Special Section on Drug Metabolism and the Microbiome

RNA-Seq Quantification of Hepatic Drug Processing Genes in Germ-Free Mice^S

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ABSTRACT

Intestinal bacteria have been shown to be important in regulating host intermediary metabolism and contributing to obesity. However, relatively less is known about the effect of intestinal bacteria on the expression of hepatic drug-processing genes in the host. This study characterizes the expression of hepatic drug-processing genes in germ-free (GF) mice using RNA-Seq. Total RNA were isolated from the livers of adult male conventional and GF C57BL/6J mice ($n = 3$ per group). In the livers of GF mice, the mRNA of the aryl hydrocarbon receptor target gene *Cyp1a2* was increased 51%, and the mRNA of the peroxisome proliferator-activated receptor α (PPAR α) target gene *Cyp4a14* was increased 202%. Conversely, the mRNA of the constitutive androstane receptor (CAR) target gene

Cyp2b10 was decreased 57%, and the mRNA of the pregnane X receptor target gene *Cyp3a11* was decreased 87% in GF mice. Although other non-Cyp phase-1 enzymes in the livers of GF mice were only moderately affected, there was a marked down-regulation in the phase-2 enzymes glutathione S-transferases p1 and p2, as well as a marked up-regulation in the major bile acid transporters Na⁺-taurocholate cotransporting polypeptide and organic anion-transporting polypeptide 1b2, and the cholesterol transporter ATP-binding cassette transporter *Abcg5/Abcg8*. This study demonstrates that intestinal bacteria regulate the expression of a large number of drug-processing genes, which suggests that intestinal bacteria are responsible for some individual differences in drug responses.

Introduction

“We may be born 100% human but will die 90% bacterial—a truly complex organism!” (Goodacre, 2007). As this statement reflects, the human body has 10 times more bacterial cells than human cells. These bacteria grow and divide inside the intestinal lumen alongside ingested food, drugs, bile, and gastrointestinal secretions. To survive, intestinal bacteria have to metabolize food, bile, and other substances to extract energy from them. In general, the host liver provides an ideal environment for oxidation and conjugation reactions, making polar and

high-molecular-weight metabolites; the intestinal bacteria provide an environment suited for reduction and hydrolysis reactions, making nonpolar and lower-molecular-weight metabolites (Sousa et al., 2008). These intestinal bacterial enzymes metabolize drugs as well as some endobiotic substances such as conjugated hormones, bilirubin, and bile acids.

Intestinal bacterial metabolism of orally administered drugs can alter their efficacy and clearance. For example, a specific intestinal bacteria *eggerthella lenta* has the genetic machinery needed to inactivate the cardiac glycoside digoxin, so antibiotic administration increases serum digoxin concentrations (Lindenbaum et al., 1981; Saha et al., 1983). Bacteria in the colon cleave the prodrug sulfasalazine to 5-aminosalicylic acid (an anti-inflammatory drug) and sulfapyridine (an antibiotic), so ampicillin administration decreases the concentration of sulfapyridine in circulation (Houston et al., 1982). Hepatic phase-1 drug-metabolizing enzymes perform oxidation, reduction, and hydrolysis reactions of drugs, and phase-2 drug-metabolizing enzymes perform conjugation reactions. Although the action of hepatic enzymes generally makes the drugs more hydrophilic, the enzymes of intestinal bacteria often make the drug more hydrophobic by deconjugating the conjugated drug metabolites,

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ABBREVIATIONS: Abc, ATP-binding cassette transporter; AhR, aryl hydrocarbon receptor; Ak, aldo-keto reductase; Aldh, aldehyde dehydrogenase; Aox, aldehyde oxidase; CAR, constitutive androstane receptor; Ces, carboxylesterase; CV, conventional; Dh, dehydrogenase/reductase family; Ent, equilibrative nucleoside transporter; Fmo, flavin monooxygenase; GF, germ-free; Gst, glutathione-S-transferase; Mdr, multidrug resistant transporter; Mrp, multidrug resistance-associated protein; Nat, *N*-acetyl transferases; Nrf2, nuclear factor erythroid 2-related factor; Ntcp, Na⁺-taurocholate cotransporting polypeptide; Oatp, organic anion-transporting polypeptide; POR, NADPH-cytochrome P450 reductase; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; Sdr, short-chain dehydrogenase/reductase family; Sult, sulfotransferase; UDPGA, uridine dinucleotide phosphate-glucuronic acid; Ugt, UDP-glucuronosyltransferase.

favoring intestinal uptake and increasing the half-life of drugs (Stojančević et al., 2013).

