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Developmental Regulation of Phase-I Drug Metabolizing Genes in Mouse Liver

Lai Peng
lai.peng@uconn.edu

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Lai Peng, PhD

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Liver is a critical organ for drug metabolism and is the primary site for the expression of Phase-I drug metabolizing enzymes, which mainly catalyze the oxidation, reduction, and hydrolysis of xenobiotics. However, in the fetus, the major function of liver is hematopoiesis. From infants to adults, dramatic changes occur in liver functions and the Phase-I gene expression, which can markedly affect the metabolism of drugs in newborns and children. Characterization of the expression profiles and regulatory mechanisms of liver development and Phase-I genes is needed for more rational drug therapy of pediatric patients. An animal model is indispensable for studying the mechanisms of postnatal development of Phase-I genes.

In this dissertation, we systemically characterized the model of mouse liver during postnatal development and examined the ontogenic expression of all major Phase-I drug metabolizing genes by RNA-sequencing. Gene ontology analysis demonstrated that cell proliferation and immune activation pathways were active in liver at neonatal ages and different metabolic processes were matured at different stages during development, with the age of day 25 as the approximate time of maturation for the liver transcriptome. Significant functional transitions in liver accompanied the ontogeny of Phase-I genes. Four ontogenic expression patterns were identified among the 71 significantly expressed cytochrome P450s, which categorized genes into neonatal-,

adolescent-, adolescent/adult-, and adult-enriched groups. Diverse expression patterns were also elucidated in another 11 families of Phase-I genes, which provided a valuable foundation for further mechanistic studies.

Long non-coding RNAs (lncRNAs) and farnesoid X receptor (FXR) mediated regulation of gene expression were explored and addressed. The developmental expression patterns of lncRNAs were initially characterized, which strongly suggested the involvement of lncRNAs in the control of liver ontogeny. Comparisons of liver transcriptome between wild-type and age-matched FXR-null mice revealed a critical role of FXR in promoting proper liver maturation and in control of Phase-I gene expressions during development.

In conclusion, the present dissertation has built up a valuable platform for the study of liver developmental gene expression and regulation. Our results also provide novel insights into identifying and further understanding the molecular targets for safe and efficacious drug treatments in children.

Developmental Regulation of Phase-I Drug Metabolizing Genes in Mouse Liver

Lai Peng

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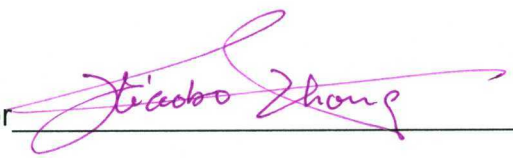
Doctor of Philosophy Dissertation

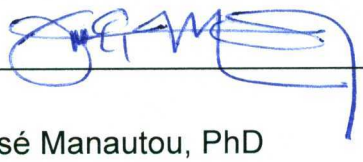
Developmental Regulation of Phase-I Drug Metabolizing Genes in Mouse Liver

Presented by

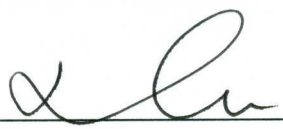
Lai Peng, B.S.

Major Advisor 

Xiao-bo Zhong, PhD

Associate Advisor 

José Manautou, PhD

Associate Advisor 

Xiuling Lu, PhD

University of Connecticut

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List of Abbreviations

Abbreviation	Full name
3' RACE	rapid amplification of cDNA 3' end
ADH or Adh	alcohol dehydrogenase
ADME	Absorption, distribution, metabolism and excretion
AhR	aryl hydrocarbon receptor
AKR or Akr	aldo-keto reductase
ALDH or Aldh	aldehyde dehydrogenase
AOX or Aox	aldehyde oxidase
apo	apolipoprotein
bp	base pairs
CAR	constitutive androstane receptor
CES or Ces	carboxylesterase
ChIP	chromatin immunoprecipitation
ChIP-Seq	chromatin immunoprecipitation coupled with sequencing
DPYD or Dpyd	dihydropyrimidine dehydrogenase
EPHX or Ephx	epoxide hydrolase
FDR-BH	Benjamini-Hochberg-adjusted false discovery rate
FMO or Fmo	flavin monooxygenase
FPKM	fragments per kilobase of exon per million reads mapped
FXR	farnesoid X receptor
GH	growth hormone
GO	gene ontology
IR	inverted repeat
lincRNA	long intergenic non-coding RNA
lncRNA	long non-coding RNA
LXR	liver X receptor
miRNA	micro RNA
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NC	non-coding
NCBI	National Center for Biotechnology Information
NcoR1	nuclear receptor co-repressor 1
ncRNA	non-coding RNA
NQO or Nqo	quinone oxidoreductase
Nrf2	nuclear factor E2 p45-related factor 2
P450	cytochrome P450
PC	protein-coding
PCR	polymerase chain reaction
PON or Pon	paraoxonase
POR or Por	NADPH-cytochrome P450 oxidoreductase

PPARα	peroxisome proliferator-activated receptor alpha
PXR	pregnane X receptor
RNA-Seq	RNA sequencing
rRNA	ribosomal RNA
SDR	short-chain dehydrogenases/ reductases
SHP	small heterodimer partner
Smrt	silencing mediator of retinoic acid and thyroid hormone receptors
UTR	untranslated region
WT	wild-type
XDH or Xdh	xanthine oxidoreductase

Chapter 1 : Introduction

1.1 Developmental Pharmacology

Drug treatment is aimed to obtain therapeutic effect and alleviate diseases, preferably with minimal side effects. The rational use of drugs requires the identification of sources of variability in drug response, quantifying the impact of the variability and modifying the drug dose to compensate the influence. Human heterogeneity that leads to altered drug response and toxicity profile can be physically-endowed (e.g., age, gender, genotype), physically-acquired (e.g., pregnancy, disease), behaviorally-linked (e.g., smoking, diet), and environmentally-induced (e.g., chemical exposure) (Rogers, 1994). One of the physically-endowed factors is ontogeny, which is known as all the developmental events that occur during the existence of a living organism, usually from the time of fertilization of the egg to the organism's mature form. Ontogeny is closely related to studies of embryology and developmental biology, and the terms are sometimes used interchangeably. While all the sources of variability have the potential to influence drug response in various ways, the effect of age or ontogeny is remarkable, and will be the focus of this thesis.

Clinical Problems

Infants and children are far different from adults in medical perspectives. Dr. Abraham Jacobi, often referred to as the father of American Pediatrics, wrote more than 100 years ago, “Pediatrics does not deal with miniature men and women, with reduced doses and the same class of disease in smaller bodies, but… has its own independent range and horizon.” (Kearns et al., 2003) In neonatal life, there are important age-dependent changes in body composition, in weight/size and in the maturation of hepatic and renal functions. With the accumulation of

knowledge on normal growth and development, we are getting increasingly aware that developmental changes significantly affect the responses to medications and an age-appropriate pharmacotherapy is needed.

However, frequently prescribed drugs for children, such as medications to treat asthma, seizures, psychiatric disorders, gastrointestinal problems and sedatives, are largely not labeled for pediatric use. Most of them contain pediatric disclaimers, like “no controlled studies have been conducted in pediatric patients” or “Safety and effectiveness in children have not been established” (Cote et al., 1996). Current practice medicine on pediatric patients is fully relied on physician’s experience without a solid support of scientific knowledge. When physicians prescribe a medicine to pediatric patients, in many cases, they use a reduced dose from FDA’s guidance on adult patients. However, how much they should reduce is completely relied on their experience. Before they make a prescription, they may not know whether patients have appropriate enzymes in their liver to metabolize the drug, because the expression levels of drug metabolizing enzymes in pediatric livers, particularly in neonatal livers, is unclear and what mechanisms control the gene expression is also unknown. Lack of the basic scientific knowledge is one of the major reasons that pediatric patients have a much greater risk to facing challenges on adverse drug reactions than adult patients. There is an urgent need to provide such basic knowledge to physicians. The problem of “unapproved” use of “approved” drugs in pediatrics has left children as therapeutic orphans. The importance of generating information on the developmental metabolic, pharmacokinetic and pharmacodynamic data needed for safe and effective drug use in children cannot be overemphasized.

Variations in drug responses during development

Drugs undergo five stages following administration: 1) absorption into the body, e.g. from the intestine; 2) distribution to their site of action in the body; 3) target interaction, e.g. binding to cellular receptors or ion channels; 4) metabolic processing; 5) excretion from the body.

Theoretically, factors that influence any stage of the pathway can affect the overall drug response. Variations in drug responses can be classified into two major groups. The first is those related to the pharmacokinetic properties of drugs, such as drug metabolizing enzymes and drug transporters, which affect how the drug is handled by the body. The second is those influencing pharmacodynamic properties of drugs, including drug targets such as enzymes, receptors and ion channels, and their associated pathways, which determine the drug's effect on the body.

Pharmacodynamics

Little information exists about the human ontogeny on the interaction between a drug and its targets, the biochemical and physiological effect of a drug on the body, i.e., the pharmacodynamics. For example, the apparent developmental differences in the pharmacodynamics of morphine in neonates are directly associated with the reduced formation of its glucuronide metabolite at early ages (de Wildt et al., 1999b). However, data on certain drugs appear to support true age-dependent differences in pharmacodynamics. Assays in peripheral blood monocytes cultures from subjects of different ages have demonstrated that cyclosporine has much lower IC50 and IC90 in infants than in older subjects (Marshall and Kearns, 1999). In addition, vitamin K-dependent proteins (protein C and prothrombin fragments 1+2) showed significantly lower plasma concentrations in prepubertal patients than in adults that treated with warfarin and exhibited similar pharmacokinetic parameters (Takahashi et al., 2000). Clearly, age-dependent differences in pharmacodynamics have implications and need to be considered in pediatric pharmacotherapy.

Absorption, Distribution and Renal Elimination

When a drug is administered by non-intravenous route, the process of absorption happens as the compound overcomes chemical, physical, mechanical and biological barriers before being distributed in the body. The oral route is the principal route for drug administration in

children. The developmental differences in the physiological composition and function of the stomach, intestine and biliary tract can alter the rate or extent of drug absorption (Allegaert et al., 2008). Generally, the prolonged emptying and reduced intestinal motility slow down the rate of drug absorption and potentially delay the onset of drug action in neonates and young infants (van den Anker, 2010).

Once a drug enters the systemic circulation, it must be distributed into interstitial and intracellular fluids. Age-dependent changes in body composition alter the physiological spaces into which a drug will distribute. The proportion of body weight assigned to fat, protein, intra- and extra-cellular water changes most prominently in early life (Rakhmanina and van den Anker, 2006). The volume of distribution at steady state for intravenous bolus administration of propofol is significantly lower in neonates, compared to older children (Allegaert et al., 2007).

Although most drugs are distributed through simple passive diffusion along concentration gradients, the expression of transporters capable of producing a biological barrier also contributes. P-glycoprotein is an example of efflux transporters capable of limiting cellular uptake of selected xenobiotics, which is expressed at different tissue sites (e.g., the blood-brain barrier, hepatocytes, enterocytes and tumors). Studies in animals demonstrated developmentally regulated P-glycoprotein expression which may affect drug distribution (Mahmood et al., 2001; Rosati et al., 2003), but this has not been systematically studied in humans.

For drugs that undergo extensive renal elimination, it is necessary to take into consideration the developmental changes in renal function when selecting an age-appropriate dose regimen. Both the glomerular filtration rate and tubular secretion increases rapidly during the first two weeks of life and then rises steadily until adult values are reached at 6 to 12 months of age (Kearns et al., 2003). Treatment of preterm neonates with gentamicin may need to increase the dosage interval from 12 hours to 18 hours to prevent accumulation of drugs to potentially toxic

serum concentrations (Mulhall et al., 1983). Prenatal exposure to certain medications, such as betamethasone and indomethacin, may alter the normal pattern of renal maturation in neonates (van den Anker et al., 1994). Thus for drugs primarily eliminated by kidney, treatment regimens need to reflect both development and treatment-associated maturational changes in kidney function.

Drug Metabolism

Drug metabolism is the process of biotransformation, generally converting lipophilic chemicals, which are readily absorbed from gastrointestinal tract and other sites, into hydrophilic chemicals, which are readily excreted in urine or bile. While, in most cases, metabolism reduces the ability of a drug to produce a pharmacological action, it can also generate metabolites that have more significant pharmacological or toxic effect, for instance, in the case of prodrugs. Metabolism is the most critical step in ADME process of a drug, because it is the only step that alters the chemical property of the drug.

The biotransformation is catalyzed by various drug metabolizing enzymes which are mainly expressed in the liver. The functional maturation of liver during postnatal development affects the expression of drug metabolizing enzymes, and profound changes of drug metabolizing enzyme activities during development impact drug efficacy and the risk of adverse events in neonates and young children. Numerous children treated with the antibiotic chloramphenicol suffered from “gray baby syndrome”. The symptoms include vomiting, grey color of the skin and cardiovascular collapse. This is due to the immature level of UDP glucuronosyltransferse in infants to metabolize the drug, resulting in the drug or metabolites accumulation to toxic levels. The use of benzyl alcohol as a preservative in intravenous fluids has caused gasping syndrome or even death in newborns, especially premature infants. And this is because of the immature level of glycine N-acetyltransferase in infants (Hines, 2013). The drug cisapride used to control gastric reflux in neonates can lead to drug-induced long QT syndrome. Cisapride is mainly

metabolized and inactivated by CYP3A4. The low activity of CYP3A4 during the perinatal period results in toxic levels of the drug, which was later withdrawn from the market. These examples of tragic adverse events signal the importance of research on developmental expression of drug metabolizing enzymes.

A recent review has summarized the patterns of human drug metabolizing enzyme ontogeny into three classes based on both *in vitro* studies using human liver tissue banks and *in vivo* studies in pediatric patients undergoing drug therapy (Hines, 2013). Class 1 enzymes are highly expressed in the fetus and within a few days to as long as two years after birth, their expression is either silenced or reduced to very low levels, examples of which are FMO1 and CYP3A7. Enzymes belonging to the second class are expressed at relatively constant levels throughout development, and the level may or may not increase modestly within the first year after birth. CYP2B6, CYP2C19 and SULT1A1 all exhibit this developmental pattern. Class 3 enzymes are the largest group. They are expressed at low levels in the fetus, and significant increases in expression to mature levels occur within a few weeks to one or two years after birth. In this group, CYP2D6 is an example of early maturation (a few weeks after birth), and CYP3A4 matures a few years after birth, whereas FMO3 and CYP2C9 adult expressions are not observed until post-puberty. The evaluation and classification of drug metabolizing enzymes into three ontogeny classes may be utilized to suggest enzyme functions and regulation. The enzymes within the same class may share functional significance in specific time windows during development. Some common regulatory mechanisms are also likely among enzymes belonging to the same class.

Examples of pharmacokinetic studies provide insight into the ontogeny of drug metabolism by specific Phase-I enzymes. The plasma clearance of intravenously administered midazolam is primarily a function of hepatic CYP3A4 and CYP3A5 activity, and the activity level increases from 1.2 to 9 ml/min/kg during the first three months of life (de Wildt et al., 1999a). The

clearance of carbamazepine from plasma, which depends largely on CYP3A4, is greater in children (aged 3-10 years) than in adults (Riva et al., 1985), which requires higher weight-adjusted doses of the drug to achieve therapeutic levels. The apparent half-life of phenytoin, primarily metabolized by CYP2C9, is prolonged to approximately 75 hours in preterm infants, but decreases to about 20 hours in term infants and to approximately 8 hours after the second week of life (Loughnan et al., 1977). Furthermore, the CYP1A2 catalyzed caffeine demethylation in adolescent girls appears to decline to levels as in adults when girls reach Tanner stage 2, whereas this occurs at Tanner stage 4 or 5 in adolescent boys, thus demonstrating a gender-based difference in CYP1A2 ontogeny (Kearns et al., 2003).

Phase-II drug metabolizing enzymes are involved in synthetic reactions including glucuronidation, sulfation, methylation or acetylation. These conjugation pathways seem to display much less isoform-specific ontogeny in early life than Phase-I enzymes (Allegaert et al., 2008). Glucuronidation is of critical roles in drug clearance, as it dramatically increases the water solubility of drugs for subsequent elimination through urine or feces. The metabolite of tramadol undergoes glucuronidation, and the ratio of glucuronidated tramadol metabolite to total tramadol metabolite was significantly lower in early compared to late neonatal life in a study conducted by Allegaert *et al*. (22% versus 33% respectively, $p<0.0001$). Similar lower conjugation level for paracetamol in urine samples of neonates was also observed by these researchers. It is also worth mentioning that drug metabolizing enzymes with substantial extra-hepatic expression (e.g., CYP3A4, CYP3A5, and isoforms of UGT) may alter the predicted pharmacokinetics based on the liver expression levels.

Previous studies have identified windows of hyper-variability during human drug metabolizing enzyme ontogeny (Hines, 2013). Constitutional, environmental, genetic factors, age and comorbidity can all have an impact on drug response during development. It is therefore critical to understand not only the ontogeny but also the regulatory mechanisms of

developmental changes in order to provide individualized drug therapy in pediatrics that is safe and effective. Hence, this thesis utilizes mouse as a model to study liver ontogeny and tries to elucidate the critical factors involved in developmental regulation, with a focus on Phase-I drug metabolizing enzymes.

1.2 The Study of Phase-I Drug Metabolizing Enzymes

Drug-metabolizing enzymes play a central role in the elimination of drugs in the body (Nishimura and Naito, 2006). The metabolic reactions of hydrolysis, reduction, and oxidation usually expose or introduce a functional group to a drug that may be further converted to a water-soluble conjugate. The reactions of hydrolysis, reduction, and oxidation are often called Phase-I reactions, and the conjugation reactions are often referred to as Phase-II reactions. The enzymes involved in hydrolysis, reduction, and oxidation of drugs are classified as Phase-I metabolizing enzymes (Parkinson and Ogilvie, 2008). The major Phase-I enzymes to be studied in this thesis include hydrolysis enzymes of carboxylesterases (CES), Paraoxonases (PON), and epoxide hydrolases (EPHX); reduction enzymes of aldo-keto reductases (AKR), quinone oxidoreductase (NQO), and dihydropyrimidine dehydrogenase (DPYD); and oxidation enzymes of alcohol dehydrogenases (ADH), aldehyde dehydrogenases (ALDH), aldehyde oxidases (AOX), flavin monooxygenases (FMO), cytochrome P450s (P450), NADPH-cytochrome P450 oxidoreductase (POR), and xanthine oxidoreductase (XDH).

Animal Model

Multiple factors contribute to the lack of appropriate pediatric studies. Children have a relatively small market share, and adequate research funding from government, industry, and health care providers is often lacking. There may be potential long-term adverse effects in pediatric clinical trials, which can lead to fear of legal liability. Also, parents are usually reluctant

to allow their children to be research subjects. These ethical, financial and logistical considerations limit the clinical drug studies in children. On the other hand, pursuing studies of drug safety and efficacy in developing animals has several theoretical advantages. Animal developmental study may point out age-specific risks or toxicities not apparent from adult animal or human studies, and help guide initial dosing range for pediatric clinical trials. The shorter life spans and generation time of laboratory animals allow more rapid detection of potential long-term consequences of early life drug exposure. More importantly, they permit the study of mechanisms by invasive physiologic, histologic and biochemical measurements that are not achievable in human studies in infants and children. Powerful genetic tools have been developed in animal models, like mice, which help examine the effects of specific genes on the resulting phenotype (Berde and Cairns, 2000).

We will use an animal model, mouse instead of human samples to study the developmental regulation of Phase-I genes in this thesis. Several limitations exist in studies of the developmental regulation of drug metabolizing enzymes with human samples. The first limitation is the ethical and technical issues in recruiting human subjects and obtaining suitable human samples of different ages during development. Secondly, variations in human metabolic capacity, which may be caused by genetic or environmental factors, can interfere with studies aimed to reveal the regulatory mechanisms that are only due to age. Furthermore, mechanistic loss-of-function or gain-of-function strategies are not applicable directly in human samples. Animal models are advantageous in overcoming these limitations.

While not all mouse Phase-I genes have been shown to metabolize specific substrate, more than half of the P450 isoforms exhibit sequence homology to human isoforms (Moscovitz and Aleksunes, 2013). Several laboratories, including us, have examined the expression of some Phase-I genes at early ages in mouse or rat liver, including P450s (Hart et al., 2009; Cui et al., 2012c), *Ces* (Zhu et al., 2009), *Akr* (Pratt-Hyatt et al., 2013), *Adh* and *Aldh* (Smolen et al.,

1990; Alnouti and Klaassen, 2008), *Pon* (Li et al., 1997), and *Fmo* (Falls et al., 1995; Cherrington et al., 1998; Janmohamed et al., 2004). Developmental expression patterns of some examined Phase-I genes in mice and rats are similar to those in humans.

The enzyme activities in animal models might not be exactly the same as those in humans, but the molecular events in regulating these developmental changes can be quite indicative as the mechanisms of basic biology are highly conserved among species, and the molecular mechanisms are what we focus on. For example, in the event of nuclear receptors regulating P450 gene expression, it is generally considered that AhR is responsible for the mRNA induction of family 1, CAR for family 2, PXR for family 3, and PPARα for the mRNA induction of family 4 of P450s, and this principle is consistent between human and mouse, indicating strong homology in regulatory mechanism of gene expression.

Novel technology: RNA-Sequencing

Previous studies quantified Phase-I gene expression on the mRNA level by either Northern blot, RT-PCR, microarrays, or multiplex suspension bead arrays (Hart et al., 2009; Li et al., 2009a), which only provide relative quantification of a given gene. These technologies detect mRNA levels by probe hybridization and fluorescence signal intensity, which cannot compare expression levels among various Phase-I genes, because different probes may have different hybridization efficiency. With the development of next-generation sequencing technologies, such as RNA sequencing (RNA-Seq), it is possible to define a whole transcriptome with low background noise, no upper limit for quantification, and a high degree of reproducibility for both technical and biological replicates (Mortazavi et al., 2008; Nagalakshmi et al., 2008). More importantly, RNA-Seq quantifies the true abundance of mRNA molecules in biological samples and enables comparison of the expression of all genes (Malone and Oliver, 2011). Furthermore, RNA-Seq has the power to quantify expression levels of alternative transcripts of the same gene

and to identify novel transcripts efficiently (Pan et al., 2008; Wang et al., 2008; Malone and Oliver, 2011).

In this thesis, taking advantage of the powerful RNA-Seq technique, we will discuss the whole liver developmental transcriptome in Chapter 2, which is the physiological base for the maturation of Phase-I drug metabolizing enzymes. In Chapter 3, we will examine and compare all of the P450 isoforms and their alternative transcripts during liver development, which is the most important family of Phase-I genes. In Chapter 4, we will investigate the ontogeny of several other major families of Phase-I genes involved in hydrolysis, reduction, and oxidation of xenobiotics in liver. In Chapter 5, we will take a further step to explore the regulation of Phase-I genes during liver maturation.

1.3 Developmental Regulation and Farnesoid X Receptor

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product, involving steps of transcription, RNA processing, translation, etc. In most case, the product of a gene is a protein with structural or catalytic functions, whereas non-protein coding genes can generate functional RNAs, such as ribosomal RNA (rRNA), micro RNA (miRNA), and long non-coding RNA (lncRNA).

The genetic information is stored in the DNA sequence of a gene, which is wrapped around histone proteins to form the higher order structure of DNA, the nucleosome and chromosome. The transcription of DNA to RNA is initiated by the transcription machinery containing a group of transcription factors and the RNA polymerase. Nuclear receptors, as an important class of transcription factors, can sense molecular signals in the cell and directly bind to DNA to regulate gene expression. The non-sequence changes in DNA structure, also known as epigenetics, can influence transcription. For example, the DNA methylation at CpG islands and post-translational

modifications to histones may alter the chromatin conformation and interact with transcription machinery to regulate transcriptional activity.

The transcription produces a primary RNA transcript that is complementary to the DNA sequence. The primary mRNA has to undergo a series of modifications to become a mature mRNA. These include 5' capping, which adds a 7-methylguanosine to the 5' end of the transcript and have the function to protect the RNA from degradation and aid in the mRNA transport to cytoplasm. Another modification is 3' cleavage and polyadenylation. When the RNA is transcribed close to the end, there is usually a polyadenylation signal in the sequence, which initiates the binding of polyadenylation factors and allows cleavage at the 3' end of the transcript and addition of a poly-A tail containing ~200 adenines. The tail also protects the RNA from degradation and may interact with other proteins to facilitate mRNA export and translation. A very important modification of the eukaryotic pre-mRNA is splicing, which removes intron sequences and keeps the coding exons connected together for translation. In certain cases, some intron sequences can be retained or exons removed in the mature mRNA, a process called alternative splicing that creates different transcripts from a single gene. Occasionally, a process call RNA editing occurs that can make discrete changes to specific nucleotide sequences within an RNA, including insertion, deletion, or nucleotide base substitution. The processing of lncRNAs is generally the same as mRNA, while the processing of small ncRNAs can be quite different. For examples, miRNAs are first transcribed with a cap and poly-A tail, and then cleaved by Drosha in the nucleus and Dicer in the cytoplasm to generate mature miRNAs.

Mature mRNAs must be transported from the nucleus to the cytoplasm where they bind to ribosomes and are translated into amino acid sequences. The polypeptides may then undergo conformational changes and translocations, becoming the functional molecules at their targeted site. Post-translational modifications, such as phosphorylation and ubiquitination, may either

increase the activity of a protein or mark the protein for degradation. A schematic summary of the gene expression and regulation process is illustrated in Fig. 1.1.

Regulation of Phase-I gene expression

The changes in Phase-I gene expression and enzyme activity can affect individual responses to drugs, both in terms of therapeutic effect as well as adverse reactions. Regulation of Phase-I gene activity can happen at each step of the gene expression process. Among these steps, transcription is a critical determinant of the gene expression level. Studies have shown the strong correlation between mRNA expression and enzyme activity for most cytochrome P450s in human liver (Rodriguez-Antona et al., 2001). Numerous mechanisms have evolved for regulating transcription. At DNA level, these include promoters, enhancers, silencers and insulators. Various protein factors interact with these DNA elements to modulate transcription rates, such as general transcription factors, activators, repressors, and chromatin remodeling complex. From fetus to adult, the inherited genomic DNA sequence in cells of an individual is normally the same. So the changes of drug metabolizing gene expression during postnatal liver development are largely regulated by epigenetic factors. And here in this thesis, we will characterize the expression of lncRNAs in mouse liver, in order to identify their regulatory roles during development. We will also focus on a critical hepatic nuclear receptor, farnesoid X receptor, in regulating the ontogeny of liver and Phase-I gene expression.

Long non-coding RNAs

LncRNAs have important regulatory roles in multiple steps of gene expression. Due to the ability of lncRNA to interact with both DNA and proteins, it can serve as a guide to promote interactions between targeted DNA sequence and transcription factors or chromatin modulators, a process that regulates gene expression. For example, the lncRNA HOTAIR functions to guide the Polycomb Repressive Complex 2 to the HOXD gene locus, resulting in HOXD gene silencing during embryonic development (Rinn et al., 2007). LncRNA can also target miRNAs in

post-transcriptional regulation. The linc-MD1 lncRNA functions as a competing endogenous RNA to repress miR-133 and miR-135 activity and aid in muscle differentiation (Cesana et al., 2011). Considering the pervasive expression and diverse function of lncRNAs, it may also play a role in regulating the expression of Phase-I metabolizing enzymes. However, few studies have explored the involvement of lncRNAs in regulation of drug metabolizing genes.

Farnesoid X Receptor

Nuclear receptors are a class of cellular proteins that are responsible for sensing various hormonal or molecular signals. In response, these receptors work together with other proteins to regulate the expression of specific genes, thereby controlling the development, homeostasis, and metabolism of the organism. FXR is one member of the nuclear receptor superfamily with the gene symbol NR1H4. It was first cloned from rat liver in 1995, and considered as an “orphan” nuclear receptor because the natural ligands for FXR were unknown at that time, although it could be activated by high concentrations of farnesol (Forman et al., 1995). Bile acids were later identified as the endogenous ligand of FXR in 1999 (Wang et al., 1999).The FXR protein shares the common structure with other nuclear receptors, including the DNA-binding domain in the N-terminal region and the ligand-binding domain in the C-terminal region. Like many other non-steroid hormone nuclear receptors, FXR binds to specific DNA response elements as a heterodimeric complex with the retinoid X receptor. The most recognized FXR response element is an inverted repeat separated by one nucleotide (IR1), detected in many FXR target genes (Zhu et al., 2011).

The metabolism of cholesterol to bile acids and their subsequent excretion in the feces is the primary pathway for the body to eliminate cholesterol. As bile acids travel down the small intestine, ~95% are reabsorbed and transported back to the liver via the enterohepatic circulation. Since approximately 5% of bile acids are eliminated in each cycle, the liver must synthesize an equivalent amount to maintain a constant bile acid pool. Bile acids are

physiologically important because of their detergent-like properties that facilitate lipid absorption. However, these same properties also make bile acids highly cytotoxic when their blood or cellular levels increase. As a sensor of bile acid levels, FXR has an important role in maintaining bile acid homeostasis, regulating all aspects of bile acid metabolism. Cholesterol 7α-hydroxylase, also known as CYP7A1, is the rate-limiting enzyme in the classic pathway of bile acid synthesis. Activation of FXR by increased bile acids level induces one of its target genes, the small heterodimer partner (SHP), which can dimerize and inactivate the transcription factors that upregulate CYP7A1 expression, resulting in a decrease in CYP7A1 expression and bile acid synthesis (Goodwin et al., 2000). FXR also regulates genes involved in 1) bile acid secretion from hepatocytes to bile canaliculi, e.g., BSEP and MRP2; 2) bile acid uptake from the intestine or from the blood into the hepatocytes, e.g., ASBT, IBABP, and NTCP; 3) bile acid conjugation and detoxification, e.g., SULT2A1, UGT2B4, and BACS (Lee et al., 2006).

Patients on bile acid supplementation were found to display low high-density lipoprotein (HDL) cholesterol levels (Leiss and von Bergmann, 1982), and mouse model with FXR knockout was distinguished from wild-type by elevated serum and hepatic cholesterol and triglycerides, and a pro-atherogenic serum lipoprotein profile (Sinal et al., 2000). These suggested a role of FXR in control of lipid metabolism and further studies identified several FXR targeted genes in this process. Apolipoprotein (apo) A-I is the major protein component of HDL in plasma, the expression of which can be suppressed by FXR activation through either direct binding or induction of SHP (Claudel et al., 2005). FXR also induces the expression of the phospholipid transfer protein, an enzyme involved in HDL remodeling, and thereby modulates HDL levels. ApoC-II is an activator of lipoprotein lipase, a key enzyme in the clearance of serum triglyceride. And FXR has been found to bind and induce the expression of apoC-II, thus facilitating triglyceride metabolism (Kast et al., 2001).

More and more data have also suggested a link between FXR and glucose homeostasis. Initially in type II diabetic patients, treatment with bile acid sequestrants were shown to lower plasma glucose level. In primary rat hepatocytes, D-glucose increased FXR mRNA in a dose- and time-dependent manner, whereas in animal models of diabetes, FXR expression was decreased (Duran-Sandoval et al., 2004). In addition, activation of FXR in mice results not only in hypoglycemia, but also in altered hepatic expression of phosphoenolpyruvate carboxykinase, which catalyzes the initial step of gluconeogenesis, and glucose-6-phosphatase, which catalyzes the last committed step of this process (Zhang et al., 2006).

In addition to the identified roles of FXR as a key metabolic regulator, it has been found to be important for liver regeneration. FXR promotes liver repair after injury, and activation of FXR is able to alleviate age-related defective liver regeneration, probably through activating Foxm1b expression, which is required for the timely expression of many cell cycle regulators during regeneration (Chen et al., 2011). Hepatocyte-specific deletion of FXR delays liver regeneration in mice, which is associated with delayed cyclin D1 activation (Borude et al., 2012).

Furthermore, studies using chromatin immunoprecipitation coupled with sequencing (ChIP-Seq) have characterized the genome-wide FXR binding sites, which were detected near previously unknown target genes with novel functions, including diverse cellular signaling pathways, apoptosis, autophagy, hypoxia, inflammation, RNA processing, metabolism of amino acids, and transcriptional regulators (Lee et al., 2012). However, the role of FXR in liver maturation and developmental regulation of Phase-I genes has not been explored. In the immediate neonatal period, bile acids are required for the newborns to absorb milk. There is a surge of serum and liver bile acids levels after birth (Cui et al., 2012a), so the increased activation of FXR immediately after birth may play a role in regulating neonatal gene expression. Most of the known liver functions regulated by FXR are established during postnatal liver development, such as bile acid circulation and lipid metabolism, and disorders like fatty liver

may have roots in the neonatal period(Beath, 2003). So understanding the role of FXR in liver maturation may provide important insights on liver physiology and diseases. Hence, this thesis will utilize FXR knockout mouse model to study the involvement of FXR in regulation of liver maturation and Phase-I gene ontogeny.

