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RNA-Seq provides new insights on the relative mRNA abundance of antioxidant components during mouse liver development

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Abstract

Mammals have developed a variety of antioxidant systems to protect them from the oxygen environment and toxic stimuli. Little is known about the mRNA abundance of antioxidant components during postnatal development of the liver. Therefore, the purpose of this study was to compare the mRNA abundance of antioxidant components during liver development. Livers from male C57BL/6J mice were collected at 12 ages from prenatal to adulthood. The transcriptome was determined by RNA-Seq with transcript abundance estimated by Cufflinks. RNA-Seq provided a complete, more accurate, and unbiased quantification of the transcriptome. Among 33 known antioxidant components examined, three ontogeny patterns of liver antioxidant components were observed: (1) Prenatal-enriched, in which the mRNAs decreased from fetal livers to adulthood, such as metallothionein and heme oxygenase-1; (2) adolescent-rich and relatively stable expression, such as peroxiredoxins; and (3) adult-rich, in which the mRNA increased with age, such as catalase and superoxide dismutase. alternative splicing of several antioxidant genes, such as *Keap1*, *Glx2*, *Gpx3*, and *Txnrd1*, were also detected by RNA-Seq. In summary, RNA-Seq revealed the relative abundance of hepatic antioxidant enzymes, which are important in protecting against the deleterious effects of oxidative stress.

Graphical Abstract

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Conflicts of interest

The authors declare no competing financial interests and conflict of interest.

Keywords

RNA-Seq; ontogeny; mouse liver; antioxidant

1. Introduction

One of the major challenges in a biological system during development is to maintain cellular redox homeostasis through balancing the rate and magnitude of oxidant generation and oxidant detoxification [1]. An imbalance in reactive oxygen species (ROS) production and adaptive antioxidant capacity will result in oxidative stress, a pathogenic stage that serves as both a cause and consequence of numerous acute toxicities and chronic diseases [2].

Newborns and especially preterm infants are susceptible to oxidative stress [3] and a delay in the development of protective pathways increases the risk of oxidative stress-induced pathogenesis in newborns [4]. Antioxidant mechanisms are crucial for prevention of oxidative stress-induced pathogenesis in newborns.

In general, there are three cellular antioxidant defense systems to protect against oxidative stress: low-molecular-weight antioxidants, enzymatic-antioxidant pathways, and free-ion sequestration. The major endogenous low-molecular-weight antioxidants include glutathione (GSH) and bilirubin.

GSH serves as the most abundant cellular thiol resource, and provides a buffer system to maintain cellular redox status. GSH is a tripeptide synthesized by gamma-glutamylcysteine synthetase and glutathione synthetase. GSH reduces hydrogen and is also oxidized to its disulfide form (GSSG). GSSG is reduced to GSH through glutathione reductase using NADPH as the co-factor. Bilirubin, which is formed by biliverdin reductase, is the major degradation product of heme catabolism. Bilirubin reduces oxidative stress and becomes oxidized to biliverdin. The enzymatic antioxidant pathways include catalase, peroxiredoxin, and glutathione peroxidase. Catalase reduces two molecules of hydrogen peroxide into water, producing one molecule of singlet oxygen. Catalase is localized in peroxisomes and is found in every organ with particularly high concentrations in liver. Glutathione peroxidase (*Gpx*) reduces hydrogen peroxide to water and spontaneously oxidizes GSH into GSSG. The

enzyme activity of *Gpx* is about one-tenth of catalase in multiple organs (i.e., liver, kidney, spleen, and lung) [5], indicating a minor role of *Gpx* in detoxifying ROS. In addition, hydrogen peroxide can be reduced by peroxiredoxin (*Prdx*), resulting in oxidation of *Prdx* from the reduced form (*Prdx*-SH) to the oxidized form (*Prdx*-S-S-*Prdx*). *Prdx*-S-S-*Prdx* can be reduced to *Prdx*-SH through thioredoxin and thioredoxin reductase. If *Prdx* is further oxidized into the sulfinic acid form (*Prdx*-SOOH), it can be reduced by sulfiredoxin.

