day after birth and then decreased to prenatal levels in adulthood (Fig. 5A). *Gstz1* is required for the metabolism of maleylacetoacetate (the

Fig. 2. Hierarchical clustering of hepatic ontogeny of Phase II conjugating enzymes in male C57BL/6J mice. Livers from C57BL/6J mice of ages –2 to 60 days were used for RNA-seq quantification. 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 solid lines separate the expression profiles into three groups of perinatal-, adolescent-, and adult-predominant expression.

Fig. 3. Hepatic ontogeny of *Ugt* mRNAs in male C57BL/6J mice. Livers from C57BL/6J mice of ages -2 to 60 days were used for RNA-seq quantification. (A) The y-axis represents mRNAs expressed as FPKM. $n = 3$, mean $\pm$ S.E. (B) Pie charts representing relative mRNA expression of each *Ugt* isoform as the percentage of total *Ugt* transcripts.

penultimate step in the catabolism of phenylalanine and tyrosine) and α -halo acids, a carcinogenic contaminant of chlorinated water (Lim et al., 2004). *Gstp1/p2* play important and complicated roles in the metabolism of xenobiotics (Henderson and Wolf, 2011). *Gstz1*, *Gstp1*, and *Gstp2* had adult-predominant expression patterns, whereas mRNA expression of *Gsto1* fluctuated during postnatal liver development (Fig. 5A).

Before birth (day -2), *mGst3* was the most abundant *Gst* in liver, followed by *Gstp1*, *-t1*, and *-m1* (Fig. 5B). On day 1, *mGst1* replaced *mGst3* as the most abundant *Gst* isoform, followed by *Gsta3*, *-z1*, and *-p1*. On day 25, *Gstm1* was the most abundant, followed by *mGst1*, *Gstt1*, and *Gsta3*. On day 60, *Gstp1* and *mGst1* were the two most abundant *Gst* isoforms, followed by *Gstm1* and *Gsta3* (Fig. 5B).

Hepatic Ontogeny of *Nat*, Methyltransferase, and AA-Conjugating Enzymes in Male C57BL/6J Mice. Acetylation catalyzed by the arylamine *N*-acetyltransferases *Nat1*, *Nat2*, and *Nat3* is a major biotransformation pathway for arylamine and hydrazine drugs, as well as many carcinogens (Sim et al., 2008). *Nat1* and *Nat2* mRNAs were

expressed at low levels before birth, increased gradually during postnatal liver development, and reached the highest levels at day 60 (Fig. 6A). *Nat3* mRNAs were undetectable in mouse liver (unpublished data). *Nat8* is a newly identified enzyme for mercapturic acid synthesis via acetylation of the cysteine *S*-conjugates (Veiga-da-Cunha et al., 2010). Hepatic mRNA expression of *Nat8* changed remarkably during development: *Nat8* mRNA was barely detectable through day 25 and increased rapidly starting from day 30, reaching the highest expression on day 60.

Catechol *O*-methyltransferase (*Comt*) catalyzes the methylation of large amounts of phenolic compounds. Hepatic *Comt* mRNA was moderately expressed at day -2 before birth and surged during two stages of postnatal development: perinatal stage (from day 1 to day 3) and postweaning stage (from day 20 to day 25) (Fig. 6B). Nicotinamide *N*-methyltransferase (*Nnmt*) is a cytosolic enzyme that catalyzes the *N*-methylation of nicotinamide, pyridines, and other structural analogs (Aksoy et al., 1994). The most dramatic ontogenic change in methyltransferases was observed for *Nnmt*; hepatic *Nnmt* mRNA was very

Fig. 4. Hepatic ontogeny of *Sult* mRNAs in male C57BL/6J mice. Livers from C57BL/6J mice of ages -2 to 60 days were used for RNA-seq quantification. (A) The y-axis represents mRNAs expressed as FPKM. $n = 3$, mean $\pm$ S.E. (B) Pie charts representing relative mRNA expression of each *Sult* isoform as the percentage of total *Sult* transcripts.