Fig. 1.1

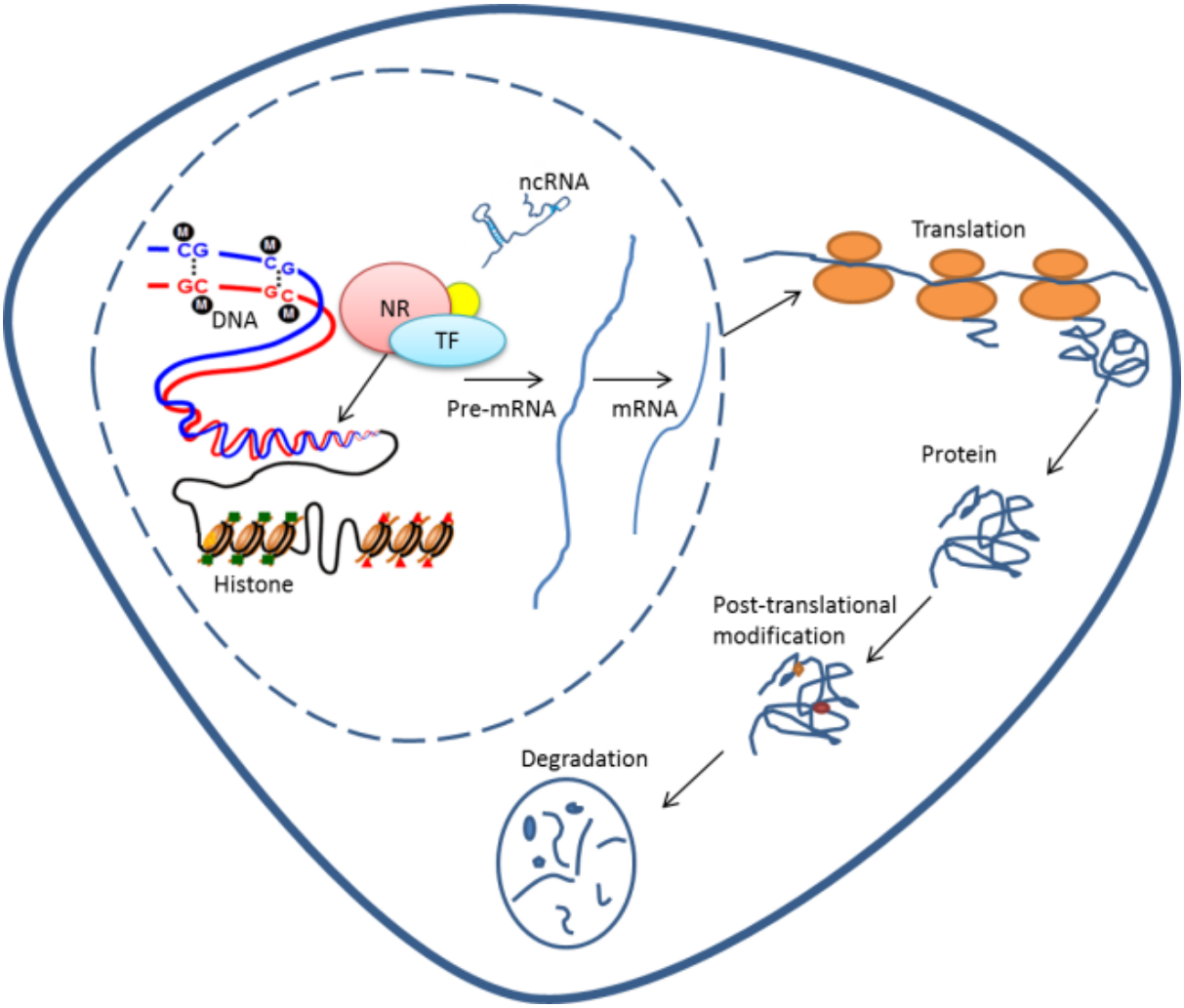

ncRNA
Translation
DNA
NR
TF
Pre-mRNA
mRNA
Protein
Histone
Post-translational
modification
Degradation

Fig. 1.1 Illustration of Gene Expression and Regulation.

Commonly in mammalian cells, the genetic information is encoded in DNA and flows to RNA and protein. DNA sequences are essential in determining the gene activity. At transcriptional level, epigenetic signatures, such as DNA methylation and histone modifications, can alter the chromatin conformation or interact with nuclear receptors (NR) and other transcription factors (TF) to influence the activity of RNA synthesis. The transcribed pre-mRNA undergoes RNA processing to generate mature messenger RNA, which is transported out of the nucleus and translated into protein. Post-transcriptionally, the steps of RNA processing, translation and the stability of RNA and protein are all under tight control and can affect the gene activity through regulation. One group of RNA is not translated into protein and designated as non-coding RNA (ncRNA), which plays important roles in modulating gene expression at multiple steps, e.g., through interaction with transcription or RNA degradation machinery.

Chapter 2 : Developmental Programming of Protein-coding and Long Non-coding RNAs in Mouse Liver

2.1 Abstract

The liver is a vital organ with critical functions in metabolism, protein synthesis and immune defense. Most of the liver functions are not mature at birth and many changes happen during postnatal liver development, which may underlie the ontogenic expression of Phase-I drug metabolizing genes and lead to differential vulnerabilities at different developmental stages. However, the details of what changes occur in liver after birth, at what developmental stages they occur, and the regulation of the developmental process are not clearly known. Long non-coding RNAs have been known to be involved in organ development and cell differentiation. Here, we analyzed the transcriptome of mouse liver from perinatal to adult ages by RNA-Sequencing, with an attempt to understand the role of lncRNAs in liver maturation. We found around 15,000 genes expressed, including about 2,000 lncRNAs. Most lncRNAs were expressed at a lower level than protein coding RNAs. Both protein-coding genes and long non-coding RNAs displayed three major ontogenic patterns: enriched expression at neonatal, adolescent, or adult stages. Neighboring protein coding and non-coding RNAs showed the trend to exhibit highly correlated ontogenic expression patterns. Gene ontology (GO) analysis of over-expressed genes during development revealed that cell proliferation and immune activation related processes were enriched at neonatal ages, tissue organization pathways were enriched at adolescent ages and livers at day 25 matured close to adult levels. GO analysis of under-expressed genes showed the immunity in liver was immature at neonatal ages and different metabolic processes matured at different stages during development. These data revealed

significant functional transition during postnatal liver development and indicate the potential importance of lncRNAs in liver maturation.

2.2 Introduction

The liver is the largest internal organ in the body, taking up about 2-4% of body weight in humans. It is also a vital blood reservoir by virtue of its large vascular capacity, which contains 10 to 15 percent of total blood volume. There are mainly five types of cells in liver. The hepatocytes are the parenchymal cells of the liver, and their prominent Golgi system and rough endoplasmic reticulum enable them to synthesize and secrete a variety of proteins. The hepatocytes also initiate the formation and secretion of bile. The endothelial cells lining the sinusoids serve as a barrier between the blood and hepatocytes. The cholangiocytes are the epithelial cells of the bile duct, and contribute to bile secretion through release of bicarbonate and water. Two other cell types line the sinusoids: the Kupffer cells, which are resident macrophages, and the stellate cells, which store fat and vitamin A.

As part of the digestive system, a matured liver plays a major role in nutrient homeostasis, including the synthesis, metabolism and transport of carbohydrates, proteins and fats. Venous blood from the stomach and intestine flows through the liver by the portal vein before entering systemic circulation. Thus liver is the first organ to encounter and deal with ingested drugs, environmental toxicants and intestinal bacteria. Bioactivation, detoxification and filtration of particulates in immune defense are also critical functions of the liver (Jaeschke, 2008). Whereas in utero, fetal liver is the major hematopoietic organ, generating blood cells, and hematopoiesis is active in liver even shortly after birth (Takeuchi and Miyajima, 2006). Definitive hematopoiesis requires a specific microenvironment in the organ. Therefore, from birth to maturity, there are dramatic changes happen in liver to achieve the organ growth and functional transition. And the

differences between neonatal and adult livers have been implicated in clinical issues. Disorders of iron metabolism such as hemochromatosis having severe effects in neonates including fulminant liver failure may be due to the active hematopoiesis in neonatal liver (Beath, 2003). Age-related sensitivity to drugs is at least partly attributable to differences in hepatic metabolic activity (Pineiro-Carrero and Pineiro, 2004).

Mouse liver originates from the gut endoderm on embryonic day 8.5, and epigenetic markings, such as unwinding of the chromatin by FoxA transcription factors, contribute to the competence of embryonic liver development (Zaret, 2002). Signals such as FGF-1, FGF-2 and BMP4 from the cardiac mesoderm specify the foregut endoderm to begin expressing liver-specific genes. One day later, cells forming the hepatic endoderm assume a columnar morphology and are ready to form the liver bud. Expression of homeobox and prospero-related homeobox 1 genes are essential to the formation of liver bud. By embryonic day 15, hepatoblasts begin to differentiate into hepatocytes and bile-duct epithelial cells. HNF-6, HNF-1β and the Notch/Jagged signaling pathway induce differentiation toward a biliary epithelial lineage, while HNF-4α followed by C/EBPα produces mature hepatocytes (Nejak-Bowen and Monga, 2008; Si-Tayeb et al., 2010). The differentiation process is also driven by the secretion of oncostatin M by blood cells in fetal liver (Kamiya et al., 1999). Although extensive researches have been done and advanced knowledge has accumulated on embryonic liver development, many aspects remain unknown in the postnatal liver development, such as the timeline of liver functional switch, the factors regulating liver growth and the mechanisms driving the organ maturation.

Many previous studies have focused on examination of protein coding genes during liver development without a special attention on long non-coding RNAs (lncRNAs). lncRNAs are non-protein-coding RNA transcripts longer than 200 nucleotides (Kapranov et al., 2007). They are alternatively spliced, contain 5'-capping and 3'-polyadenylation just like protein-coding RNAs,

but have few or no open reading frames (Cabili et al., 2011a). They either locate in the intergenic regions, called long intergenic noncoding RNAs (lincRNAs), or partially overlap with protein coding genes and can be transcribed from sense or antisense strand of the gene (Pauli et al., 2012). Comparative analysis has indicated that lncRNAs are evolutionarily conserved, especially at the promoter region (Guttman et al., 2009). The expression level of lncRNAs are usually lower than protein coding RNAs (Cabili et al., 2011b; Pauli et al., 2012), and they are expressed in tissue-specific and developmentally regulated manners (Amaral et al., 2008; Cabili et al., 2011b; Pauli et al., 2012). Some specific lncRNAs are transcriptionally regulated by key transcription factors in a mechanism similar to their neighborhood protein coding genes (Guttman et al., 2009). lncRNAs have been demonstrated to serve as signal, decoy, guide or scaffold to regulate various biological processes (Wang and Chang, 2011), including the cell cycle (Hung et al., 2011), pluripotency, X-inactivation (Ogawa et al., 2008), cell differentiation (Pauli et al., 2012), and organ development and maturation (Cesana et al., 2011; Hu et al., 2011; Alvarez-Dominguez et al., 2014). LncRNAs have also been associated to liver disease progress (Spizzo et al., 2012; Shi et al., 2013; Li et al., 2014). Yet whether long non-coding RNAs are involved in regulation of postnatal liver development has not been explored before.

In the current study, we used RNA-Sequencing (RNA-Seq) to quantify the liver transcriptome during the developmental period from perinatal stage to adult. RNA-Seq allows the analysis of the whole transcriptome with lower background noise, higher sensitivity, and a high degree of reproducibility compared to traditional technologies (Mortazavi et al., 2008; Nagalakshmi et al., 2008). More importantly, RNA-Seq quantifies the true abundance of RNA molecules in biological samples and enables the comparison of expression of all genes in multiple samples (Malone and Oliver, 2011). It also provides the chance for us to investigate the expression of long non-coding RNAs during liver maturation. Our results revealed the dynamic transcriptional changes happened in liver during postnatal development with specific transition

and maturation ages defined, and also suggested the potential importance of lncRNAs in liver maturation, which may facilitate future investigations to identify mechanistic roles of lncRNAs in regulation of gene expression during liver maturation.

2.3 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. The use of these mice was approved by the Institute of Laboratory Animal Resources. Breeding pairs were established at 4:00 p.m. and separated at 8:00 a.m. the following day. The body weights of the females were recorded each day to determine pregnancy status. Livers (n = 3) from male offspring 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 day 3, 5, 10, 15, 20, 30, 45, and 60 (collected around 9:00 a.m.), which represents periods of prenatal (day -2), neonatal (day 0 to 10), juvenile (day 15 to 30), and young adult (day 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. Livers were immediately frozen in liquid nitrogen after removal and stored at -80°C.

Total RNA Extraction

Total RNA was isolated by using RNAzol B reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. RNA concentrations were quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at a wavelength of 260 nm. The integrity of the total RNA was evaluated on an Agilent 2100 Bioanalyzer (Agilent

Technologies Inc., Santa Clara, CA) and the samples with RNA integrity values larger than 7.0 were accepted for sequencing library construction.

cDNA Library Construction

The cDNA libraries from all samples were prepared by using an Illumina TruSeq RNA sample prep kit (Illumina, San Diego, CA). Three micrograms of total RNA were used as the RNA input based on the manufacturer's recommendation. mRNAs were isolated from the total RNAs by poly-A selection using poly-T primers. RNA fragmentation, first and second strand cDNA syntheses, end repair, adaptor ligation, and PCR amplification were performed according to the manufacturer's protocol. Fragments of the cDNA library ranged from 220 to 500 bps with an average size at 280 bp (including 120 bp adapter sequences). Quality of the cDNA libraries was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) before sequencing.

RNA-Seq

RNA-Seq was performed on an Illumina HiSeq 2000 instrument by the Genome Sequencing Facility at the University of Kansas Medical Center. Clusters of the cDNA libraries were generated on a TruSeq flow cell and sequenced for 100 bp paired end reads (2 × 100) with a TruSeq 200 cycle SBS kit (Illumina, San Diego, CA). A PhiX bacteriophage genome and a universal human reference RNA were used as controls and sequenced in parallel with other samples to ensure that 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 approximately 1% as an internal quality control.

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 by Illumina's Real Time

Analysis software. The output bcl files were converted to qseq files by Illumina's BCL Converter 1.7 software and subsequently converted to FASTQ files for downstream analysis. For the analysis of protein-coding and non-coding genes at all 12 ages, FASTQ files were aligned with Tophat 1.4.1. Custom GTF was supplied to the program, which was compiled from Ensembl 66 genes and selected (non-overlapping) Noncode v3.0 models (Bu et al., 2012). Cufflinks 1.3.0 (Trapnell et al., 2010) was used to quantitate expression levels with the same custom GTF file provided. The RNA abundance was expressed as the number of fragments per kilobase of exon per million reads mapped (FPKM). For the analysis of samples at the 6 representative ages of day -2, 1, 5, 20, 25 and 60, the RNA-Seq reads from the FASTQ files were mapped to the mouse reference genome (GRCm38/mm10) by Tophat 2.0.8. The output files in BAM format were analyzed by Cufflinks 2.1.1 to estimate the transcript abundance.

Gene Ontology Analysis

Statistically significant differential expression in this analysis were defined using the following criteria 1) the gene mean FPKM>1 across the ages; 2) fold change for the average FPKM of the three replicates >1.5 between compared samples; 3) Benjamini-Hochberg adjusted p-values from t-test <0.05. Lists of differentially expressed genes at ages of day -2, 1, 5, 20 and 25 compared to day 60 in mice were supplied to High-Throughput GoMiner for biological interpretation (Zeeberg et al., 2005). Significantly enriched GO categories were selected with a false discovery rate (FDR) <0.05.

Data Visualization and Statistics

For the visualization of developmental gene expression patterns in liver, protein-coding and non-coding genes were separated and hierarchically clustered (Pearson correlation distance, average linkage). ANOVA was used to test for significant difference in expression during development. P-values were adjusted using Benjamini-Hochberg algorithm with a threshold of 0.05. Protein-coding genes were from Ensembl 66, and non-coding genes included Ensembl

non-coding and Noncode non-overlapping annotations. Ensembl t/ r/ sn/ sno/ mi/ misc-RNAs were removed from the final list of non-coding RNAs, and RNA-Seq used only poly-T beads selected RNAs for sequencing, so the list mainly contained long non-coding RNAs. For Pearson correlation coefficient-based heat map visualization, the average FPKM of the three replicates at each age were used to calculate the Pearson's r values between different ages.

2.4 Results

Transcriptome of Protein Coding and Long Non-coding RNAs in Mouse Postnatal Liver Development

RNA-Seq generated an average of 175 million (from 172 to 179 million) 100 bp paired end reads per sample for the 36 samples from 12 different ages of day -2, 0, 1, 3, 5, 10, 15, 20, 25, 30, 45, and 60 (n=3) with a mean of 83% (from 75% to 88%) of the reads mapped to the mouse reference genome (NCBI37/mm9). The mapped reads were annotated to protein coding genes by Ensembl66 and non-coding genes by Ensembl66 and Noncode v3 as the reference annotation databases. Because the sequencing libraries were constructed from poly-T bead selected RNAs, the detected non-coding RNAs were mainly lncRNAs, but some small non-coding RNAs, such as tRNAs, rRNAs, snRNAs, snoRNAs, and miRNAs, were also detectable. FPKM is used to represent gene expression level. The distribution of gene expression levels revealed that the majority of lncRNAs were expressed at a lower level than protein coding genes with a sharp distribution peak around FPKM=1 in all 12 ages. As an example, distribution curves at day 5 are shown in Fig. 2.1. These lower expression levels were consistent with previous reports in zebrafish and humans (Cabili et al., 2011b; Pauli et al., 2012), suggesting a general property of lncRNAs. When the genes with FPKM > 1 in any age were considered as the genes expressed during liver postnatal maturation, as showed in Table 2.1, a total of 15,244

Table 2.1 Protein-coding and long non-coding genes expressed during liver postnatal maturation

Gene class	Annotated database	Total annotated genes	Expressed in liver	Percentage of expressed genes	Differentially expressed in liver	Percentage of differentially expressed genes in liver
Protein coding RNAs	Ensembl66	23,196	12,990	56%	10,768	83%
Long non-coding RNA	Ensembl66	9,322	1,194	13%	952	80%
	Noncode v3.0	18,267	738	4%	498	67%
	Total lncRNAs	27,589	1,932	7%	1,450	75%
Other non-coding RNA*	Ensembl66	5,481	322	6%		
Total		56,266	15,244	27%		

* Other non-coding RNAs include Ensembl66 annotated t-RNAs, r-RNAs, miRNAs, snRNAs, snoRNAs, and miscRNAs

(27%) genes among the 56,266 annotated mouse genes by both annotation databases were expressed in liver over the 12 ages, including 12,990 (56%) protein-coding genes, 1,932 (%) lncRNAs, and 322 other small non-coding RNAs.

Developmental Changes of Protein Coding and Long Non-coding RNAs in Mouse Postnatal Liver Development

The Pearson correlation coefficient between any two samples of different ages was used to assess the similarity and differences of gene expression profiles over the 12 ages (Fig. 2.2). High correlation was found among the days, which correspond to liver development for perinatal (day -2, 0), neonatal (day 1, 3, 5), adolescent (day 10, 15, 20, 25), and young adult (day 30, 45, 60) with significant changes between each developmental stage.

ANOVA tests identified 10,768 (83%) of the 12,990 expressed protein-coding genes and 1,450 (75%) of the 1,932 expressed lncRNAs, which had significantly differential expression levels during postnatal liver development (Table 2.1). Hierarchical clustering analysis was performed on these differentially expressed genes to reveal their developmental expression patterns (Fig. 2.3). Three major patterns were identified for both protein-coding (Fig. 2.3A) and lncRNA genes (Fig. 2.3B) with Cluster 1, enriched at neonatal ages; Cluster 2, enriched at adolescent ages; and Cluster 3, enriched at adult ages. The proportion of genes belong to each pattern group was similar between protein-coding and lncRNA genes, with Cluster 1 as the largest group (>70% of differentially expressed genes), followed by Cluster 3 and Cluster 2. Genes in these three groups together accounted for over 95% of all differentially expressed genes.

The emerging molecular functions of lncRNAs include regulation of gene expression in *cis* (on neighboring genes) or in *trans* (distantly located genes) (Wang and Chang, 2011). One expectation from the in *cis* regulation in our samples is that the expression of lncRNAs and their neighboring gene loci should be correlated across the developmental ages to share the same

cluster patterns. To test this, we analyzed the correlation of ontogenic expression patterns between pairs of neighboring genes by examining the distribution of their correlation *R* values (Fig. 2.4). As a control, the distribution of *R* value for 10,000 random selected protein-coding gene (PC) pairs showed a peak around 0. Since the correlation of expression between a lncRNA (NC) and its PC neighbor may result from a true *cis* effect of the NC on its PC neighbor, or merely proximal transcriptional activity in the surrounding open chromatin, we also compared the correlation between neighboring PC-PC and PC-NC pairs. Two different stringency levels for selecting neighboring pairs were used. The lower stringency for PC-PC selected 21,300 pairs, including every nearest PC neighbor for every PC gene without filters, and the lower stringency for PC-NC pairs selected 19,923 pairs, including every nearest PC neighbor for every NC gene. The higher stringency limited neighboring gene pairs to a 10 kb distance from each other, and at least one gene must be expressed, which reduced the number of pairs to the scale of thousands. At low stringency, the distribution of correlation *R* for both PC-PC and PC-NC pairs showed a peak around 0.2. At high stringency, which is a more reasonable criterion, the distribution peak of *R* values for PC-NC was around 0.8, while that for PC-PC was still around 0.2. This result demonstrated that PC-NC neighboring genes tended to exhibit high correlation in ontogenic expression patterns.

Fig. 2.1

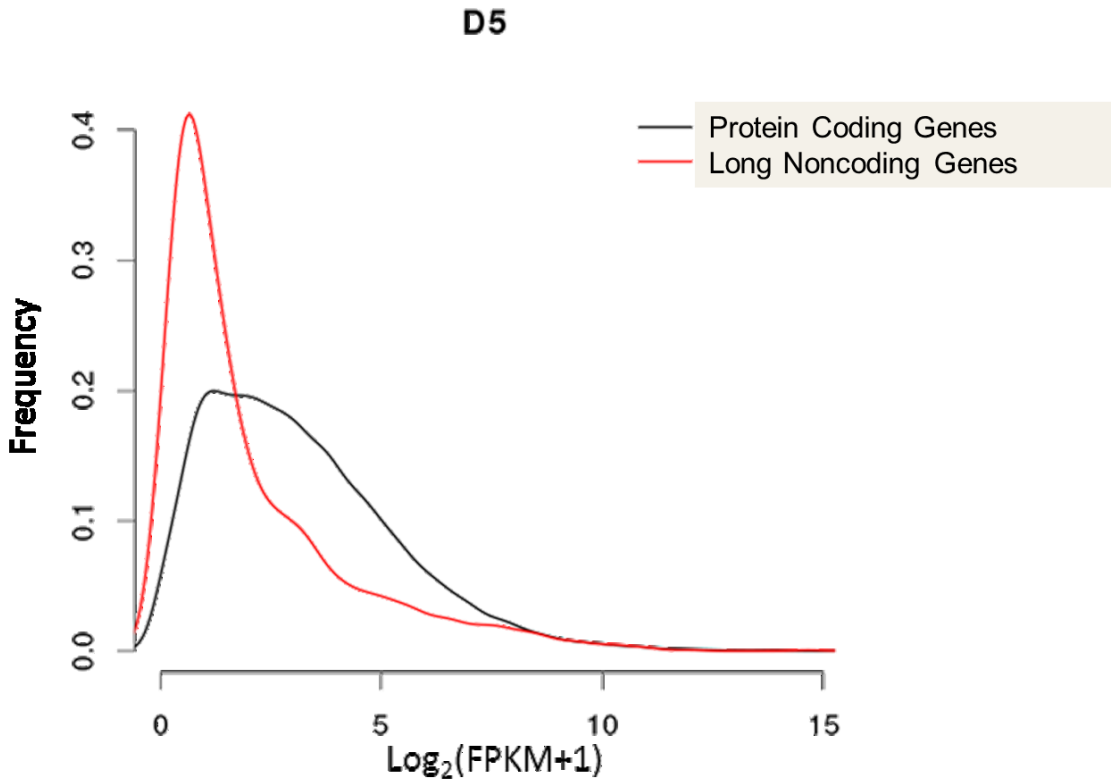


Fig. 2.1 Distribution of Gene Expression for Protein-coding and Long Nonconding RNAs.

This figure uses data from the age of day 5 as an example, and the FPKM value for each gene was the average FPKM of three individual animals

Fig. 2.2

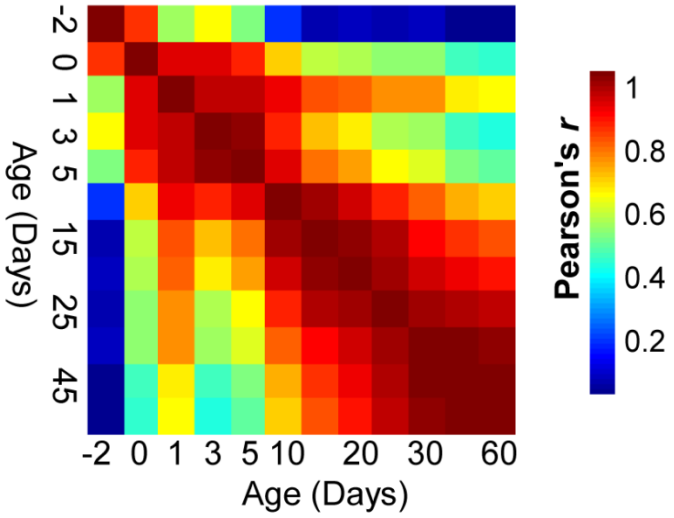


Fig. 2.2 The similarity of gene expression profiles between any two samples of different ages represented by Pearson Correlation Coefficient.

Fig. 2.3

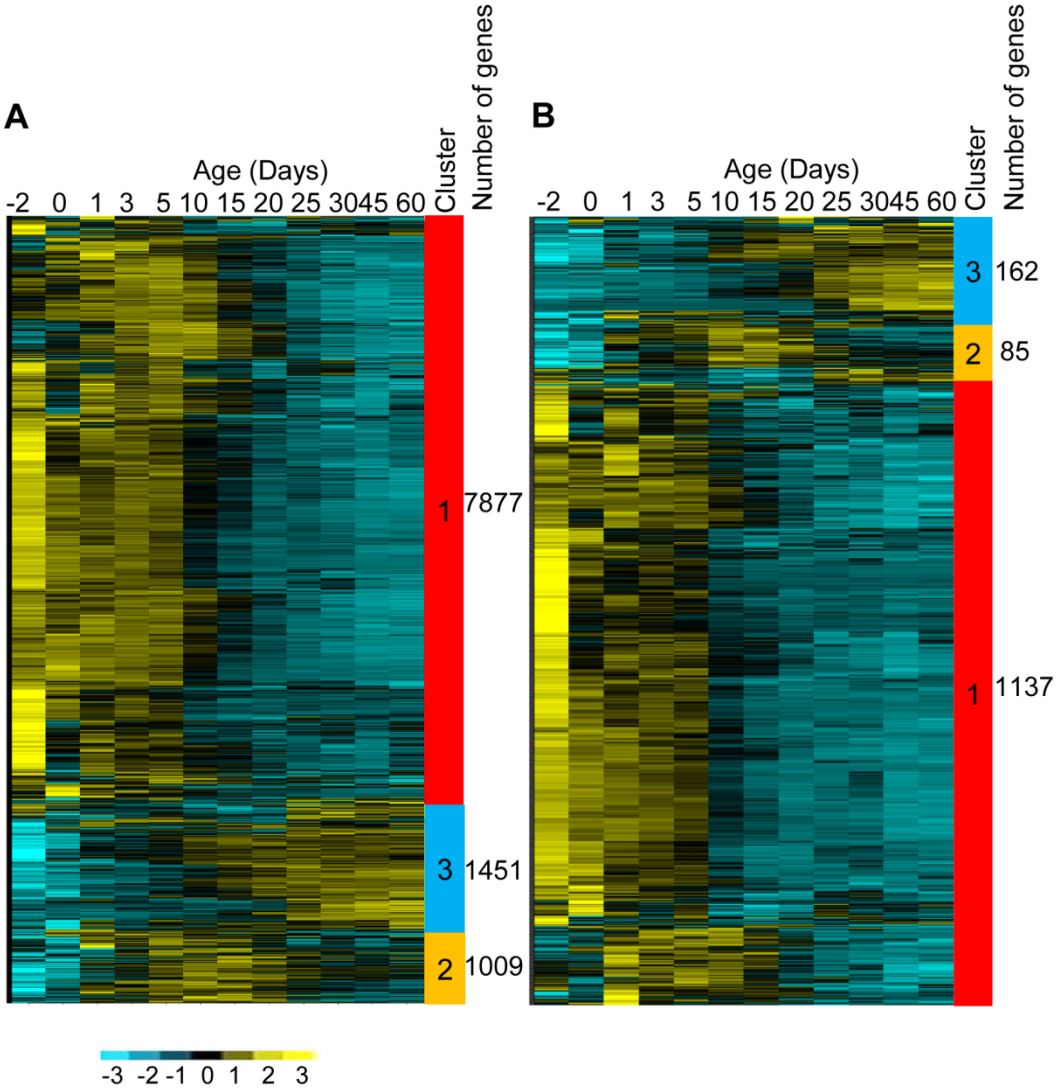

A
Age (Days)
-2 0 1 3 5 10 15 20 25 30 45 60
Cluster
Number of genes
1 7877
3 1451
2 1009
B
Age (Days)
-2 0 1 3 5 10 15 20 25 30 45 60
Cluster
Number of genes
3 162
2 85
1 1137
-3 -2 -1 0 1 2 3

Fig. 2.3 Developmental gene expression patterns during postnatal liver maturation.

Heat maps are drawn for all significantly differentiated expressed protein-coding (A) and long non-coding (B) genes during development in liver. Differential expressions were detected by ANOVA with adjusted *p*-values < 0.05. For each gene, $\log_2$(FPKM+1) over the ages were converted to z-scores, and the scale bar indicates z-score values. Genes are hierarchically clustered, and the color bar on the right shows the major trends of gene expression: high relative expression at early ages (neonatal, Cluster 1), intermediate ages (adolescent, Cluster 2) and older ages (adult, Cluster 3). The number of protein-coding and long non-coding genes in each major cluster are also listed on the right side.

Fig. 2.4

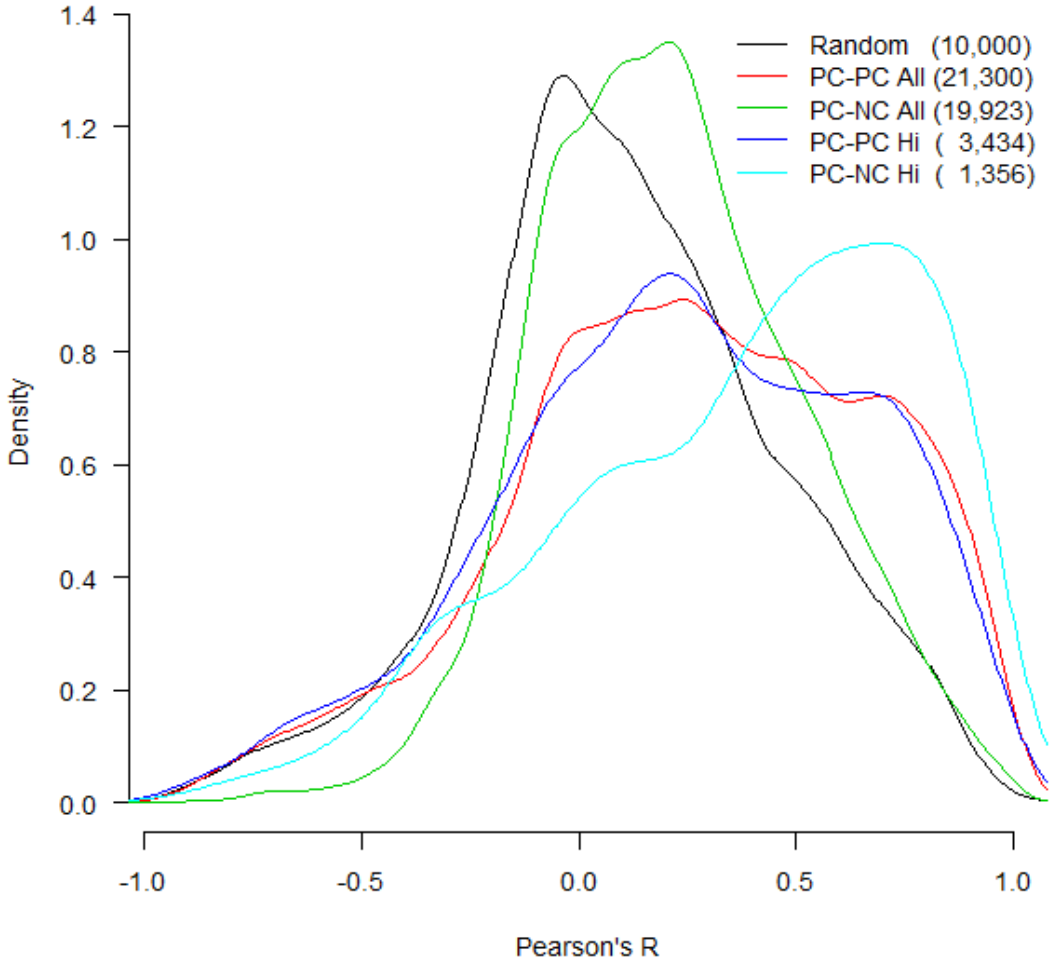


Fig. 2.4 Distribution of Correlation R Value for Expression Patterns between Pairs of Neighboring Genes.

Random are gene pairs selected at random; PC-PC All means every nearest PC (protein-coding) neighbor for every PC gene; PC-NC All means every nearest PC neighbor for every NC (long non-coding) gene; PC-PC Hi means PC-PC neighbor pairs within 10kb of each other, and at least one gene in the pair is expressed; PC-NC Hi means PC-NC neighbor pairs within 10kb of each other, and NC must be expressed. The number of gene pairs in each group is shown in the parenthesis.

Biological Interpretation of the Liver Developmental Transcriptome in Mouse Liver

In order to examine progress of liver functional maturation during postnatal development, six ages from the four different developmental stages at day -2 (perinatal), day 1 and day 5 (neonatal), day 20 and day 25 (adolescent), and day 60 (adult) were selected to analyze the liver transcriptome in more details. Day 60 was used as a matured reference transcriptome. Gene expression at each of the younger ages was compared to that of day 60 samples for differential expression. Statistically significant differential expression for both over-expressed and under-expressed genes were identified in each younger age and Gene Ontology (GO) analysis were performed to identify the most significantly affected pathways during liver maturation.