Transient metal ions (especially Fe^{2+} and Cu^{+}) facilitate generation of hydroxyl free radicals from hydrogen peroxide through the Fenton-like reaction. Thus, free ion-binding proteins are important in preventing oxidative stress. Ferritin serves to store, deposit, and transfer iron in a non-toxic form [6]. Some heavy metals, including cadmium and mercury, readily bind to thiol groups of proteins, leading to disruption of redox homeostasis and protein damage. Metallothioneins are a family of low-molecular-weight cysteine-rich (22%–33%) proteins that bind endogenous and exogenous heavy metals. Metallothioneins are highly inducible upon heavy ion overload and metallothionein-bound metals are generally considered as non-toxic [7].

The *Nrf2* antioxidant pathway is known to be the master defense pathway against oxidative stress [8]. Under quiescent conditions, *Nrf2* interacts with the actin-anchored protein *Keap1*, largely localized in the cytoplasm to maintain low basal expression of *Nrf2*-regulated genes. Under oxidative conditions, *Nrf2* is released from *Keap1* and translocates to the nucleus to activate the expression of several dozen cytoprotective genes that enhance cell survival [9]. Activation of *Nrf2* induces a panel of genes that promote low-molecular-weight antioxidants, enzymatic-antioxidant pathways, and freeion sequestration [10].

The next generation mRNA sequencing (RNA-Seq) provides a “true-quantification” of transcripts and an unbiased detection of transcript variants [11, 12]. We successfully used this technology to define developmental changes of cytochrome P450s [13, 14], transporters [15], phase II drug metabolizing enzymes [16], epigenetic enzymes in liver [17], and genes involved in hepatic energy metabolism [18, 19]. The purpose of this study was to quantify the mRNA abundance and transcript variants of the 35 major antioxidant genes in the liver for their relative abundance during development.

2. Materials and Methods

2.1. Animals

Eight-week old C57BL/6J breeding pairs of mice were purchased from Jackson Laboratory (Bar Harbor, ME). They were housed in an AAALAC accredited facility at the University of Kansas Medical Center. All animals were given *ad libitum* access to water and standard rodent chow (Harlan Teklad 8604, Harlan Teklad, Madison, WI). Breeders were bred overnight and separated the next morning. Pups were weaned at 21-days of age. Livers were collected at the following 12 ages: day –2 (GD17.5 embryos from pregnant mothers were removed for tissue collection), day 0 (right after birth and before the start of suckling), day 1 (24 hours after birth), 3, 5, 10, 15, 20, 25, 30, 45, and 60 (collected approximately at 8–10 am). Due to potential variations caused by the estrous cycle in maturing adult female mice,

only male livers were used for this study (n = 3 per age, randomly selected from multiple litters). Livers were frozen immediately in liquid nitrogen and stored at -80°C .

2.2. RNA isolation

Total RNA was isolated using RNAzol Bee reagent (Tel-Test Inc., Friendswood, TX) per the manufacturer's protocol. RNA concentrations were quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at a wavelength of 260 nm. Integrity of the total RNA samples was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) and the samples with RNA integrity values above 7.0 were used for the RNA-Seq.

2.3. cDNA library preparation

The cDNA libraries from total RNA samples were prepared by an Illumina TruSeq RNA sample prep kit (Illumina, San Diego, CA). Three micrograms of total RNA were used as the RNA input according to recommendations of the manufacturer's protocol. The mRNAs were selected from the total RNAs by purifying the poly-A containing molecules using poly-T primers. The RNA fragmentation, first and second strand cDNA syntheses, end repair, adaptor ligation, and PCR amplification were performed according to the manufacturer's protocol. The average size of the cDNA libraries was approximately 160 bp (excluding the adapters). The cDNA libraries were validated for RNA integrity and quantity using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) before sequencing.

2.4. RNA-Sequencing

The cDNA libraries were clustered onto a TruSeq paired-end flow cell and sequenced for 100 bp paired-end reads (2×100) using a TruSeq 200 cycle SBS kit (Illumina, San Diego, CA). For the initial run, a phi X 174 (PhiX) bacteriophage genome as well as a universal human reference RNA sample were used as controls on the Illumina HiSeq2000 sequencer (KUMC – Genome Sequencing Facility), and sequenced in parallel with other samples to ensure the data generated for each run are 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.

