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Role of farnesoid X receptor in establishment of ontogeny of phase-I drug metabolizing enzyme genes in mouse liver

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Abstract   The expression of phase-I drug metabolizing enzymes in liver changes dramatically during postnatal liver maturation. Farnesoid X receptor (FXR) is critical for bile acid and lipid homeostasis in liver. However, the role of FXR in regulating ontogeny of phase-I drug metabolizing genes is not clear. Hence, we applied RNA-sequencing to quantify the developmental expression of phase-I genes in both *Fxr*-null and control (C57BL/6) mouse livers during development. Liver samples of male C57BL/6 and *Fxr*-null mice at 6 different ages from prenatal to adult were used. The *Fxr*-null showed an overall effect to diminish the "day-1 surge" of phase-I gene expression, including cytochrome P450s at neonatal ages. 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*), and epoxide hydrolase (*Ephx*); reduction: aldoketo reductase (*Akr*), quinone oxidoreductase (*Nqo*), and dihydropyrimidine dehydrogenase (*Dpyd*); and oxidation: alcohol dehydrogenase (*Adh*), aldehyde dehydrogenase (*Aldh*), flavin monooxygenases (*Fmo*), molybdenum hydroxylase (*Aox* and *Xdh*), cytochrome P450 (P450), and cytochrome P450 oxidoreductase (*Por*). The data also suggested new phase-I genes potentially targeted by FXR. These results revealed an important role of FXR in regulation of ontogeny of phase-I genes.

1. Introduction

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 phase-I drug-metabolizing enzymes mature during this period in mouse liver[1,2]. Changes in expression of some phase-I enzymes during liver maturation in humans have also been reported, including cytochrome P450 (P450s)[3–6], carboxylesterase (CES)[7,8], paraoxonase (PON)[9,10], alcohol dehydrogenase (ADH)[11], and flavin monooxygenase (FMO)[12–14]. 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[15–19]. 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.

Farnesoid X receptor (FXR) is a bile acid sensing nuclear receptor in maintenance of 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[20]. FXR also plays a role in liver regeneration[21]. Dysfunction of FXR may lead to development of digestive system diseases[22] and alcoholic liver disease[23]. The role of FXR in regulating certain drug metabolizing enzyme genes has also 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 and MRP3) xenobiotics[24,25]. A functional FXR polymorphism is common (2.5%–12%), and has been associated with changes in FXR-target gene expression in humans[26,27]. Understanding the regulatory mechanism of FXR on hepatic expression of drug metabolizing enzyme genes in newborns will greatly help us to understand the natural and aberrant development of drug metabolizing enzyme genes in pediatric pharmacology.

In the current study, we used RNA-sequencing to quantify hepatic phase-I metabolizing enzyme gene expression during the developmental period from perinatal stage to adult in both wild-type and a well-established Fxr knockout mouse model[20]. FXR has been confirmed to be knocked out in whole body in this Fxr-null model. Our results revealed the significant differences in Fxr-null livers compared to control livers during postnatal development, and suggested novel roles of FXR in the regulation of phase-I gene developmental expression.

2. Materials and methods

2.1. 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[1,2]. An Fxr-deficient mouse model[28] was used to examine the role of FXR in determination of ontogeny of phase-I drug metabolizing enzyme genes in mouse liver. These knockout mice were bred under standard conditions in the Office of Animal Care Facility at the University of Connecticut (Storrs, CT, USA). The use of these mice was approved by the University of Connecticut's Institutional Animal Care and Use Committee. Liver samples ($n=3$) from wild-type and $Fxr^{-/-}$ mice were collected at the following 6 ages: day –2 (gestational day

17.5), day 1 (exactly 24 h 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 °C.

2.2. 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[2].

2.3. RNA-Seq data analysis

For comparison of phase-I genes between wild-type and Fxr-knockout samples at the 6 ages of days –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.