Intestinal bacteria, in addition to their direct effects on drug metabolism, can also alter the expression of the hepatic drug-metabolizing enzymes of the host. Bacterial infections are known to down-regulate the expression and activities of drug-metabolizing enzymes such as the cytochrome P450 enzymes (Morgan, 1997). Endotoxin of common Gram-negative bacteria injected into rats decrease hepatic drug metabolism and cytochrome P450 expression (Ueyama et al., 2005). Oral antibiotics such as ciprofloxacin can alter the metabolism of other drugs coadministered to the host (Xie et al., 2003). Ciprofloxacin decreases the intestinal bacteria that make the secondary bile acid lithocholic acid, thus decreasing activation of the nuclear receptor pregnane X receptor and lowering Cyp3a expression in livers (Staudinger et al., 2001).

The drug-metabolizing capacity of an individual varies not only because of polymorphisms in genes encoding host drug-metabolizing enzymes and chemicals that induce or inhibit these enzymes but also probably because of individual differences in intestinal bacterial species. Further, therapeutic modulation of intestinal bacteria by probiotics, prebiotics, and fecal microbiota transplantation has the potential to alter the drug-metabolizing capacity of the host, and thus to affect the pharmacokinetics and pharmacodynamics of orally administered drugs taken simultaneously by the host. Thus, there is a need to identify drug-metabolizing enzymes that are altered by intestinal bacteria.

Studying germ-free (GF) mice that have no intestinal bacteria will reveal target genes that are likely to be regulated by intestinal bacteria. GF mice are born and raised inside sterile isolators and receive sterile food, water, and bedding. Previous studies have demonstrated that certain drug-metabolizing enzymes are altered in the livers of GF mice (Bjorkholm et al., 2009; Toda et al., 2009), which provided the first evidence that the xenobiotic-processing pathways of the host are targeted by intestinal microbiota. However, there has yet to be a systematic and quantitative determination of all drug-processing genes, including the phase-I and phase-II drug-metabolizing enzymes and the transporters in the liver. RNA-Seq provides a “true quantification” of transcripts and thus is an unbiased method of quantifying and comparing the mRNA abundance of multiple genes (Cui et al., 2012). Therefore, this study determined the alterations in hepatic drug-metabolizing enzymes at the transcriptome level in GF mice as compared with conventional (CV) mice by comparing the mRNA of hepatic phase-1, phase-2 drug-metabolizing enzymes and transporters in the livers of GF- and CV-male mice using RNA-Seq.

Materials and Methods

Animals. All mice used in the studies were males, between 2 and 3 months of age ($n = 3/\text{group}$), which were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility at the University of Kansas Medical Center with a 14/10-hour light/dark cycle, a temperature and humidity-controlled environment, and ad libitum access to water. The initial breeding colony of GF C57BL/6J/UNC mice was established with mice purchased from the National Gnotobiotic Rodent Resource Center (University of North Carolina, Chapel Hill). All CV mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and received autoclaved rodent diet and autoclaved water for a week before and during the study. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Reagents. The monoclonal mouse anti-rat Cyp2b1/2b2 antibody, which also detects mouse Cyp2b10, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A Cyp3a11 antibody was a generous gift from Dr. Xiaochao Ma (University of Pittsburgh). Secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents, unless indicated otherwise, were purchased from Sigma-Aldrich.

Tissue Collection. All mice were killed and tissue was collected between 9:00 AM and noon to minimize the variations in drug-metabolizing enzyme gene expression due to the circadian rhythm (Zhang et al., 2009).

RNA Isolation. Total RNA was isolated from tissues using RNA Bee reagent (Tel-Test, Friendswood, TX) following the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. The quality of the RNA was assessed by running the sample on a denaturing agarose gel and visualizing two discrete 18S and 28S ribosomal RNA bands, with the 28S band double the intensity of the 18S band.

cDNA Library Preparation and RNA Sequencing. The cDNA library preparation and sequencing of the transcriptome were performed with the help of the University of Kansas Medical Center (KUMC) Genome Sequencing Facility. The cDNA libraries from the total RNA samples ($n = 3/\text{group}$) were prepared using an Illumina TruSeq RNA sample prep kit (Illumina, San Diego, CA). The average size of the cDNAs were approximately 160 base pairs (bp) (excluding the adapters). The cDNA libraries were validated for RNA integrity and quantity using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) before sequencing. The cDNA libraries were clustered onto a TruSeq paired-end flow cell and sequenced (2×50 bp) using a TruSeq SBS kit (Illumina) on the Illumina HiSeq2000 sequencer (KUMC Genome Sequencing Facility) with a multiplex strategy of four samples per lane.

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 software on a Dell PC attached to a 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 mm10 reference genome, and the splice junctions were identified by TopHat. The output files in BAM (binary sequence alignment) format were analyzed by Cufflinks to estimate the transcript abundance and the differential expression (Cuffdiff, FDR-BH<0.05). The mRNA abundance was expressed in FPKM (fragments per kilobase of exon per million reads mapped).

Western Blot Analysis. Western blot analysis of Cyp2b10 and Cyp3a11 was performed as previously described elsewhere with minor modifications (Renaud et al., 2011). Liver homogenates were prepared in radioimmunoprecipitation assay buffer (Sigma-Aldrich). Protein concentrations were determined using BCA assay reagents according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The samples were subjected to polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and probed with the respective primary and secondary antibodies. Membranes were stripped and reprobed with β -actin antibody as the loading control. Proteins were detected using chemiluminescence (Pierce Biotechnology). Intensities of protein bands were quantified using Image J software (National Institutes of Health, Bethesda, MD).