2.5. RNA-Seq data analysis

After the sequencing platform generated the sequencing images, the pixel-level raw data collection, image analysis, and base calling were performed by Illumina's Real Time Analysis (RTA) software on a Dell PC attached to the HiSeq2000 sequencer. The base call files (*.BCL) were converted to qseq files by the Illumina's BCL Converter and the qseq files were subsequently converted to FASTQ files for downstream analysis. The RNA-Seq reads from the FASTQ files were mapped to the mouse reference genome (NCBI37/mm10) and the splice junctions were identified by TopHat. The output files in BAM (Binary Alignment/Map) format were analyzed by Cufflinks to estimate the transcript abundance. The mRNA abundance was expressed in FPKM (fragments per kilobase of exon per million reads mapped).

2.6. Data visualization and statistics

The antioxidant genes were retrieved from the Cufflinks output for further analysis. Significant expression during at least one age of liver development was determined using the drop-in-deviance F test of the fitted FPKM data. The *p*-values were adjusted for extra Poisson variation and corrected for false discovery by the Benjamini-Hochberg method (FDR-BH) with a threshold of 0.05. A two-way hierarchical clustering dendrogram was generated by JMP v. 9.0 (SAS, Cary, NC) to determine the expression patterns of the mRNAs during liver development. The differential gene expression during liver development was determined by one-way analysis of variance (ANOVA) in SPSS v. 16 and statistically significant differences were considered at $p < 0.05$ (Duncan's multiple range test). The FPKM values were $\log_2$ transformed to achieve normal distribution prior to ANOVA.

3. Results

RNA-Seq generated an average of 175 million reads per sample and more than 80% of the reads were mapped to the mouse genome by TopHat. The mRNA ontogeny of antioxidant components was determined in livers of mice at 12 ages. These components include the genes involved in GSH synthesis and recycling, the Keap1-Nrf2 pathway, antioxidant enzymes such as superoxide dismutase (Sod) and catalase, nonenzymatic components such as thioredoxin and glutaredoxin, and nucleoredoxin. In addition, transcript variants of antioxidant components were analyzed.

3.1. Ontogeny of genes involved in GSH synthesis, bilirubin synthesis, and the Keap1-Nrf2 pathway.

Figure 1A illustrates the changes in abundance during ontogeny of the mRNAs responsible for glutathione synthesis, including *Gclc* (glutamate cysteine ligase catalytic subunit), *Gclm* (glutamate cysteine ligase modifier), and glutathione synthetase (*Gss*) as well as glutathione reductase (*Gsr*). *Gclc* mRNA was low on Day-2 and gradually increased to a peak value by 60 days of age. In contrast, *Gclm* mRNA was highest at Day-2 and gradually decreased 56% by 60 days of age. *Gsr* mRNA abundance peaked at Day 5 and decreased 64% by 60 days of age. *Gss* mRNA was low throughout the developmental stages.

Heme oxygenase is the enzyme that catalyzes heme into biliverdin, the precursor of the antioxidant bilirubin. Figure 1B depicts changes of heme oxygenase 1 (*Homx1*) during ontogeny. *Homx1* mRNA was high at Day-2 and gradually decreased to 7% of peak level on Day 25 and remained low through Day 60.

Figure 1C shows the ontogeny changes of *Nrf2*, *Keap1*, and *Nrf2*-target gene NADPH quinone oxidoreductase 1 (*Nqo1*). *Nrf2* mRNA abundance dropped to 42% of prenatal level at birth, increased to prenatal level at Day 1, and kept constant thereafter. The FPKM for *Keap1* mRNA was relatively constant, but was 18% higher at 3 and 5 days of age. There was no significant change in the abundance of *Nrf2* and *Keap1* mRNA during development. *Nqo1* mRNA was low at Day-2 and even lower at later ages.

3.2. Ontogeny of catalase and superoxide dismutase

The ontogeny of the antioxidant enzyme catalase and superoxide dismutase is shown in Figure 2. Catalase increased with age, gradually increased to 189% of prenatal level at Day 10, and reached 6-fold of prenatal level at Day 60. *SODs* are a family of enzymes that catalyze the biotransformation of superoxide ($O_2^{\cdot-}$) into oxygen and hydrogen peroxide. *Sod1* is located in the cytoplasm, *Sod2* in the mitochondria, and *Sod3* in extracellular fluid. *Sod1* had the highest mRNA expression in the *Sod* gene family. *Sod2* mRNA was the lowest with a FPKM of 14 at Day -2 and slightly increased through Day 60. *Sod3* mRNA was low before birth, increased between Day 1 and Day 20, and then decreased to prenatal level at 60 days of age.