low until day 10; thereafter it increased rapidly and reached a high level on day 60 (Fig. 6B). Arsenic (+3 oxidation state) methyltransferase (*As3mt*) is the arsenic methyltransferase that protects against arsenic toxicity by converting inorganic arsenic to the methylated metabolite (Sumi and Himeno, 2012). Hepatic *As3mt* mRNAs were moderately altered during development; *As3mt* mRNA was at twice the amount in adult than in fetal stage. Thiopurine *S*-methyltransferase (*Tpmt*) plays an important role in the metabolism and detoxification of some thiopurine drugs used in pediatric leukemia, rheumatoid arthritis, and inflammatory bowel disease (Mladosievcova et al., 2011). After birth, hepatic *Tpmt* mRNA gradually increased to the highest level on day 15 and then slightly decreased to 3-fold the fetal level on day 60 (Fig. 6C).

Bile acid-CoA ligase (*Bal/Slc27a5*) and bile acid-CoA:AA *N*-acyltransferase (*Baat*) are two key enzymes for conjugation of bile acids with glycine and taurine (Clayton, 2011). Glycine-*N*-acyltransferase (*Glyat*) catalyzes glycine conjugation, a Phase II detoxification process for endogenous chemicals and xenobiotics, such as salicylic

acid, benzoic acid, and methylbenzoic acid (Badenhorst et al., 2012). Before birth, *Bal* was already expressed moderately, and its expression was markedly increased during postnatal development, reaching the highest levels on day 20, and fluctuated thereafter (Fig. 6D). Before birth, hepatic mRNA expression of *Baat* and *Glyat* was very low (Fig. 6E). After birth, these 2 AA conjugation enzymes were increased at different developmental stages: *Glyat* mRNA surged from day 0 to day 3 and increased to adult levels on day 25. In contrast, hepatic *Baat* mRNA remained low until day 10, after which it surged from day 10 to day 20 and maintained at high levels thereafter.

γ -Glutamyltransferase 1 (*Ggt1*) plays a key role in the γ -glutamyl cycle, a pathway for the synthesis and degradation of GSH as well as drug and xenobiotic detoxification. *Ggt1* catalyzes the formation of cysteine conjugates of xenobiotics. Cysteine conjugate- β -lyase 1 (*Ccbl1*) and *Ccbl2* catalyze the metabolism of cysteine conjugates of certain halogenated alkenes and alkanes, which often results in the formation of reactive metabolites leading to nephrotoxicity (Cooper and Pinto, 2006).

Fig. 5. Hepatic ontogeny of *Gst* mRNAs in male C57BL/6J mice. Livers from C57BL/6J mice of ages -2 to 60 days were used for RNA-seq quantification. (A) The y-axis represents mRNAs expressed as FPKM. $n = 3$, mean $\pm$ S.E. (B) Pie charts representing relative mRNA expression of each *Gst* isoform as the percentage of total *Gst* transcripts.

Hepatic mRNA expression of *Ccbl1* fluctuated during the first 20 days of postnatal development, surged 4-fold from day 20 to day 25 (after weaning), and stayed constant thereafter (Fig. 6E). In contrast, hepatic *Ccbl2* mRNA increased 23-fold from 2 days before birth (day -2) to 1 day after birth (day 1) and decreased markedly starting from day 10, reaching day 0 level at adulthood (Fig. 6E).