2.4. Data visualization and statistics

In this study, we consider genes with FPKM >1 as expressed. ANOVA was used to test for significant difference in expression between wild-type and Fxr-null mice. P values were adjusted using Benjamini-Hochberg algorithm with a threshold of 0.05. 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.

3. Results

3.1. Phase-I gene ontogeny in $Fxr^{-/-}$ mice

For all the P450 genes, significant differences in total mRNA expression between wild-type and $Fxr^{-/-}$ mice were found at ages of days –2, 1 and 5 (Fig. 1A). The expression of total P450s was higher before birth and lower after birth till day 5 in $Fxr^{-/-}$ mice than in wild-type. There was a surge in expression of P450 genes around birth. From days –2 to day 1, the total P450 mRNA level increased more than 12-fold in wild-type mice, yet this increase was less than 5-fold in $Fxr^{-/-}$ mice (Fig. 1A). The total P450 mRNAs reached similar levels from days 20 to 60 in both wild-type and Fxr-null mice. A similar change in total mRNA expression of non-P450 phase I genes between wild-type and $Fxr^{-/-}$ mice was found in Fig. 1B.

When examining the composition of different families of phase-I genes within this total expression, we also found dramatic changes around birth and significant differences between wild-type and Fxr-null mice (Fig. 2). At day –2, the mRNA percentage of Akr and Aldh were much lower and the percentage of Adh and Ces were much higher in Fxr-null mice compared with wild-type mice. The percentage of P450 mRNAs in all phase-I genes nearly

A FPKM of total P450s

Age (days)	FPKM of total P450s in WT	
	Mean	SEM (n=3)
-2	578	28
1[#]	7162[#]	144
5	6574	618
20	13599	590
25	13067	614
60	14696	1822
Age (days)	FPKM of total P450s in $Fxr^{-/-}$	
	Mean	SEM (n=3)
-2	939	93
1	4410	421
5	4303	221
20	12355	134
25	11216	323
60	12760	67

B FPKM of total non-P450 Phase I

Age (days)	FPKM of total phase-I in WT	
	Mean	SEM (n=3)
-2	1725	60
1[#]	10650[#]	122
5	10649	977
20	22585	883
25	24340	801
60	25496	2008
Age (days)	FPKM of total phase-I in $Fxr^{-/-}$	
	Mean	SEM (n=3)
-2	2590	176
1	7631	604
5	9131	492
20	23840	215
25	22193	471
60	23511	200