3.3. Ontogeny of glutathione peroxidase, peroxiredoxin, thioredoxin, and glutaredoxin

The relative abundance of glutathione peroxidases (*Gpx*) is depicted in the top panels of Figure 3. *Gpx1* mRNA was the most and *Gpx3* was the least abundant *Gpx* isoform. *Gpx1* mRNA was 60-fold and 200-fold higher than *Gpx4* and *Gpx3* before birth, decreased 65% after birth, and remained constant until Day 60. *Gpx2* mRNA abundance was minimal throughout the 60-day period (data not shown). The mRNA of *Gpx3* and *Gpx4* were relatively stable with age. *Gpx3* mRNA increased 1.6-fold from Day -2 to Day 60; while *Gpx4* mRNA decreased 61% from Day -2 to Day 60.

The enzymes responsible for oxidized protein repair, including peroxiredoxin (*Prdx1-6*), thioredoxin, and glutaredoxin are shown in the Fig. 3B and 3C. At Day -2, the highest expression was *Prdx2*, which decreased rapidly after Day 10 to 5% of prenatal level. *Prdx3* was the second most abundant *Prdx* at birth, but the lowest in adults. *Prdx5* was low at Day -2, but became most abundant *Prdx* isoform by Day 60. *Prdx1* and *Prdx6* mRNA were also low and moderately increased after birth. Thus, *Prdx2* was more abundant in fetal liver, but *Prdx1*, 5, and 6 were predominant in adult livers.

In general, the mRNAs of thioredoxin and glutaredoxin did not show any marked change with age. The abundance of *Txn1* and *Txn2* was maintained throughout development with *Txn2* mRNA 50% to 2-fold higher than *Txn1*. *Txnip* was higher at birth, which gradually decreased to 65%. For glutaredoxins, *Glrx* was highest at Day 1, decreased to 60% at Day 10, and was maintained at this level. *Srxn1* was low at Day -2 and slightly increased in adult mice. *Glrx2* and *Nxn* mRNA levels were low and did not change during development.

3.4. Relative abundance of major antioxidant components during development

The relative abundance of the mRNAs of 33 antioxidant enzyme genes during development is shown in Fig. 4. At Day -2, *Gpx1* (58%) and *Prdx2* (19%) were the most predominantly expressed, and others were less expressed: *Prdx3* (2%), *Sod1* (2%) while *Cat*, *Gclm*, *Txn1*, *Txn2*, *Prdx1*, *Prdx4-6* were about 1%. At Day 5, the relative abundance of *Gpx1* and *Prdx2* decreased from 58% and 19% to 35% and 9%, respectively; while *Txn2* increased from 1% to 6%; *Prdx4* and *Prdx5* increased to 5%; and *Prdx6* and *Txn1* increased to 3%. At Day 20, *Gpx1* mRNA was still the most abundant (51%), but *Prdx2* mRNA decreased to a low level (1%). Compared with the mRNA of other antioxidant genes, *Cat* mRNA was relatively more abundant (7%), followed by *Prdx1* and *Txn2* (4%). At adulthood (Day 60), the mRNA of

Gpx1 was still the most abundant (49%), followed by *Cat* (9%), *SOD* (7%), *Prdx5* (6%), and *Prdx1* (4%). In general, the mRNA of antioxidant genes was most abundant at Day -2, when total FPKM values were higher than 6,600. In contrast, the total FPKM value of antioxidant genes decreased to 3,000 FPKM from Day 0 to Day 60.

3.5. Patterns of expression of antioxidant genes during liver development

Figure 5 is a heat map that shows the pattern of abundance of the antioxidant genes during development. Most of the antioxidant genes were enriched during the perinatal stage. During the adolescent period, peroxiredoxins (*Prdx1*, *Prdx4*, *Prdx6*), *Sod3*, and *Txn2* were expressed at their highest level. In adult mice, *Cat*, *Sod1*, *Sod2*, *Gclc*, *Prdx5*, and *Srxn1* were expressed at their highest level.