Statistical Analysis. Data are presented as mean $\pm$ S.E.M. Significant differences between CV and GF mice were determined by Cuffdiff, FDR-BH<0.05.

Results

Alterations in mRNA Expression of Hepatic Phase-1 Drug-Metabolizing Enzymes in GF Mice Compared with CV Mice

Enzymes involved in phase-1 drug metabolism catalyze hydrolysis, reduction, and oxidation reactions. As compared with the livers of CV mice, carboxylesterases and cytochrome P450 enzymes are the most differentially regulated hepatic phase-1 drug-metabolizing enzymes in the livers of GF mice.

Carboxylesterases. Carboxylesterase (Ces) is an important family of enzymes that hydrolyzes drugs and other xenobiotics (Slatter et al., 1997). Ces1c and Ces3a were the Ces with the highest mRNAs in the livers of both CV and GF mice, and neither enzyme was differentially regulated by the absence of intestinal microbiota (Fig. 1A). Compared with CV mice, GF mice have decreased levels of Ces2a (39%), Ces3b (23%), and Ces4a (40%) mRNA but increased Ces1g mRNA (42%). The mRNA of other Ces (10 out of 14) were the same between the livers of CV and GF mice (Fig. 1A).

Choline Esterase. The mRNAs of choline esterases, acetylcholine esterase, and butyrylcholine esterase were similar in the livers of CV and GF mice (Supplemental Table 2).

Fig. 1. Gene expression of (A) *Ces*, (B) *Akr*, and (C) *Aldh*. Total RNA was isolated from livers of adult male CV and GF C57BL/6 mice ($n = 3$ per group) and quantified by RNA-Seq as described in *Materials and Methods*. *Differential expression determined using Cuffdiff (FDR-BH < 0.05). Dark blue and red bars represent CV and GF male mice, respectively. FPKM, fragments per kilobase of exon per million reads mapped.

Paraoxonase. The mRNAs of three paraoxonases—Pon1, 2, and 3—were detected in the livers of mice and were similar in CV and GF mice (Supplemental Table 2).

Alkaline Phosphatase. Three types of alkaline phosphatase were detected in the liver; of these, the mRNA of Alpl (tissue-nonspecific form) was 1.5-fold higher in the livers of GF mice compared with CV mice (Supplemental Table 2).

β -Glucuronidase. The mRNA of β -glucuronidase was similar in the livers of CV and GF mice (Supplemental Table 2).

Aldo-Keto Reductase. Aldo-keto reductase (*Akr*) comprises NADPH-dependent oxido-reductase enzymes, which reduce aldehydes to alcohols. *Akr1c6* was the highest expressed *Akr* in livers of CV mice, and its mRNA was not altered by the absence of intestinal bacteria. GF mice had increased *Akr1c20* (30%) and *Akr1d1* (56%) mRNA and decreased *Akr1c19* mRNA (31%) in the liver as

compared with CV mice. The mRNAs of the other *Akr* enzymes (6 out of 9) were quantitatively similar in the livers of both groups of mice (Fig. 1B).

Dehydrogenase/Reductase Family. The mRNA of four members of the short-chain dehydrogenase/reductase family (*Sdr*) family was expressed in the livers of the mice; the mRNA of *Sdr9c7* was decreased 60% in the livers of GF mice compared with CV mice (Supplemental Table 2). Nine dehydrogenase/reductase family (*Dhrs*) enzymes were expressed in the liver. The mRNA of *Dhrs9*, although lowly expressed in the livers of CV mice, was decreased 80% in the livers of GF mice (Supplemental Table 2).

Aldehyde Dehydrogenase. Aldehyde dehydrogenase (*Aldh*) enzymes catalyze the oxidation of aldehydes to carboxylic acids using NAD^+ as a cofactor. *Aldh1a1* and *Aldh2* were the most highly expressed *Aldh* in the livers of both CV and GF mice. *Aldh3a2* mRNA was increased (54%) and *Aldh1b1* mRNA was decreased (29%) in the livers of GF mice compared with CV mice. The mRNAs of other *Aldhs* (9 out of 11) were expressed at similar levels in CV and GF mice (Fig. 1C). The mRNAs of dihydropyrimidine dehydrogenase and dimeric dihydrodiol dehydrogenase were similar in the livers of CV and GF mice (Supplemental Table 2).

Epoxide Hydrolase, NAD(P)H-Quinone Oxidoreductase, and Carbonyl Reductase. The mRNA levels of different epoxide hydrolases, NAD(P)H-quinone oxidoreductases (*Nqo*), and carbonyl reductases were similar in livers of CV and GF mice (Fig. 2, A–C).