[#]Day 1 surge in both P450s and non-P450 phase-I in WT, but not in $Fxr^{-/-}$ mice.
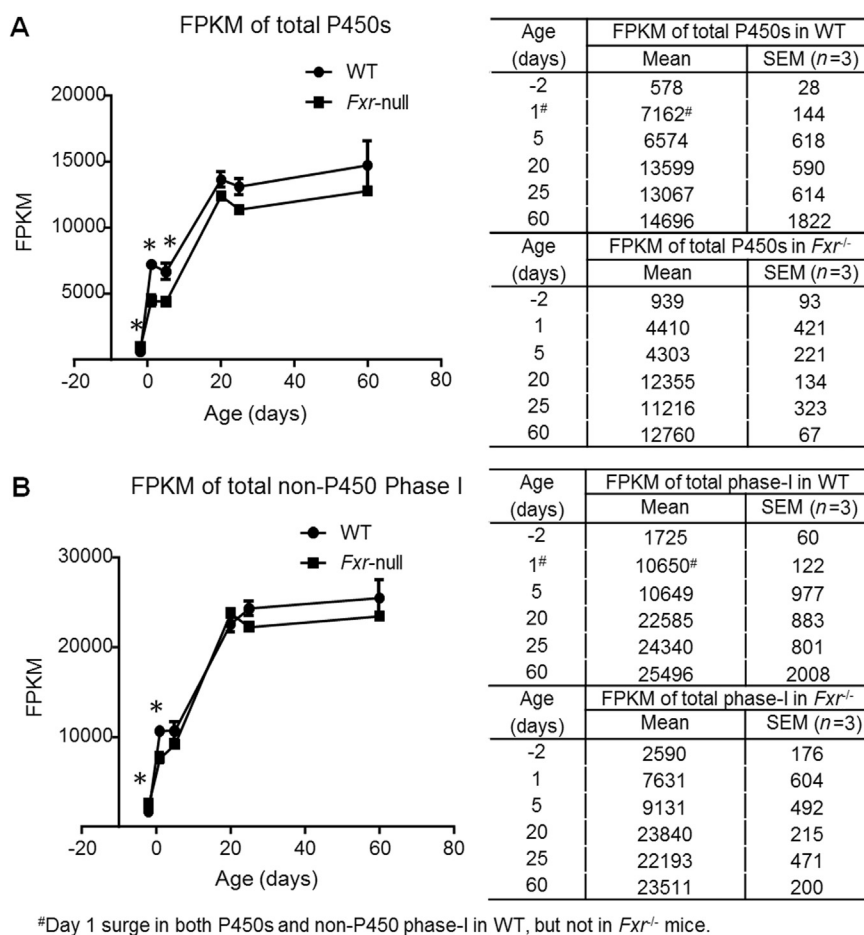
Figure 1 Total mRNA levels of the 102 P450 genes (A) and 185 non-P450 phase-I genes (B) in the mouse liver during development. RNA-Seq was done for liver mRNAs of wild-type C57BL/6 and Fxr-null male mice at 6 ages from 2 days before birth to 60 days after birth. The FPKM values of all genes in the group at each age were added and plotted to show the developmental pattern of total mRNAs. Bars represent the mean FPKM and SEM of three individual animals. Star sign indicates significant difference of expression between wild-type and Fxr-null mice with adjusted $P<0.05$.

doubled from days –2 to 1 in wild-type mice, but the P450 percentage was much lower in Fxr-null mice at days 1 and 5. Ces was a family of phase-I genes that showed dramatically higher percentage of expression in Fxr-null mice at perinatal stage of days –2, 1 and 5 (Fig. 2). It is possible that the loss of FXR activated other nuclear receptors or transcription factors in compensation that induced Ces and $Aldh$ expression at neonatal ages. When the age got older, for example at days 25 and 60, both the total mRNA and the composition of different families of phase-I gene expression became similar in wild-type and Fxr-null mice.

After examining the overall effect of Fxr knockout on phase-I gene expression 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 large to small distance in Fig. 3. The genes at the upper part showed

more difference in their developmental expression patterns caused 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 absence of FXR[29]. But in our results, induction of $Cyp3a11$ mRNA only happened at day 20 after birth, and $Fxr^{-/-}$ does not significantly alter CYP3A11 ontogenic trend (Fig. 4).