3.6. Novel isoforms of antioxidant variants during liver development

Figure 6 illustrates transcript variants in livers of mice at various ages. For the 4 transcript variants of *Glxr2*, variant 1 (NM_001038592), which is the longer transcript, was the major isoform in mouse liver throughout development. Other variants of *Glxr2* (NM_001038593, NM_023505, NM_001038594) were very lowly expressed throughout liver development and no apparent change in ontogeny was observed. Four *Keap1* transcription variants (NM_001110305, NM_001110306, NM_001110307, and NM_016679) encode the same protein. NM_001110307 was the predominant variant, which was highest between Day 1 and Day 5 (5–10 FPKM). *Gpx3* had two transcription variants: variant 1 (NM_001083929) represents the longest transcript, which was highest on Day 1 of age and decreased thereafter to 17% of prenatal level at Day 60 of age. Variant 2 (NM_008161) was lowly expressed at all ages during liver development in mice. *Txnrd1* had four transcription variants: variant 1 (NM_001042513) was the abundant transcript and maintained stable abundance throughout liver development. Compared with variant 1, variant 2 (NM_015762) was also moderately expressed, while the other variants (NM_001042523, NM_001042514) were lowly expressed. *Gpx4* had two transcription variants: variant 1 (NM_008162) was the dominant variant with abundance markedly higher than variant 2 (NM_001037741) throughout development stages. *Txnrd3* had 2 variants with variant NM_001178058 predominant, while variant NM_001178058 was lowly expressed throughout liver development.

4. Discussion

Changes in the expression of antioxidant components are important in affecting the body defense against various toxic stimuli, because these changes are critical in regulating free radical scavenge as well as the repair of oxidized proteins during liver development [20, 21]. The present study clearly shows three patterns of abundance of 33 antioxidant genes during development, adding to the evolving knowledge on antioxidant enzymes and other non-enzymatic proteins protecting against oxidative stress in the liver.

GSH is the most abundant cellular thiol and directly reduces hydrogen peroxide to water at the expense of oxidizing GSH to its disulfide (GSSG), either non-enzymatically or through glutathione peroxidase (*Gpx*). GSSG is reduced to GSH by glutathione reductase (*Gsr*). The *Gpx*-GSH pathway is also a major intracellular mechanism that prevents lipid peroxidation

[22], DNA fragmentation [23], and protein adduction [24]. Age-related changes in GSH antioxidant components are associated with various diseases [25, 26], including liver diseases [27]. In rat liver, GSH content, *Gsr* and *Gpx* activity are reported to be low during the perinatal period and activity increases to adult levels by late post-weaning [28]. In contrast, the present study shows in mice that the abundance of *Gsr* and *Gpx* mRNA is high at neonatal ages, decreases during the perinatal period, and remains constant to the adult period (Fig. 1A and Fig. 3A).

The *Nrf2* antioxidant pathway is a master regulator of the defense system against oxidative stress [29]. *Nrf2* is a transcription factor that positively regulates the basal and inducible expression of a large battery of antioxidant genes [10]. During development, *Nrf2* and *Keap1* are stably expressed with no apparent change during ontogeny. Given the fact that the activity of *Nrf2* is regulated through dissociation from Keap1, the mRNAs of *Nrf2* target genes are most relevant to indicate *Nrf2* activity during development. However, some of the *Nrf2* target genes exhibit diverse expression patterns during development. For example, *Nqo1* shows a slight decrease from prenatal to adulthood, *Gss* remains constantly low at all ages, *Gclm* was increased from prenatal (10 FPKM) to adult (60 FPKM), and *Gclc* decreased from 100 FPKM in prenatal to 40 FPKM after birth. Thus, *Nrf2* itself may not be the major regulator of the antioxidant genes during development.