Hepatic Ontogeny of mRNAs Encoding Enzymes Responsible for Synthesis of Cosubstrates for Phase II Conjugation Reactions in Male C57BL/6J Mice. UDP-glucose pyrophosphorylase 2 (*Ugp2*) catalyzes the synthesis of UDP-glucose, whereas UDP-glucose 6-dehydrogenase (*Ugdh*) converts UDP-glucose to UDP-glucuronate,

the cosubstrate for UDP-glucuronidation. Hepatic *Ugp2* mRNA increased 4-fold from day -2 to day 5 and slightly decreased after weaning (day 25). In contrast, hepatic *Ugdh* mRNA expression did not increase until after weaning and reached the highest level at adulthood (day 60) (Fig. 7). 3'-Phosphoadenosine 5'-phosphosulfate synthase 1 (*Papss1*) and *Papss2* catalyze the synthesis of PAPS, the sulfate donor cosubstrate for all sulfotransferases. Hepatic *Papss1* mRNA remained constantly low throughout development. In contrast, there was a day 1 surge in hepatic *Papss2* mRNA, which peaked on day 15 and decreased slightly thereafter. Glutamate-cysteine ligase catalytic (*Gclc*) and modifier (*Gclm*) subunit are enzymes for the synthesis of GSH, the

cosubstrate for all *Gst* enzymes. Interestingly, hepatic *Gclc* mRNA increased but *Gclm* mRNA decreased during liver maturation (Fig. 7).

There was a marked ontogenic switch in hepatic expression of enzymes for the synthesis of the common methyl donor *S*-adenosylmethionine. Methionine adenosyltransferase 1a (*Mat1a*) expression increased

markedly, whereas *Mat2a* and *Mat2b* mRNAs decreased moderately (48–68%) during postnatal development (Fig. 7).

Tissue Distribution of Phase II Enzymes in Adult Liver, Kidney, and Small Intestine in Male C57BL/6J Mice. The three major tissues of drug processing, namely liver, kidney, and small intestine, differed sharply in the expression of various Phase II enzymes. In liver, *Ugt2b5*, *-2b36*, and *-2b1* were the most abundant *Ugt* enzymes. In kidney, *Ugt3a2*, *-2b38*, and *-3a1* accounted for >75% of total *Ugt* transcripts. In contrast, in small intestine, *Ugt2b34* accounted for >50% of total *Ugt* transcripts, followed by *Ugt2a3* and *-1a7c* (Fig. 8A).

In liver, *Sult1a1* accounted for >65% of total *Sult* transcripts, followed by *Sult1d1* and *Sult5a1*. In kidney, *Sult1d1* accounted for >65% of total *Sult* transcripts, followed by *Sult1c2* and *Sult1a1*, with other *Sult* transcripts expressed at low levels. In small intestine, three *Sult* members, namely *Sult1b1*, *-2b1*, and *-1d1*, accounted for almost all *Sult* transcripts (Fig. 8B).

Fig. 6. (A-E) Hepatic ontogeny of transcripts of *Nat*, methyltransferase, and AA-conjugating enzymes in male C57BL/6J mice. Livers from C57BL/6J mice of ages -2 to 60 days were used for RNA-seq quantification. The y-axis represents mRNAs expressed as FPKM. $n = 3$, mean $\pm$ S.E.

Fig. 7. Hepatic ontogeny of mRNAs of genes encoding enzymes for the synthesis of cosubstrates for Phase II conjugation in male C57BL/6J mice. Livers from C57BL/6J mice of ages -2 to 60 days were used for RNA-seq quantification. The y-axis represents mRNAs expressed as FPKM. $n = 3$, mean $\pm$ S.E.

Compared with *Ugt* and *Sult* enzymes, the expression of *Gst* family members was more diversified among the three tissues (Fig. 9). In liver, *mGst1*, *Gstp1*, *Gstm1*, and *Gsta3* were the most abundant *Gst* transcripts. In kidney, *Gstm1*, *Gsta2*, *mGst3*, and *Gstz1* were the most abundant *Gst* transcripts. In contrast, in small intestine, *Gsta1*, *Gstm3*, *mGst3*, and *Gsto1* were the most highly expressed *Gst* transcripts.