3.2. Developmental expression of known FXR target phase-I 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.
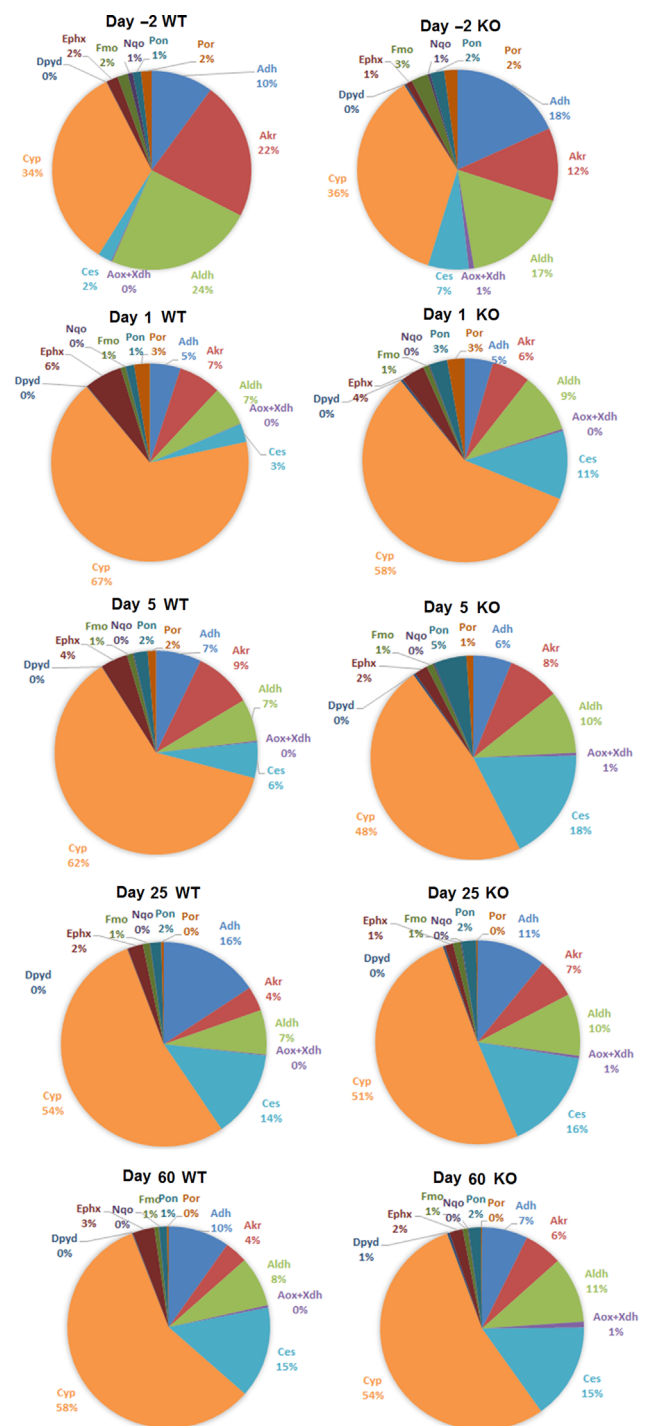
Figure 2 Percentages of FPKM values of each phase-I family at ages of days –2, 1, 5, 25, and 60 in both wild-type and *Fxr*-null mice. WT, wild-type and KO, *Fxr*-null.