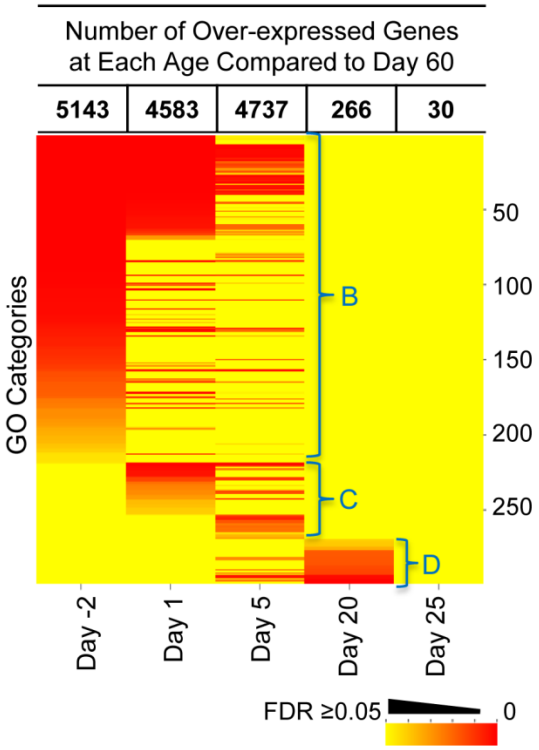
Fig. 2.5A shows a heat map for GO categories with significantly over-expressed genes in early ages. The numbers of over-expressed genes at each younger age compared to Day 60 were also listed. There was a clear developmental transition of enriched biological processes in these over-expressed genes as indicated by the brackets B, C and D. GO categories in bracket B were specific to perinatal and neonatal stages, and representative GO categories in this group were listed in Fig. 2.5B. Enriched biological processes at this stage were mainly involved in cell cycle and basic cellular metabolism, suggesting active cell proliferation in fetal and newborn livers. GO categories involved in blood cell development and homeostasis also showed up at this stage, which corresponded to the hematopoietic function of fetal liver. Typical GO categories in bracket C, which were specific to neonatal livers, were shown in Fig. 2.5C. The major over-expressed pathways at this stage were related to blood cell generation and immune activation. So the neonatal liver still retained residual hematopoietic function from fetal liver. And with exposure to the environment after birth, the immune system became activated. Representative GO categories enriched at day 20 were shown in Fig 2.5D, which were pathways involved in tissue structure organization, and also include a few metabolic processes

of alcohol and sulfur compounds. At day 25, there were no significant GO categories with gene over-expression compared to day 60.

Fig. 2.6A shows a heat map for GO categories with significantly under-expressed genes in early ages. The numbers of under-expressed genes at each younger age compared to Day 60 were also listed. There were fewer under-expressed genes as the age became closer to day 60. Day 25 samples had very few significant GO categories, indicating the liver was quite mature at this age. The majority of pathways with gene under-expression were different kinds of metabolic processes. One group of them matured relatively late during development (significant under-expression until day 20, see bracket B in Fig. 2.6A), including metabolic processes of isoprenoids, fatty acid, sterol, cholesterol, amine, organic acid, aromatic compounds, peptide, glutathione and cellular modified amino acids (Fig. 2.6B). Under-expressed GO categories specific to ages of day -2, 1 and 5 were biological processes involved in metabolic processes of xenobiotics, tryptophan, vitamin, small molecule catabolism, and carboxylic acid biosynthesis, as well as immuno response processes, such as humoral immune response, immune effector process, leukocyte mediated immunity, T cell and B cell mediated immunity (Fig. 2.6C). The representative GO categories that matured early during development (day 1) were listed in Fig. 2.6D, which included pathways of critical nutrients metabolism (glucose, lipid, and amino acids), bile acids transport, and blood coagulation. These results revealed important details of hepatic functional transition and maturation in postnatal liver development.

Fig. 2.5

A



B

GO Categories	FDR		
	Day -2	Day 1	Day 5
Cell cycle phase	0.0000	0.0000	0.0015
Mitosis	0.0000	0.0001	0.0032
DNA replication	0.0000	0.0002	0.0032
Chromatin modification	0.0000	0.0011	0.0186
Chromosome Organization	0.0000	0.0000	0.0029
RNA processing	0.0000	0.0000	0.0000
mRNA transport	0.0000	0.0011	>0.05
Ribosome biogenesis	0.0000	0.0000	0.0447
Macromolecule methylation	0.0000	0.0308	0.0473
Microtubule cytoskeleton organization	0.0000	0.0013	0.0461
Cellular macromolecular complex assembly	0.0000	0.0000	0.0148
Centrosome cycle	0.0017	>0.05	>0.05
Erythrocyte differentiation	0.0041	0.0003	>0.05
Leukocyte homeostasis	0.0109	>0.05	>0.05
B cell receptor signaling pathway	0.0131	0.0050	0.0033
Leukocyte activation	0.0461	0.0009	0.0016

Fig. 2.5

C

GO Categories	FDR	
	Day 1	Day 5
Leukocyte migration	0.0018	0.0232
Mast cell degranulation	0.0035	>0.05
Erythrocyte development	0.0060	>0.05
Actin filament based movement	0.0247	>0.05
Neutrophil chemotaxis	0.0247	0.0424
Hemopoiesis	0.0254	>0.05
B cell activation	0.0257	>0.05
Cell activation	0.0004	0.0014
Phagocytosis	0.0454	0.0286
T cell activation	>0.05	0.0235

D

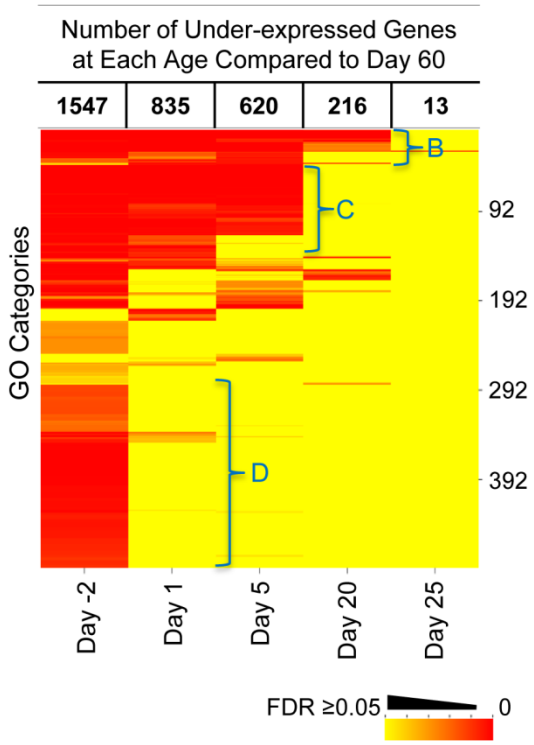
GO Categories	FDR	
	Day 5	Day 20
Cell migration	0.0233	0.0000
Extracellular matrix organization	0.0218	0.0135
Localization of cell	>0.05	0.0141
Steroid metabolic process	0.0269	0.0167
Blood vessel development	>0.05	0.0171
Cell substrate adhesion	>0.05	0.0174
Fibrinolysis	>0.05	0.0180
Tissue remodeling	>0.05	0.0346
Sulfur compound metabolic process	>0.05	0.0386
Alcohol metabolic process	>0.05	0.0402

Fig. 2.5 Gene Ontology Analysis for Over-expressed Genes during Development Compared to Day 60.

(A) Heat map for GO categories with significant gene over-expression at ages of day -2, 1, 5, 20 and 25. The color represents the false discovery rate (FDR) of each GO category. The numbers on the right are the cumulative number of GO categories. The three brackets B, C and D identify groups of GO categories enriched at specific ages during development. Representative GO categories in each bracket are show in the corresponding panels of (B), (C) and (D).

Fig. 2.6

A



B

GO Categories	FDR			
	Day -2	Day 1	Day 5	Day 20
Isoprenoid metabolic process	0.0000	0.0000	0.0000	0.0030
Fatty acid metabolic process	0.0000	0.0000	0.0000	0.0000
Sterol metabolic process	0.0000	0.0000	0.0001	0.0000
Cholesterol metabolic process	0.0000	0.0000	0.0001	0.0000
Amine metabolic process	0.0000	0.0000	0.0000	0.0345
Organic acid catabolic process	0.0000	0.0000	0.0000	0.0192
Cellular aromatic compound metabolic process	0.0000	0.0000	0.0000	0.0234
Peptide metabolic process	0.0000	0.0000	0.0000	0.0240
Glutathione metabolic process	0.0000	0.0000	0.0000	0.0254
Cellular modified amino acid metabolic process	0.0000	0.0000	0.0000	0.0185

Fig. 2.6

C

GO Categories	FDR		
	Day -2	Day 1	Day 5
Xenobiotic metabolic process	0.0000	0.0000	0.0000
Tryptophan metabolic process	0.0001	0.0004	0.0002
Vitamin metabolic process	0.0000	0.0000	0.0000
Small molecule catabolic process	0.0000	0.0000	0.0001
Carboxylic acid biosynthetic process	0.0000	0.0000	0.0000
Humoral immune response	0.0000	0.0000	0.0000
Immune effector process	0.0000	0.0000	0.0005
Leukocyte mediated immunity	0.0000	0.0000	0.0025
T cell mediated cytotoxicity	0.0001	0.0049	0.0076
B cell mediated immunity	0.0000	0.0000	0.0131

D

GO Categories	FDR	
	Day -2	Day 1
Glycerolipid metabolic process	0.0000	0.0005
Chylomicron remnant clearance	0.0007	0.0153
Tyrosine metabolic process	0.0007	0.0153
Blood coagulation	0.0000	>0.05
Hemostasis	0.0000	>0.05
Urea metabolic process	0.0018	>0.05
Glucose metabolic process	0.0031	>0.05
Cellular carbohydrate metabolic process	0.0034	>0.05
Bile acid and bile salt transport	0.0067	>0.05
Lipid storage	0.0043	>0.05
Glycine metabolic process	0.0215	>0.05

Fig. 2.6 Gene Ontology Analysis for Under-expressed Genes during Development Compared to Day 60.

(A) Heat map for GO categories with significant gene under-expression at ages of day -2, 1, 5, 20 and 25. The color represents the false discovery rate of each GO category. The numbers on the right are the cumulative number of GO categories. The three brackets B, C and D identify groups of GO categories enriched at specific ages during development. Representative GO categories in each bracket are show in the corresponding panels of (B), (C) and (D).

2.5 Discussion

In this study, RNA-Seq analysis of liver developmental transcriptome at 12 ages in mice resulted in two major findings. First, the developmental expression of lncRNAs in mouse liver was characterized, which may play an important role in the regulation of liver maturation. Second, the ontogenic expression patterns for protein-coding genes were defined, which revealed the timeline for liver growth and functional transition during postnatal development.

In our data, approximately 40% of all annotated genes were found expressed in liver during postnatal development. And around 80% of the expressed genes showed differential expression across the ages, indicating a dynamic nature of developmental transcriptome. An interesting result was that the majority of the genes showed decreased expression as the liver became mature (Fig. 2.3A). Pathway analysis of the differentially expressed genes demonstrated that most of the highly expressed genes at early ages belonged to diverse biological processes of cell proliferation, basic cellular metabolism, cellular structure assembly, and hematopoiesis (Fig. 2.5B). However, as the age grew, the liver cells became more static and functionally more specialized in metabolism. Thus the expressed genes changed to a more concentrated group.

Non-coding RNAs have been found to be pervasively expressed in the genome, and long non-coding RNAs are an important class involved in a variety of biological functions. The molecular mechanisms for long non-coding RNAs to regulate gene expression are largely base on their ability to form complex secondary structures and specifically interact with proteins like transcription factors and chromatin modifiers, either blocking or facilitating their activity in transcription (Wang and Chang, 2011). Our result of long non-coding RNA ontogenic expression patterns (Fig. 2.3B), which was the same as protein-coding RNAs, strongly suggested the implication of long non-coding RNAs in regulation of hepatic gene transcription during postnatal development. Neighboring protein-coding and long non-coding RNA pairs exhibited higher

correlations in ontogenic expression than neighboring protein-coding gene pairs under the same selection criteria, indicating that in *cis* regulation by lncRNAs may be an important mechanism of developmental gene expression in mouse liver. This was the first attempt to explore the potential role of long non-coding RNAs in liver maturation, which clustered long non-coding RNAs into different pattern groups and provided foundations and clues for study of their molecular functions.

Our GO analysis revealed the details of biological changes happened in liver. At perinatal stage (day -2 to 5), the liver underwent rapid cell proliferation and growth. And blood cell development was still active in liver (Fig. 2.5B). At neonatal stage (day 1 and 5), there were extensive immune cell activations as the newborns were exposed to the environment and started food intake, which introduced enormous challenges to the immune system. But the immunity in liver was not matured at this stage (Fig. 2.6C), which may explain the common diagnosis of sepsis in neonates (Diehl-Jones and Askin, 2003). At day 20, the liver went through changes of cell adhesion and tissue structure maturation (Fig. 2.5D). Some metabolic pathways were over-expressed at this age, and this might correspond to the cause of increased resistance to certain drugs in young children (Pineiro-Carrero and Pineiro, 2004).Genes in most metabolic processes were under-expressed at young ages (Fig. 2.6). And the ones that matured at earlier ages were in pathways for the metabolism of the most critical nutrients, like glucose, lipid and amino acids (Fig. 2.6D). The critical function of blood coagulation also matured immediately after birth to prevent life-threatening hemorrhage. As the mice were usually weaned and separated from the mother three weeks after birth, this was the time that most of the liver functions became mature. And at day 25, very few genes were still differentially expressed compared to day 60.

The functional maturation of the liver organ is the physiological base for the maturation of Phase-I drug metabolizing enzymes. As we can see, there are dramatic functional transitions

during postnatal liver development, especially in metabolic pathways, which suggested significant changes in the expression of Phase-I enzymes. In the following chapters, we will analyze the ontogeny of major Phase-I genes and explore possible regulatory mechanisms of their developmental expression.

Chapter 3 : RNA-Seq Reveals Dynamic Changes of mRNA Abundance of Cytochrome P450s and their Alternative Transcripts during Mouse Liver Development

3.1 Abstract

Cytochrome P450s are a superfamily of enzymes that have critical functions in liver to catalyze the biotransformation of numerous drugs. However, the functions of most P450s are not mature at birth, which can markedly affect the metabolism of drugs in newborns. Therefore, characterization of the developmental profiles and regulatory mechanisms of P450 expression is needed for more rational drug therapy of pediatric patients. An animal model is indispensable for studying the mechanisms of postnatal development of the P450s. Hence we used RNA-Seq to provide a “true-quantification” of mRNA expression of all P450s in mouse liver during development. Liver samples of male C57BL/6 mice at 12 different ages from prenatal to adulthood were used. Total mRNAs of the 103 mouse P450s displayed two rapid increasing stages after birth, reflecting critical functional transition of liver during development. Four ontogenic expression patterns were identified among the 71 significantly expressed P450s, which categorized genes into neonatal-, adolescent-, adolescent/adult-, and adult-enriched groups. The 10 most highly expressed subfamilies of mouse P450s in livers of adult mice were Cyp2E, -2C, -2D, -3A, -4A, -2F, -2A, -1A, -4F, and -2B, which showed diverse expression profiles during development. The expression patterns of multiple members within a P450 subfamily were often classified to different groups. RNA-Seq also enabled the quantification of known transcript variants of CYP2C44, CYP2C50, CYP2D22, CYP3A25, and CYP26B1, and identification of novel transcripts for CYP2B10, CYP2D26, and CYP3A13. In conclusion, this

study reveals the mRNA abundance of all the P450s in mouse liver during development, and provides a foundation for mechanistic studies in the future.

3.2 Introduction

Cytochrome P450s (P450s) have been identified in all domains of life: animals, plants, fungi, protists, bacteria, archaea, and even viruses. More than 18,000 distinct P450 proteins are known (Nelson, 2009). According to the genomic data base, there are 57 functional P450s identified in humans, and 102 P450s in mice (Nelson et al., 2004). Genes encoding P450 enzymes, and the enzymes themselves, are designated as CYP, followed by a number indicating the gene family, a letter indicating the subfamily, and another number for the individual gene. For example, CYP3A4 is the P450 enzyme belonging to the gene family 3, subfamily A, and designated as number 4. The current nomenclature guidelines suggest that members of the same P450 family share >40% amino acid sequence identity, while members of one subfamilies must share >55% amino acid sequence identity.

In the catalytic cycle of P450s, the heme iron converts between the states of ferric (Fe3+) and ferrous (Fe2+), facilitating the essential electron transfer from the substrate to molecular oxygen. The flavoprotein POR is a critical co-factor in this process as it directly passes the electron from NADPH through FAD to FMN in the POR protein and finally to P450 enzymes. It is the only electron donor for all microsomal P450s, and alteration in POR activity can affect P450-catalyzed drug oxidation (Hart et al., 2008).

There are 18 families of human P450 enzymes. The vast majority of these enzymes, which are found in families 1, 2, and 3, function in metabolism of xenobiotics, solely oxidizing chemicals that are not normally constituents of the body. They are the most important phase I drug-metabolizing enzymes, contributing ~80% of phase I reactions (Evans and Relling, 1999),

in the forms of hydroxylation, epoxidation, heteroatom (S-, N-) oxygenation, heteroatom (O-, S-, and N-) dealkylation, oxidative group transfer and so on. P450s in family 4 are involved mainly in the metabolism of fatty acids and eicosanoids. And other families of P450s have important roles in many endogenous pathways, such as steroidogenesis (e.g., CYP11/17/19), biosynthesis of cholesterol and bile acids (e.g., CYP7/8/27), and metabolism of vitamin D and retinoic acid (e.g., CYP24/26/27) (Parkinson and Ogilvie, 2008). Due to their broad substrate specificity, it is possible that two or more P450s contribute to the metabolism of a single compound, or a single P450 catalyzes two or more metabolic pathways for the same drug. Yet sensitive probe substrates can also be found to monitor P450 activity, and specific enzyme inhibitors can be used as controls for assessing enzymatic activities (Table 3.1). Previous studies have demonstrated that the P450 mRNAs in family 1-4 are inducible by ligands of four classes of xenobiotic receptors, namely the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the peroxisome proliferator-activated receptor alpha (PPARα) (Petrick and Klaassen, 2007). Although these receptors have overlapping targets, it is generally considered that AhR is responsible for the mRNA induction of family 1, CAR for family 2, PXR for family 3, and PPARα for the mRNA induction of family 4 of P450s. P450 induction may be associated with a loss of therapeutic effectiveness, which is a particular concern when using drugs that have a narrow therapeutic index and treating a life-threatening illness. The function, inhibition and inducibility of mouse and human P450 orthologs are quite conserved. Table 3.1 listed examples of specific substrate reactions and inhibitors of P450 enzymes in mice (Bogaards et al., 2000; Hrycay and Bandiera, 2009).

P450s catalyze the oxidation of organic substances, including the biotransformation of numerous endobiotics (e.g. steroids, fatty acids, and eicosanoids) as well as the detoxification or bioactivation of a variety of xenobiotics (e.g. drugs, chemical carcinogens, and environmental contaminants) (Nebert and Gonzalez, 1987; Danielson, 2002). P450s are the major enzymes

Table 3.1 Specific substrate reactions and inhibitors of P450 enzymes in mice

P450 Enzymes	Characteristic Metabolic Reaction	Inhibitors
CYP1A2	Methoxyresorufin O-demethylation	Furafylline
CYP2A5	Coumarin 7-hydroxylation	8-Methoxypsoralen
CYP2B10	Pentoxyresorufin O-depentylation	Pilocarpine
CYP2C29	Tolbutamide p-methyl hydroxylation	Menadione
CYP2D22	Bufuralol 1'-hydroxylation	Quinine
CYP2E1	4-Nitrophenol ortho-hydroxylation	4-Methylpyrazole
CYP3A11	Testosterone 6β-hydroxylation	Ketoconazole
CYP4A12	Arachidonic acid ω-hydroxylation	HET0016

involved in the metabolism and bioactivation of drugs, accounting for about 75% of drug biotransformation (Guengerich, 2008). Liver expresses the largest number of individual P450 enzymes (Hrycay and Bandiera, 2009). However, most P450s in the liver are expressed at low levels at birth. The expression of P450s changes during liver development, which has been categorized into several different developmental patterns, and considerable interindividual variability occurs in the immediate postnatal period (Hines and McCarver, 2002; Hines, 2007). Low P450 expression in liver during postnatal development is thought to be responsible for the substantial pharmacokinetic differences between newborns and adults, and thus contributes to differences in therapeutic efficacy and adverse drug reactions in pediatric patients (Blake et al., 2005; Hines, 2008). One example is that the low CYP3A4 in neonatal livers results in a low capacity to oxidize cisapride, which can result in QT prolongation in pediatric patients (Pearce et al., 2001; Treluyer et al., 2001). An in-depth understanding of the regulation of the ontogeny of human P450s is needed for safer and more effective drug therapy for pediatric patients.

The paucity of suitable tissue samples and limitations due to ethical and technical issues have made it difficult to study the mechanisms controlling the ontogenic expression of P450s in human liver (Rowell and Zlotkin, 1997). Animal models would be advantageous in overcoming these difficulties and minimizing the influence of genetic variations and the environment. In recent years, the mouse and rat have surpassed many other laboratory animals as the experimental models of choice for the study of physiology, metabolism, and disease (Muruganandan and Sinal, 2008; Hrycay and Bandiera, 2009). Advantages of these models include rapid growth, easy maintenance, and the development of genetic manipulation techniques for mechanistic studies with gain-of-function and loss-of-function strategies. Several researchers have examined the ontogenic gene expression profiles of a few P450s in mouse or rat liver (Choudhary et al., 2004; Alcorn et al., 2007; Cherala et al., 2007; Hart et al., 2009; Li et

al., 2009a). Developmental expression patterns of some P450s in mice and rats are similar to that in humans.

Previous studies quantified P450 gene expression at the mRNA level by either microarrays or multiplex suspension arrays (Hart et al., 2009; Li et al., 2009a), which only provides relative quantification of a given P450. These technologies detect mRNA levels by probe hybridization and fluorescence signal intensity, which cannot compare expression levels among various P450s, because different probes may have different hybridization efficiency. With the development of next-generation sequencing technology, RNA-Seq can define a transcriptome with low levels of background noise, no upper limit for quantification, and a high degree of reproducibility for both technical and biological replicates (Mortazavi et al., 2008; Nagalakshmi et al., 2008). More importantly, compared to microarrays, RNA-Seq quantifies the true abundance of mRNA molecules in biological samples, and enables the comparison of expression levels of all genes (Malone and Oliver, 2011). Furthermore, RNA-Seq has the power to quantify expression levels of alternative transcripts of the same gene and to identify novel transcripts efficiently (Pan et al., 2008; Wang et al., 2008; Malone and Oliver, 2011). In the current study, RNA-Seq was used to systematically quantify the abundance of 103 mouse P450 mRNAs in liver during development to define the ontogenic profiles of these P450 mRNAs, with an additional focus on the identification and quantification of alternative transcripts. Therefore, the purpose of this study was to generate comprehensive information on the ontogeny of the mRNAs of the P450 family of enzymes in the livers of mice, which will provide a foundation for determining the regulatory mechanisms controlling the various transcription patterns of P450s during liver development.

3.3 Materials and Methods

Animals, Total RNA Extraction, Sequencing Library Construction, and RNA-Seq

The source and breeding of animals, RNA extraction, library construction, RNA-Seq and FASTQ data file collection were the same as those in Chapter 2.

RNA-Seq Data 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 1.2. The output files in BAM (binary sequence alignment) format were analyzed by Cufflinks 1.0.3 to estimate the transcript abundance (Trapnell et al., 2010).

Data Visualization and Statistics

The P450 genes (103 in total) were retrieved from Cufflinks output for further analysis. Significant gene expression was determined by the drop-in-deviance F test of the fitted FPKM data to a generalized linear model with a Poisson link function, a statistic designed to measure the significance of a gene's measured FPKM value relative to a zero FPKM value. The p-values were adjusted for extra Poisson variation and corrected for false discovery by the Benjamini-Hochberg method (FDR-BH) with a threshold set at 0.05 for significant expression (Benjamini and Hochberg, 1995). A two-way hierarchical clustering dendrogram was generated by JMP (version 9.0; (SAS Institute, Cary, NC) to determine the expression patterns of the P450 mRNAs during liver development.

Validation of Alternative mRNA Transcripts

Alternative splicing events in the CYP2B10 and CYP2D26 were validated by reverse transcription and end-point PCR. Total RNA (5 μg) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers in a final volume of 50 μl. End-point PCRs with reaction volumes of 20 μl were performed. Primer sequences are listed in Table 3.2. The PCR products were separated on a 2% agarose gel. Bands of the expected

sizes were excised and purified with a Qiagen QIAquick gel extraction kit (Valencia, CA). Subsequently, DNA was sequenced with sequencing primers (Table 3.2) by ACGT Inc. (Wheeling, IL). The alternative polyadenylation of CYP3A13 mRNA was validated by Rapid Amplification of cDNA 3'-End (3'RACE) using RNA ligase-mediated rapid amplification of cDNA ends (FirstChoice RLM-RACE kit; Ambion Inc., Austin, TX) as described before (Li et al., 2012). Primer sequences are shown in Table 3.2.

3.4 Results

Total Expression and Proportions of Individual P450s during Liver Development

RNA-Seq generated an average of 175 million 100 bp paired-end reads per sample for the 36 samples from 12 different ages (n=3). More than 80% of the reads from each sample were mapped to the mouse reference genome (NCBI37/mm9) by TopHat 1.2 (data not shown). The FPKM values were highly reproducible in the triplicate samples at each age as indicated by the small standard errors. If the Benjamini-Hochberg adjusted drop-in-deviance F-test p-value (FDR-BH) in at least one of the 12 ages were less than 0.05 for a P450 gene, then that gene was considered to be expressed in liver during development. Of the 103 mouse P450s, 71 genes were expressed during liver development.

Total FPKM values of all P450 mRNAs increased about 25-fold during postnatal liver development from day -2 (479) to day 60 (12,345), with two surges followed by a plateau stage after each surge (Fig. 3.1A). Fig. 3.1B shows the composition of the P450s, represented as percentage of the total P450 mRNA FPKM, at the beginning and end ages of these surge and plateau stages, i.e. day -2, 1, 10, 20, and 60. The first surge occurred from day -2 (FPKM, 479) to day 1 (5,926) with more than a 10-fold increase of total P450 mRNA FPKM values. During the first surge, the top 10 highly expressed P450 genes changed dramatically, with only 2 genes

Table 3.2 Primer sequences for validation of novel transcripts identified by RNA-Seq

Gene	Method	Amplification	Sequence (5' to 3')	PCR product
Cyp2b10	End-point PCR	From exon 6 to exon 9 (including cassette exon)	F: ATGGCTTCCTGCTCATGCTCAAGT	411 bp
			R: GACAAATGCGCTTTCCCACAGACT	
	Sequencing	From exon 7 to exon 9	AGTGCCACACAGAGTGACCAAAGA	
Cyp2d26	End-point PCR	From exon 5 to cassette exon	F: ACTACACATCCCTGGTTTGCCTGA	520 bp
			R: AGCCTCTGAGCACCTTCTCTTGTA	
	Sequencing	From exon 6 to cassette exon	TGATTGACCTGTTCATGGCAGGGA	
	End-point PCR	From cassette exon to exon 9	F: TACAAGAGAAGGTGCTCAGAGGCT	327 bp
			R: TAGGGCTCTGGAGTAACTGGCATT	
	Sequencing	From exon 8 to cassette exon	CATGAAGGCCTCGTGCTTCACAAA	
Cyp3a13	3' RACE	cDNA synthesis	GCGAGCACAGAATTAATACGACTCACTATAGGT12VN	
		Outer PCR	F: AAGTTGCTCTTGTCAGAGTCCTGC	
			R: GCGAGCACAGAATTAATACGACT	
		Inner PCR	F: CACTGTCCAGCCTTGTAAGGAAAC	
			R: CGCGGATCCGAATTAATACGACTCACTATAGG	
		Sequencing	AAGTTGCTCTTGTCAGAGTCCTGC	

(*Cyp2d22* and *Cyp2d10*) in the top 10 P450s at both day -2 and day 1. During the first plateau stage, although the total P450 mRNA FPKM values were relatively constant between day 1 and 10, the individual P450 mRNAs were altered, with CYP2E1 the most abundant at day 10. The second surge occurred between day 10 and 20, with nearly a 2-fold increase of total P450 FPKM values from 6,139 to 11,087. The individual P450 mRNAs, which increased during the second surge from day 10 to 20, were similar, because 6 P450s appeared at both ages in the top 10 highly expressed P450s, and the top 3 are the same in an order of CYP2E1, CYP3A11, followed by CYP2D26. During the second plateau stage of day 20 to 60, the individual P450 mRNAs continued to change, and became more diverse. At day 60, the top 10 highly expressed P450s consisted of only 74% of the total P450 mRNAs, and many additional P450s were expressed.

Expression Patterns of P450 Genes during Liver Development

P450 genes with a maximum over minimum expression ratio less than two among the twelve studied ages were to be excluded for this party of the study. The data indicate that all 71 significantly expressed P450 genes had larger than two-fold differences in expression during development. Two-way hierarchical clustering analysis of the 71 P450 genes revealed 4 distinct Groups (Fig. 3.2A). The sum of the FPKM values in each group was plotted against age in Fig. 3.2B. Group 1 (neonatal-enriched) contained CYP2D26, CYP3A16, CYP3A41a/b, CYP3A44, CYP4A10, CYP4A14,CYP4A31, CYP4A32, CYP4F16, CYP4F18, CYP4F39, CYP20A1, and CYP39A1. The P450 genes in this group increased markedly after birth and reached a peak around day 1 of age, and then decreased and remained relatively low after day 30. Group 2 genes (adolescent-enriched) consisted of CYP2A22, CYP2B9, CYP2B13, CYP2B23, CYP2C37, CYP2C40, CYP2C68, CYP2C69, CYP2D37-ps, CYP2E1, CYP2G1, CYP4F15, CYP7A1, and CYP26B1. The P450 genes in this group increased rapidly after birth, reached maximal expression around day 20, and then decreased to lower levels in adult mice. Group 3 genes

(adolescent/adult-enriched) included CYP1A2, CYP2A4, CYP2A5, CYP2A12, CYP2B10, CYP2C29, CYP2C39, CYP2C50, CYP2C54, CYP2C55, CYP2C70, CYP2D22, CYP2J6, CYP3A13, CYP3A25, CYP3A59, CYP4B1, CYP4F14, and CYP17A1. The mRNA of the P450s in this group were expressed at very low levels at birth, but increased substantially between day 10 and 25, and reached a plateau in adult mice. Group 4 P450s (adult-enriched) consisted of CYP2C38, CYP2C44; CYP2C67, CYP2D9, CYP2D10, CYP2D11, CYP2D12, CYP2D13, CYP2D34, CYP2D40, CYP2F2, CYP2J5, CYP2R1, CYP2U1, CYP3A11, CYP4A12A/B, CYP4F13, CYP4V3, CYP7B1, CYP8B1, CYP26A1, CYP27A1, and CYP51. The P450s in this group were expressed lowly 2 days before birth, gradually increased after birth with a rapid increase around 25 days of age, and reached highest expression in adults (45 and 60 days of ages). The largest correlation distance with respect to neighboring ages for the four groups (as shown by the clade separation on the dendrogram scale) was observed between 20 and 25 days of age.

Fig. 3.1

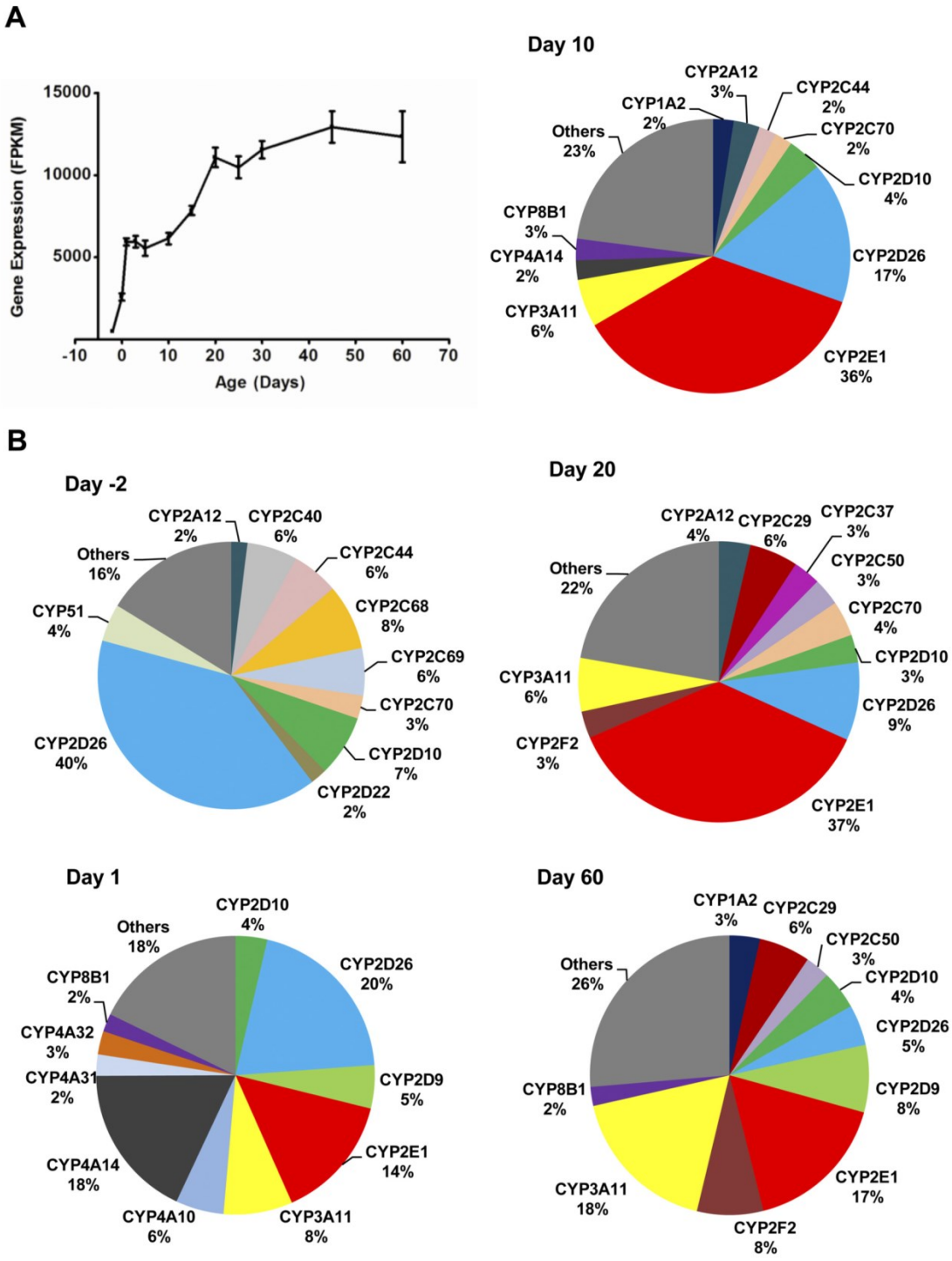

A
Gene Expression (FPKM)
15000
10000
5000
-10
0
10
20
30
40
50
60
70
Age (Days)
Day 10
CYP2A12 3%
CYP2C44 2%
CYP1A2 2%
CYP2C70 2%
Others 23%
CYP2D10 4%
CYP8B1 3%
CYP4A14 2%
CYP2D26 17%
CYP3A11 6%
CYP2E1 36%
B
Day -2
CYP2A12 2%
CYP2C40 6%
Others 16%
CYP2C44 6%
CYP51 4%
CYP2C68 8%
CYP2C69 6%
CYP2C70 3%
CYP2D26 40%
CYP2D10 7%
CYP2D22 2%
Day 20
CYP2A12 4%
CYP2C29 6%
CYP2C37 3%
Others 22%
CYP2C50 3%
CYP2C70 4%
CYP2D10 3%
CYP3A11 6%
CYP2D26 9%
CYP2F2 3%
CYP2E1 37%
Day 1
CYP2D10 4%
Others 18%
CYP2D26 20%
CYP8B1 2%
CYP4A32 3%
CYP2D9 5%
CYP4A31 2%
CYP2E1 14%
CYP4A14 18%
CYP4A10 6%
CYP3A11 8%
Day 60
CYP1A2 3%
CYP2C29 6%
CYP2C50 3%
Others 26%
CYP2D10 4%
CYP2D26 5%
CYP8B1 2%
CYP2D9 8%
CYP2E1 17%
CYP3A11 18%
CYP2F2 8%

Fig. 3.1 Total Expression and Proportions of Individual P450s during Liver Development.