The distribution of *Nat* mRNAs was less tissue-specific compared with other Phase II enzymes. In all three tissues of liver, kidney, and small intestine, *Nat8* was the most abundant, followed by *Nat2* and *Nat1* (unpublished data). *Nat1* was only expressed in liver and kidney, *Nat2* was highest in small intestine, whereas *Nat8* was predominant in kidney (Fig. 10A). Regarding methyltransferases, *Comt* and *Nnmt* were liver-predominant, *Tpmt* was kidney-predominant, whereas *As3mt* expression was less variable among the three tissues (Fig. 10A).

Among the AA conjugation enzymes, *Bal* and *Baat* were highly liver-specific, whereas *Glyat* was only expressed in liver and kidney (Fig. 10B). *Ggt1* was only expressed in kidney and intestine, *Ccbl2*

was liver-predominant, whereas *Ccbl1* was less variable among the three tissues (Fig. 10B).

The tissue distribution of enzymes for the synthesis of cosubstrates was generally less variable compared with Phase II enzymes (Fig. 10C). Tissues with the highest expression were liver for *Ugp2*, *Gclc*, and *Mat1a*; kidney for *Gclm*, *Papss1*, *Mat2a*, and *Mat2b*; and small intestine for *Ugdh* and *Papss2* (Fig. 10C).

Real-Time PCR Validation of RNA-Seq Data. Consistent with RNA-seq data (Figs. 5A and 6), real-time PCR data showed that hepatic mRNA expression of *mGst3* was substantially downregulated during postnatal development, whereas *Nat8* mRNA was barely detectable until day 25, but surged from day 25 to day 60 (Fig. 11).

Discussion

The present study, for the first time, provides comprehensive quantitative analysis of developmental regulation of mRNA expression

A Ugts

B Sults

Fig. 8. Tissue distribution of mRNAs of genes encoding *Ugt* and *Sult* enzymes in adult male C57BL/6J mice. (A) Pie charts representing relative mRNA expression of each *Ugt* isoform as the percentage of total *Ugt* transcripts in liver, kidney, and small intestine, respectively, from C57BL/6J mice 60 days old. $n = 2$, mean. (B) Pie charts representing relative mRNA expression of each *Sult* isoform as the percentage of total *Sult* transcripts in liver, kidney, and small intestine, respectively, from C57BL/6J mice 60 days old. $n = 2$, mean.

Fig. 9. Tissue distribution of mRNAs of genes encoding *Gst* enzymes in adult male C57BL/6J mice. Pie charts representing relative mRNA expression of each *Gst* isoform as the percentage of total *Gst* transcripts in liver, kidney, and small intestine, respectively, from C57BL/6J mice 60 days old. $n = 2$, mean.

of all major Phase II enzymes, including enzymes involved in glucuronidation, sulfation, GSH conjugation, acetylation, methylation, and AA conjugation in mouse liver. Many of these Phase II enzymes display marked ontogenic changes in mRNA expression in liver, with a majority of them induced during postnatal liver development. In adulthood, these Phase II enzymes have distinct expression levels in

Fig. 10. Tissue distribution of mRNAs of genes encoding other Phase II enzymes and enzymes responsible for the synthesis of cosubstrates for Phase II conjugation reactions in adult male C57BL/6J mice. (A and B) Stacked column charts representing relative mRNA expression of each Phase II enzyme as the percentage of total transcripts of this enzyme in liver, kidney, and small intestine from C57BL/6J mice 60 days old. $n = 2$, mean. (C) Stacked column chart representing relative mRNA expression of each cosubstrate-synthesizing enzyme as the percentage of total transcripts of this enzyme in liver, kidney, and small intestine from C57BL/6J mice 60 days old. $n = 2$, mean.

the three major tissues of drug processing, namely liver, kidney, and small intestine.