Therefore, we examined the developmental expression pattern of several known 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 HNF4α and LRH-1 and blocks their activation of *Cyp7a1* and *Cyp8b1* transcription.
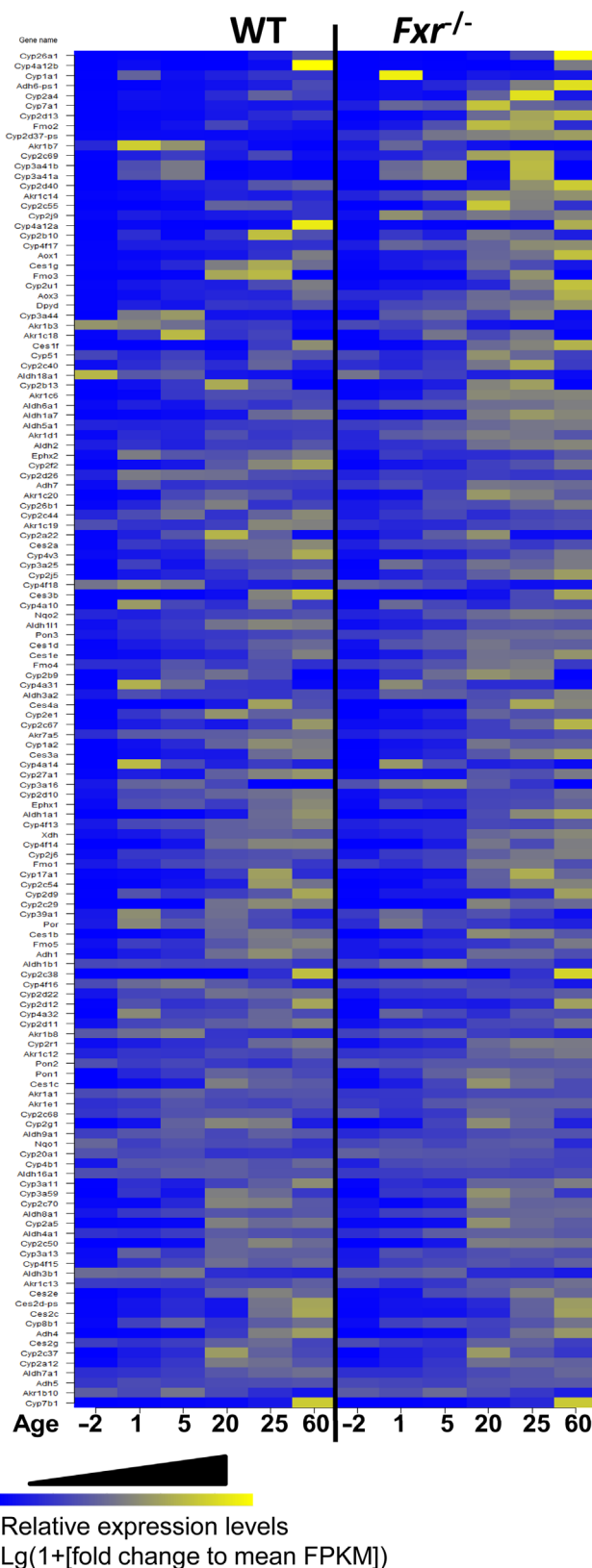

Figure 3 Developmental expression patterns of phase-I genes in livers of wild-type and *Fxr*−/− mice. Heat map of expression profiles are drawn for all expressed phase-I genes (FPKM > 1). The genes are listed in an order based on the distance of ontogenic pattern between WT and *Fxr*−/− samples, with the ones at the top showing patterns most altered in *Fxr*-knockout mice.
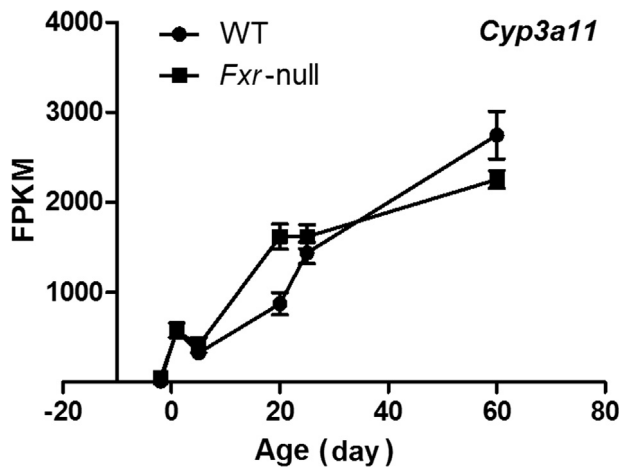
Figure 4 Expression patterns of CYP3A11 during liver development. Data are expressed as mean FPKM and SEM of three individual animals.

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. 5A). 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. 5B). So the regulation of FXR on *Cyp8b1* gene was relatively weak, and there might be other critical 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. 5C). And the result indicated that the induction of CYP7A1 at day 60 was mainly due to failed suppression from the FXR intestinal pathway. Taken together, these data provided new insights for the role of FXR in regulation of its target gene expression.