(A) Total mRNAs of the 103 mouse P450 genes in liver during development. 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 103 mouse P450 genes at each age were added and plotted to show the developmental pattern of total P450 mRNAs. Bars represented the mean ± S.E.M. of three individual animals. (B) Individual P450 mRNAs (shown as percentages of total P450 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 the top 10 P450 mRNAs at each age are listed in alphabetical order, and the rest were grouped as “Others.”

Comparison of Transcript Abundance of Some P450 Subfamilies during Liver Development

The 103 mouse P450 genes belong to 16 families and 39 subfamilies. Ontogenic patterns of the major P450 subfamilies in liver are summarized in Fig. 3.3.

CYP1A subfamily. The CYP1A subfamily has two members, CYP1A1 and CYP1A2. CYP1A1 mRNA was expressed at very low levels in liver at all ages. CYP1A2 had a developmental pattern belong to group 3 (adolescent/adult-enriched) (Fig. 3.3A).

CYP2A subfamily. The CYP2A subfamily consists of five members, CYP2A4, CYP5, CYP12, CYP22, and CYP2AB1. Except for CYP2AB1, the other four genes in liver were expressed with the adolescent or adolescent/adult-enriched patterns (Fig. 3.3B).

CYP2B subfamily. The CYP2B subfamily contains five members, CYP2B9, -10, -13, -19, and -23. CYP2B9 and CYP2B13 contributed the most to the expressed levels of this subfamily with an adolescent-enriched pattern (Fig. 3.3C).

CYP2C subfamily. The CYP2C subfamily includes 16 members, CYP2C29, -37, -38, -39, -40, -44, -50, -53-ps, -54, -55, -65, -66, -67, -68, -69, and -70. CYP2C29, -37, -50, -69, and -70 were the most highly expressed members in this P450 subfamily in livers of mice. CYP2C69 was neonatal-enriched, CYP2C37 was adolescent-enriched, and CYP2C29, -50, and -70 were adolescent/adult-enriched (Fig. 3.3D).

CYP2D subfamily. The CYP2D subfamily consists of 10 members, CYP2D9, -10, -11, -12, -13, -22, -26, -34, -37-ps, and -40. Three genes, CYP2D9, -10, and -26, were expressed to a higher extend than the other CYP2D genes. CYP2D26 increased remarkably after birth and remained at a consistent level until day 20, and decreased after 30 days of age (neonatal-enriched). CYP2D9 was expressed lowly until day 20 and increased markedly to reach a plateau at 30 days of age (adolescent/adult-enriched). CYP2D10 increased gradually throughout development (Fig. 3.3E).

Fig. 3.2

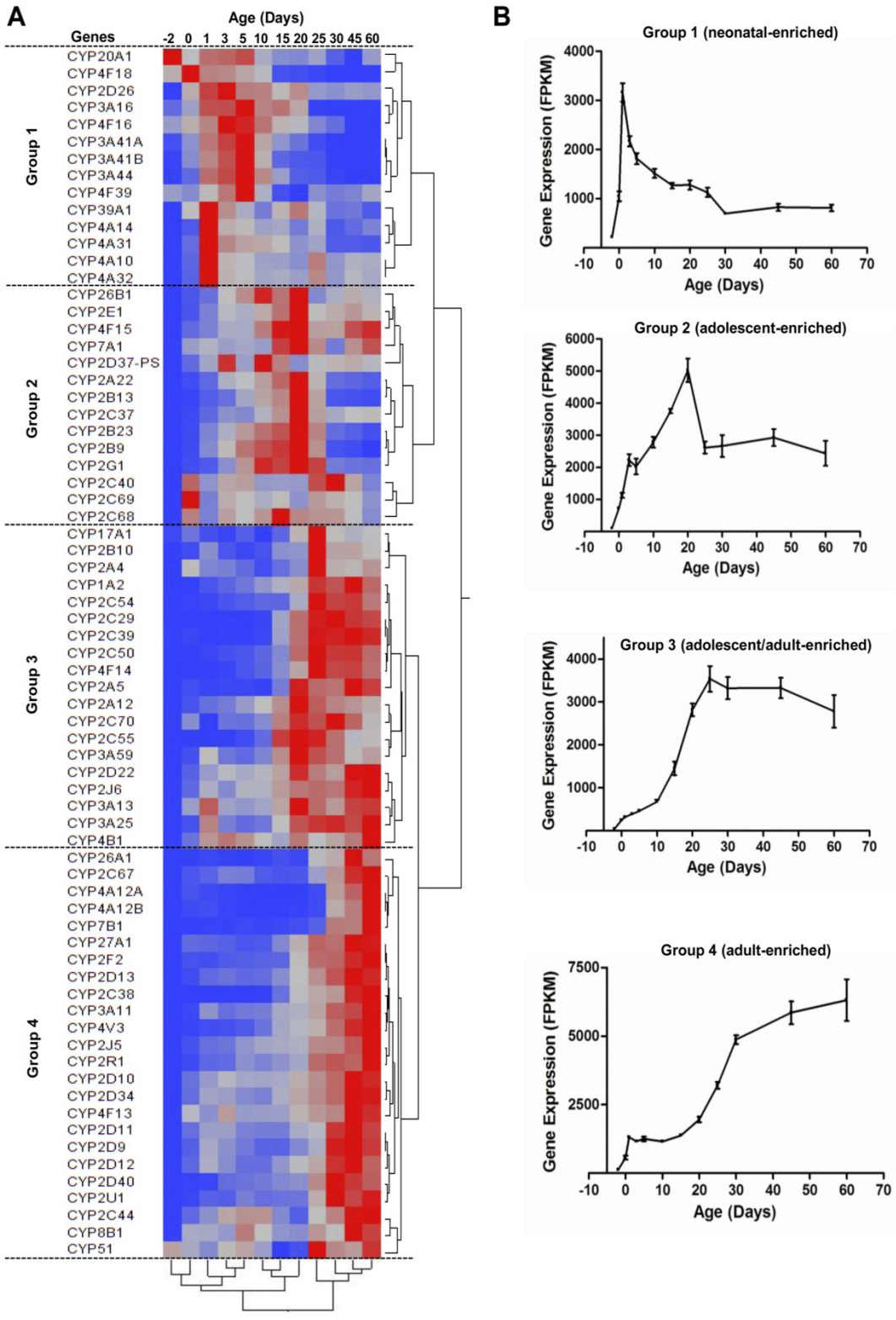

A
Genes
Age (Days)
-2 0 1 3 5 10 15 20 25 30 45 60
Group 1
CYP20A1
CYP4F18
CYP2D26
CYP3A16
CYP4F16
CYP3A41A
CYP3A41B
CYP3A44
CYP4F39
CYP39A1
CYP4A14
CYP4A31
CYP4A10
CYP4A32
Group 2
CYP26B1
CYP2E1
CYP4F15
CYP7A1
CYP2D37-PS
CYP2A22
CYP2B13
CYP2C37
CYP2B23
CYP2B9
CYP2G1
CYP2C40
CYP2C69
CYP2C68
Group 3
CYP17A1
CYP2B10
CYP2A4
CYP1A2
CYP2C54
CYP2C29
CYP2C39
CYP2C50
CYP4F14
CYP2A5
CYP2A12
CYP2C70
CYP2C55
CYP3A59
CYP2D22
CYP2J6
CYP3A13
CYP3A25
CYP4B1
Group 4
CYP26A1
CYP2C67
CYP4A12A
CYP4A12B
CYP7B1
CYP27A1
CYP2F2
CYP2D13
CYP2C38
CYP3A11
CYP4V3
CYP2J5
CYP2R1
CYP2D10
CYP2D34
CYP4F13
CYP2D11
CYP2D9
CYP2D12
CYP2D40
CYP2U1
CYP2C44
CYP8B1
CYP51
B
Group 1 (neonatal-enriched)
Gene Expression (FPKM)
Age (Days)
Group 2 (adolescent-enriched)
Gene Expression (FPKM)
Age (Days)
Group 3 (adolescent/adult-enriched)
Gene Expression (FPKM)
Age (Days)
Group 4 (adult-enriched)
Gene Expression (FPKM)
Age (Days)

Fig. 3.2 Expression Patterns of P450 Genes during Liver Development.

(A) Hierarchical clustering of expression profiles for 71 significantly expressed P450 mRNAs in livers of male C57BL/6 mice. Significant expression was determined by the FDR-BH with a threshold of _0.05. 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 lines categorize the expression profiles into four groups. (B) Changes in total FPKM values of the P450 mRNAs in each group on the basis of the hierarchical clustering in A through different ages during development. Bars represent the mean ± S.E.M. of three individual samples.

CYP2E subfamily. The CYP2E subfamily has only one member, CYP2E1, which was one of the most abundant P450s expressed in liver. CYP2E1 increased sharply from day -2 to day 5, and then continually increased to a peak at day 20, followed by a decrease and then increase to a consistently high level thereafter (Fig. 3.3F).

CYP2F subfamily. The CYP2F subfamily also contains only one member, CYP2F2, which was expressed at a low level until 15 days of age and thereafter gradually increased (Fig. 3.3G).

CYP3A subfamily. The CYP3A subfamily includes 9 members, CYP3A11, -13, -16, -25, -41A, -41B, -44, -57, and -59. CYP3A11 was the most abundantly expressed gene in this subfamily, which gradually increased throughout postnatal development (Fig. 3.3H). Several CYP3A members, including CYP3A16, -41A, -41B, and -44, were neonatal-enriched with the highest expressed levels around 5-days of age (Fig. 3.3H).

CYP4A subfamily. The CYP4A subfamily has 8 members, CYP4A10, -12A, -12B, -14, -29-ps, -30b-ps, -31, and -32. Four members in this subfamily, CYP4A10, -14, -31, and -32, had a neonatal-enriched expression pattern with the highest expression at 1 day of age. The other two members, CYP4A12A and CYP4A12B, were not expressed until day 30, and then increased rapidly in adult mice (Fig. 3.3I).

CYP4F subfamily. The CYP4F subfamily contains 9 members, CYP4F13, -14, -15, -16, -17, -18, -39, -40, and -41-ps. CYP4F13, -14, and -15 were expressed in liver and they appeared to have an adolescent or adolescent/adult enriched pattern (Fig. 3.3J). CYP4F16, -18, and -39 were also expressed, but at very low levels.

Ontogeny of Known Transcript Variants of P450s during Liver Development

Among the 103 mouse P450 genes, six have known alternative transcripts annotated in the NCBI database, including CYP1A1 (two isoforms), CYP2C44 (two isoforms), CYP2C50 (three

Fig. 3.3

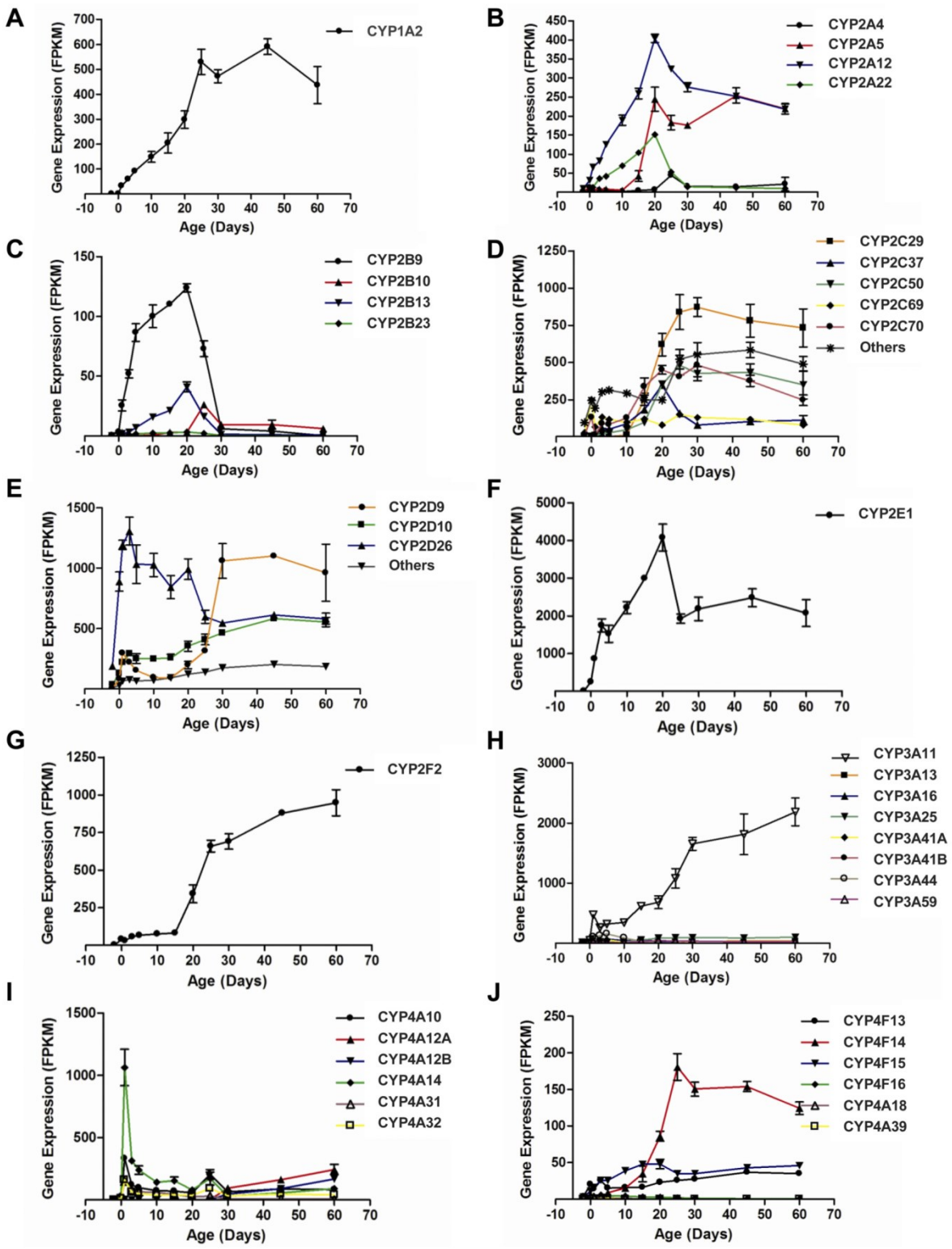

A
CYP1A2
B
CYP2A4
CYP2A5
CYP2A12
CYP2A22
C
CYP2B9
CYP2B10
CYP2B13
CYP2B23
D
CYP2C29
CYP2C37
CYP2C50
CYP2C69
CYP2C70
Others
E
CYP2D9
CYP2D10
CYP2D26
Others
F
CYP2E1
G
CYP2F2
H
CYP3A11
CYP3A13
CYP3A16
CYP3A25
CYP3A41A
CYP3A41B
CYP3A44
CYP3A59
I
CYP4A10
CYP4A12A
CYP4A12B
CYP4A14
CYP4A31
CYP4A32
J
CYP4F13
CYP4F14
CYP4F15
CYP4F16
CYP4A18
CYP4A39
Gene Expression (FPKM)
Age (Days)

Fig. 3.3 Expression Patterns of the Selected P450 Subfamilies during Liver Development.

(A) CYP1A; (B) CYP2A; (C) CYP2B; (D) CYP2C; (E) CYP2D; (F) CYP2E; (G) CYP2F; (H) CYP3A; (I) CYP4A; and (J) CYP4F. Data are presented as mean FPKM ± S.E.M. of three individual samples.

isoforms), CYP3A25 (two isoforms), and CYP26B1 (two isoforms). CYP1A1 was expressed very lowly in liver. RNA-Seq was able to provide the ontogenic patterns of the annotated alternative transcripts of the five P450 genes that were significantly expressed in liver (Fig. 3.4A-E).

For the two CYP2C44 transcripts, NM_001167905 lacks the last exon of NM_001001446 and has an alternative polyadenylation site. NM_001001446 was the major isoform in mouse liver throughout development. Its mRNA was high at birth and reached a peak around 5 and 10 days of age, decreased between 10 and 20 days of age, and increased again and reached a high expression plateau after 45-days of age (Fig. 3.4A). NM_001167905 was not expressed throughout liver development.

Cyp2c50 has three transcripts. NM_001167875 lacks the fifth exon of NM_134144. NM_001167877 is a truncated isoform with the coding region containing only the first 4 exons of NM_134144. NM_134144 was the major isoform in mouse liver throughout development. Its expression profile was a typical Group 3 pattern (adolescent/adult-enriched) (Fig. 3.4B). NM_001167875 and NM_001167877 were very lowly expressed throughout liver development.

The two CYP2D22 transcripts encode the same protein, but NM_019823 has 6 less nucleotides at the end of the first exon than NM_001163472, which results in a shorter 5'-UTR. NM_019823 was the major transcript of Cyp2d22 expressed throughout liver development. The mRNA of NM_019823 increased rapidly from 2 days before birth to 3 days after birth, and then remained relatively consistent with a small peak at 20-days of age. The mRNA of NM_001163472 remained consistently low at all ages (Fig. 3.4C).

Cyp3a25 has two transcripts. NR_030782 is a non-coding RNA with a skipped sixth exon of NM_019792. The expression profiles of these two transcripts were similar during development, but NM_019792 had higher expression levels at all postnatal ages. Both transcripts had a "day

Fig. 3.4

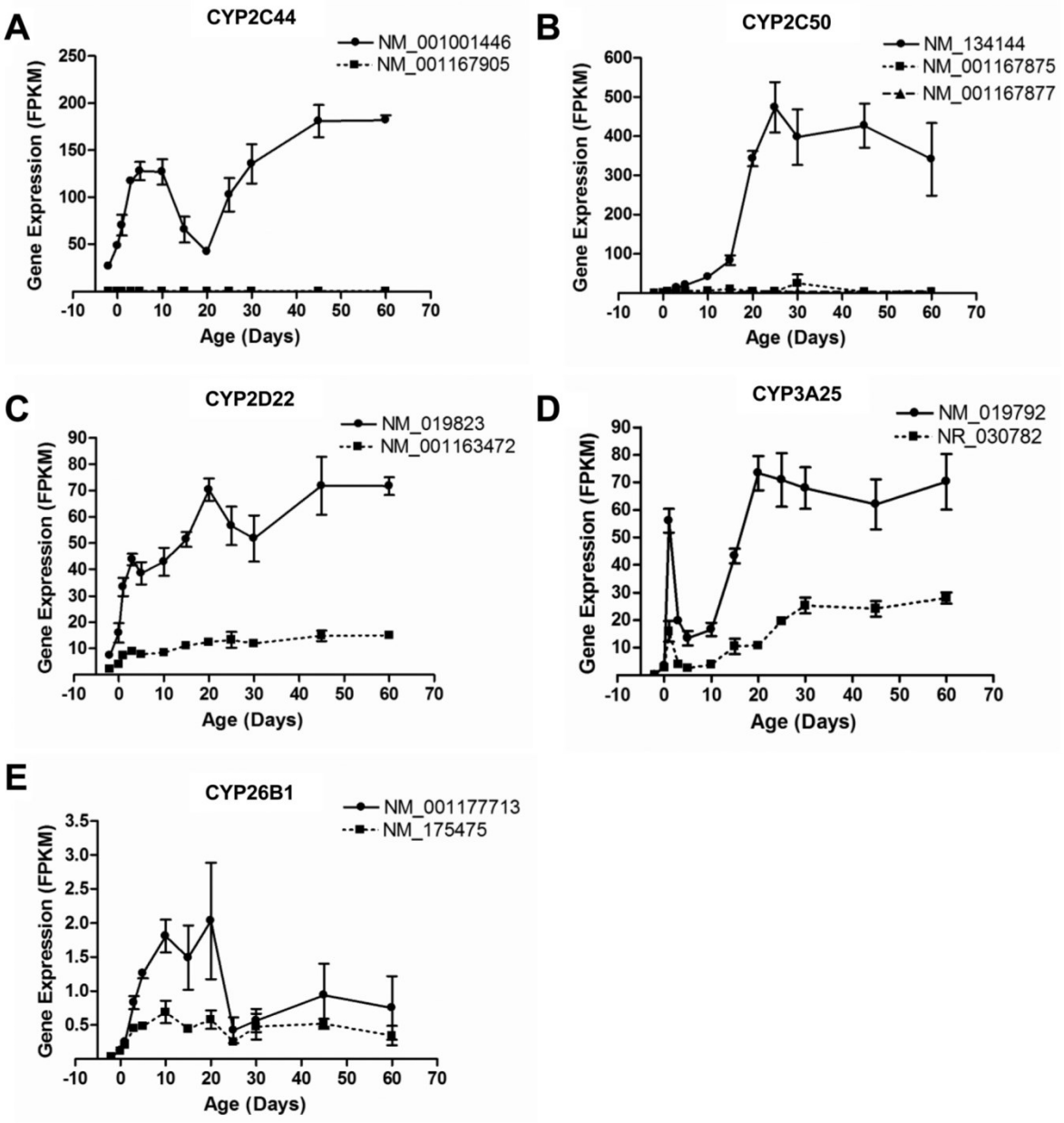


Fig. 3.4 The mRNAs of Known Alternative Transcripts of Five P450s that Were Significantly Expressed during Liver Development.

(A) CYP2C44; (B) CYP2C50; (C) CYP2D22; (D) CYP3A25; and (E) CYP26B1. The transcripts are labeled with their NCBI transcript identification number. Data are presented as mean FPKM ± S.E.M. of three individual samples.

1 surge", which decreased markedly by 5 days of age and then rapidly increased between 10 and 20 days of age, and reached a plateau at 20 days of age (Fig. 3.4D).

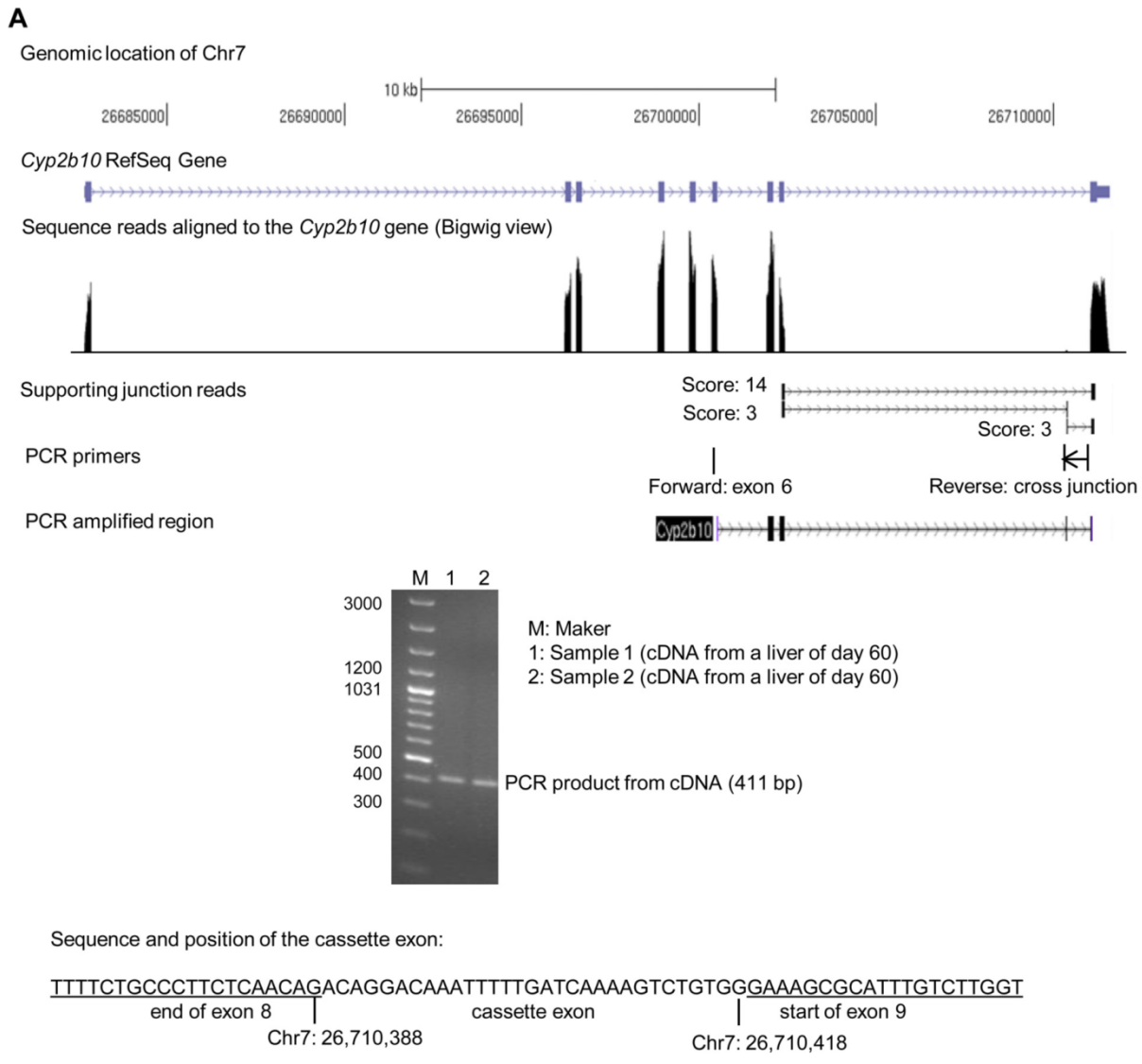
The two CYP26B1 transcripts encode the same protein, but have different transcription start sites. NM_001177713 is shorter than NM_175475 in the 5'-UTR. Both transcripts were expressed at very low levels during mouse liver development (Fig. 3.4E).

Identification of Novel Transcripts of P450s in Liver by RNA-Seq

Potential novel transcripts of P450s, which are not annotated in the NCBI database, were identified by examining sequencing results in the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway). Alignment results from TopHat were converted to BigWig files and uploaded to the browser to display the distribution of reads in the genome. Junction files were also used to determine alternative splicing events. In the browser, each junction consists of two connected blocks, where each block is as long as the maximal overhang of any read spanning the junction. The score is the number of alignments spanning the junction.

The *Cyp2b10* RefSeq gene in assembly of NCBI37/mm9 was located on chromosome 7 between 26,682,683 and 26,711,643 with 9 exons. A tiny peak of read alignment between the peaks of exon 8 and 9 was observed around chromosome position 26,710,000 in the Bigwig view (Fig. 3.5A). Two junctions with a score of 3 indicated alternative splicing events that could generate a cassette exon in this region. The cassette exon was validated by endpoint PCR using cDNAs from two liver samples of 60-day-old mice. The forward PCR primer mapped to exon 6 and the reverse PCR primer mapped across the junction between the cassette exon and exon 9 (Table 3.1), which produced an amplicon containing the cassette exon with a size of 411 bp in all samples examined (Fig. 3.5A). Sequencing of the PCR product revealed the presence of a 31-bp cassette exon, which could alter the protein translation reading frame when retained in the transcript.

Fig. 3.5



Sequence and position of the cassette exon:

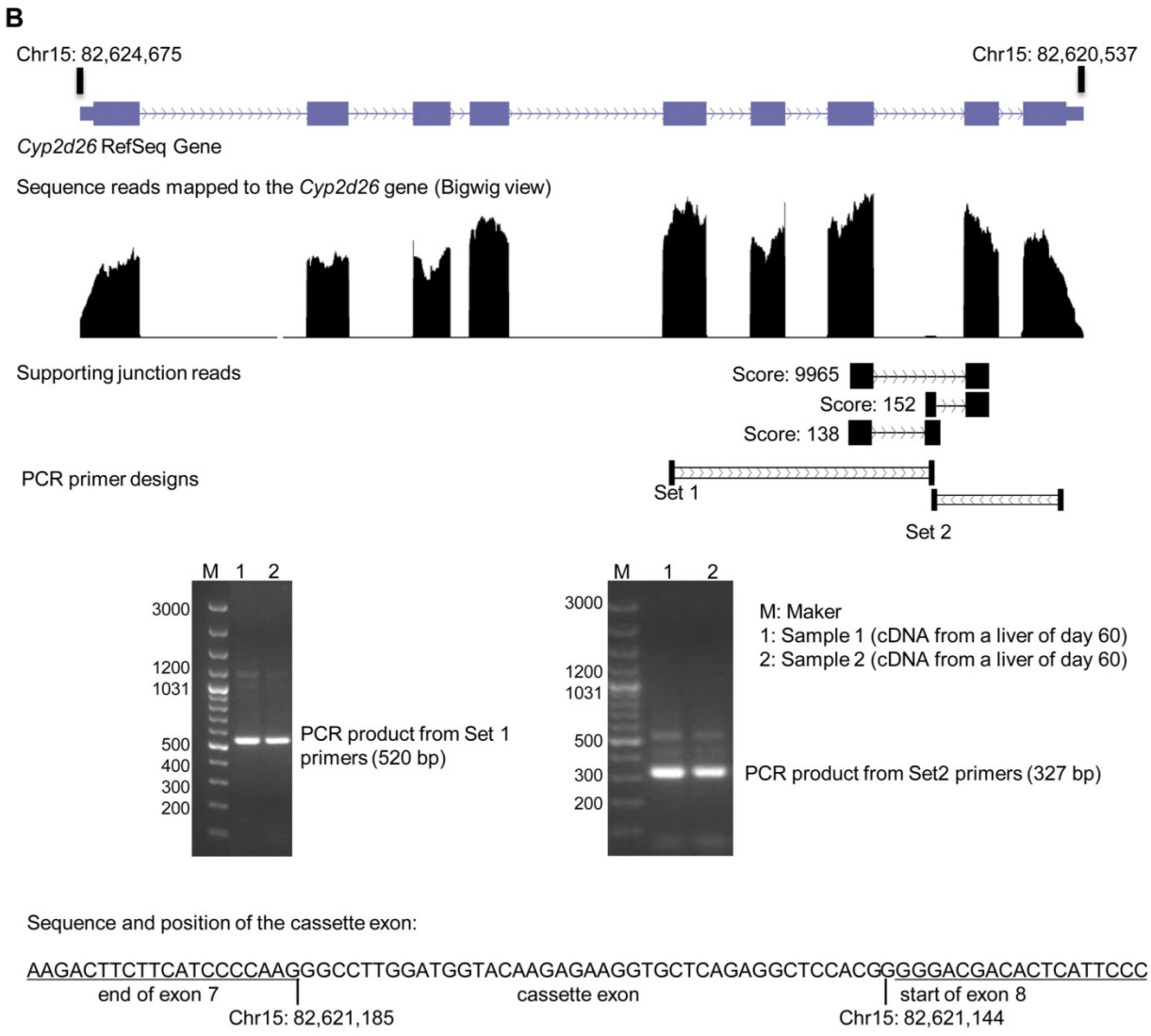
TTTTCTGCCCTTCTCAACAGACAGGACAAATTTTTGATCAAAAGTCTGTGGGAAAGCGCATTTGTCTTGGT

end of exon 8 | cassette exon | start of exon 9

Chr7: 26,710,388

Chr7: 26,710,418

Fig. 3.5



Sequence and position of the cassette exon:

AAGACTTCTTCATCCCCAAGGGCCTTGGATGGTACAAGAGAAGGTGCTCAGAGGCTCCACGGGGGACGACACTCATTCCC

end of exon 7 | cassette exon | start of exon 8

Chr15: 82,621,185 Chr15: 82,621,144

Fig. 3.5

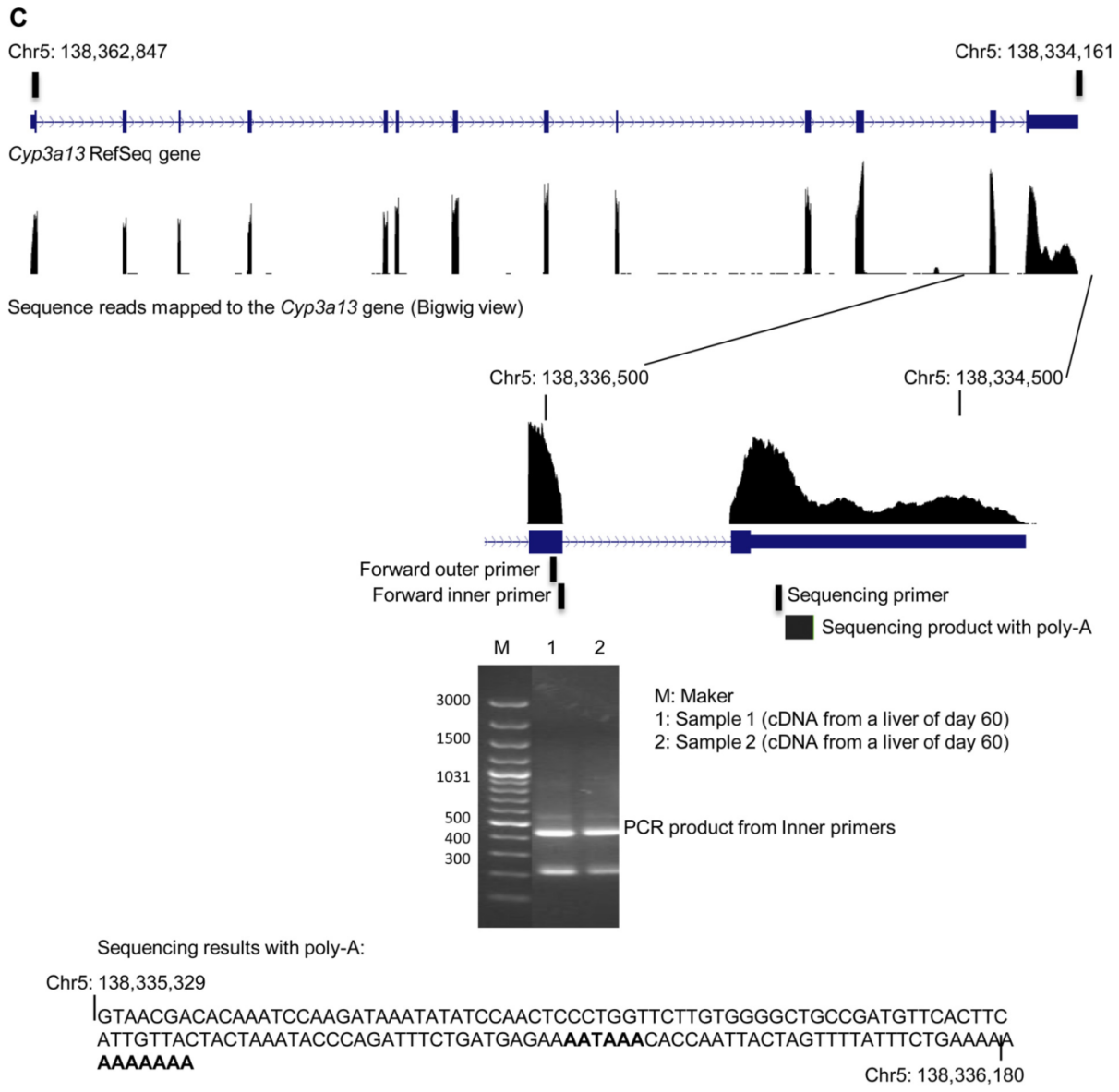

C
Chr5: 138,362,847
Chr5: 138,334,161
Cyp3a13 RefSeq gene
Sequence reads mapped to the Cyp3a13 gene (Bigwig view)
Chr5: 138,336,500
Chr5: 138,334,500
Forward outer primer
Forward inner primer
Sequencing primer
Sequencing product with poly-A
M 1 2
3000
1500
1031
500
400
300
M: Maker
1: Sample 1 (cDNA from a liver of day 60)
2: Sample 2 (cDNA from a liver of day 60)
PCR product from Inner primers
Sequencing results with poly-A:
Chr5: 138,335,329
GTAACGACACAAATCCAAGATAAATATATCCAACTCCCTGGTTCTTGTGGGGCTGCCGATGTTCACTTC
ATTGTTACTACTAAATACCCAGATTTCTGATGAGAAAATAAACACCAATTACTAGTTTTATTTCTGAAAAA
AAAAAAA
Chr5: 138,336,180

Fig. 3.5 Identification of Novel Transcripts of P450s in Liver by RNA-Seq.