The present data on hepatic ontogeny and tissue distribution of *Sult* enzymes are consistent with a previous report (Alnouti and Klaassen, 2006). Nevertheless, RNA-seq data illustrate the relative abundance of various *Sult* family members during liver development and among adult tissues, which were not known previously. It is noteworthy that although *Sult2a* isoforms are largely silenced in adult male mouse livers, they remain actively transcribed in adult female mouse livers (Alnouti and Klaassen, 2006). Additionally, although *Sult3a1* is not expressed in adult male mouse livers, it is expressed appreciably in livers of adult female mice (Alnouti and Klaassen, 2006).

The present data on hepatic ontogeny and tissue distribution of *Gst* enzymes are largely consistent with previous reports (Knight et al., 2007; Cui et al., 2010), with the exception of *mGst3*. In the present

Fig. 11. Real-time PCR validation of RNA-seq data on ontogenic mRNA expression of *mGst3* and *Nat8* in livers of male C57BL/6J mice. Total RNAs from livers of male C57BL/6J mice of ages -2, 1, 5, 10, 20, 25, 45, and 60 days were used for real-time PCR validation. $n = 3$, mean $\pm$ S.E. The y-axis represents mRNAs calculated by the comparative computed tomography method, which determines the amount of target normalized to the geometric mean of *Gapdh* and *Ncu-g1*. * $P < 0.05$ vs. day -2.

study, results from both RNA-seq and real-time PCR showed that *mGst3* was the most abundant *Gst* in mouse liver before birth, and *mGst3* was substantially downregulated during postnatal liver development (Figs. 5A and 11). In contrast, a previous study showed that hepatic *mGst3* mRNA increased moderately (~ 1 -fold) from day -2 to day 0 and remained unchanged thereafter (Cui et al., 2010). The reason for such apparent difference in hepatic ontogeny of *mGst3* in mice remains unclear, but appears unlikely due to strain difference, because C57BL/6 mice were used in these two studies. In humans, *mGST3* mRNA expression is low in adult liver, but appears to be high in fetal liver (Jakobsson et al., 1997). Thus, apparently, both humans and mice have ontogenic downregulation of *mGST3* during postnatal liver development. *mGst3* has been implicated in the synthesis of leukotriene C4 and possesses GSH-dependent peroxidase activity (Jakobsson et al., 1997).

The present study illustrates the first ontogenic patterns of mRNA expression of *Ugt* enzymes in mouse liver. Physiologic functions change dramatically from fetal liver to adult liver. Adult liver is the major organ to metabolize AAs and lipids, to maintain gluconeogenesis, to synthesize serum proteins, and to detoxify metabolic wastes and xenobiotics. In contrast, fetal liver has fewer metabolic functions and is a major organ for hematopoiesis. The perinatal surge of *Ugt1a1* is consistent with the key role of *Ugt1a1* in glucuronidation of bilirubin and prevention of hyperbilirubinemia (Kadakol et al., 2000). In contrast, the rapid increase in hepatic mRNA expression of many *Ugt2b* family members during weaning (day 20 to day 30) (Fig. 3) is consistent with their important role in the glucuronidation of steroid hormones and xenobiotics (Bock, 2010). *Ugp2* catalyzes the synthesis of UDP-glucose, whereas *Ugdh* converts UDP-glucose to UDP-glucuronate, the cosubstrate for UDP-glucuronidation. UDP-glucose also serves as the precursor for glycogen synthesis. The increased need for glucuronidation of endogenous chemicals and the postnatal depletion

of hepatic glycogen might be the driving force of the adolescent-predominant expression pattern of *Ugp2* (Fig. 7), whose expression decreases moderately after the replenishment of glycogen storage in adolescent liver (Lopez et al., 1999). In contrast, the delayed surge in hepatic expression of *Ugdh* right after weaning (Fig. 7) coincides with marked increases in ingestion of xenobiotics and glucuronidation of these xenobiotics after weaning.