3.3. 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[31]. And in our *Fxr*-null mice, the *Pon1* mRNA level was induced during development (Fig. 6A), which further supported the role of FXR in repression of PON1 expression, probably through intestinal FXR function. In
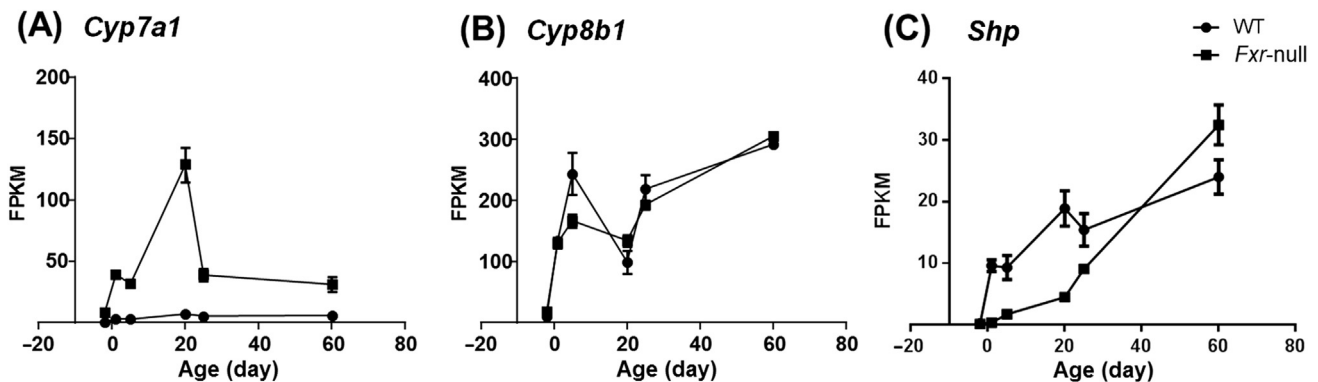
On the other hand, activated FXR in the intestine can induce intestinal FGF-15 (FGF-19 in human), which circulates to liver and bind FGFR4 to activate JNK phosphorylation pathway and suppress *Cyp7a1* and *Cyp8b1* expression[30]. Our results demonstrated that *Cyp7a1* mRNA level was significantly induced at all

Figure 5 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.
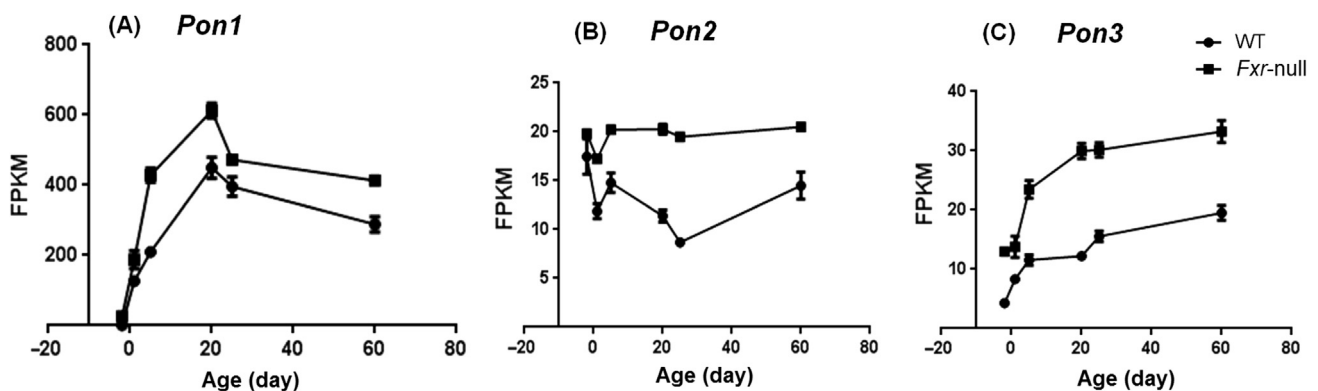