(A) Identification of a cassette exon in CYP2B10 mRNA. (B) Identification of a cassette exon in CYP2D26 mRNA. (C) Identification of an alternative polyadenylation site in CYP3A13 mRNA. RNA-Seq reads of a day 60 sample were aligned to the reference genome mm9 by TopHat and viewed as distribution peaks by the UCSC genome browser. Output junction reads from TopHat are also shown. The cassette exons or alternative polyadenylation was validated by endpoint PCR or 3'-RACE. The PCR products are shown by gel electrophoresis and further sequenced by single-pass DNA sequencing with a designed primer. The sequence was aligned back to the P450 gene regions.

A similar cassette exon peak and alternative splicing junctions were also found in the browser view of the *Cyp2d26* gene, which is located on the minus strand of chromosome 15 between 82,620,537 and 82,624,675 (Fig. 3.5B). The cassette exon was located between exon 7 and 8, and validated by endpoint PCR with two sets of primers. Primer set 1 bound to exon 5 and the cassette exon, which amplified a fragment with an expected size of 520 bp. Primer set 2 bound to the cassette exon and exon 9, which amplified a fragment with an expected size of 327 bp. The cassette exon sequence contained 42 nucleotides, which would change the protein product if retained in the transcript.

Thirteen peaks of sequencing reads were observed along the RefSeq *Cyp3a13* gene. The first twelve exons were covered by a single peak. However, in exon 13, a region with high-level reads followed by a region with low-level reads was found. A sharp decrease in the sequencing reads occurred around chromosome position 138,335,200 in the 3'-UTR of *Cyp3a13* (Fig. 3.5C). The RNA-Seq reads ended at coordinate 138,334,161 of chromosome 5, which matched with the termination of this gene in the reference sequence. This suggested an alternative polyadenylated mRNA transcript in the 3'-UTR region of *Cyp3a13*. To validate the shorter 3'-UTR transcript, we performed 3'RACE-PCR with two RNA samples from livers of 60-day old mice. The forward PCR primers, shown in Fig. 3.5C, together with reverse primers provided by the RLM-RACE kit, generated a band that was sequenced using the *Cyp3a13*-specific sequencing primer (Table 3.1). Sequencing results revealed a poly(A)-containing sequence that uniquely mapped to the 3'-UTR of *Cyp3a13*. Termination of the shorter 3'-UTR transcript occurred at chromosome location 138, 336,180, which was more than 2kb upstream of the end of the reference gene sequence. In addition, an AATAAA sequence was noted at 23 bases upstream of the termination site, which might serve as a recognition signal for the poly(A) polymerase complex.

3.5 Discussion

In the current study, RNA-Seq was used to truly quantify mRNA abundance of the 103 P450 genes in mouse liver during postnatal development. Compared to other commonly used methods for RNA quantification, such as microarray, branched DNA, or real-time PCR, RNA-Seq directly counts sequence reads of the nucleotide molecules in biological samples, which is the only method for true quantification of mRNA expression. Expression of a transcript is represented by FPKM (fragments per kilobase of exon per million reads mapped), which normalizes sequencing depths between different samples and sizes between various genes, allowing direct comparison of mRNAs among various transcripts in a genome-wide scale. As shown in Fig. 3.1, the quantitative abundance of mRNAs encoded by the 103 P450 genes was determined in mice at 12 ages, which has not been reported previously.

From perinatal through neonatal to adult life, the total FPKM values of all P450 genes increased about 25 fold with two rapidly increasing stages (Fig. 3.1A). The first surge occurred from 2 days before birth to 1 day after birth. Not only the total abundance, but also the composition of expressed P450s was markedly altered during this period, when the pup was born and lost the direct physiological connection with the mother. Multiple hormones were known to influence the expression of drug metabolizing enzymes. During pregnancy, maternal hormones cross the placenta into the baby. After birth, the loss of maternal hormones may be a contributing factor to the changes in the expression of some P450s. The new born mice also began to be exposed to the environment, which contains all kinds of xenobiotics. A critical function of P450s is xenobiotic metabolism, thus the first surge of P450 expression may provide protections to the newborns against environmental toxic compounds. The second surge occurred from day 10 to day 20, with a rapid increase in abundance of the P450 mRNAs. But the expression of each P450 was similar from 10 to 20 days of age, as the expression of most P450s increased simultaneously. This period is also the most rapidly growing stage of postnatal

liver development, as studies have shown that cell proliferation in mouse liver is high until 20 days of age, and then the hepatic architecture begins to resemble the adult liver (Apte et al., 2007).

Hierarchical clustering of significantly expressed P450s revealed four hepatic ontogenic expression patterns (Fig. 3.2). The expression profiles of these patterns were similar to a previous study of P450 ontogeny in mice (Hart et al., 2009), with some genes enriched at younger ages and some enriched at older ages. However, the previous study examined only a few P450s, whereas the present study included all the expressed P450s in the liver of mice, and therefore generated a more comprehensive summary of ontogenic patterns. Whereas, the earlier study concluded there were three patterns of P450 ontogeny in livers of mice (Hart et al., 2009), the present study concludes there are four patterns. Clustering of the P450s from 12 developmental ages indicates that the largest correlation distance with respect to neighboring ages is between 20 and 25 days of age, which spanned the period when the diet changes from milk to chow. Because P450s have important functions in metabolizing chemicals absorbed from the gastrointestinal tract, it is reasonable to anticipate this change in P450 expression with a change in food intake.

The most highly expressed P450 mRNAs in liver of mice were from the CYP1-4 families, which belong to the xenobiotic-metabolizing P450s. They displayed changes in expression during development. Some P450 subfamilies were primarily expressed during the neonatal stage, whereas others were mainly expressed during the adolescent or adult stage of development. Even within each subfamily, individual members were expressed with various developmental patterns. In the CYP2D subfamily, CYP2D26 mRNA reached its maximum expression during the neonatal period, whereas CYP2D9 reached its maximum expression during the adolescent/adult period (Fig. 3.3E). Similarly in the CYP3A subfamily, CYP3A16, CYP3A41A, CYP3A41B, and CYP3A44 were maximally expressed during the neonatal period,

whereas CYP3A11 was maximally expressed in the adult mice (Fig. 3.3H). A similar developmental switch also exists in human CYP3A enzymes (Schuetz et al., 1994; Stevens et al., 2003; Leeder et al., 2005). Thus mice might serve as a good model to study the mechanisms controlling the developmental switches of P450 expression in liver. Of note, it is difficult to directly compare our data in mice with other previously published data in humans. Our study determined levels of mRNA transcripts of P450s, whereas most other published studies determined protein concentrations and enzyme activities of human P450s.

Because RNA-Seq has the ability to examine the transcriptome at the resolution of a single base (Wang et al., 2009), it can quantify the mRNAs of genes as well as their individual transcript variants (Fig. 3.4). The known transcript variants of the P450s might be translated into different proteins, as in CYP2C44 and CYP2C50, or those that differ only in the UTR regions of the transcripts, as in CYP2D22 and CYP26B1. Because transcript variants can alter protein functions, mRNA stability, subcellular localization, and translation efficiency of the transcripts (Pesole et al., 2000), identification of the predominant transcripts of P450s and their developmental expression patterns might help to predict variations in protein activity.

Research has shown that 92-94% of human genes undergo alternative splicing, promoter usage, and polyadenylation (Wang et al., 2008). Only six out of the 103 mouse P450 genes in the NCBI database have annotated transcript variants, so alternative splicing P450 transcripts in mice is probably an underexplored area. With the advantage of RNA-Seq, the present study identified several novel transcript variants in CYP2B10 and CYP2D26, even though they are expressed at very low levels (very small peaks in BigWig view of Fig. 3.5A and 3.5B). When the cassette exons are translated, the resultant P450 enzymes will have different protein sequences, which might lead to a difference in enzyme activities. The CYP3A13 transcripts, with the shorter 3'-UTR, appeared to be the predominant transcript of this gene. A similar shorter 3'-UTR transcript has been reported for the human *CYP3A4* gene (Li et al., 2012). Although the

CYP3A4 transcript with shorter 3'-UTR doesn't change amino acid sequences, it is more stable and can translate to protein more efficiently than the CYP3A4 transcript with the longer 3'-UTR (Li et al., 2012). The mouse *Cyp3a13* gene might serve as a model to study how alternative polyadenylation events are regulated. Further studies are needed to investigate the functional significance of these novel transcripts.

P450 proteins and enzyme activities were not determined in this study due to technique limitations. Specific antibodies against most of the mouse P450 proteins, specific substrates, and inhibitors of many individual mouse P450 enzymes are not available. Technological breakthroughs in proteomics and metabolomics are essential to study the ontogeny of P450 proteins.

In summary, the present study describes the ontogeny of the mRNAs of all P450s in the mouse liver during development. These data provide the fundamental knowledge for studying mechanisms of regulation of the transcription of P450 genes in liver during development. A previous study from our laboratories indicates that the developmental regulation of *Cyp3a* gene expression in mouse liver may be due to dynamic changes of histone modifications during postnatal liver maturation (Li et al., 2009b). No doubt, epigenetics and various hepatic transcription factors play an important role in regulating ontogenic expression of P450 enzymes (Kyrmizi et al., 2006). Studies on the networking of transcription factors and epigenetic signatures in liver may shed light on the regulatory mechanisms of the observed P450 developmental patterns. These mechanisms may be of considerable importance in understanding the kinetics of xenobiotic metabolism during the neonatal period.

Chapter 4 : RNA-Sequencing Quantification of Hepatic Ontogeny of Phase-I Enzymes in Mice

4.1 Abstract

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

4.2 Introduction

Drug-metabolizing enzymes play a central role in the elimination of drugs from the body. The metabolic reactions of hydrolysis, reduction, and oxidation usually introduce a small functional group to the substrate or convert an existing functional group to a new group that may be further conjugated with a large and bulky water-soluble co-substrate. The reactions associated with hydrolysis, reduction, and oxidation are often called Phase-I reactions, and the reactions associated with conjugation are often referred as Phase-II reactions.

Non-P450 Oxidative Enzymes

The oxidation of alcohols to aldehydes and carboxylic acids is sequentially catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase. ADHs are a group of zinc-containing, cytosolic enzymes that facilitate the interconversion between alcohols and aldehydes (or ketones) with the reduction of NAD^+ to NADH, or oxidation of NADH to NAD^+. In humans, ADH isozymes in family 1 are responsible for the oxidation of ethanol and other small, aliphatic alcohols, whereas ADH4 preferentially oxidizes large aliphatic and aromatic alcohols. The substrates for ADH5 are preferentially long-chain alcohols, aromatic alcohols and also include formaldehyde. The function of ADH6 is not clear, and ADH7 is not expressed in adult human liver. Different families of ALDHs have different subcellular locations, and the ALDHs that are responsible for oxidizing simple aldehyde, such as the metabolite of ethanol, are mitochondrial enzymes.

Molybdozymes are flavoprotein enzymes consisting of two ~150 kDa subunits, each of which contains FAD, molybdenum, and two iron–sulfur (Fe_2S_2) centers. There are two major molybdozymes that participate in the biotransformation of xenobiotics: aldehyde oxidase and xanthine oxidoreductase. In contrast to P450s, which generally catalyze the oxidation of carbon

atoms with a high electron density, AOX and XDH catalyze the oxidation of electron-deficient carbons (i.e., double-bonded carbon atoms in nitrogen heterocycles). XDH contributes significantly to the first-pass elimination of several purine derivatives (e.g., 6-mercaptopurine and 2, 6-dithiopurine), and limits the therapeutic effects of these cancer chemotherapeutic agents. Physiologically, XDH catalyzes the sequential oxidation of hypoxanthine to xanthine and uric acid. A rare genetic disorder in XDH can cause xanthinuria, with the accumulation of xanthine. AOX plays an important physiological role in the catabolism of biogenic amines and catecholamines. Molybdozymes are located in the cytosol, and they transfer electrons to molecular oxygen, which can generate reactive oxygen species and lead to oxidative stress and lipid peroxidation.

Flavin-containing monooxygenases are a group of enzymes that catalyze chemical reactions via the bound cofactor flavin. These reactions involve oxidation of heteroatoms, particularly nucleophilic atoms such as the nitrogen of amines and the sulfur of thiols. FMOs and P450s have several properties in common. They are both micorosomal enzymes that require NADPH and O_2, and many of the reactions catalyzed by FMOs can also be catalyzed by P450s. To distinguish FMO and P450 *in vitro*, it has been noticed that FMOs are more heat labile and catalyze reaction at optimum pH (8-10) higher than that for most P450 (pH 7-8). The regulatory controls for FMOs in liver microsomes are different from P450s. Phenobarbital treatment is found to suppress FMO1 expression in rats while it induces P450s. Additionally, the AhR agonist TCDD induces FMO2 and FMO3 mRNA levels in mice (Tijet et al., 2006). Several other oxidative Phase-I enzymes are also involved in the biotransformation of xenobiotics, such as amine oxidases in brain and peroxidases in white blood cells, but they are either not expressed or not functionally critical in liver, so are not discussed in this thesis.

Phase-I Enzymes in Reduction

A variety of xenobiotics contain a carbonyl function (R-CHO and R_1-CO-R_2) that undergoes reduction *in vivo*, which is generally catalyzed by NAD(P)H-dependent reductases that belong to one of two superfamilies, the aldo-keto reductases and the short-chain dehydrogenases/ reductases (SDRs). The cytosolic and microsomal carbonyl reductase of SDRs in human play a role in the reduction of carbonyl-containing xenobiotics. The AKRs are a superfamily of cytosolic enzymes that reduce both xenobiotic and endobiotic compounds. Various members of the AKR family have been implicated in the reduction of carbonyl-containing xenobiotics, such as acetohexamide, befunolol, haloperidol and numerous aromatic and aliphatic aldehydes (Rosemond and Walsh, 2004). AKR7A is one of the AKRs induced by Nrf2 following oxidative stress, exposure to electrophiles, or depletion of glutathione. The AKRs mainly function in reduction, but may also have a role as oxidizing enzymes in dihydrodiol dehydrogenation (Burczynski and Penning, 2000).

Quinone oxidoreductases are cytosolic flavoproteins catalyzing the reduction of quinones to hydroquinones. There are two closely related NQOs in human, NQO1 and NQO2, which have different substrate specificities, and can be distinguished on their differential inhibition by dicoumarol and quercetin, respectively. When quinones undergo one-electron reduction by POR to semiquinone radicals, the reactive metabolites can cause oxidative stress by reacting with oxygen to form reactive oxygen species and lead to damage to proteins and DNA through alkylation (Bolton et al., 2000). In contrast, the two-electron reduction of quinones by NQOs produces hydroquinones, which are usually stable enough to undergo glucuronidation or sulfonation. The role of NQOs as a protective antioxidant or a pro-oxidant activator depends on the properties of the resultant hydroquinone, which may undergo one-electron oxidation to form the reactive semiquinone. This has been the mechanism of certain anticancer agents like mitomycin C, because NQO1 is often overexpressed in tumor cells. Antioxidant response

element and xenobiotic response element have been found in the regulatory region of NQO1, which can be induced through AhR activation.

Dihydropyrimidine dehydrogenase is an NADPH-requiring homodimeric protein located mainly in liver cytosol, where it catalyzes the reduction of anticancer drug 5-fluorouracil and related pyrimidines. One metabolite of the antiviral Sorivudine can covalently bind to DPYD and irreversibly inactivate the enzyme. Therefore, co-administration of 5-fluorouracil with Sorivudine may increase the blood concentration of 5-fluorouracil to toxic or even lethal levels (Kanamitsu et al., 2000). Several genetic polymorphisms also result in a partial or complete loss of DPYD activity, and assessing an individual's DPYD genotype (DNA sequence) or phenotype (DPYD activity in peripheral blood cells) is recommended before 5-fluorouracil therapy to adjust the dosage on an individual basis.

Phase-I Enzymes in Hydrolysis

The hydrolysis of xenobiotic esters and amides in humans is largely catalyzed by two carboxylesterases called CES1 and CES2, both highly expressed in the liver and mainly associated with the endoplasmic reticulum. CES1 generally catalyzes the hydrolysis of xenobiotics with a small alcoholic leaving group (as in the case of delapril), while CES2 generally catalyzes the hydrolysis of xenobiotics with a small acidic or large alcoholic leaving group (as in the case of procaine), although there are also overlapping substrates for both enzymes. The hydrolysis of irinotecan by CES2 produces the active agent SN-38, which is responsible the anticancer and toxic effects of irinotecan. High levels of CES2 in the intestine are, at least in part, causing SN-38 generation and diarrhea after irinotecan treatment (Satoh et al., 2002). In addition to hydrolyzing xenobiotics, CES hydrolyze numerous endogenous compounds, such as palmitol-CoA, retinyl ester, platelet-activating factor and other esterified lipids.

Paraoxonases catalyze the hydrolysis of a broad range of organophosphates, organophosphinites, aromatic carboxylic acid esters, cyclic carbonates, and lactones. Both humans and mice express three paraoxonases, namely PON1, PON2 and PON3. PON1 and PON3 are present in liver microsomes and plasma, while PON2 is expressed in several tissues but not present in plasma. Plasma PON1 has been suggested to protect against atherosclerosis by hydrolyzing specific derivatives of oxidized cholesterol or phospholipids in atherosclerotic lesions. And PON1 knockout mice are predisposed to atherogenesis (Draganov and La Du, 2004). In addition to differences in tissue distribution, PONs in mice respond differently to oxidative stress, in which PON1 is decreased, PON3 is increased and PON2 is not affected.

Epoxide hydrolases are found in the microsomal fraction of virtually all tissues, catalyzing the trans-addition of water to alkene epoxides and arene oxides, which can form during P450-dependent oxidation of aliphatic alkenes and aromatic hydrocarbons, respectively. EPHXs play an important role in detoxifying electrophilic epoxides that might otherwise bind to proteins and nucleic acids and cause cellular toxicity and genetic mutations, and they are normally distributed parallel with P450s in tissues to presumably ensure the rapid detoxification of epoxides generated in oxidative metabolism. The potentially toxic metabolites are converted to the corresponding dihydrodiols, which are usually less reactive and easier to excrete. In mammals, two forms of EPHX are mainly involved in hydrolyzing xenobiotics, the microsomal epoxide hydrolase encoded by *Ephx1* and the soluble epoxide hydrolase encoded by *Ephx2.* In rodents, EPHX1 is under regulatory control by PPARα and inducible following peroxisome proliferator treatment, whereas EPHX2 is inducible by Nrf2 in response to oxidative stress.

Many prodrugs are designed to be hydrolyzed by hydrolytic enzymes to release the active drug. Enzymes with such function also include cholinesterase (e.g., for propranolol ester) and alkaline phosphatase (e.g., for fosphenyoin). With the development of recombinant DNA technology, numerous peptides, such as peptide hormones and monoclonal antibodies, have

been used as therapeutic agents. And the activity of peptidases is having more and more clinical implications in metabolizing these drugs.

Liver is the major organ for Phase-I drug metabolism, but before birth, the liver functions as a hematopoietic organ. A functional transition occurs in liver after birth, and most of the drug-metabolizing enzymes mature during this period. Changes in expression of some Phase-I enzymes during liver maturation in humans have been reported, including P450s (Stevens et al., 2003; Koukouritaki et al., 2004; Stevens et al., 2008; Croom et al., 2009), CES (Yang et al., 2009; Zhu et al., 2009), PON (Cole et al., 2003; Huen et al., 2009), ADH (Smith et al., 1971), and FMO (Cherrington et al., 1998; Koukouritaki et al., 2002; Hines, 2006). The dynamic changes in the ontogenic expression of these genes are thought to be responsible for the substantial pharmacokinetic differences between newborns and adults, and this contributes to differences in therapeutic efficacy and adverse drug reactions in pediatric patients (Kearns et al., 2003; Blake et al., 2005; Hines, 2007; Hines, 2008; Hines, 2013). An in-depth understanding of the regulatory mechanisms of the ontogeny of Phase-I enzymes is needed for safer and more effective drug therapy for pediatric patients.

Several limitations exist in studies of the developmental regulation of Phase-I enzymes with human samples. The first limitation is the ethical and technical issues in recruiting human subjects and obtaining suitable human samples (Rowell and Zlotkin, 1997). Secondly, variations in human metabolic capacity, which may be caused by genetic or environmental factors, can interfere with studies aimed to reveal the regulatory mechanisms that are only due to age. Furthermore, mechanistic loss-of-function or gain-of-function strategies are not applicable directly in human samples. Animal models are advantageous in overcoming these limitations. In recent years, mice and rats have surpassed other laboratory animals as the experimental models of choice for the study of physiology, metabolism, and disease (Muruganandan and Sinal, 2008; Hrycay and Bandiera, 2009). Advantages of these models include rapid growth,

easy maintenance, and the development of genetic manipulation techniques for mechanistic studies with gain-of-function and loss-of-function strategies. Several laboratories, including us, have examined the ontogenic gene expression profiles in mouse or rat liver for some Phase-I genes, including P450s (Hart et al., 2009; Cui et al., 2012c), *Ces* (Zhu et al., 2009), *Akr* (Pratt-Hyatt et al., 2013), *Adh* and *Aldh* (Smolen et al., 1990; Alnouti and Klaassen, 2008), *Pon* (Li et al., 1997), and *Fmo* (Falls et al., 1995; Cherrington et al., 1998; Janmohamed et al., 2004). Developmental expression patterns of some Phase-I genes in mice and rats are similar to those in humans.

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

In this report, RNA-Seq was used to systematically quantify the mRNA expression of major non-P450 Phase-I genes in mouse liver during postnatal maturation to define the ontogenic profiles of these mRNAs. The groups included enzymes catalyzing reactions in hydrolysis (carboxylesterase, paraoxonase, and epoxide hydrolase), reduction (aldo-keto reductase, quinone oxidoreductase, and dihydropyrimidine dehydrogenase), and oxidation (alcohol

dehydrogenase, aldehyde dehydrogenase, flavin monooxygenases, molybdenum hydroxylase, and cytochrome P450 oxidoreductase). The purpose of this study was to generate comprehensive information on the ontogeny of mRNAs of Phase-I genes in livers of mice, which will form the foundation for determining the regulatory mechanisms controlling the various transcription patterns of Phase-I genes during liver maturation.

4.3 Material and Methods

Animals, Total RNA Extraction, Sequencing Library Construction, RNA-Seq and RNA-Seq data analysis

The source and breeding of animals, RNA extraction, library construction, RNA-Seq, FASTQ data file collection and RNA-Seq data analysis were the same as those in Chapter 3.

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 ($\log[E(FPKM_i|AGE)] = \beta_0 + \beta_j \,\mathrm{AGE}_{\mathrm{ij}}$ where AGE is an indicator variable representing the 12 ages, j=1..11) and reduced ($\log[E(FPKM_i|AGE)] = \beta_0$) 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.

4.4 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. If the Benjamini-Hochberg adjusted *p* value of the drop-in-deviance F test (FDR-BH) for gene expression in at least one of the 12 ages was less than 0.05 for a Phase-I gene, then that gene was considered to be expressed in liver during maturation. Table 4.1 lists the number of genes in each category that are significantly expressed at various developmental stages of prenatal (day -2), neonatal (day 5), adolescence (day 25), and adult (day 60) as well as the total number of genes expressed during liver development (Day -2 to Day 60) in each Phase-I family. Of the 186 mouse Phase-I genes, 136 genes were expressed in liver during maturation, but only about half of them (64) were expressed in prenatal liver. A significant change in expression of Phase-I enzyme genes was observed around birth. The

Table 4.1 Number of expressed genes in each Phase-I family in mouse liver at a specific age of prenatal (day -2), Neonatal (day 5), adolescence (day 25), and adult (day 60), as well as during development (Day -2 to Day 60)

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

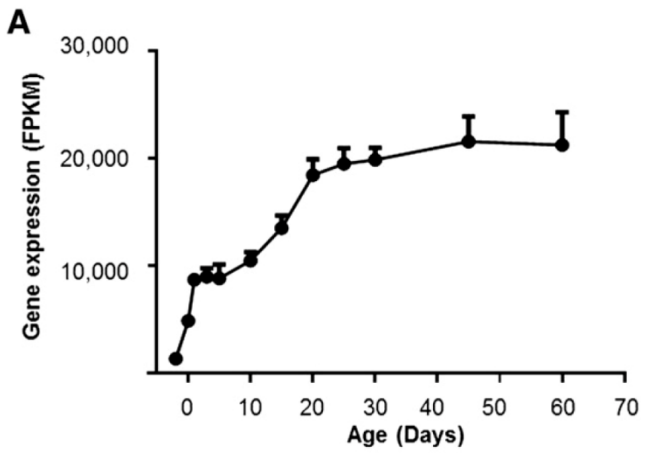
Numbers in the brackets represent total number of genes in the family

number of the expressed genes increased to 112 in neonatal liver at day 5. The total expression levels of all Phase-I mRNAs represented as cumulative FPKM values increased approximately 15 fold during postnatal liver maturation from day -2 (FPKM = 1,383) to day 60 (21,285) with two surges (Fig. 4.1A), the first of which with six fold increase from day -2 (1,383) to day 1 (8,736) and the other with 1.8 fold increase from day 10 (10,511) to day 20 (18,482), with each surge followed by a relatively stable value of cumulative FPKM. Fig. 4.1B shows the composition of the Phase-I families represented as percentages of the total FPKM value for each stage at prenatal (day -2), neonatal (day 5), adolescence (day 25), and adult (day 60). The major Phase-I families expressed in prenatal liver were P450 (35%), *Aldh* (25%), *Akr* (21%), and *Adh* (10%) are. After birth, there is a further increase in the relative proportions of the mRNAs of the P450s (from 35% prenatal levels to about 60% after birth), and other highly expressed Phase-I families include *Ces*, *Adh*, and *Aldh*.

Ontogeny of Genes of Phase-I Enzymes Involved in Hydrolysis Reactions

Carboxylesterases: CESs are enzymes that participate in the hydrolysis and trans-esterification of a variety of esters and amides. Five families of *Ces* genes are annotated in the NCBI reference sequence database for mice. mRNA expression of four families of *Ces* (*Ces1*-*Ces4*) was detected in mouse liver samples. The most abundant family in adult liver was *Ces1*, and it consisted up to 58% of the total liver *Ces* mRNAs at age day 60 (Fig. 4.2A). 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. 4.2B). The *Ces2* family was moderately expressed, with *Ces2a* and *Ces2e* being the major members. The expression of *Ces2a* gradually increased after birth till day 60, and *Ces2e* reached its adult levels at around day 25 (Fig. 4.2C). The *Ces3* family was also highly expressed in adult liver. *Ces3a* and *-3b* exhibited similar developmental patterns with a rapid increase in expression between day 20

Fig. 4.1



Age (Days)	Total FPKM	
	Mean	SEM (n=3)
-2	1,383	46
0	4,917	321
1	8,736	237
3	9,014	465
5	8,871	751
10	10,511	465
15	13,534	674
20	18,482	853
25	19,547	850
30	19,907	645
45	21,605	1347
60	21,285	1756

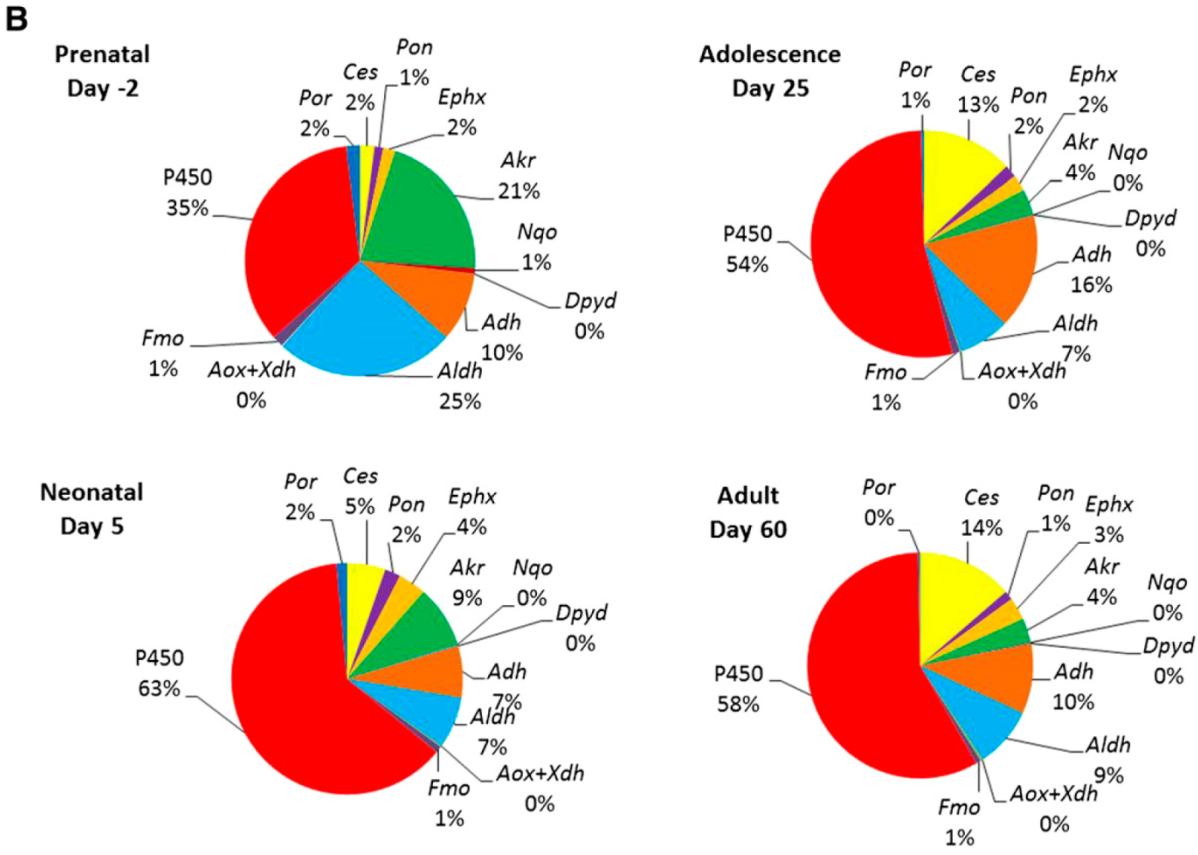


Fig. 4.1 Total Expression and Proportions of Individual Phase-I Families during Liver maturation.

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

and day 30 (Fig. 4.2D). *Ces4* family was expressed at a low level in liver, and it did not show significant difference in expression during development. In total, 13 *Ces* genes had significant changes in expression during development. A two-way hierarchical clustering analysis revealed that the mRNAs of these *Ces* were all low in pre- and neonatal ages, but markedly increased to adult levels around 20-30 days after birth (Fig. 4.2E).

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 *Pon*s 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. 4.3A). At the prenatal age day -2, *Pon2* mRNA level was higher than *Pon1* and *Pon3*. After birth, the *Pon2* mRNA expression first increased till day 5 then decreased till day 25, and then went up again to reach adult level at day 60. *Pon3* had overall increased expressions after birth with small fluctuations at adolescent ages (Fig. 4.3B).

Epoxide Hydrolases: *Ephx*s 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. 4.4).