Other Reductases. The mRNA of glutathione reductase, thioredoxin reductase, and cytochrome b5 reductase were similar in the livers of CV and GF mice (Supplemental Table 2).

Molybdenum Hydroxylases. The four different molybdenum hydroxylases were expressed similarly in livers of CV and GF mice. The mRNA of two enzymes involved in the synthesis of the molybdenum cofactor were also similar in livers of CV and GF mice (Supplemental Table 2).

Xanthine Oxidoreductase and Amine Oxidases. The mRNA of xanthine oxidoreductase and monoamine oxidase A and B as well as the mRNA of seven other amine oxidases were similar in the livers of CV and GF mice (Supplemental Table 2).

Aldehyde Oxidase. Aldehyde oxidase (*Aox*) is an important class of cytosolic drug-metabolizing enzymes with broad substrate specificity. For example, *Aox1* plays a role in ethanol-induced liver injury (Shaw and Jayatilke, 1990). *Aox3* was the highest expressed *Aox* in the livers of mice, and its mRNA was similar in the livers of CV and GF mice. Compared with the CV mice, *Aox1* mRNA was reduced by about one-third in GF mice (Fig. 2D).

Alcohol Dehydrogenase. The mRNA levels of alcohol dehydrogenases were similar in the livers of CV and GF mice (Supplemental Fig. 1).

Peroxidases. The mRNA of prostaglandin synthases was similar in livers of CV and GF mice. Nine different glutathione peroxidases were expressed in the livers of the mice, and they were all similarly expressed in CV and GF mice except for the mRNA of glutathione peroxidase 6, which was expressed 4-fold higher in the livers of GF mice compared with CV mice. There were six peroxiredoxin enzymes expressed similarly in the livers of CV and GF mice (Supplemental Table 2).

Flavin Monooxygenase. Flavin monooxygenase (*Fmo*) comprises flavin adenine dinucleotide-containing monooxygenases that require NADPH to oxidize nucleophilic nitrogen, sulfur, and phosphorous atoms of a xenobiotic. *Fmo1* and *Fmo5* are the two most highly expressed *Fmo* in the livers of mice. The GF mice had increased *Fmo2* (39%) and *Fmo5* (38%) mRNA in livers compared with CV mice, whereas the mRNA of *Fmo1* and 4 were similar in the livers of GF and CV mice (Fig. 2E).

Fig. 2. Gene expression of (A) epoxide hydrolase (Eph), (B) NAD(P)H-quinone oxidoreductase (Nqo), (C) carbonyl reductase (Cbr), (D) aldehyde oxidase (Aox), and (E) flavin monooxygenase (Fmo). Total RNA was isolated from the livers of adult male CV and GF C57BL/6 mice ($n = 3$ per group) and quantified by RNA-Seq as described in *Materials and Methods*. *Differential expression determined using Cuffdiff (FDR-BH < 0.05). Dark blue and red bars represent CV and GF male mice, respectively. FPKM, fragments per kilobase of exon per million reads mapped.

NADPH-Cytochrome P450 Oxidoreductase. NADPH-cytochrome P450 oxidoreductase (POR) is essential for passing electrons from NADPH to cytochrome P450 enzymes (Cyp) located in the endoplasmic reticulum. There is only one POR for the many Cyp enzymes in the liver, and GF mice had increased POR mRNA levels (46%) in the liver compared with CV mice (Fig. 3A).

Cytochrome P450 Enzyme. Cyp is the largest family of drug-metabolizing enzymes in the liver, responsible for most hepatic phase-I drug metabolism. Cyp enzymes are heme containing and catalyze the mono-oxygenation of xenobiotics. Cyp enzymes are divided into families and subfamilies based on amino acid homology. The first three families, namely, Cyp1, Cyp2, and Cyp3, are involved in xenobiotic metabolism. The Cyp4 family is important for ω -hydroxylation of fatty acids and prostaglandins, but its members also play a role in xenobiotic metabolism (Hsu et al., 2007). The following describes the expression of the Cyp1, Cyp2, Cyp3, and Cyp4 families in the livers of CV and GF mice:

1. Cyp1 family: GF mice had increased Cyp1a2 mRNA (51%) in livers as compared with CV mice (Fig. 3A). Cyp1a1 mRNA was not significantly expressed in the livers of either CV or GF mice (data not shown).
2. Cyp2a subfamily: Among the Cyp2a subfamily, Cyp2a5 was highly expressed in the livers of CV and GF mice. GF mice had increased Cyp2a5 (143%) and Cyp2a22 mRNA (33%) in the liver compared with CV mice. Of note, Cyp2a5 is an aryl hydrocarbon receptor (AhR) target gene and is responsible for the metabolism of drugs and xenobiotics such as halothane, nicotine, and aflatoxin B1. The mRNA of Cyp2a4 and Cyp2a12 were similar (two of six Cyp2a) in the livers of CV and GF mice (Fig. 3A).
3. Cyp2b subfamily: The Cyp2b subfamily was generally lowly expressed in livers of both CV and GF mice. In the livers of GF mice, the mRNA of Cyp2b9 was higher (7454%) but Cyp2b10 mRNA was lower (57%) than in livers of CV mice (Fig. 3A).