Figure 6 Expression patterns of (A) *Pon1*, (B) *Pon2*, and (C) *Pon3* during liver development. Data are expressed as mean FPKM and SEM of three individual animals.
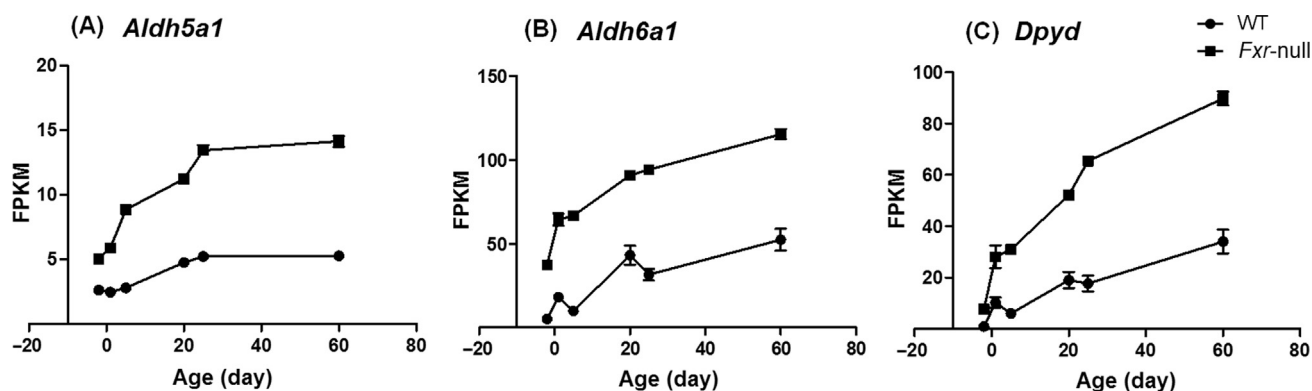
Figure 7 Expression patterns of (A) *Aldh5a1*, (B) *Aldh6a1*, and (C) *Dpyd* during liver development. Data are expressed as mean FPKM and SEM of three individual animals.

addition, the induction of *Pon2* and *Pon3* mRNA in *Fxr*-null mice was also observed (Fig. 6B and C). *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. 7). ChIP-Seq study identified FXR binding on *Aldh5a1* gene[32]. So the mRNA basal expression of these phase-I genes during development 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.

4.   Discussion

In this study, comparison of ontogeny of hepatic phase-I genes in wild-type and *Fxr*-knockout mice by RNA-Seq revealed important roles of FXR in regulating developmental expression of phase-I genes. 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 baby mice take milk from their mother, which has a high fat content. Bile acids are necessary for the absorption of fat, and they are mainly synthesized and secreted at the immediate perinatal period to facilitate milk absorption. As the endogenous ligands for FXR, bile acids activated FXR signaling may be important for regulating perinatal gene expression in liver. Studies have shown that FXR functions to initiate the expression of major bile acid transporters in newborn mice[33]. FXR is critical for the "day 1 surge" pattern of *Ntcp*, *Bsep*, and *Mdr2* in newborn mouse liver. And here in our results, FXR is also critical for the "day 1 surge" of total phase-I expression (Fig. 1). The total mRNA of P450s was expressed significantly lower at newborn ages (days 1 and 5) in *Fxr*-null mice.

For the phase-I gene expressions most altered by the loss of FXR, *Cyp26a1* mRNA 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. 3). *Cyp4* gene family is known to be regulated by PPAR receptors, and FXR can influence PPAR expressions[34]. So this may also be an indirect effect of *Fxr* knockout. Many genes identified in this project may be indirectly regulated by FXR. 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[35]. 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 ontogeny of phase-I genes.

Quantifying the developmental expression is an initial step to study ontogeny of phase-I genes. It utilized advanced technology to generate an overall picture of gene expression, and revealed potential fields of interest that await further researches. For example, 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 development gene expression. Characterization of functional FXR polymorphisms in human and their roles in bile acid homeostasis will help to understand the important roles of FXR. Altogether, these studies would enable a more profound understanding of liver development and ontogeny of phase-I genes, and provide a foundation to assist researchers in understanding developmental susceptibility and improving the safety and effectiveness of pharmacotherapy in children.

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