Ontogeny of Genes of Phase-I Enzymes Involved in Reduction Reactions

Aldo-keto reductases: AKRs are a group of cytosolic enzymes that catalyze the reduction of aldehydes and ketones to primary and secondary alcohols, respectively (Jin and Penning, 2007). Mouse *Akr* genes consist of two families, *Akr1* and *Akr7*, with a total of 16 genes. *Akr1* is the larger family with 5 subfamilies (*Akr1a*-*e*) and 15 genes. The RNA-Seq data showed 14 out of the 16 mouse *Akr* genes were significantly expressed during liver maturation, and all the

Fig. 4.2

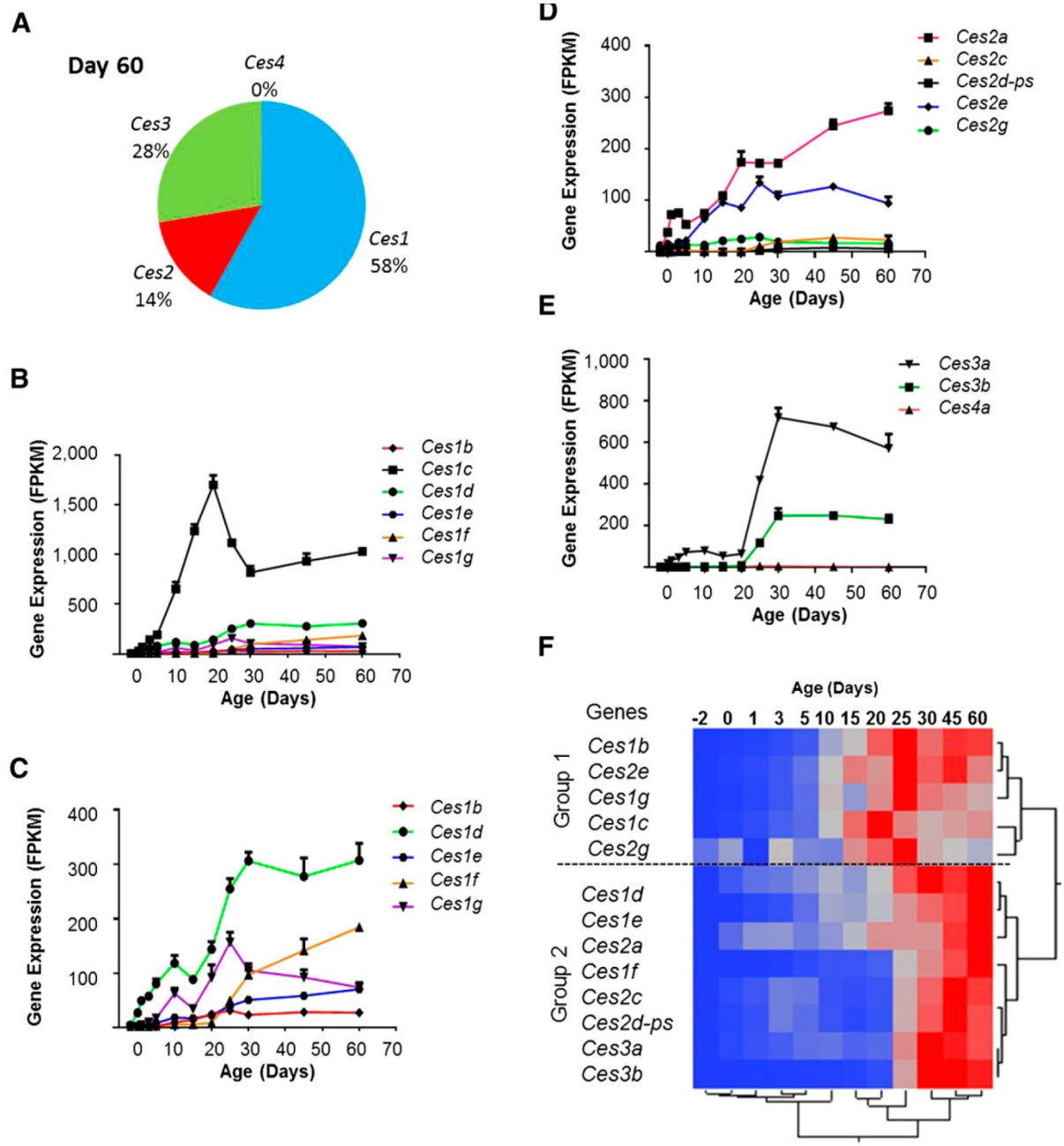

A
Day 60
Ces4
0%
Ces3
28%
Ces1
58%
Ces2
14%
B
Gene Expression (FPKM)
Age (Days)
Ces1b
Ces1c
Ces1d
Ces1e
Ces1f
Ces1g
C
Gene Expression (FPKM)
Age (Days)
Ces1b
Ces1d
Ces1e
Ces1f
Ces1g
D
Gene Expression (FPKM)
Age (Days)
Ces2a
Ces2c
Ces2d-ps
Ces2e
Ces2g
E
Gene Expression (FPKM)
Age (Days)
Ces3a
Ces3b
Ces4a
F
Age (Days)
Genes
-2 0 1 3 5 10 15 20 25 30 45 60
Group 1
Ces1b
Ces2e
Ces1g
Ces1c
Ces2g
Group 2
Ces1d
Ces1e
Ces2a
Ces1f
Ces2c
Ces2d-ps
Ces3a
Ces3b

Fig. 4.2 Expression of *Ces* mRNAs during Liver Development in Male C57BL/6 Mice.

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

Fig. 4.3

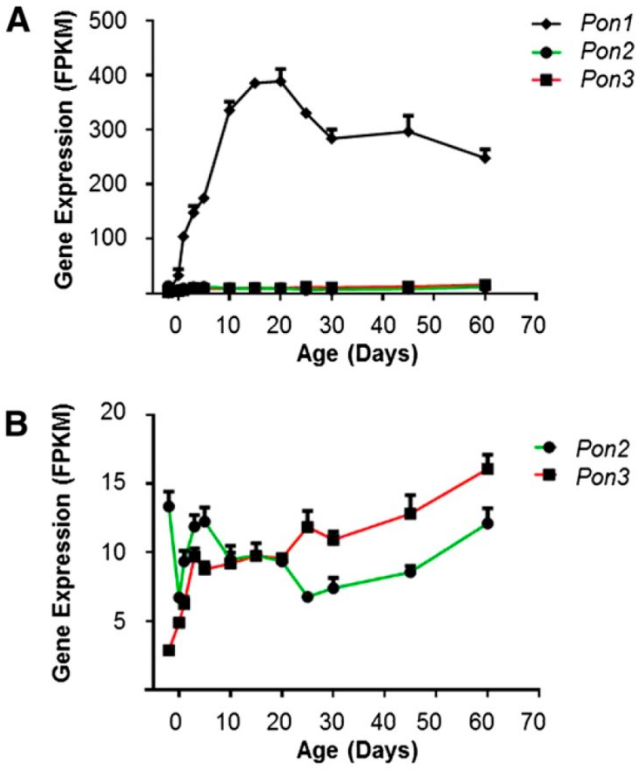


Fig. 4.3 Expression of *Pon* Genes during Liver Development.

(A) All three *Pon* ontogenic mRNA expression patterns; (B) *Pon1* was removed to enlarge *Pon2* and *Pon3* expression patterns. Data are expressed as mean FPKM and SEM of three individual animals.

Fig. 4.4

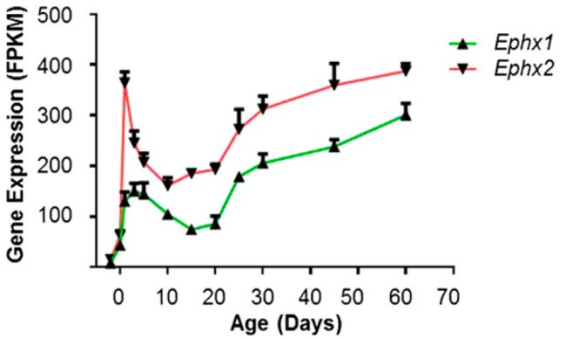


Fig. 4.4 Expression Patterns of *Ephx1* and *Ephx2* during Liver Development.

expressed *Akr*s exhibited significant differential expressions across the ages. The cumulative FPKM values of all expressed *Akr* mRNAs increased 2-3 fold during postnatal liver development (Fig. 4.5A). The composition of *Akr* mRNAs also changed with age, evidenced by alterations in individual *Akr* genes. At day -2, *Akr1a4* was the most abundant member, which accounted for 65% of the total *Akr* mRNAs. After birth, *Akr1a4* mRNA slightly increased, but its proportion in *Akr* mRNAs decreased (Fig. 4.5B-C). *Akr1b7* was highest during the neonatal stage. It accounted for 28% of the total *Akr* mRNA at day 1, but was undetectable after day 20. *Akr1c6* was one of the major *Akr* genes in liver. It was lowly expressed at birth, gradually increased to a peak at day 30, and then slightly decreased to adult levels around day 45. The mRNA expression profile of *Akr7a5* was similar to the profile of total *Akr* mRNAs, so the percentage of *Akr7a5* mRNA was relatively constant during development (10~15 %) (Fig. 4.5B-C). Two-way hierarchical clustering analysis of the differentially expressed *Akrs* revealed two major patterns (Fig. 4.5D). Unlike *Ces* genes, which all had low expression at the neonatal stage, some *Akr* genes displayed neonatal enriched expression (Group 1). In Group 2, *Akr* genes however showed relatively low expression during the neonatal ages. These genes were a diverse group with some genes enriched at the adolescent stage (e.g. *Akr1c20*) and some at the adult stage (e.g. *Akr1c12*).

Quinone oxidoreductase: NQO1 and NQO2 perform two-electron reduction of quinones to hydroquinones. The RNA-Seq results demonstrated that the expression of *Nqo1* was relatively stable during development, with two small drops around birth and day 25. *Nqo2* mRNA was expressed higher than *Nqo1* in mouse liver 5 days after birth. *Nqo2* showed two periods of increased expression during development; one from day 0 to day 15, and the other from day 30 to day 60 (Fig. 4.6A).

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

Fig. 4.5

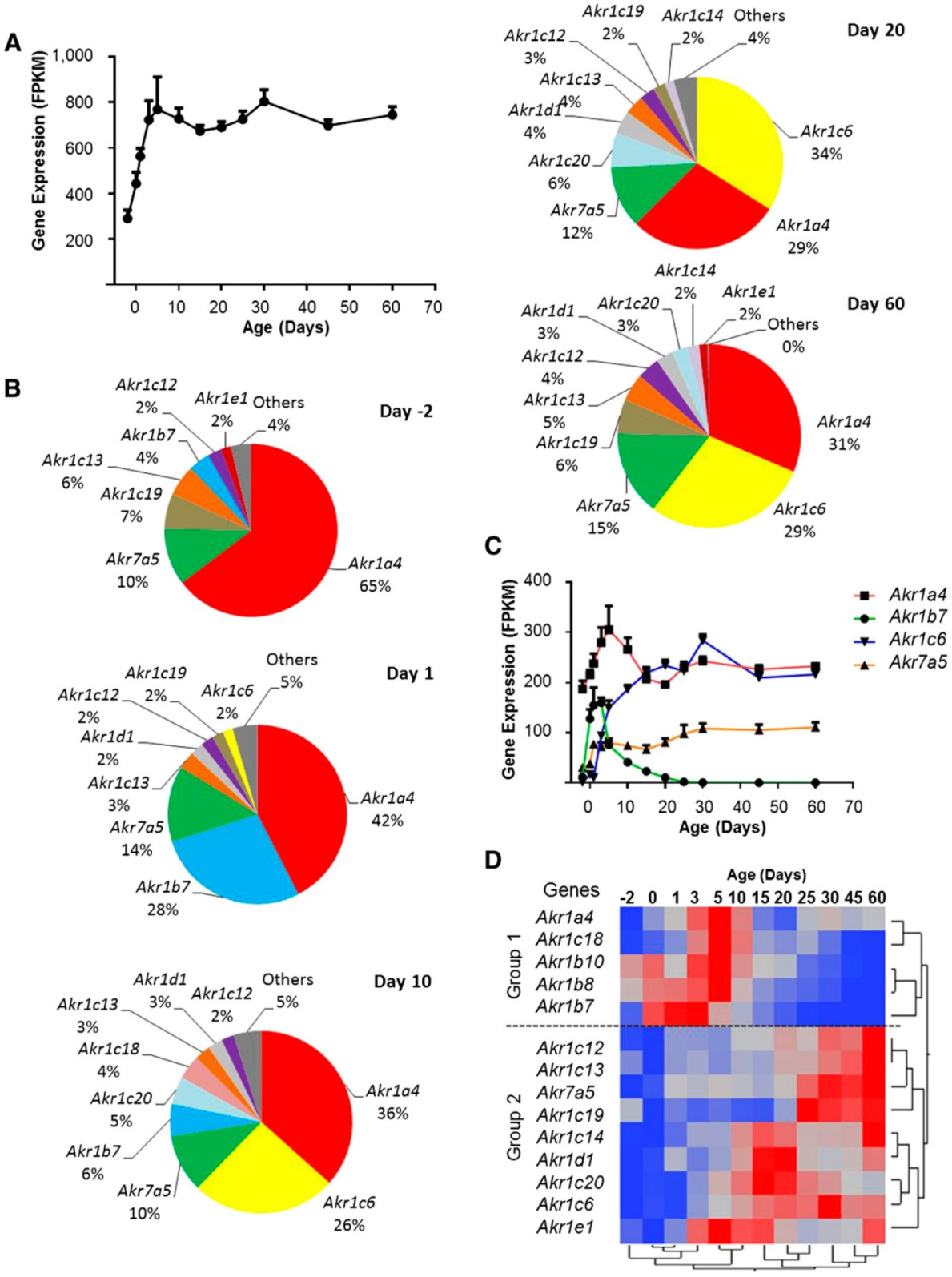

A
Gene Expression (FPKM)
1,000
800
600
400
200
0 10 20 30 40 50 60 70
Age (Days)
B
Day -2
Akr1c12 2%
Akr1e1 2%
Others 4%
Akr1b7 4%
Akr1c13 6%
Akr1c19 7%
Akr7a5 10%
Akr1a4 65%
Day 1
Others 5%
Akr1c19 2%
Akr1c6 2%
Akr1c12 2%
Akr1d1 2%
Akr1c13 3%
Akr7a5 14%
Akr1b7 28%
Akr1a4 42%
Day 10
Akr1d1 3%
Others 5%
Akr1c13 3%
Akr1c12 2%
Akr1c18 4%
Akr1c20 5%
Akr1b7 6%
Akr7a5 10%
Akr1c6 26%
Akr1a4 36%
Day 20
Akr1c19 2%
Akr1c14 2%
Others 4%
Akr1c12 3%
Akr1c13 4%
Akr1d1 4%
Akr1c20 6%
Akr7a5 12%
Akr1c6 34%
Akr1a4 29%
Day 60
Akr1c14 2%
Akr1e1 2%
Akr1d1 3%
Akr1c20 3%
Others 0%
Akr1c12 4%
Akr1c13 5%
Akr1c19 6%
Akr7a5 15%
Akr1a4 31%
Akr1c6 29%
C
Gene Expression (FPKM)
400
300
200
100
0 10 20 30 40 50 60 70
Age (Days)
Akr1a4
Akr1b7
Akr1c6
Akr7a5
D
Genes
Age (Days)
-2 0 1 3 5 10 15 20 25 30 45 60
Group 1
Akr1a4
Akr1c18
Akr1b10
Akr1b8
Akr1b7
Group 2
Akr1c12
Akr1c13
Akr7a5
Akr1c19
Akr1c14
Akr1d1
Akr1c20
Akr1c6
Akr1e1

Fig. 4.5 Expression Patterns of *Akr* Genes during Liver Development.

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

Fig. 4.6

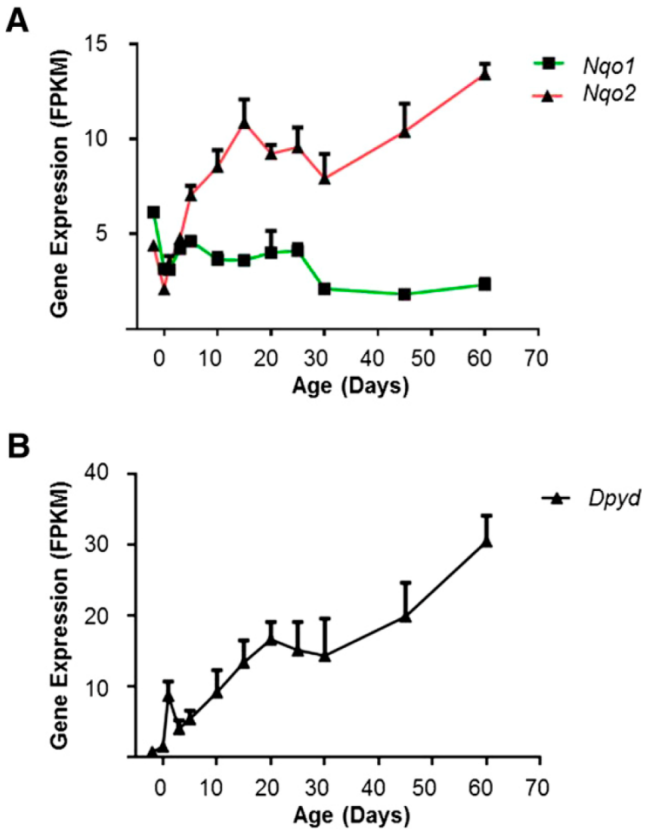


Fig. 4.6 Expression Patterns of *Nqo* and *Dpyd* Genes during Liver Development.

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

expression of *Dpyd* showed a marked increase at day 1, dropped slightly till day 3, and then went up gradually to adult levels at day 60, with a small plateau between day 20 and day 30 (Fig. 4.6B). The adult mRNA level of *Dpyd* was about 30 times higher than newborns.

Ontogeny of Genes of Phase-I Enzymes Involved in Oxidation Reactions

Alcohol dehydrogenases: ADHs metabolize a wide spectrum of substrates, including ethanol, retinol, other aliphatic alcohols, hydroxysteroids, and lipid peroxidation products (Duester et al., 1999). 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. *Adh1* mRNA was the most highly expressed, consisting of over 90% of *Adh* mRNAs in adult liver (Fig. 4.7), followed by *Adh5* and then *Adh4*. *Adh6* and *Adh7* were minimally expressed in liver. *Adh*s generally had lower expression at younger ages and reached stable mature levels at day 30 or earlier. *Adh1* and *Adh5* also showed a peak of expression around day 0 or day 1 (Fig. 4.7).

Aldehyde dehydrogenase: ALDHs are a group of enzymes that catalyze the oxidation and detoxification of aldehydes. There were total 20 *Aldh* genes in mouse genome, and 15 of these genes were significantly expressed at mRNA levels during liver maturation. Of these 15 genes, 14 showed differential expression across the ages. The total mRNA of *Aldh* genes increased almost linearly over fivefold from 2 days before birth to 60 days after birth (Fig. 4.8A). *Aldh1l1* and *Aldh2* were the major *Aldh* genes expressed at all ages of liver development. *Aldh1b1*, *Aldh4a1*and *Aldh9a1* accounted for a high percentage of total *Aldh* mRNAs at the prenatal and adolescent stages, whereas *Aldh1a1* matured later and became highly expressed only at the adult stage (Fig. 4.8B). Two-way hierarchical clustering analysis of the differentially expressed *Aldh*s also demonstrated diverse ontogenic patterns of individual *Aldh*s. Similar to *Akr*s, Group 1 *Aldh* genes had enriched expression at the perinatal stage, and genes in Group 2 were expressed higher at the adult stage than at the perinatal stage (Fig. 4.8C).

Fig. 4.7

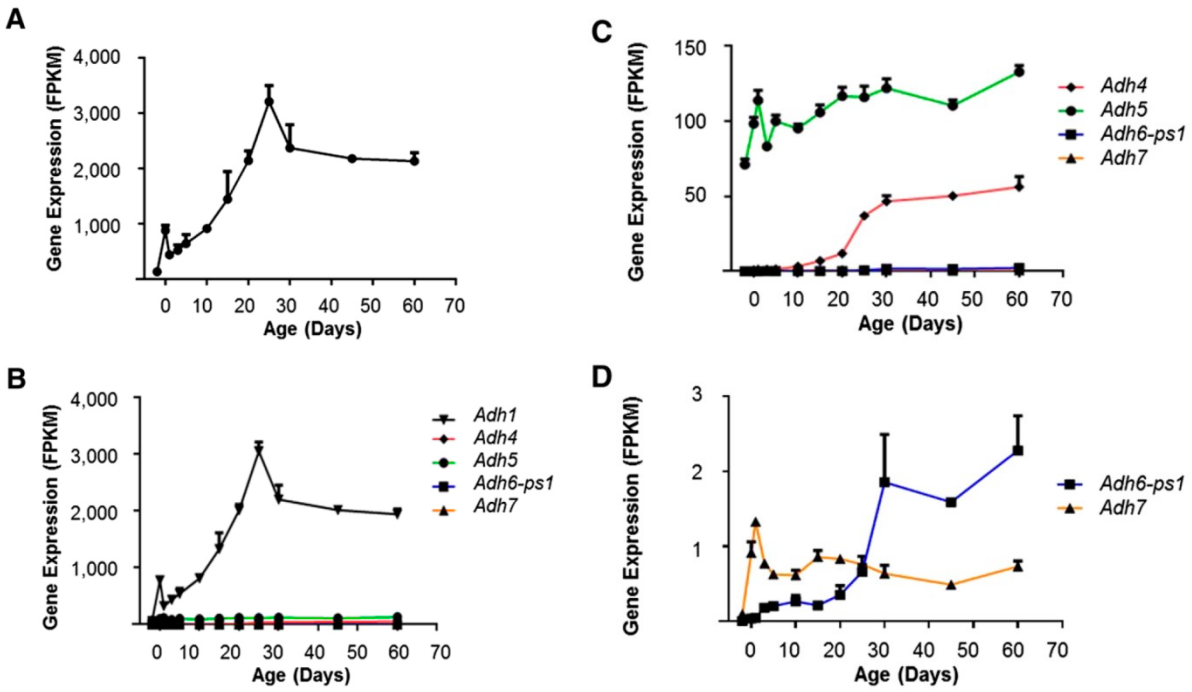


Fig. 4.7 Expression Patterns of *Adh* Genes during Liver Development.

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

Fig. 4.8

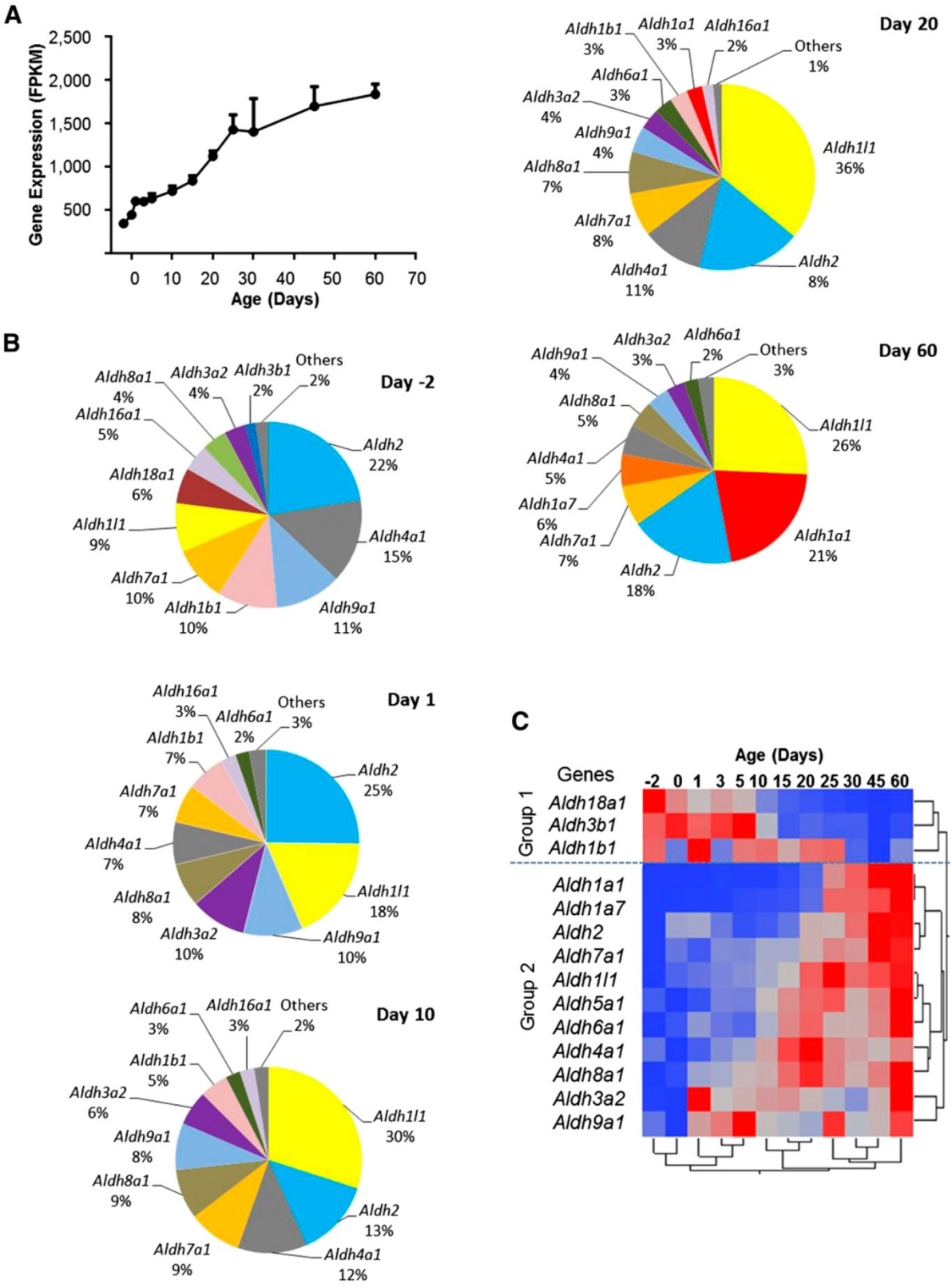

A
Gene Expression (FPKM)
2,500
2,000
1,500
1,000
500
0 10 20 30 40 50 60 70
Age (Days)
B
Day -2
Aldh8a1 4%
Aldh3a2 4%
Aldh3b1 2%
Others 2%
Aldh16a1 5%
Aldh2 22%
Aldh18a1 6%
Aldh4a1 15%
Aldh1l1 9%
Aldh7a1 10%
Aldh1b1 10%
Aldh9a1 11%
Day 1
Aldh16a1 3%
Others 3%
Aldh6a1 2%
Aldh1b1 7%
Aldh2 25%
Aldh7a1 7%
Aldh4a1 7%
Aldh1l1 18%
Aldh8a1 8%
Aldh3a2 10%
Aldh9a1 10%
Day 10
Aldh6a1 3%
Aldh16a1 3%
Others 2%
Aldh1b1 5%
Aldh1l1 30%
Aldh3a2 6%
Aldh9a1 8%
Aldh8a1 9%
Aldh2 13%
Aldh7a1 9%
Aldh4a1 12%
Day 20
Aldh1b1 3%
Aldh1a1 3%
Aldh16a1 2%
Others 1%
Aldh6a1 3%
Aldh3a2 4%
Aldh9a1 4%
Aldh1l1 36%
Aldh8a1 7%
Aldh7a1 8%
Aldh2 8%
Aldh4a1 11%
Day 60
Aldh9a1 4%
Aldh3a2 3%
Aldh6a1 2%
Others 3%
Aldh8a1 5%
Aldh1l1 26%
Aldh4a1 5%
Aldh1a7 6%
Aldh1a1 21%
Aldh7a1 7%
Aldh2 18%
C
Age (Days)
Genes
-2 0 1 3 5 10 15 20 25 30 45 60
Group 1
Aldh18a1
Aldh3b1
Aldh1b1
Group 2
Aldh1a1
Aldh1a7
Aldh2
Aldh7a1
Aldh1l1
Aldh5a1
Aldh6a1
Aldh4a1
Aldh8a1
Aldh3a2
Aldh9a1

Fig. 4.8 Expression Patterns of *Aldh* Genes during Liver Development.

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

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 *Fmo*s are annotated in the mouse genome. The mRNA expression of *Fmo5* increased gradually with age, and was the most abundant in adult males, followed by *Fmo1*, which showed a rapid increase of expression from birth to day 5, and then slightly increased to a peak at day 25 and decreased to adult expression levels at day 30. The expression of *Fmo3* became detectable at 10 days after birth, increased to peak levels at day 25, and then dropped to negligible levels after day 30 in male mice (Fig. 4.9A). *Fmo2* and *Fmo4* were very lowly expressed in liver during maturation, and *Fmo2* was mainly detected at the adolescent stage from day 10 to day 20 (Fig. 4.9B).

Molybdenum hydroxylases: Mammalian molybdenum hydroxylases require FAD and molybdenum cofactor for their catalytic activity. There are two major molybdenum hydroxylases participating in the metabolism of xenobiotics: aldehyde oxidases (AOX) and xanthine oxidoreductase (XDH) (Parkinson and Ogilvie, 2008). Four *Aox* genes are annotated in the mouse genome and two of them were expressed during liver maturation. *Aox3* was the major *Aox* in mouse liver. Its mRNA level increased over 10 fold from day -2 to day 3, remained stable from day 3 to day 25, and then went up about 5 fold to adult level at day 60. *Aox1* was expressed at lower level than *Aox3*, and also showed an increase of expression after day 25 (Fig.10A). *Xdh* mRNA expression gradually increased about 10 fold from day -2 to day 20, and then remained relatively stable (Fig. 4.10B).

NADPH-cytochrome P450 oxidoreductase: POR transfers electrons from NADPH to cytochrome P450s for their catalytic function. The expression of *Por* mRNA rose sharply around birth, and decreased to about half the level of day 1 by day 5. From day 5 to day 20, *Por* expression was relatively stable. Then at day 25, it decreased again to about half the level of day 20 and remained at that level till maturity (Fig. 4.11).

Fig. 4.9

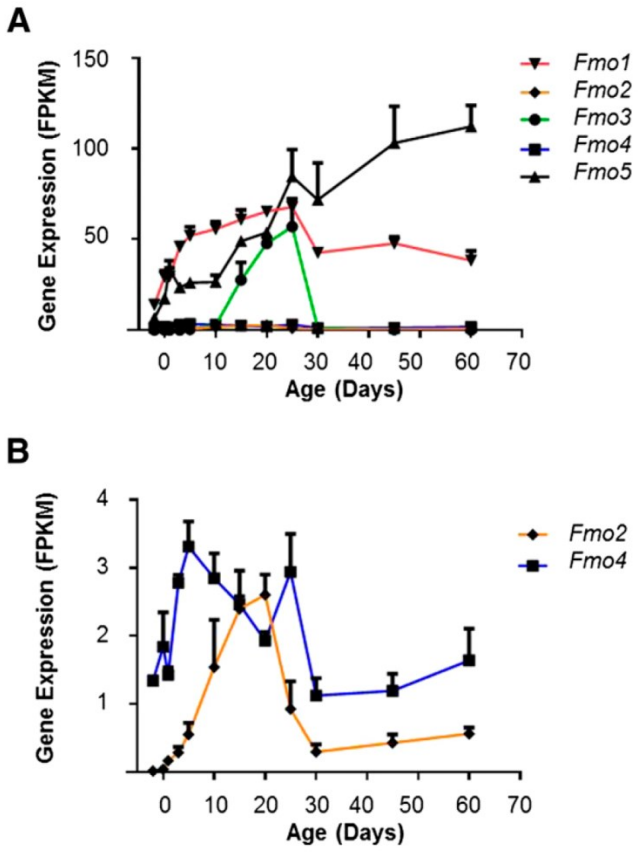


Fig. 4.9 Expression of *Fmo* Genes during Liver Development.

(A) All five *Fmo* ontogenic mRNA expression patterns; (B) *Fmo1,3,5* are removed to enlarge *Fmo2* and *Fmo4* expression patterns.

Fig. 4.10

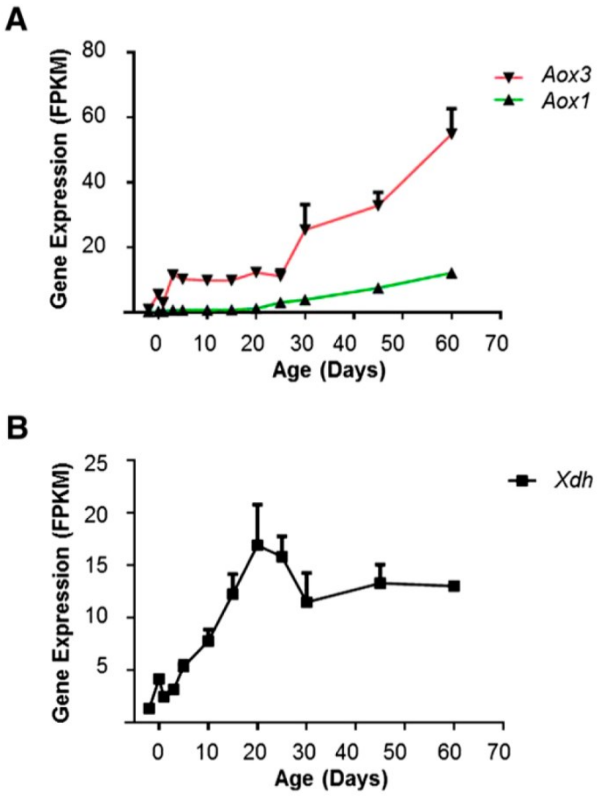


Fig. 4.10 Expression of Molybdenum Hydroxylases during Liver Development.

(A) mRNA ontogenic patterns of aldehyde oxidase *Aox1* and *Aox3*; (B) mRNA ontogenic patterns of *Xdh*.

Fig. 4.11

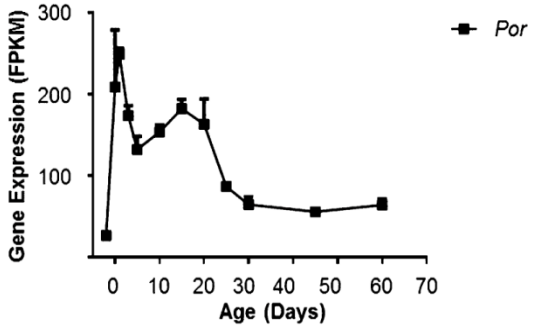


Fig. 4.11 mRNA Expression Pattern of *Por* during Liver Development.

4.5 Discussion

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

From perinatal through neonatal to adult liver, the total FPKM values of Phase-I mRNAs increase 15 fold with two rapidly increasing stages (Fig. 4.1A), which may reflect functional transition of liver from a hematopoietic organ to a metabolic organ during liver maturation. The first surge occurs from 2 days before birth to 1 day after birth. During that period of time, the total FPKM values increase 8 fold and the composition of each Phase-I family also changes dramatically. P450s are the most changed Phase-I enzymes during this perinatal surge, indicating an urgent need for liver to deal with exposure to xenobiotics immediately after birth. The second surge occurs from day 10 to day 20 with a rapid increase in abundance of Phase-I mRNAs. This period is also the most rapidly growing stage of postnatal liver maturation. After day 25 (adolescence), the total FPKM levels and composition of the Phase-I enzymes are consistent till day 60 (adult). These data indicate that during the developmental period from neonatal to adolescence, hepatic expression of many Phase-I enzyme genes change dramatically. Expectedly, the ability to metabolize xenobiotics including drugs by Phase-I enzymes should also be changed dramatically in this period of time. Determination of the

mechanisms in regulation of the Phase-I gene expression during this developmental period will provide insight for understanding drug metabolism in pediatric patients.