The present study, for the first time, illustrates ontogenic patterns of mRNA expression of Phase II enzymes for acetylation and methylation in mouse liver. Hepatic mRNA expression of *Nat* enzymes involved in xenobiotic metabolism, such as *Nat1*, *Nat2*, and *Nat8*, is increased with liver maturation (Fig. 6). *Comt* is important in the metabolism of hormones (e.g., catecholamines and estrogens) and a large number of dietary phenolic compounds (Zhu, 2002). The two surges in *Comt* expression during perinatal (day 1 to day 3) and postweaning (day 20 to day 25) might correspond to the markedly increased need for the metabolism of hormones and dietary phenolic compounds, respectively, during postnatal development. *Nnmt* catalyzes the *N*-methylation of nicotinamide, pyridines, and other structural analogs; its expression increases substantially with liver maturation. Interestingly, hepatic *NNMT* is markedly downregulated in liver cancer patients (Kim et al., 2009). The regulatory mechanism of *Nnmt* expression during liver development and carcinogenesis warrants further investigation.

The present data suggest that neonatal mouse livers might differ considerably from adult livers regarding the metabolism of GSH conjugates. GSH conjugation is initiated by transfer of GSH onto an electrophilic acceptor (such as the anticancer drug cisplatin and toxicants ethylene dibromide and acrylamide), followed either by direct excretion of the GSH conjugates from the hepatocytes into bile, or by the hydrolysis of glutamate by *Ggt* to form cysteinyl-glycine-*S*-conjugates. Certain dipeptidases present in kidney and liver can hydrolyze the cysteinyl-glycine-*S*-conjugates to cysteine *S*-conjugates. *Nat8* catalyzes the acetylation of the cysteine *S*-conjugates in liver or kidney, leading to the formation of mercapturic acids, which are more polar and water-soluble than the original electrophiles and are readily excreted in urine or bile by multidrug resistance proteins. In the absence of acetylation, cysteinyl conjugates are unstable, undergoing β -elimination catalyzed by *Ccbl*, with formation of toxic sulfur derivatives (Monks et al., 1990). Compared with adult mouse livers, the newborn mouse livers have much higher expression of *Ggt1* and *Ccbl2* (Fig. 7) but very low expression of *Nat8* (Fig. 6), which might result in a higher formation of toxic metabolites of certain GSH conjugates in newborns via the *Ggt-Ccbl2* β -elimination pathway.

RNA-seq provides the first genome-wide approach of true quantification of transcripts, which allows quantitative comparison of the abundance of various members within each gene family in various tissues. Such knowledge may be particularly useful for the study of the physiologic function of a given Phase II enzyme when using mice with targeted deletion of this gene. For example, *Sult1a1*, a major *Sult* with broad substrate specificity (Hempel et al., 2007), accounts for $>60\%$ of the total *Sult* transcripts in mouse liver, but only accounts for a very small percentage of total *Sult* transcripts in kidney and small intestine. Therefore, loss of *Sult1a1* is expected to markedly affect the sulfation of certain chemicals in liver, but may have minimal effect on the sulfation of these chemicals in kidney and small intestine. In contrast, loss of *Sult1d1* (a key enzyme for catecholamine sulfation) (Shimada et al., 2004) and *Ugt2b34* may markedly affect the sulfation and glucuronidation of chemicals in kidney and small intestine, respectively, because *Sult1d1* accounts for $>70\%$ of total *Sult* transcripts in kidney, whereas *Ugt2b34* accounts for $>50\%$ of total *Ugt* transcripts in small intestine.