Our previous genome-wide analyses of developmental changes of hepatic transcriptome indicate that in the prenatal and neonatal group, *c-Myc* has the highest number of associated upstream regulators, followed by p53 [18]. *Prdx2*, *Gclc*, and *Gclm* have been reported as direct target genes induced by *c-Myc* to ameliorate oxidative stress [30]. In contrast, *c-Myc* has been reported as a *Nrf2*-interacting protein that negatively regulates phase II genes through electrophile responsive elements [31]. p53 mediates the induction of metallothionein by metals [32]. Moreover, direct interaction between *Nrf2* and *p21* (*Cdkn1a*), the p53-target gene upregulates the *Nrf2*-mediated antioxidant response [33]. Additionally, activation of *NF-κB* induces hepatic expression of *Nqo1* and *Gpx1* [34]. *c-Myc*, p53, *p21*, and *NF-κB* are expressed at much higher levels in perinatal livers than adult livers [18]. Thus, higher perinatal levels of *c-Myc*, p53, *p21*, and *NF-κB* may help explain the generally higher expression of most antioxidant genes, particularly *Prdx2*, *Mt1*, *Mt2*, *Nqo1*, and *Gpx1* in perinatal livers than adult livers.

There are two types of antioxidant defense systems in the organism: enzyme reaction systems and non-enzymatic systems. Antioxidant enzymes, including *Sod* and *Cat*, produce a long-acting antioxidant and detoxification effects and *Sod*-null mice are susceptible to age-related diseases [35]. Both *Cat* and *Sod1* abundance increases with age, indicating increasing capacity of these enzymatic antioxidant systems with age.

The endogenous reductants are thioredoxins and glutaredoxins, small ubiquitous proteins with two redox-active cysteines in their active centers [36]. *Prdx2* showed a similar developmental pattern as *Mt1* and *Mt2*, which decreased from 1,300 FPKM in prenatal to about 200 FPKM in adult mice, while other *Prdx* isoforms including *Prdx1*, 3, 4, and 5 are lowly expressed throughout development. The relative abundance of *Txn2* and *Txn1* is more than *Txnrd1–3* and remained stable throughout development. Heme oxygenase is the

enzyme that catalyzes heme into biliverdin, the precursor of the antioxidant bilirubin. Expression of *homx1* in mouse liver decreased during development. This pattern was also observed in rat ontogeny [37], implicating decreased heme catabolism and bilirubin synthesis during liver development [38].

The next generation of mRNA sequencing (RNA-Seq) provides a “true-quantification” of transcripts and makes the comparison of mRNA abundance among multiple genes possible [11, 12]. The present study shows that *Gpx1* is the predominant antioxidant gene at all developmental ages in liver of mice. In the present study, of the 33 antioxidant genes quantified, *Gpx1* mRNA consists of 50% of the abundance of mRNA, indicating the crucial role of GSH-*Gpx1* pathway in antioxidant defense during development and adulthood.

The next generation of mRNA sequencing (RNA-Seq) also uncovers quantitative information on transcription variants that encodes different isoforms of protein. These different isoforms may have distinct cellular localizations and involve in diverse physiological functions. For *Glrx2*, NM_001038592 encodes isoform a (*Glrx2a*), and NM_001038593, NM_001038594, and NM_023505 encode a shorter isoform b (*Glrx2b*) that lacks amino acid residues 1 – 33. There is no report on functional differences of mouse *Glrx2a* and *Glrx2b*. Human *Glrx2a* functions in mitochondrial redox homeostasis and is important for the protection against and recovery from oxidative stress [39]. Human *Glrx2b* localizes in the cytosol and nucleus and potentially involved in tumor progression [40] and osteoclastogenesis [41]. NM_001038592 is the predominant transcript of *Glrx*, indicating that *Glrx2a* is the predominant enzyme isoform throughout development. Txnrd1 has four transcript variants resulting from alternative splicing at the 5' end: NM_001042513, NM_001042514, and NM_015762 encode the common 55 kDa form, and NM_001042523 encodes a N-terminally elongated form of 67 kDa [42]. The multitude of *Txnrd1* transcripts with different 5'-ends probably reflect alternative post-transcriptional regulation as well as regulation by cell- or growth condition-specific switches to transcription from alternative promoters [42]. Among glutathione peroxidase (*Gpx*) superfamily, *Gpx4* is unique because it reduces peroxides of complex lipids as well as hydroperoxide-associated membranes. *Gpx4* transcript variant NM_008162 encodes the mitochondrial form (*m-Gpx4*), and NM_001037741 encodes the nuclear form (*n-Gpx4*). *M-Gpx4* was shown to be dispensable in mouse embryonic development but essential for sperm development [43]. The predominant expression of NM_008162 indicates abundant *m-Gpx4* as opposed to *n-Gpx4*. For most of antioxidant related genes, tissue distribution and the function of protein products of their splicing variants is unknown. The present study revealed the expression patterns in mouse liver, and provided novel insights of their role in development.