The ontogenic patterns of many Phase-I genes we found in this study are consistent with the previous findings in mice and humans by other researchers. For example, the high expression of carboxylesterase 1 (*Ces1*) in our results was consistent with the literature that *Ces1* was the major expressed carboxylesterase gene in mouse liver (Holmes et al., 2010). Another study demonstrated that the expression of mouse *Ces1* and *Ces2* in the liver were markedly lower in newborns than in adults and increased gradually to levels of adult animals in 2 to 4 weeks (Zhu et al., 2009). Our data confirmed these, and provided more details on changes with age and more information about individual members of each gene family (Fig. 4.2). Adult humans also express higher levels of *CES1* and *CES2* than children and fetus (Yang et al., 2009). As a former study suggested that the *CES* genes from mouse and human had evolutionally conserved transcriptional regulatory mechanisms (Hosokawa et al., 2007), these ontogeny data in mice are expected to provide important resources for interpreting the developmental regulation of CESs in humans.

A study showed that lower levels of PON1 enzyme persisted in young children till at least 7 years of age (Huen et al., 2009), which was similar to the ontogenic pattern of *Pon1* mRNA expression in mice. Another group found that the plasma PON1 activity in mice reached a plateau in 3 weeks after birth (Li et al., 1997), which is similar to our data of *Pon1* mRNA expression in mouse liver (Fig. 4.3). A transgenic mouse strain that lacks endogenous *Pon1*, but with the human *PON1* gene, exhibited a similar developmental pattern of expression as wild-type mice, indicating conserved developmental regulatory elements between mouse and human *PON1* (Cole et al., 2003), making our data helpful for mechanistic studies.

Quinones are highly reactive molecules. They can undergo one-electron reduction, commonly catalyzed by NADPH-cytochrome P450 reductase, and generate semiquinone

radicals, which are reactive metabolites themselves and may cause oxidative stress by redox cycling. The resultant reactive oxygen species can lead to DNA damage, lipid peroxidation, membrane damage, cytotoxicity, and neoplasia. NQO1 and NQO2 compete for the above reaction and catalyze the two-electron reductive metabolism of quinones to produce stable hydroquinones, which are removed by glucuronidation or sulfonation (Long and Jaiswal, 2000; Parkinson and Ogilvie, 2008). Quinones are ubiquitous in nature and human exposure to quinones occurs through diet, airborne pollutants, and drugs (Monks and Jones, 2002). Therefore, quinone oxidoreductase has a highly important role in developmental toxicology. The present study is the first demonstration of the developmental expression of these genes. NQO1 and NQO2 are two closely related flavoproteins. Although they have overlapping substrate specificities, significant differences exist in relative affinities for various substrates (Das et al., 2006). Our results also revealed the differential expression of *Nqo1* and *Nqo2* mRNAs during development (Fig. 4.6A). The changes of expression during liver maturation may have strong toxicity impact in children. Further studies are needed to address the ontogeny of NQO enzymes and their significance in human health.

Although *FMO3* is the most highly expressed FMO family member in adult human liver, it demonstrated gender-specific expression in mice, and was not detectable in male liver of mice (Falls et al., 1995). Our data only examined mRNA expression in male animals and was consistent with this result. *Fmo3* was expressed equally in male and female mice, even at 4 weeks of age (Cherrington et al., 1998), and then after puberty, the gender difference appeared due to sex steroids (Falls et al., 1997). We also showed the detailed time window when the shutdown of *Fmo3* happened, which was between day 25 and day 30 (Fig. 4.9A). *Fmo1* in mice showed increased expression after birth, unlike human, in which *Fmo1* was most abundant in fetal liver and absent after birth (Hines, 2006). Interestingly, *Fmo3* was not expressed immediately after birth in mice, and appeared after 10 days of age, which is similar in humans

(Hines and McCarver, 2002). Thus, neonatal mice may serve as a model to study the mechanism of the delayed onset of *FMO3* expression in humans.

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

POR is the only electron donor for all microsomal P450s, and alteration in POR activity can affect P450-catalyzed drug oxidation (Hart et al., 2008). Our previous study had revealed the ontogenic mRNA expression of all P450s in mouse liver, and a large number of P450 genes had increased expression after birth (Peng et al., 2012). Here we showed hepatic mRNA expression of *Por*, which could influence P450 activities, actually decreased during postnatal development (Fig. 4.11). Thus, the level of *Por* needs to be taken into considerations when we study the developmental enzyme activity of P450s.

Protein levels and enzyme activities of Phase-I genes were not determined in this study due to extensive workload and technical limitations. Specific antibodies, substrates and inhibitors for many individual mouse Phase-I enzymes are not available. Yet compared with the few studies

that measured protein expression or enzyme activities of certain Phase-I genes in mouse liver during development, such as CES (Zhu et al., 2009), PON (Cole et al., 2003), EPHX (Rouet et al., 1984), and FMO (Cherrington et al., 1998), our mRNA expression patterns were fairly indicative of the protein or enzyme activity levels. But whether the mRNA expression can be extrapolated to the protein expression and enzymatic activity of the Phase-I enzymes remain to be validated. For future studies, applying certain pediatric drugs to the mice of different developmental stages and checking the pharmacokinetics of these drugs to see if the metabolism pattern can be predicted by the current gene expression may be informative. Technological breakthroughs in proteomics and metabolomics are essential to the study of ontogeny of Phase-I enzymes on the protein contents and metabolite levels in the future.

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

Chapter 5 : FXR in Regulation of Postnatal Liver Development and Phase-I Gene Expressions

5.1 Abstract

The functions of liver, including the expression of Phase-I drug metabolizing enzymes, are not mature at birth, and hepatic gene expressions change dramatically during liver maturation. Farnesoid X receptor is critical for bile acid and lipid homeostasis in liver. However, the role of FXR in regulating liver maturation and Phase-I drug metabolizing gene ontogeny is not clear. Hence, we applied RNA sequencing to quantify the developmental transcriptome in both FXR-null and its control (C57BL/6) mouse livers during development, with a focus on Phase-I genes. Liver samples of male C57BL/6 and FXR-null mice at 6 different ages from prenatal to adult were used. Similarity analysis of global gene expression indicated that liver maturation was delayed in FXR-null mice at adolescent stage. FXR knockout mice showed prolonged activation of neonatal-specific pathways and delayed maturation of multiple metabolic pathways compared to wild-type mice. Surprisingly, knockout of FXR induced heart, kidney and many other organ developmental processes in liver. The loss of FXR also led to increased transcription of 90% of the differentially expressed transcription regulators during liver maturation. Among the 185 Phase-I genes from 12 different families, 136 were expressed, and differential expression during development occurred in genes from all 12 Phase-I families, including hydrolysis: carboxylesterase (*Ces*), paraoxonase (*Pon*), epoxide hydrolase (*Ephx*); reduction: aldoketo reductase (Akr), quinone oxidoreductase (*Nqo*), dihydropyrimidine dehydrogenase (*Dpyd*); and oxidation: alcohol dehydrogenase (*Adh*), aldehyde dehydrogenase (*Aldh*), flavin monooxygenases (*Fmo*), molybdenum hydroxylase (*Aox* and *Xdh*), cytochrome P450 (P450), and cytochrome P450 oxidoreductase (*Por*). The data also suggested new Phase-I genes

potentially targeted by FXR. These results indicate an important role of FXR in regulation of liver maturation and Phase-I genes ontogeny.

5.2 Introduction

The liver is the largest internal organ in the body, taking up about 2-4% of body weight in humans. As part of the digestive system, a matured liver plays a major role in nutrient homeostasis, including the synthesis, metabolism and transport of carbohydrates, proteins and fats. Venous blood from the stomach and intestine flows through the liver by the portal vein before entering systemic circulation. Thus liver is the first organ to encounter and deal with ingested drugs, environmental toxicants and intestinal bacteria. Bioactivation, detoxification and filtration of particulates in immune defense are also critical functions of the liver (Jaeschke, 2008). Whereas in utero, fetal liver is the major hematopoietic organ, generating blood cells, and hematopoiesis is active in liver even shortly after birth (Takeuchi and Miyajima, 2006). Definitive hematopoiesis requires a specific microenvironment in the organ. Therefore, from birth to maturity, there are dramatic changes happen in liver to achieve the organ growth and functional transition. And the differences between neonatal and adult livers have been implicated in clinical issues. Disorders of iron metabolism such as hemochromatosis having severe effects in neonates including fulminant liver failure may be due to the active hematopoiesis in neonatal liver (Beath, 2003). Age-related sensitivity to drugs is at least partly attributable to differences in hepatic metabolic activity (Pineiro-Carrero and Pineiro, 2004).

Phase-I drug metabolizing enzymes catalyze the oxidation, reduction and hydrolysis of xenobiotics. A functional transition occurs in liver after birth, and most of the drug-metabolizing enzymes mature during this period. Changes in expression of some Phase-I enzymes during liver maturation in humans have been reported, including P450s (Stevens et al., 2003;

Koukouritaki et al., 2004; Stevens et al., 2008; Croom et al., 2009), CES (Yang et al., 2009; Zhu et al., 2009), PON (Cole et al., 2003; Huen et al., 2009), ADH (Smith et al., 1971), and FMO (Cherrington et al., 1998; Koukouritaki et al., 2002; Hines, 2006). The dynamic changes in the ontogenic expression of these genes are thought to be responsible for the substantial pharmacokinetic differences between newborns and adults, and this contributes to differences in therapeutic efficacy and adverse drug reactions in pediatric patients (Kearns et al., 2003; Blake et al., 2005; Hines, 2007; Hines, 2008; Hines, 2013). An in-depth understanding of the regulatory mechanisms of the ontogeny of Phase-I enzymes is needed for safer and more effective drug therapy for pediatric patients.

The Farnesoid X receptor (FXR) is a bile acids-activated transcription factor and a member of the nuclear receptor superfamily (Zhu et al., 2011). In the immediate neonatal period, bile acids are required for the newborns to absorb milk. There is a surge of serum and liver bile acids levels after birth (Cui et al., 2012a), so the increased activation of FXR immediately after birth may be important for regulation of hepatic gene expression at early ages. Previous studies have identified FXR as a key metabolic regulator. It is critical for bile acids, lipid and glucose homeostasis (Sinal et al., 2000; Claudel et al., 2005), and even plays a role in liver regeneration (Chen et al., 2011). Most of these liver functions regulated by FXR are established during postnatal liver development, and disorders like fatty liver may have roots in the neonatal period(Beath, 2003). Thus understanding the role of FXR in liver maturation may provide important insights on liver physiology and diseases.

The role of FXR in regulating certain drug processing genes has been established. Bile acids activate FXR in the intestine and liver, which induces the expression of PXR, facilitating the detoxification of lithocholate and xenobiotics by inducing CYP3A, Phase-II enzymes (e.g. sulfotransferase 2A1), and transporters (e.g. MRP2, MRP3) xenobiotics (Kliewer et al., 2002; Jung et al., 2006). FXR also maintains bile acid homeostasis by feedback inhibition of

expression of important genes in bile acid synthesis, for example, CYP7A1, which is the rate-limiting enzyme in the classic pathway of bile acid synthesis, and CYP8B1, which is required for the synthesis of cholic acid. Functional FXR polymorphism is common (2.5-12%), and has been associated with changes in FXR-target gene expression in humans (Marzolini et al., 2007; Kovacs et al., 2008). Understanding the regulatory mechanism of FXR on hepatic expression of drug processing genes in newborns will greatly help us to understand the natural and aberrant development of drug processing genes in pediatric pharmacology.

In the current study, we used RNA-Sequencing to quantify the liver transcriptome during the developmental period from perinatal stage to adult, in both wild-type and FXR knockout mice, and focused on the analysis of Phase-I gene ontogeny. RNA-Seq allows the analysis of the whole transcriptome with lower background noise, higher sensitivity, and a high degree of reproducibility compared to traditional technologies (Mortazavi et al., 2008; Nagalakshmi et al., 2008). More importantly, RNA-Seq quantifies the true abundance of RNA molecules in biological samples and enables the comparison of expression of all genes in multiple samples (Malone and Oliver, 2011). Our results revealed the significant differences in FXR-null livers compared to control livers during postnatal development, and also suggested novel roles of FXR in the regulation of liver maturation and Phase-I gene developmental expression.

5.3 Materials and Methods

Animals

We used C57BL/6 mice as the wild-type animals. The animal purchase, breeding and tissue collection for wild-type mice were performed as previously described in Chapter 2. Breeding pairs of *Fxr-/-* mice on C57BL/6 background were kindly provided by Dr. Grace Guo from University of Kansas Medical Center. These knockout mice were bred under standard

conditions in the Office of Animal Care Facility at the University of Connecticut. The use of these mice was approved by the University of Connecticut's Institutional Animal Care and Use Committee. Liver samples (n=3) from *Fxr-/-* mice were collected at the following 6 ages: day -2 (gestational day 17.5), day 1 (exactly 24 hours after birth), and days 5, 20, 25, and 60 (collected at approximately 9:00 AM). These ages represent the periods of prenatal (day -2), neonatal (days 1 and 5), juvenile (days 20 and 25), and young adult (day 60). Due to potential variations caused by the estrous cycle in maturing female mice, only male livers were used in this study. The livers were immediately frozen in liquid nitrogen after removal and stored at -80℃.

Total RNA Extraction, Sequencing Library Construction, and RNA-Seq

RNA extraction, library construction, RNA-Seq and FASTQ data file collection were performed as previously described in Chapter 2.

RNA-Seq Data Analysis

For comparison of wild-type and FXR knockout samples at the 6 ages of day -2, 1, 5, 20, 25 and 60, the RNA-Seq reads from the FASTQ files of these two types of mice were mapped to the mouse reference genome (GRCm38/mm10) by Tophat 2.0.8. The output files in BAM format were analyzed by Cufflinks 2.1.1 to estimate the transcript abundance.

Gene Ontology Analysis

Statistically significant differential expression in this analysis were defined using the following criteria 1) the gene mean FPKM>1 across the ages; 2) fold change for the average FPKM of the three replicates >1.5 between compared samples; 3) Benjamini-Hochberg adjusted p-values from t-test <0.05. Lists of differentially expressed genes at ages of day -2, 1, 5, 20 and 25 compared to day 60 in FXR knockout mice were supplied to High-Throughput GoMiner for biological interpretation (Zeeberg et al., 2005). Significantly enriched GO categories were selected with a FDR <0.05.

Data Visualization and Statistics

ANOVA was used to test for significant difference in expression during development. P-values were adjusted using Benjamini-Hochberg algorithm with a threshold of 0.05. For Pearson correlation coefficient-based heat map visualization, the average FPKM of the three replicates at each age were used to calculate the Pearson's r values between different ages. For the visualization of nuclear receptors and core transcription factors expression in liver, significant differential expressions were defined in the same way as in GO analysis. To study the distance of Phase-I gene ontogenic pattern between WT and *Fxr*$^{-/-}$ samples, the average FPKM values from three individual animals were used. For each gene, the FPKM values at the 12 ages (6 ages per genotype) were divided by the mean FPKM of the 12 ages before calculating distance in order to normalize the difference of expression level among genes.

5.4 Results

Biological Interpretation of the Liver developmental Transcriptome in Fxr$^{-/-}$ *Mice*

To further understand the regulation of postnatal liver development, we quantified the developmental transcriptome in nuclear receptor FXR knockout mice as well as wild-type mice at the same ages of day -2, 1, 5, 20, 25 and 60. Our RNA-Seq data viewed in the genome browser validated that there was no functional FXR mRNA generated in the knockout samples (Fig. 5.1).

The similarity of gene expression at the 6 ages was analyzed in both wild-type and *Fxr-/-* samples (Fig. 5.2). Consistent with Fig. 2.2, correlation heat map of only 6 ages also displayed clear developmental stages (Fig. 5.2A). But the correlation heat map for *Fxr-/-* samples exhibited dramatic differences in developmental stage transition (Fig. 5.2B). The outstanding feature was that the difference between adolescent (day 20 and 25) and neonatal (day 1 and 5)

Fig. 5.1

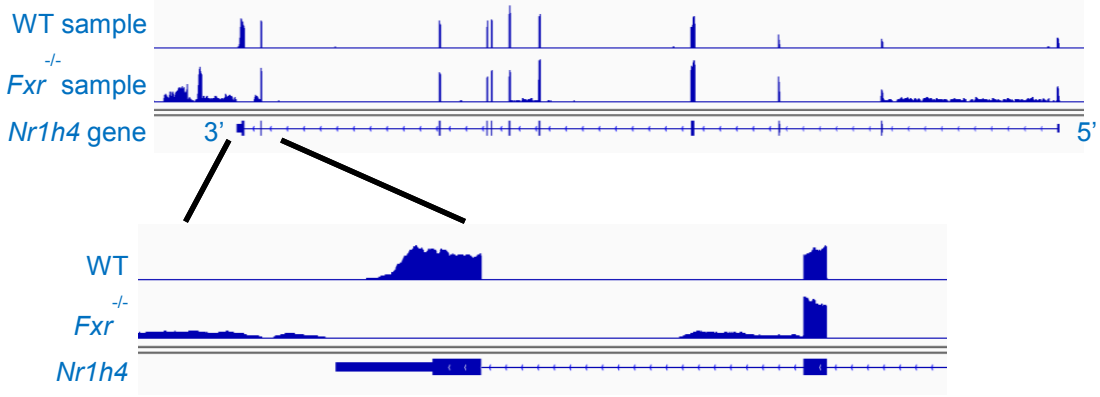


Fig. 5.1 RNA-Seq Validation of *Fxr-/-* Mouse Model.

IGV browser view shows that there are not sequencing reads mapped to the last exon of Fxr gene, which is consistent with the animal model created by Sinal et al.

Fig. 5.2

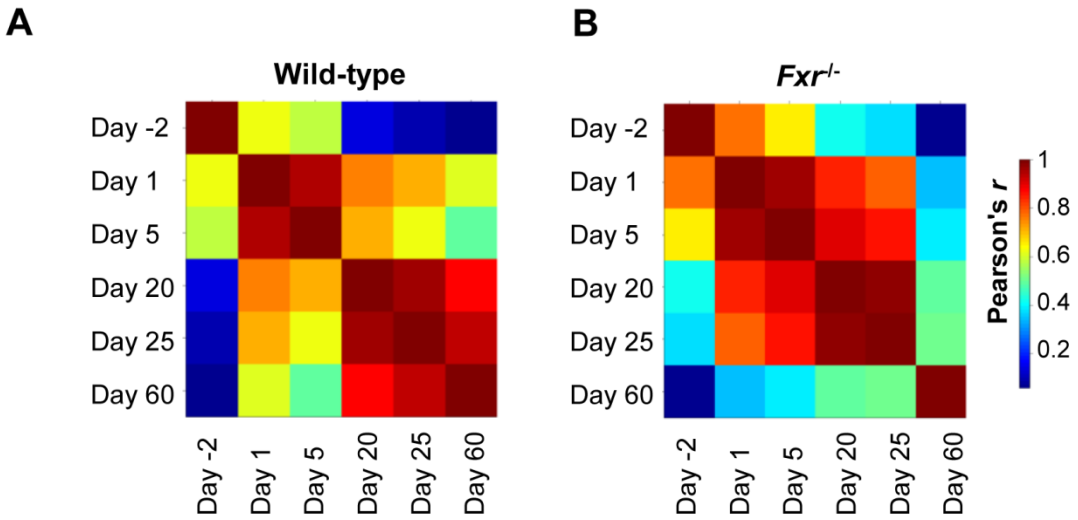


Fig. 5.2 Similarity of Gene Expression during Development in Wild-type (A) *and Fxr-/-* (B) Mice.

Pearson correlation coefficient-based heat map are drawn to present the similarity of gene expression profiles based on all expressed genes over 6 different ages.

stages was diminished. The knockout of FXR seemed to delay the postnatal maturation of liver. And the GO analysis further proved this.

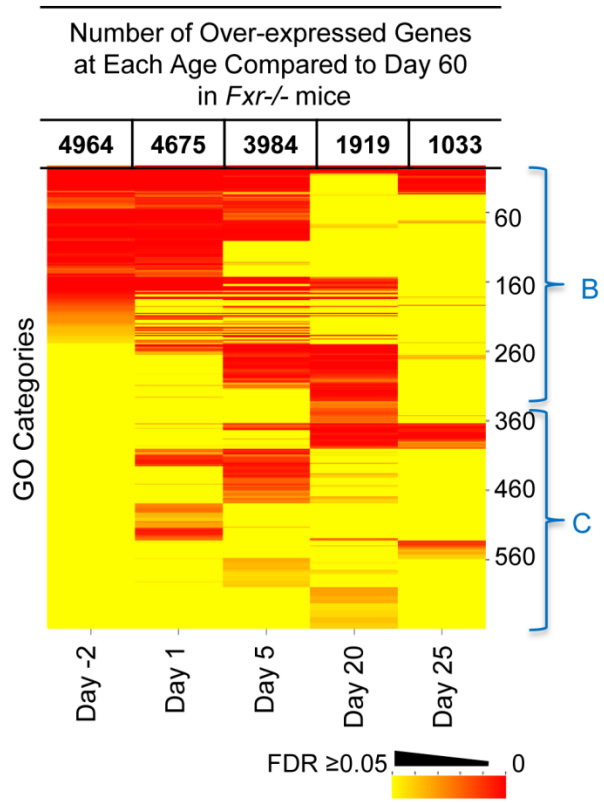
At the ages of day 20 and 25 compared to day 60, there were a lot more differentially expressed genes in *Fxr-/-* samples than in wild-type samples (Fig. 5.3A and 5.4A). The enriched GO categories for over-expressed genes in *Fxr-/-* samples could generally be divided into two groups (bracket B and C in Fig. 5.3A). One group was similar GO categories that showed up in the analysis of wild-type samples, but they were not as age-specific as in wild-type and also enriched at later ages in knockout samples (Fig. 5.3B). The other group contained GO categories not appeared in wild-type samples, and they were mainly pathways for other organ development instead of the liver (Fig. 5.3C). This result indicted that the nuclear receptor FXR might have important roles to maintain the liver identity during postnatal development. The enriched GO categories for under-expressed genes in *Fxr-/-* samples were similar to that in wild-type samples, including all kinds of metabolic processes. But many of them showed delayed maturation and were continually enriched at later ages. Examples were given in Fig. 5.4B.

Effects of FXR Knockout on the Expression of Hepatic Nuclear Receptors and Core Transcription Factors

Previous studies on the regulation of liver-specific genes have identified a core group of hepatic regulators, which form a complex and plastic hepatic transcription factor network during liver development (Kyrmizi et al., 2006). To assess the role of FXR in this network and the effect of FXR knockout on the expression of hepatic transcription regulators, we examined the developmental expression of core transcription factors and all nuclear receptor family members in livers of wild-type and *Fxr-/-* mice. A total of 53 genes were summarized into the transcription regulator group (Table 5.1), 39 of them were expressed in liver during development (mean FPKM>1), and 30 of them had significant differential expression between wild-type and *Fxr-/-*

Fig. 5.3

A



B

GO Categories	FDR				
	Day -2	Day 1	Day 5	Day 20	Day 25
Cytoskeleton organization	0.0000	0.0000	0.0000	0.0008	0.0133
DNA replication	0.0000	0.0000	0.0000	0.0005	0.0000
Cell cycle phase	0.0000	0.0000	0.0000	0.0006	0.0000
Cell division	0.0000	0.0000	0.0000	0.0077	0.0000
Mitosis	0.0000	0.0000	0.0000	>0.05	0.0000
Centrosome cycle	0.0000	0.0000	0.0001	>0.05	>0.05
Erythrocyte differentiation	0.0000	0.0000	0.0001	>0.05	>0.05
Hemopoiesis	0.0000	0.0000	0.0001	0.0409	>0.05
Leukocyte activation	0.0002	0.0000	0.0000	0.0096	>0.05
Mast cell activation	0.0020	0.0009	0.0009	0.0267	>0.05
Leukocyte migration	0.0251	0.0141	0.0000	0.0009	>0.05
Phagocytosis	0.0251	0.0068	0.0015	0.0000	>0.05
Steroid metabolic process	>0.05	>0.05	>0.05	0.0008	0.0012
Tissue remodeling	>0.05	>0.05	>0.05	0.0054	0.0111

Fig. 5.3

C

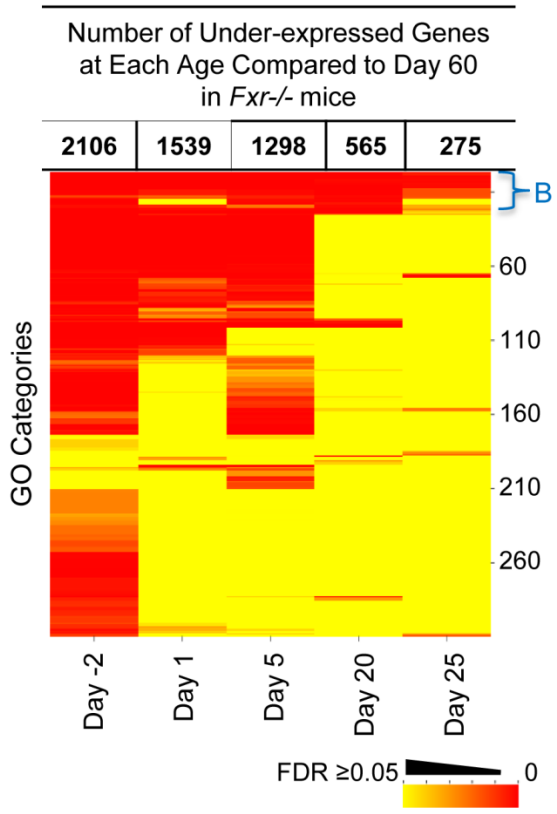
GO Categories	FDR				
	Day -2	Day 1	Day 5	Day 20	Day 25
Head development	>0.05	0.0077	0.0211	>0.05	>0.05
Body morphogenesis	>0.05	0.0088	0.0065	>0.05	>0.05
Axon regeneration	>0.05	>0.05	0.0280	>0.05	>0.05
Neuron development	>0.05	>0.05	0.0249	0.0443	>0.05
Female gonad development	>0.05	0.0232	>0.05	>0.05	>0.05
Face development	>0.05	0.0214	>0.05	>0.05	>0.05
Embryo development	>0.05	0.0356	>0.05	>0.05	>0.05
Odontogenesis	>0.05	>0.05	0.0428	>0.05	0.0364
Heart development	>0.05	>0.05	>0.05	0.0431	>0.05
Muscle system process	>0.05	>0.05	0.0016	0.0018	>0.05
Epithelium development	>0.05	>0.05	>0.05	0.0005	>0.05
Adrenal gland development	>0.05	>0.05	>0.05	0.0233	>0.05
Kidney development	>0.05	>0.05	>0.05	0.0035	0.0013
Glomerulus development	>0.05	>0.05	0.0337	0.0315	0.0083
Male gonad development	>0.05	>0.05	>0.05	0.0167	0.0151

Fig. 5.3 Gene Ontology Analysis for Over-expressed Genes during Development Compared to Day 60 in *Fxr-/-* Mice.

(A) Heat map for GO categories with significant gene over-expression at ages of day -2, 1, 5, 20 and 25. The color represents the false discovery rate of each GO category. The numbers on the right are the cumulative number of GO categories. The brackets B and C are used to label different groups of GO categories. Representative GO categories in each bracket are show in the corresponding panels of (B) and (C). The FDR values with red color in panel (B) indicate that the same GO category has a FDR>0.05 in age-matched wild-type samples.

Fig. 5.4

A



B

GO Categories	FDR				
	Day -2	Day 1	Day 5	Day 20	Day 25
Amine metabolic process	0.0000	0.0000	0.0000	0.0000	0.0033
Organic acid catabolic process	0.0000	0.0000	0.0000	0.0000	0.0008
Cellular modified amino acid metabolic process	0.0000	0.0000	0.0000	0.0013	0.0010
Xenobiotic metabolic process	0.0000	0.0000	0.0000	0.0006	0.0018
Tryptophan metabolic process	0.0032	0.0137	0.0053	0.0004	0.0187
Cellular lipid metabolic process	0.0000	0.0000	0.0000	0.0000	0.0293
Fatty acid metabolic process	0.0000	0.0000	0.0000	0.0000	0.0429

Fig. 5.4 Gene Ontology Analysis for Under-expressed Genes during Development Compared to Day 60 in *Fxr-/-* Mice.

(A) Heat map for GO categories with significant gene under-expression at ages of day -2, 1, 5, 20 and 25. The color represents the false discovery rate of each GO category. The numbers on the right are the cumulative number of GO categories. The bracket B is used to label a group of GO categories. Representative GO categories in the bracket are show in panel (B). The FDR values with red color in panel (B) indicate that the same GO category has a FDR>0.05 in age-matched wild-type samples.

Table 5.1 Expression summary of hepatic transcription regulator in wild-type and *Fxr-/-* mice

Status	Genes	Count
Increased in *Fxr-/-* samples	*Esr1, Rora, Nr3c2, Nr1d2, Onecut1, Thrb, Nr6a1, Nr1i3, Rarb, Nr2c2, Rorc, Nr2f2, Foxa1, Pparg, Rara, Nr5a2, Nr1h4, Foxa2, Nr3c1, Hnf1a, Esrra, Ppard, Ppara, Nr1i2, Foxa3, Rxra, Cebpa*	27
Decreased in *Fxr-/-* samples	*Nr0b2, Nr2c1, Nr1h3*	3
Not significantly changed between wild-type and *Fxr-/-* samples	*Rxrg, Rarg, Hnf1b, Thra, Nr1d1, Rxrb, Nr1h2, Nr2f6, Hnf4a*	9
Not expressed in liver	*Nr2e1, Rorb, Nr0b1, Nr2e3, Esr2, Nr5a1, Nr4a3, Nr4a2, Vdr, Hnf4g, Esrrb, Nr2f1, Esrrg, Nr4a1*	14

Fig. 5.5

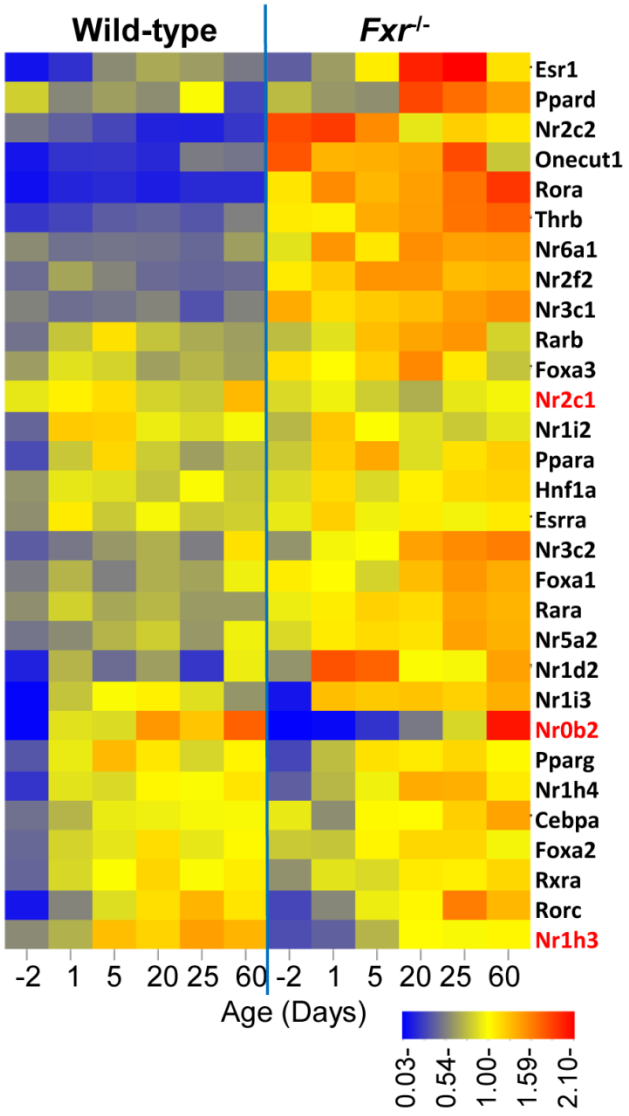


Fig. 5.5 The Effect of *Fxr-/-* on Developmental Expression of Nuclear Receptors and Core Transcription Factors in Liver.

Heat map of expression profiles are drawn for all nuclear receptors and main hepatic transcription factors (genes labeled on the right) with significant differential expression between wild-type and *Fxr*$^{-/-}$ samples. For each gene, the value of $\log_2$(1+[fold change to mean FPKM]) over the ages are calculated to show the trends of expression. The three gene names in red color have higher expression in wild-type samples than in *Fxr*$^{-/-}$ samples.

mice for at least one age during development (Fig. 5.5). Within the differentially expressed genes, only 3 showed decreased expressions in the knockout samples, including *Nr2c1*, *Nr1h3* and the known FXR target gene *Nr0b2*, and the rest 27 genes were all induced in the knockout samples. The increased expression of most hepatic transcription regulators in *Fxr-/-* mice was probably a result of compensatory effects for the loss of FXR, and suggested widespread interactions within the transcription regulatory network where regulators cooperated in determining the hepatic phenotype.