4. Cyp2c subfamily: Cyp2c29 was the highest expressed among all the Cyp2c subfamily members in the livers of mice, and it was not differentially regulated in the livers of GF mice. However, interestingly, for most other Cyp2c mRNAs that are expressed at intermediary and low levels, the absence of intestinal bacteria resulted in an increase in their mRNA expression, including Cyp2c38, Cyp2c39, Cyp2c40, Cyp2c50, Cyp2c54, Cyp2c67, Cyp2c68, and Cyp2c69. The mRNA of Cyp2c55 was decreased, and other Cyp2c mRNAs were similar in the livers of CV and GF mice (Fig. 3B).
5. Cyp2d subfamily: Among the Cyp2d subfamily members, Cyp2d9 was the most highly expressed in livers of mice. GF mice generally had minimal alterations in the mRNAs of the Cyp2d family, except for a moderate increase in Cyp2d13 and 2d37-ps mRNAs (Fig. 3C).
6. Cyp2e and Cyp2f: Cyp2e1 and Cyp2f2 were both highly expressed in livers of CV mice, and their mRNA levels were similar in GF and CV mice (Fig. 4A).
7. Cyp3a subfamily: Cyp3a11 was the highest expressed member among the Cyp3a subfamily in the livers of CV mice, and its expression was decreased the most in the livers of GF mice (87%) compared with CV mice. GF mice also have reduced mRNAs of other Cyp3a isoforms, such as Cyp3a16 (86%), Cyp3a44 (87%), and Cyp3a59 (11%). The mRNA levels of Cyp3a13, Cyp3a25, and Cyp3a41a are similar in livers of CV and GF mice (Fig. 4A).
8. Cyp4a, 4b, 4f, and 4v subfamilies: In the livers of GF mice, most of the differentially expressed Cyp4 genes were up-regulated; for example, the mRNAs of Cyp4a10, Cyp4a14, Cyp4a31, and Cyp4a32 were increased between 150% and 200%, whereas the mRNA of Cyp4a12b was increased 31% over CV mice. Cyp4f17 mRNA was decreased (38%) in GF mice, and the mRNA levels of other Cyp4 genes were similar in livers of CV and GF mice (Fig. 4B).

Peptidases, Hydrolases, and Lipoxigenases. The mRNA of four serine peptidases (Htra) were lowly expressed in the livers of mice;

Fig. 3. Gene expression of (A) POR, Cyp1a, 2a, and 2b subfamily, (B) Cyp2c subfamily, and (C) Cyp2d subfamily. Total RNA was isolated from the livers of adult male CV and GF C57BL/6 mice ($n = 3$ per group) and quantified by RNA-Seq as described in *Materials and Methods*. *Differential expression determined using Cuffdiff (FDR-BH<0.05). Dark blue and red bars represent CV and GF male mice, respectively. Cyp, cytochrome P450; FPKM, fragments per kilobase of exon per million reads mapped.

among them Htra4 was expressed 2-fold higher in the livers of GF mice compared with CV mice, whereas others were similarly expressed. There were three other peptidases, five hydrolases, and six arachidonate lipxygenases that were expressed similarly in the livers of CV and GF mice (Supplemental Table 2).

Alterations in mRNA Expression of Hepatic Phase-2 Drug Metabolizing Enzymes in GF Mice Compared with CV Mice

Phase-2 drug-metabolizing enzymes are involved in conjugation reactions. Intestinal bacteria possess enzymes that can deconjugate conjugated xeno- and endobiotics. The glutathione *S*-transferases and UDP-glucuronosyltransferases are the most altered phase-2 drug-metabolizing enzymes in the livers of GF mice compared with CV mice.

Fig. 4. Gene expression of (A) Cyp2e, 2f, and 3a subfamily and (B) Cyp4 family. Total RNA was isolated from livers of adult male CV and GF C57BL/6 mice ($n = 3$ per group) and quantified by RNA-Seq as described in *Materials and Methods*. *Differential expression determined using Cuffdiff (FDR-BH<0.05). Dark blue and red bars represent CV and GF male mice, respectively. Cyp, cytochrome P450; FPKM, fragments per kilobase of exon per million reads mapped.

Glutathione *S*-Transferase. Glutathione-*S*-transferase (Gst) enzymes catalyze the transfer of glutathione to the xenobiotic to make it more hydrophilic. Gst enzymes detoxify polycyclic aromatic hydrocarbons and other carcinogens in the diet and tobacco; therefore, polymorphisms in Gst enzymes are associated with differences in the susceptibility to carcinogens. Among Gst family members, the highest expressed Gst enzyme in the livers of mice is Gstp1. GF mice have decreased mRNA of Gsta1 (48%), Gstp1 (66%), Gstp2 (64%), and Gstm3 (32%) compared with CV mice. The gene expression of Gstp1 was the second most decreased among the phase-1 and phase-2 genes in livers of GF mice (Fig. 5A and B).