It is noteworthy that only the mRNA levels of these Phase II enzymes were investigated in this study. More studies are needed to determine whether the marked differences in mRNA expression of these Phase II enzymes during different stages of liver development and among the three adult tissues of liver, kidney, and small intestine translate into differences in their protein expression and enzymatic function. Two recent genome-scale studies indicate that in mammalian cells, mRNA levels explain ~40% of the variation in protein levels (Schwanhauser et al., 2011); however, transcript levels correlate more strongly with clinical traits than protein levels (Ghazalpour et al., 2011). It is noteworthy that the ADME of xenobiotics depends on the cellular milieu composed of various transporters and metabolic enzymes coordinately regulated by a group of transcription factors (Klaassen and Slitt, 2005). Transcript levels of ADME genes might better reflect the cellular milieu for the ADME of xenobiotics under certain pathophysiological conditions. Bisphenol A (BPA) is an important industrial and environmental chemical. Phase II conjugation reaction plays the key role in limiting the body's exposure to the parent unconjugated BPA, which is a well characterized endocrine disruptor (Taylor et al., 2011). Results from a comparative study on pharmacokinetics of BPA in neonatal and adult CD-1 mice demonstrate that neonatal mice (on day 3 and day 10) have much higher blood levels of unconjugated BPA than adult mice after oral ingestion. In contrast, postweaning (day 21) mice have blood levels of unconjugated BPA comparable to those in adult mice (Doerge et al., 2011). Glucuronidation is the major metabolic pathway for BPA in mice (Zalko et al., 2003). In parallel, the present data demonstrate that most *Ugt* enzymes are expressed at low levels in neonatal mice (day 3 and day 10) but at high, adult-comparable levels in postweaning mice (day 25). Thus, it appears that the marked differences in mRNA expression of these *Ugt* enzymes during different stages of liver development translate into differences in their protein expression and enzymatic function. Technological breakthroughs in proteomics and metabolomics are essential to conduct genome-scale comparative study to identify the sets of genes whose ontogenic expression are mainly regulated at transcriptional and posttranscriptional levels, respectively.

Although the mechanism of the ontogenic changes in hepatic expression of Phase II enzymes is still unknown, literature suggests that growth hormone (GH) and androgens play key roles in the regulation of gender- and age-specific gene expression (Waxman and Holloway, 2009; Baik et al., 2011). The female- and male-predominant expression of *Ugt1a1* and *Ugt2b1*, respectively, in mouse liver is due to suppression of *Ugt1a1* mRNA but induction of *Ugt2b1* mRNA by male-pattern GH secretion (Buckley and Klaassen, 2009). Thus, the decrease in hepatic expression of *Ugt1a1* but induction of *Ugt2b1* in male mice upon puberty (after day 25) (Fig. 3A) is likely due to the establishment of male-pattern GH secretion upon puberty. *Sult2a1/a2* has a markedly female-predominant expression in adult mouse liver due to suppressive effects of androgens and male-pattern GH secretion, as well as stimulatory effects by estrogens and female-pattern GH secretion (Alnouti and Klaassen, 2011). Thus, the marked hepatic downregulation of *Sult2a1/a2* and other *Sult2a* enzymes in male mice upon puberty (after day 25) (Fig. 4A) may be due to the increase of androgens and establishment of male-pattern GH secretion upon puberty. Similarly, *Sult1a1* and *Sult1d1* have moderately female-predominant expression in adult mouse liver (Alnouti and Klaassen, 2006) due to suppressive effects of androgens and male-pattern GH secretion (Alnouti and Klaassen, 2011). Therefore, the moderate hepatic downregulation of *Sult1a1* and *Sult1d1* in male mice upon puberty (after day 25) (Fig. 4A) may be due to the increase of androgens and establishment of male-pattern GH secretion upon puberty.

In summary, the present study provides the first knowledge about the true quantification of ontogenic patterns of mRNA expression of all major known Phase II enzymes during mouse liver development and the distribution of these mRNAs among the three key tissues of ADME, namely liver, kidney, and small intestine, in adulthood. Such knowledge will serve as the foundation for further study on the regulation of gene expression and physiologic function of these Phase II enzymes during liver development, as well as the mechanism of tissue-specific gene expression and the physiologic importance of these Phase II enzymes in these three key tissues of ADME.

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Authorship Contributions

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