Phase-I Gene Ontogeny in Fxr$^{-/-}$ Mice

After examining the global effect of FXR knockout during liver development, we took a further step to assess the ontogeny of individual Phase-I genes. Based on UCSC Genes Track mm10, there were 185 Phase-I genes in the 12 major families of Phase-I genes, and our data showed 136 of them were expressed during liver maturation in wild-type (WT) and *Fxr-/-* mice (average FPKM >1). Differential expression between wild-type and knockout mice occurred in genes from all 12 Phase-I gene families for at least one age during development, suggesting an extensive role of FXR in gene regulation. The distances of developmental pattern between WT and *Fxr$^{-/-}$* samples were calculated and Phase-I genes were listed in the order from small to large distance in Fig. 5.6. The genes at the upper part showed more difference in their developmental expression patterns by the knockout of FXR, and the top 10 most altered genes were *Cyp26a1, Cyp4a12b, Cyp1a1, Adh6-ps1, Cyp2a4, Cyp7a1, Cyp2d13, Fmo2, Cyp2d37-ps, and Akr1b7*. Changes in expression of these genes showed developmental stage-specific effects, for example, *Cyp26a1* and *Cyp4a12b* were only strongly induced or repressed at day 60, *Cyp2a4* was induced at adolescent stage, and *Akr1b7* was markedly repressed at neonatal stage in the knockout mice compared with wild-type. *Cyp3a11* is one of the most important genes in drug metabolism. Research has shown that CYP3A11 expression was induced in the

Fig. 5.6

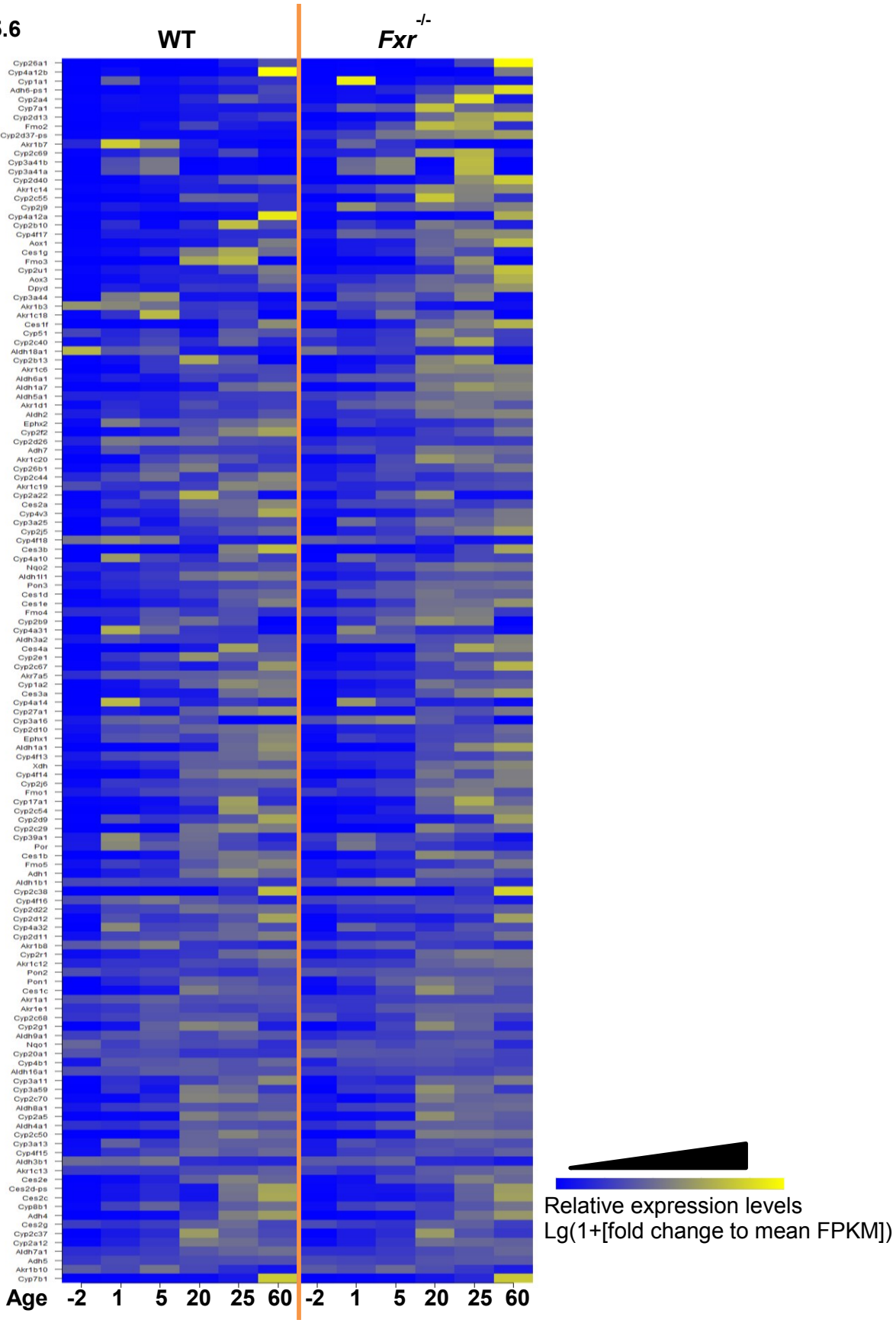

WT
Fxr -/-
Cyp26a1
Cyp4a12b
Cyp1a1
Adh6-ps1
Cyp2a4
Cyp7a1
Cyp2d13
Fmo2
Cyp2d37-ps
Akr1b7
Cyp2c69
Cyp3a41b
Cyp3a41a
Cyp2d40
Akr1c14
Cyp2c55
Cyp2j9
Cyp4a12a
Cyp2b10
Cyp4f17
Aox1
Ces1g
Fmo3
Cyp2u1
Aox3
Dpyd
Cyp3a44
Akr1b3
Akr1c18
Ces1f
Cyp51
Cyp2c40
Aldh18a1
Cyp2b13
Akr1c6
Aldh6a1
Aldh1a7
Aldh5a1
Akr1d1
Aldh2
Ephx2
Cyp2f2
Cyp2d26
Adh7
Akr1c20
Cyp26b1
Cyp2c44
Akr1c19
Cyp2a22
Ces2a
Cyp4v3
Cyp3a25
Cyp2j5
Cyp4f18
Ces3b
Cyp4a10
Nqo2
Aldh1l1
Pon3
Ces1d
Ces1e
Fmo4
Cyp2b9
Cyp4a31
Aldh3a2
Ces4a
Cyp2e1
Cyp2c67
Akr7a5
Cyp1a2
Ces3a
Cyp4a14
Cyp27a1
Cyp3a16
Cyp2d10
Ephx1
Aldh1a1
Cyp4f13
Xdh
Cyp4f14
Cyp2j6
Fmo1
Cyp17a1
Cyp2c54
Cyp2d9
Cyp2c29
Cyp39a1
Por
Ces1b
Fmo5
Adh1
Aldh1b1
Cyp2c38
Cyp4f16
Cyp2d22
Cyp2d12
Cyp4a32
Cyp2d11
Akr1b8
Cyp2r1
Akr1c12
Pon2
Pon1
Ces1c
Akr1a1
Akr1e1
Cyp2c68
Cyp2g1
Aldh9a1
Nqo1
Cyp20a1
Cyp4b1
Aldh16a1
Cyp3a11
Cyp3a59
Cyp2c70
Aldh8a1
Cyp2a5
Aldh4a1
Cyp2c50
Cyp3a13
Cyp4f15
Aldh3b1
Akr1c13
Ces2e
Ces2d-ps
Ces2c
Cyp8b1
Adh4
Ces2g
Cyp2c37
Cyp2a12
Aldh7a1
Adh5
Akr1b10
Cyp7b1
Age -2 1 5 20 25 60 -2 1 5 20 25 60
Relative expression levels
Lg(1+[fold change to mean FPKM])

Fig. 5.6 Developmental Expression Patterns of Phase-I Genes in Livers of Wild-type and *Fxr-/-* Mice.

Heat map of expression profiles are drawn for all expressed Phase-I genes (FPKM>1). And genes are listed in an order based on the distance of ontogenic pattern between WT and *Fxr*$^{-/-}$ samples, with the ones at the top showing patterns most altered by FXR knockout.

Fig. 5.7

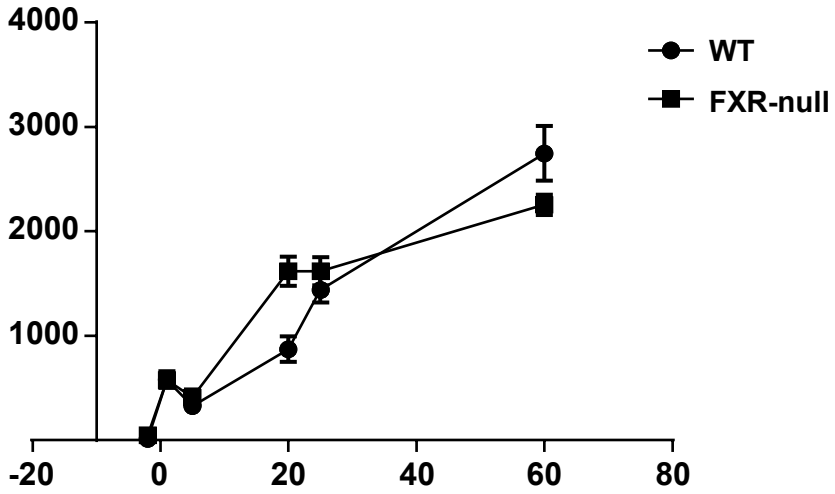


Fig. 5.7 Expression Patterns of CYP3A11 during Liver Development.

Data are expressed as mean FPKM and SEM of three individual animals.

absence of FXR (Schuetz et al., 2001). But in our results, induction of CYP3A11 mRNA only happened at day 20, and *Fxr-/-* does not significantly alter CYP3A11 ontogenic trend (Fig. 5.7).

Developmental Expression of Known FXR Target Genes

As we have found out widespread changes of gene expression in FXR-null mice, especially in those hepatic nuclear receptors and core transcription factors, it would be difficult for us to conclude on the direct role of FXR in regulation of gene transcription. Therefore, we examined the developmental expression pattern of several know FXR target genes. CYP7A1 is the rate-limiting enzyme in the classic bile acid synthesis pathway, and CYP8B1 is required for synthesis of cholic acid. The known mechanisms of FXR in regulating CYP7A1 and CYP8B1 are as follows. The activation of FXR by bile acids in the liver induces nuclear receptor SHP (Nr0b2), which interacts with HNF4 and LRH-1 and blocks their activation of *Cyp7a1* and *Cyp8b1* transcription. On the other hand, activated FXR in the intestine can induce FGF-15 (FGF-19 in human), which circulates to liver and bind FGFR4 to activate JNK phosphorylation pathway and suppress *Cyp7a1* and *Cyp8b1* expression (Claudel et al., 2005). Our results demonstrated that CYP7A1 mRNA level was significantly induced at all 6 ages in FXR knockout mice, and the induction fold change was largest at day 20, indicating the repression of CYP7A1 by FXR was more critical at day 20 (Fig. 5.8A). Although it was proposed that CYP7A1 and CYP8B1 were under the same FXR regulatory pathway, CYP8B1 mRNA level was not significantly altered in FXR-null mice (Fig. 5.8B). So the regulation of FXR on *Cyp8b1* gene was relatively weak, and there might be other regulatory factors that maintain the expression of CYP8B1 in the absence of FXR. The expression of SHP, the direct target gene of FXR in the liver pathway for regulation of CYP7A1 and CYP8B1, was only reduced at young ages in FXR-null mice, also suggesting developmental stage-specific effect of FXR (Fig. 5.8C). And the result indicated that the induction of CYP7A1 at day 60 was mainly due to failed suppression from the FXR intestinal

Fig. 5.8

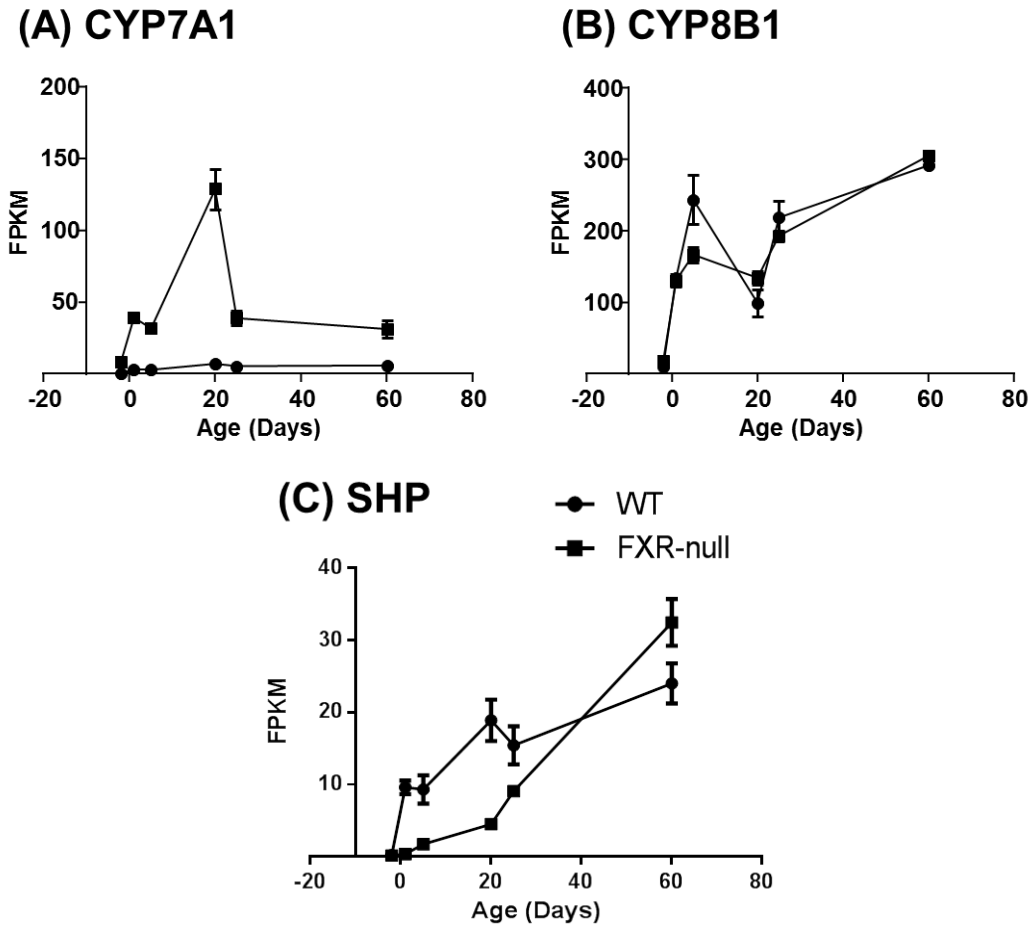


Fig. 5.8 Expression Patterns of (A) CYP7A1, (B) CYP8B1 and (C) SHP during Liver Development.

Data are expressed as mean FPKM and SEM of three individual animals.

pathway. Taken together, these data provided new insights for the role of FXR in regulation of gene expression.

Phase-I Genes Potentially Targeted by FXR

Our data also suggested new Phase-I genes potentially targeted by FXR. Previous study has shown that bile acid and FGF19 treatment decreased PON1 mRNA level (Shih et al., 2006). And in our FXR-null mice, the PON1 mRNA level was induced during development, which further supported the role of FXR in repression of PON1 expression, probably through intestinal FXR function. In addition, the induction of PON2 and PON3 mRNA in FXR-null mice was also observed (Fig. 5.9). *Pon1/2/3* genes are located next to each other in the genome and it is highly likely that they are co-regulated by FXR.

Besides *Cyp7a1*, only three other genes, *Aldh5a1, Aldh6a1* and *Dpyd,* showed significant changes of expression at all 6 ages in FXR-null mice (Fig. 5.10). ChIP-Seq study identified FXR binding on *Aldh5a1* gene (Lee et al., 2012). So the mRNA basal expression of these Phase-I genes may be repressed by FXR. However, further study is needed to validate the direct involvement of FXR in control of the expression of these genes.

5.5 Discussion

In this study, comparison of liver developmental transcriptome in wild-type and FXR knockout mice by RNA-Seq revealed two major groups of findings. First, compared with wild-type mice, the deletion of FXR showed developmental stage-specific effects on different biological processes in liver and had an overall impact to delay liver maturation at adolescent stage. Second, the loss of FXR resulted in significant changes of Phase-I gene expression from all 12 major families, and suggested new Phase-I genes potentially targeted by FXR.

The effect of FXR knockout on postnatal liver development was most significant at adolescent stage, as the pathways specifically active at neonatal stage in wild-type mice failed to be shut down at adolescent stage in *Fxr-/-* mice, and the pathways that should mature before adolescent stage remained immature in *Fxr-/-* mice (Fig. 5.3 and 5.4). The surge of liver bile acids levels during this period shown by previous studies (Cui et al., 2012a) may underlie the critical function of FXR at this developmental stage. Shown in the sample correlation heat map (Fig. 5.2), although the similarity between neonatal and adolescent stages was higher in FXR-null mice, the correlations between day 60 sample and earlier ages were still very low, indicating dramatic liver maturation processes happened between day 25 and day 60 in FXR-null mice. It would be informative to study the knockout livers during this period and elucidate how the livers overcome the loss of FXR and mature.

Our result of the multiple organ developmental processes in *Fxr-/-* mice further confirmed the role of FXR as a core hepatic nuclear receptor that defines the hepatic phenotype. Six hepatic transcription factors have been identified to form complex auto-regulatory and cross-regulatory circuits (Kyrmizi et al., 2006). And the regulatory circuits may be a more pervasive phenomenon as most of the nuclear receptors together with the core transcription factors showed altered expressions in *Fxr-/-* mice (Table 5.1). While most transcriptional regulators displayed increased expression probably to compensate FXR function, the liver X receptor α (LXRα, Nr1h3) showed significantly decreased expression at all ages. Interestingly, LXRs and FXR are known to be the yin and yang of cholesterol and fat metabolism, maintaining a balanced regulation of cholesterol and bile acid metabolism (Kalaany and Mangelsdorf, 2006). And here our results suggested a further interplay between these two nuclear receptors as FXR may be involved in the regulation of LXRα.

Postnatal liver development is largely an under-studies area. Some groups have identified certain critical factors for postnatal liver development, including β-Catenin and Yes-associated

protein (Apte et al., 2007; Septer et al., 2012). These two factors showed increased expression during postnatal liver development to promote cell proliferation, and deletion of either of them could lead to impaired liver growth. Our transcriptome data also revealed the developmental expression of β-Catenin and Yes-associated protein (data not shown). They were both induced in FXR knockout mice, which is consistent with the prolonged cell proliferation in FXR knockout livers. And the result further proved their direct roles in control of liver growth.

For the Phase-I gene expressions most altered by the loss of FXR, CYP26A1mRNA was highly induce at day 60. CYP26A1 functions in the catabolism of retinoic acid, and is normally induced by increased retinol or retinoic acid levels. It is possible that this gene induction is a result of altered metabolism in retinoic acid pathway. The second gene in the distance list, *Cyp4a12*, was markedly suppressed at day 60 (Fig. 5.6). *Cyp4* gene family is known to be regulated by PPAR receptors, and FXR can influence PPAR expressions. So this may also be an indirect effect of FXR knockout. With a complicated regulatory network, it is difficult for us to conclude on the role of FXR in some of the changes of expression patterns. ChIP-Seq technology using specific anti-FXR antibody is a powerful tool to identify genome-wide FXR binding sites. Future studies combining the current data with ChIP-Seq experiments during liver development would provide important information to understand the regulatory mechanism of FXR on Phase-I gene ontogeny.

Quantifying the developmental transcriptome is an initial step to study postnatal liver development. It utilized advanced technology to generate an overall picture of gene expression, and revealed potential fields of interest that await further researches. For example, histology and liver functional studies based on the transcriptome data would help provide a more defined concept of how the liver matures. Future studies to over-express FXR or treat the animals with FXR agonists during development may complement the loss-of-function study and help differentiate the direct and indirect effects of FXR in regulating postnatal liver development.

Analyzing the role of FXR in liver maturation is critical for studying the developmental regulation of Phase-I genes, because drug metabolism is an important liver function, and the maturation of Phase-I enzymes is an indispensable part of live maturation. Altogether, these studies would enable a more profound understanding of liver development and Phase-I gene ontogeny, and provide a foundation to assist researchers in understanding developmental susceptibility and improving the safety and effectiveness of pharmacotherapy in children.

Fig. 5.9

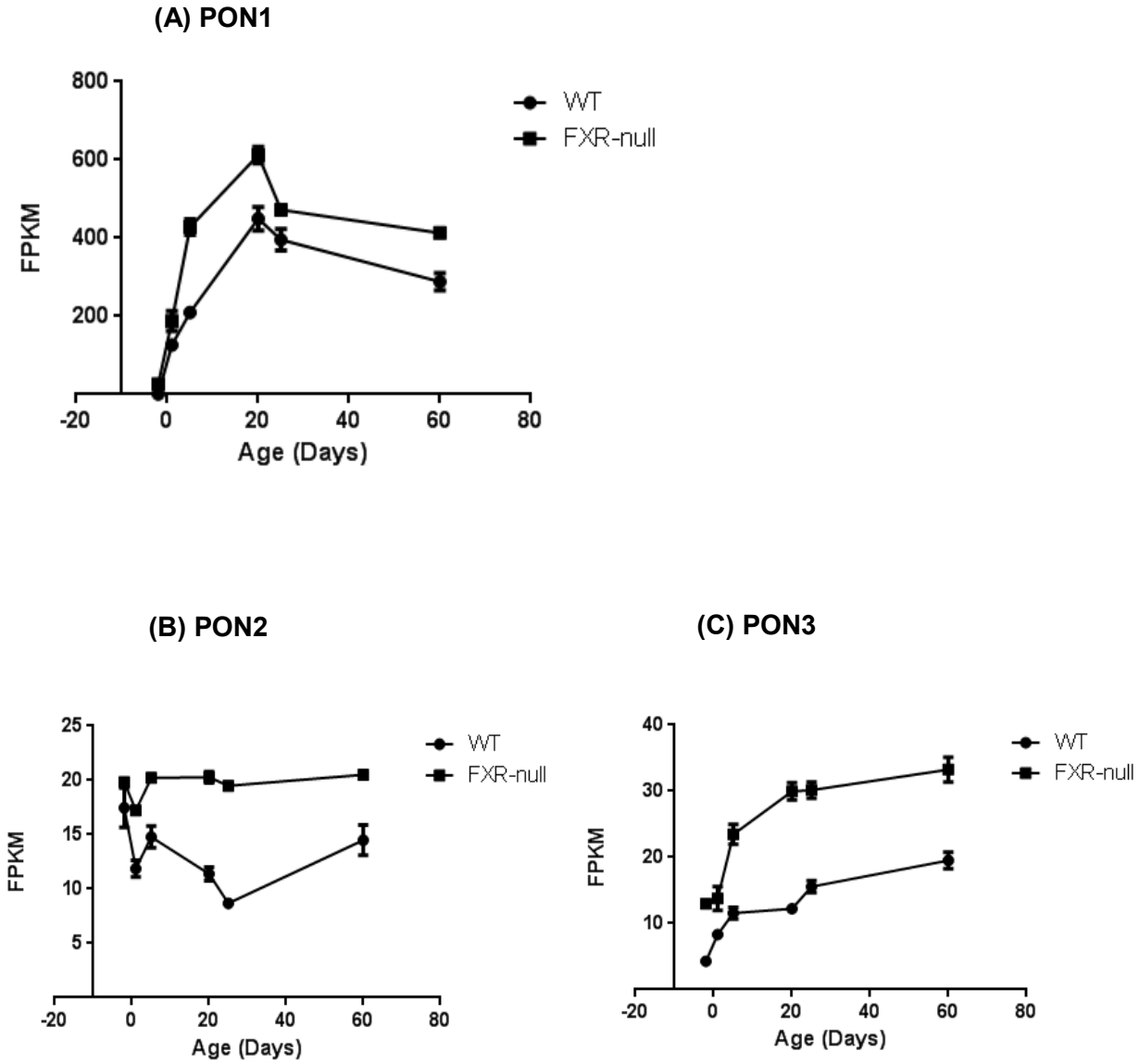


Fig. 5.9 Expression Patterns of (A) PON1, (B) PON2 and (C) PON3 during Liver Development.

Fig. 5.10

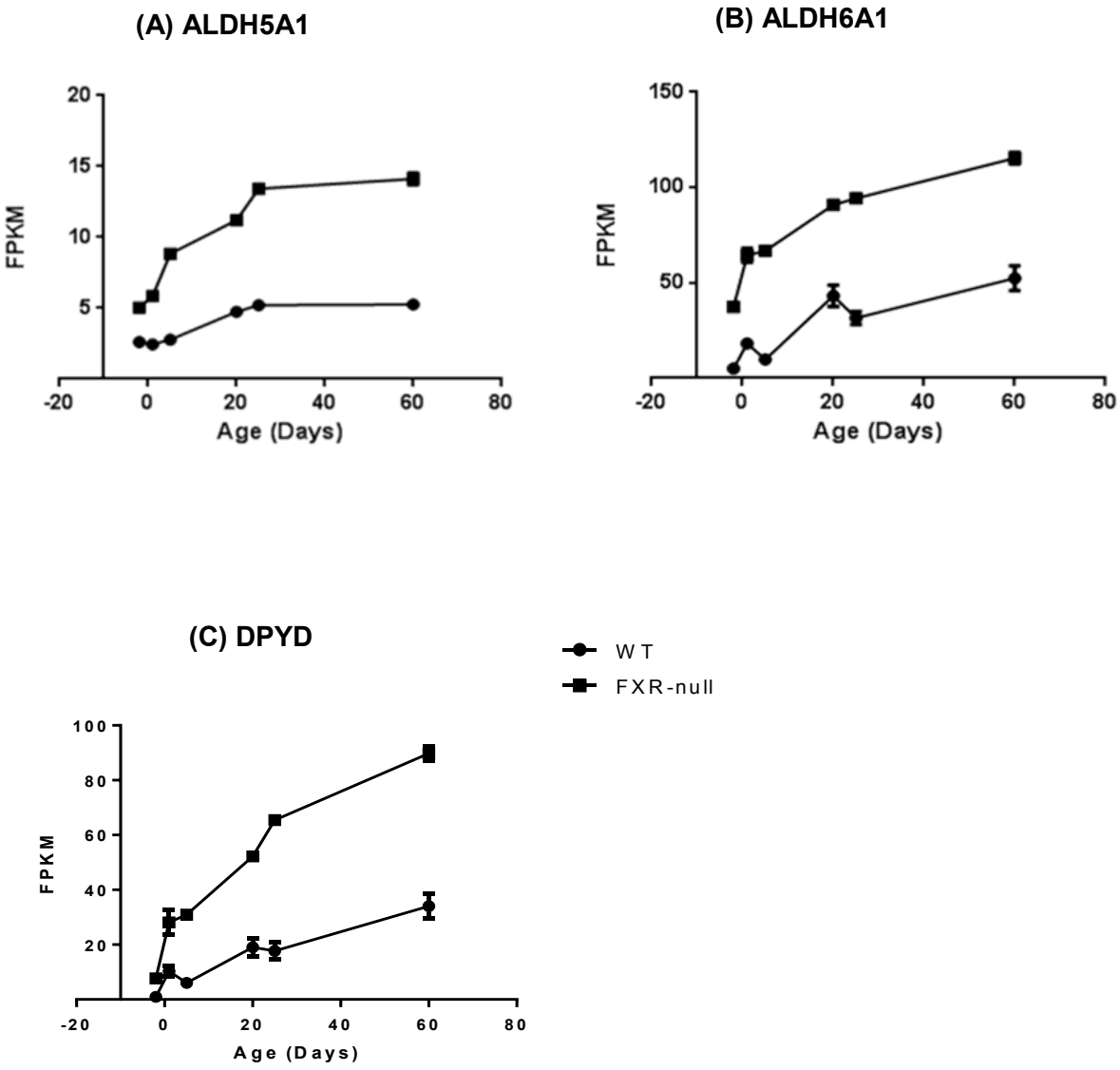


Fig. 5.10 Expression Patterns of (A) ALDH5A1, (B) ALDH6A1 and (C) DPYD during Liver Development.

Chapter 6 : Conclusion and Future Directions

6.1 Conclusion

The present dissertation has systemically characterized the model of mouse liver during postnatal development and examined the ontogenic expression signatures of all major Phase-I drug metabolizing genes. Long non-coding RNAs and nuclear receptor FXR mediated regulation of gene expression have been explored and addressed.

The major function of fetal liver is hematopoiesis, which is completely different from adult liver. Dramatic changes during development are necessary for the maturation of liver functions, and they may have significant impact on the risk of adverse drug reactions in children. Through multi-disciplinary approaches, the present study has investigated the process of functional transition during liver maturation. Different developmental stages revealed by transcriptome patterns were identified, including perinatal, neonatal, adolescent and adult, which were highlighted by changes of gene activity in various biological processes. Taking advantage of the deep sequencing technology, the developmental expression patterns of lncRNAs were initially characterized. LncRNAs exhibited the same major patterns as protein-coding RNAs, and neighboring protein-coding and long non-coding RNAs showed more correlated developmental expressions, which strongly suggested the involvement of lncRNAs in the control of liver ontogeny.

For Phase-I metabolism, a systematic study of the ontogenic expression of 103 P450 genes in mouse liver was performed, as well as another 11 families of Phase-I genes, including carboxylesterase, paraoxonase, epoxide hydrolase, which function in hydrolysis; aldo-keto reductase, quinone oxidoreductase, dihydropyrimidine dehydrogenase, which mainly catalyze reduction reaction; and alcohol dehydrogenase, aldehyde dehydrogenase, flavin

monooxygenases, molybdenum hydroxylase, and cytochrome P450 oxidoreductase, which are typical oxidative enzymes. Total Phase-I gene mRNA expression showed two rapidly increasing stages after birth, reflecting the timeline for the maturation of metabolic functions. Diverse expression patterns of Phase-I genes with enriched mRNA at different stages of development were also identified, and the patterns were highly homologous to humans, which provide an important foundation for further mechanistic studies.

FXR is an important nuclear receptor activated by bile acids. In the immediate perinatal period, newborns need to synthesize and secrete bile acids into bile to facilitate the absorption of milk. The activation of FXR around birth may be critical for hepatic gene expression at early ages. Based on our characterized model of mouse liver development, we went on to examine the role of FXR in regulation of liver and Phase-I gene ontogeny through comparison with age matched FXR-null mice. The data revealed a critical role of FXR in promoting proper liver maturation and in control of Phase-I gene expressions during development.

The details about developmental liver functional transition, showing what pathways are matured at which stage, provide evidence to prove the general idea that pediatrics are different from adult clinics, and give a foundation for us to understand the cause of clinical problems in developmental pharmacology. The study on FXR has identified a novel role of FXR in liver maturation, thus the differences in FXR activity among children can be a potential factor to help predict possible variations and susceptibilities of pediatric patients. In conclusion, through integrating different research models and technologies, the present dissertation has examined the ontogenic patterns as well as possible regulatory mechanisms for phase-I drug metabolizing gene expression during liver development, which provides novel insights into identifying and further understanding the molecular targets for safe and efficacious drug treatments in children. The results also build up a valuable model platform for the future study of developmental gene expression and regulation.

6.2 Future Directions

Factors in developmental regulation may come from endogenous, nutritional and environmental sources. Endogenous hormones are key upstream signals in regulating gene expression during perinatal and postnatal development. Growth hormone (GH) is a member of an extensive GH/prolactin family of peptides. The essential role of GH in regulating body growth and maturation of the liver is well demonstrated by the studies of lit/lit mice, which have a spontaneous mutation in the growth hormone releasing hormone receptor (Eicher and Beamer, 1976; Noshiro and Negishi, 1986). Hepatic expression of P450s is profoundly altered in lit/lit mice (Sharma et al., 1998; Waxman and O'Connor, 2006), demonstrating a critical role of GH in regulating hepatic expression of these genes. It would be interesting to examine liver developmental process in lit/lit mice to further characterize the role of growth hormone on Phase-I gene ontogeny.

We consider the current study of FXR related to the effect of nutrition, as the natural ligand for FXR is bile acid, the level of which can be influenced by food intake. In addition to maturation of the bile acid processing system during postnatal liver development, weaning and food intake will result in absorption of an increasing amount of xenobiotics. Increased absorption of xenobiotics requires the activation of detoxification systems. PXR and CAR are classic xenobiotic sensors in the liver, and are responsible for the inducible expression of multiple drug processing genes. However, the role of these xenobiotic sensors in regulation of Phase-I genes during development has not been discussed. Surprisingly, a recent study in human hepatocytes found that altered bile acid levels could affect the basal expression of multiple P450s, probably because bile acids are ligand activators of FXR, PXR and CAR (Chaudhry et al., 2013). Investigating hepatic bile acid levels and the active role of PXR and CAR during development

would be critical to understand the nutritional and environmental factors in regulation of the maturation of drug metabolizing enzymes.

There is a group of liver enriched transcription factors that are involved in liver development, many of which are regulated by GH, such as HNF3β, 3γ, 4α, and 6, CCAAT/enhancer binding protein alpha (C/EBPα), C/EBPβ, c-fos and c-jun (Osafo et al., 2005). It is clear that there is crosstalk among multiple nuclear receptors in liver. Studying the mechanism role of these nuclear receptors in regulating gene expression may help us dissect the complex interplay network among nuclear receptors and elucidate the overlapping and unique properties of their functions during development.

Hepatic gene expression is hierarchically regulated by multiple mechanisms. Nuclear receptors and other transcription factors are essential for gene regulation but lack the enzymatic activities necessary to modulate chromatin structure. These activities are catalyzed by coregulators recruited in response to different signals, including ligand-receptor binding (Gronemeyer et al., 2004). Coregulators are classified as coactivators or corepressors. Coactivators are histone acetyltransferases, methyltransferases, or their docking-partners. They are known to interact with xenosensors like PXR (Xie et al., 2009), and involved in chromatin relaxation and recruitment of the basic transcriptional machinery (Hankinson, 2005). Corepressors, such as nuclear receptor co-repressor 1 (NcoR1) and silencing mediator of retinoic acid and thyroid hormone receptors (Smrt), preferentially bind to unactivated receptors and recruit histone deacetylases and/or demethylases, leading to chromatin condensation and gene repression (Collingwood et al., 1999; Nishioka and Reinberg, 2001). When examining the differentially expressed genes in FXR-null mice compared with wild-type mice during development in our data, GO analysis identified significantly enriched biological processes of histone modifications and chromatin remodeling. We know that the DNA sequences are normally unaltered during development, but epigenetic signatures can be changed with the

environment, which may play an important role in developmental regulation. Studying the function of coregulators associated with the important hepatic nuclear receptors and the change of epigenetic signatures in the target genes will provide more insights into the molecular mechanism of gene regulation during development.

Studies have shown that drug treatment at early ages may lead to epigenetic memory that affects the drug response at later ages (Chen et al., 2012). Based on our study on normal developmental processes, future investigations about the effect of neonatal drug treatment on variations of drug response may introduce a new concept in inter-individual variations and have significant impact on research of personalized medicine. The successful completion of these related studies will generate critical knowledge that will serve as the foundation for further understanding of pediatric pharmacology in humans.

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