Short-chain fatty acids, which are intestinal bacterial metabolites, are known to induce the expression of Gstp1 in intestine (Stein et al., 1996), and it appears that they might also increase Gstp1 expression in the liver. The mRNA levels of Gstm2 (67%) and Gstm3 (67%) are increased in livers of GF mice compared with CV mice. Other Gst enzymes have similar expression in the livers of CV and GF mice. The enzyme glutamate-cysteine ligase catalytic subunit is the rate-limiting enzyme for glutathione synthesis. Glutamate-cysteine ligase catalytic subunit mRNA levels were also decreased (40%) in the livers of GF mice compared with CV mice (Fig. 5, A and B).

UDP-Glucuronosyltransferase. UDP-glucuronosyltransferase (Ugt) enzymes catalyze the transfer of glucuronic acid from the cosubstrate uridine diphosphate glucuronic acid to the xenobiotic. Among the Ugt enzymes, Ugt2b5, Ugt2b36, and Ugt2b1 were the highest expressed in livers of mice. Livers of GF mice have decreased Ugt2b35 (32%),

Ugt2b37 (10%), and Ugt2b38 (11%) mRNA compared with CV mice. The mRNA levels of other Ugt enzymes were similar in the livers of CV and GF mice (Fig. 5C).

Sulfotransferases. Sulfotransferases (Sults) catalyze the transfer of a sulfonic acid group from the cosubstrate 3'-phosphoadenosine-5'-phosphosulfate to the xenobiotic. Sult1a1 was the highest expressed Sult in the livers of mice. GF mice have increased Sult1a1 (52%), Sult1b2 (70%), and Sult1d1 (68%) mRNA levels compared with CV mice, whereas the mRNA of Sult5a1 is decreased (48%) (Fig. 6A).

N-Acetyl Transferases. The *N*-acetyl transferases (Nat) enzymes catalyze the transfer of an acetyl group from the cofactor acetyl-coenzyme A to an amino group in the xenobiotic. This conjugation makes the xenobiotic less water soluble unlike other phase-2 drug-metabolizing reactions that make them more water soluble. Nat6 was the highest expressed Nat enzyme in the liver of mice. The mRNAs of all Nat enzymes were similar in the livers of GF and CV mice (Fig. 6B).

Fig. 5. Gene expression of Gst (A, B) and Ugt (C) enzymes. Total RNA was isolated from the livers of adult male CV and GF C57BL/6 mice ($n = 3$ per group) and quantified by RNA-Seq as described in *Materials and Methods*. *Differential expression determined using Cuffdiff (FDR-BH < 0.05). Dark blue and red bars represent CV and GF male mice, respectively. FPKM, fragments per kilobase of exon per million reads mapped.

Methyl Transferases. Eight different methyl transferases were expressed in the livers of CV and GF mice. The mRNA of indolethylamine *N*-methyltransferase was 1.6-fold higher in the livers of GF mice compared with CV mice, whereas the other

Fig. 6. Gene expression of (A) Sult, (B) Nat, and (C) transcription factors in the liver. Total RNA was isolated from the livers of adult male CV and GF C57BL/6 mice ($n = 3$ per group) and quantified by RNA-Seq as described in *Materials and Methods*. *Differential expression determined using Cuffdiff (FDR-BH < 0.05). Dark blue and red bars represent CV and GF male mice, respectively. FPKM, fragments per kilobase of exon per million reads mapped.

methyltransferases were similarly expressed in CV and GF mice livers (Supplemental Table 2).

Amino Acid Conjugation. The enzymes involved in amino acid conjugation of xeno- and endobiotics were expressed similarly in the livers of CV and GF mice (Supplemental Table 2).

Phosphorylation and Other Unusual Conjugation Enzymes. Examples of enzymes involved in phosphorylation and unusual conjugation reactions include, choline phosphotransferase, hypoxanthine-guanine phosphoribosyltransferase, and nucleoside diphosphate kinase; the mRNAs of all these enzymes were similarly expressed in the livers of CV and GF mice (Supplemental Table 2).

Alterations in mRNA Expression of Xenobiotic-Sensing Transcription Factors in Liver

Hepatic transcription factors such as AhR, constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor α (PPAR α), and nuclear factor erythroid 2-related factor (Nrf2) act as xenosensors and regulate the expression of hepatic drug-metabolizing enzymes and transporters. Interestingly, GF mice have higher AhR, CAR, PPAR α , and Nrf2 mRNAs in livers than CV mice. The mRNA of PXR remains the same in the livers of GF and CV mice (Fig. 6C).