Among the 33 known antioxidant components examined, three ontogeny patterns were observed: (1) Prenatal-rich genes with mRNAs that decreased from fetal livers to adulthood (example: *Homx-1* and *Gclm*); (2) adolescent-rich and relatively stable expression (example: peroxiredoxins); and (3) adult-rich genes with mRNAs that increased with age (example: *Cat*, *Sod1*, and *Sod2*). It is interesting to note that the basal expression of more than 80% of the antioxidant genes is higher in prenatal or adolescent age than in adulthood.

In summary, the present study utilized RNA-Seq technology to profile major antioxidant components in mouse liver during development. Among the 33 antioxidant components examined, three ontogeny patterns were observed: prenatal-rich, adolescent-rich, and adult-rich expression. Transcript variants of several antioxidant genes were also detected by RNA-Seq. Thus, RNA-Seq revealed the relative abundance of hepatic antioxidant enzymes and this quantitative change during development.

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ABBREVIATIONS

Cat	Catalase
FPKM	fragments per kilobase of exon per million reads mapped
Gclc	glutamate cysteine ligase catalytic
Gclm	glutamate cysteine ligase modifier
Glrx	glutaredoxin
Gpx	glutathione peroxidase
GSH	glutathione
Gss	glutathione synthase
GSSG	Glutathione disulfide
Gsr	glutathione reductase
GTF	gene transfer format
IGV	Integrative Genomics Viewer
Homx1	heme oxygenase 1
Keap1	kelch-like ECH associating protein 1
Mt	metallothionein
Nqo1	NAD(P)H:quinone oxidoreductase 1
Nrf2	nuclear factor, erythroid derived 2, like 2
Nxn	nucleoredoxin
Prdx	peroxiredoxin

Sod	Superoxide dismutase
Srxn1	sulphiredoxin 1 homolog
Txn	thioredoxin

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Highlights:

- Three ontogeny patterns of liver antioxidant components were observed: prenatal-, adolescent- and adult-enriched
- Most of the antioxidant genes were enriched during the perinatal stage
- Prdx1, Prdx4, Prdx6, Sod3, and Txn2 were adolescent enriched
- Cat, Sod1, Sod2, Gclc, Prdx5, and Srxn1 were adult enriched
- Gpx1 had highest mRNA abundance out of 33 antioxidant enzyme genes during development

Fig. 1. The mRNA ontogeny of (A) genes involve in GSH synthesis and reduction, (B) heme oxygenase-1, and (C) genes involve in the Keap1-Nrf2 pathway. Data are expressed as mean FPKM $\pm$ SE, n = 3.

Fig. 2.
The mRNA ontogeny of (A) catalase and (B) superoxide dismutases. Data are expressed as mean FPKM $\pm$ SE, n = 3.

Fig. 3.

The mRNA ontogeny of (A) glutathione peroxidases, (B) peroxiredoxins, (C) thioredoxins and thioredoxin reductases, and (D) glutaredoxins and nucleoredoxin. Data are expressed as mean FPKM $\pm$ SE, $n = 3$.

Fig. 4. Relative proportions of mRNAs of antioxidant genes during liver development as visualized by pie charts. Four representative ages are shown, namely day -2 (prenatal), day 5 (neonatal), day 20 (adolescent), and day 60 (adult).

Fig. 5. A two-way hierarchical clustering dendrogram of the mRNAs of the 33 antioxidant genes that are significantly expressed during liver development. Data are expressed as mean FPKM, standardized, and visualized by JMP v9.0 (SAS). Red: relatively high expression; blue: relatively low expression.

Fig. 6.

The FPKM values of known mRNA isoforms of six genes involve in antioxidant defense that are significantly expressed during liver development. Data are expressed as mean FPKM $\pm$ SE (n = 3) and visualized by SigmaPlot (v11.0).