Alterations in mRNA Expression of Transporters

Uptake Transporters. In livers of both CV and GF mice, the most highly expressed basolateral uptake transporters were Na⁺-taurocholate cotransporting polypeptide (Ntcp), which transports the majority of conjugated bile acids, and organic anion-transporting polypeptide 1b2 (Oatp1b2), which transports various xenobiotics as well as unconjugated bile acids. Interestingly, in the livers of GF mice, the mRNAs of both Ntcp and Oatp1b2 were further up-regulated (46% and 61%, respectively). The Oatp1a1, organic cation transporter 1, and equilibrative nucleoside transporter 1 transporters were expressed at intermediary levels, and equilibrative nucleoside transporter 1 mRNA was 64% higher in the livers of GF mice as compared with CV mice, whereas the mRNAs of Oatp1a1 and organic cation transporter 1 were the same in CV and GF mice.

Other transporters, including Oatp1a4, Oatp2b1, organic anion transporter 2, and apical sodium dependent bile acid transporter, were expressed at relatively low levels, and there was a 64% decrease in apical sodium dependent bile acid transporter mRNA in livers of GF mice as compared with CV mice, whereas the other three transporters were similar in livers of CV and GF mice.

Efflux Transporters. In livers of both CV and GF mice, the highest expressed efflux transporters on the mRNA level were the bile acid canalicular efflux transporter bile salt export pump, and the canalicular xenobiotic efflux transporter multidrug resistance-associated protein 2 (Mrp2). The transporters that are expressed only at minimal levels were the xenobiotic efflux transporters multidrug resistant transporter 1a (Mdr1a) and Mdr1b, the basolateral efflux transporters organic solute transporters α and β , the copper efflux transporter Atp7b, as well as the aminophospholipid flippase Atp8b1.

The other transporters, including the phospholipid floppase Mdr2, the xenobiotic efflux transporters Bcrp, multidrug and toxic compound extrusion-type protein 1, Mrp3, Mrp6, as well as the sterol efflux transporter dimer ATP-binding cassette transporters g5/g8 (Abcg5/g8) and the cholesterol efflux transporter Abca1, were expressed at intermediary levels. In comparison with the livers of GF mice, the mRNA of Mrp2 increased 48%, and the mRNAs of Abcg5/8 increased about 100%, whereas the mRNAs of other efflux transporters were similar in CV and GF mice.

Alterations in Protein Levels of Cyp Enzymes

The mRNAs of two very important drug-metabolizing enzymes, Cyp2b10 and Cyp3a11, were decreased in the livers of GF mice compared with CV mice. Therefore, the protein levels of Cyp3a11 and Cyp2b10 in the livers of CV and GF mice were quantified. Similar to their mRNA, both Cyp2b10 and Cyp3a11 protein levels were decreased in the livers of GF mice compared with CV mice (Fig. 7).

Discussion

It is known that intestinal bacteria can alter the expression of some drug-metabolizing enzymes in the liver (Bjorkholm et al., 2009; Toda et al., 2009). However, previous studies only analyzed a small subgroup of host hepatic drug-metabolizing enzymes. Therefore, we analyzed the hepatic transcriptome of the GF and CV mice by RNA-Seq and comprehensively compared the mRNA levels of phase-1 and phase-2 drug-metabolizing enzymes in GF and CV mice. The absence of intestinal bacteria in mice alters the gene expression of a number of phase-1 and phase-2 drug-metabolizing enzymes.

Human CYP3A4 metabolizes more than 60% of all drugs. The mRNA of Cyp3a11, the mouse homolog of CYP3A4, decreased 87% in the livers of GF mice compared with CV mice (Fig. 4A). This together with the protein analysis by Western blotting (Fig. 8) suggests that intestinal bacteria play an important role in regulating this critical drug-metabolizing enzyme.

Fig. 7. Gene expression of uptake (A) and efflux (B) transporters. Total RNA was isolated from the livers of adult male CV and GF C57BL/6 mice ($n = 3$ per group) and quantified by RNA-Seq as described in *Materials and Methods*. *Differential expression determined using Cuffdiff (FDR-BH < 0.05). Dark blue and red bars represent CV and GF male mice, respectively. FPKM, fragments per kilobase of exon per million reads mapped.

This study provides a list of drug-metabolizing enzymes whose mRNA levels increase or decrease in the absence of intestinal bacteria (Table 1). These host drug-metabolizing genes are regulated by intestinal bacteria at the transcriptional level as their mRNA levels are altered by the absence of intestinal bacteria. Although we noticed decreased protein levels of Cyp3a and Cyp2b enzymes, further studies are needed to confirm the changes in protein levels and activities of other enzymes and transporters.

The expression of several genes decreased in GF mice, and it is likely that these genes are involved in metabolizing intestinal bacterial metabolites, so their expression is higher in the presence of intestinal bacteria. This is supported by a study that demonstrated that short-chain fatty acids can increase the expression of drug-metabolizing enzymes in human primary colon cancer cells (Sauer et al., 2007). In the presence of intestinal bacteria, the liver up-regulates some enzymes that conjugate drugs and other xeno- and endobiotics, thus increasing their elimination. Subsequently, the conjugated drugs excreted into bile will be deconjugated by intestinal bacterial enzymes, and the unconjugated drug will enter the enterohepatic circulation to be conjugated by the liver enzymes again. In the absence of intestinal bacteria, these hepatic conjugation enzymes are down-regulated.

Several genes showed increased expression in GF mice compared with CV mice. The functions of these enzymes might also be performed by intestinal bacterial enzymes, so in the presence of intestinal bacteria these enzymes would be down-regulated while they would be increased in the absence of intestinal bacteria.

Two groups have performed microarray analyses and described changes in the mRNA of hepatic drug-metabolizing enzymes in male GF NMRI and male GF IQI mice (Bjorkholm et al., 2009; Toda et al., 2009). Their results were contradictory to each other, and our observations are different from both their reports, possibly owing to differences in the strains of mice used in the studies. Supplemental Table 1 shows the list of hepatic drug-metabolizing genes that were altered in GF mice compared with CV mice in this study compared with the two previous microarray studies of GF mice.

In our study, the mRNA levels of Cyp2b9 increased markedly (7454%) whereas in the study by Toda et al. (2009) the mRNA levels of Cyp2b9 decreased in GF mice compared with controls. The mRNA

TABLE 1

List of genes that are differentially regulated at the transcription level by the presence of intestinal bacteria

Decreased in GF Mice	Increased in GF Mice
Phase-1 drug-metabolizing enzymes	
Ces2a	Ces1g
Ces3b	Akr1c20
Ces4a	Akr1d1
Akr1c19	Aldh3a2
Aldh1b1	Fmo2,5
Aox1	Cyp1a2
Cyp2b10	Cyp2a5,22
Cyp3a11,16,44,59	Cyp2b9
Cyp4f17	Cyp2c38,39,40,50,54,67,68,69
	Cyp4a10,12b,14,31,32
Phase-2 drug-metabolizing enzymes	
Gsta1	Gstt2,3
Gstp1,2	Sult1a1
Gstm3	Sult1b1
Ugt2b35,37,38	Sult1d1
Sult5a1	
Transporters	
Asbt	Ntcp
	Oatp1b2
	Ent1
	Mrp2
	Abcg5
	Abcg8

Asbt, apical sodium dependent bile acid transporter; Ent1, equilibrative nucleoside transporter 1.

of Sult1c2 and Ugt1a1 increased in GF mice in the study by Bjorkholm et al. (2009) whereas the mRNA levels were the same in GF and CV mice in our study. The gene expression of the major xenobiotic-sensing nuclear receptor PXR remained the same in the livers of GF and CV mice in our study, whereas Toda et al. (2009) reported a decrease in PXR mRNA in GF mice and hypothesized that in GF IQI mice the decreased concentrations of secondary bile acids were the reason for the decrease in gene expression of the nuclear receptor CAR and its target genes. Although Bjorkholm et al. (2009) reported that in GF NMRI mice the mRNA of the nuclear receptor CAR and its target genes increased, we did not observe an increase in all CAR target genes. In our study, the mRNA of the CAR target gene Cyp2b9 increased whereas Cyp2a4 mRNA remained the same and Cyp2b10 mRNA decreased in the livers of GF mice compared with CV mice. However, we noticed that the PXR target gene Cyp3a11 mRNA and protein decreased in GF mice, suggesting a decrease in PXR-signaling in the absence of intestinal bacteria.

The gene expression of the other xenobiotic-sensing transcription factors AhR, PPAR α , and Nrf2 increased in the livers of GF mice compared with CV mice in our study. Zhang et al. (2015) demonstrated that exposure through diet to the environmentally persistent organic pollutant 2,3,7,8-tetrachlorodibenzofuran alters intestinal bacterial composition and regulates host gene expression through AhR activation, which is evidence that intestinal bacteria communicates with AhR.

A number of hepatic drug-metabolizing enzymes are target genes for intestinal bacteria, including the major drug-metabolizing enzyme Cyp3a11. Changes in these intestinal bacteria may alter the pharmacokinetics and pharmacodynamics of orally administered drugs. The composition of intestinal bacteria and their functional properties may one day be used to help predict an individual's response to a drug. Thus, it is important to study the effect of probiotic strains of bacteria or fecal microbiota transplantation on drug-metabolizing genes to prevent potential detrimental interactions with any simultaneously ingested drugs. Altering intestinal bacteria also

Fig. 8. Protein expression of Cyp2b10 and Cyp3a11 in (A) males and (B) females. Western blot results of Cyp2b10 and Cyp3a11 in the livers of GF and CV mice. Intensities of protein bands were quantified using Image J software. *Statistically significant differences between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice, respectively. M, males.

might provide a novel approach to modifying the drug-metabolizing capacity of the liver.

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Authorship Contributions

Participated in research design: Selwyn, Cui, Klaassen.

Conducted experiments: Selwyn, Cui, Klaassen.

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Wrote or contributed to the writing of the manuscript: Selwyn, Cui, Klaassen.

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