Membrane drug transporters contribute to the disposition of many drugs. In human liver, drug transport is controlled by two main superfamilies of transporters, the solute carrier transporters (SLC) and the ATP Binding Cassette transporters (ABC). Altered expression of these transporters due to drug-drug interactions can contribute to differences in drug exposure and possibly effect. In this study, we determined the effect of rifampin on gene expression of hundreds of membrane transporters along with all clinically relevant drug transporters.

Methods: In this study, primary human hepatocytes ($n = 7$ donors) were cultured and treated for 24 h with rifampin and vehicle control. RNA was isolated from the hepatocytes, mRNA expression was measured by RNA-seq, and miRNA expression was analyzed by Taqman OpenArray. The effect of rifampin on the expression of selected transporters was also tested in kidney cell lines. The impact of rifampin on the expression of 410 transporter genes from 19 different transporter gene families was compared with vehicle control.

Results: Expression patterns of 12 clinically relevant drug transporter genes were changed by rifampin (FDR < 0.05). For example, the expressions of ABCC2, ABCB1, and ABCC3 were increased 1.9-, 1.7-, and 1.2-fold, respectively. The effects of rifampin on four uptake drug transporters (SLCO1B3, SLC47A1, SLC29A1, SLC22A9) were negatively correlated with the rifampin effects on specific microRNA expression (SLCO1B3/miR-92a, SLC47A1/miR-95, SLC29A1/miR-30d#, and SLC22A9/miR-20; $r < -0.79; p < 0.05$). Seven hepatic drug transporter genes (SLC22A1, SLC22A5, SLC15A1, SLC29A1, SLCO4C1, ABCC2, and ABCC4), whose expression was altered by rifampin in hepatocytes, were also present in a renal proximal tubular cell line, but in renal cells rifampin did not alter their gene expression. PXR expression was very low in the kidney cells; this may explain why rifampin induces gene expression in a tissue-specific manner.
INTRODUCTION

The transport of drugs in and out of tissues and cells is now recognized as an important factor in drug efficacy and toxicity (International Transporter et al., 2010; Hillgren et al., 2013). Drug transport occurs at key sites including the liver, kidney, intestine, and blood-brain barrier to regulate drug and/or metabolite distribution (International Transporter et al., 2010; Hillgren et al., 2013). Understanding the regulation of drug transporter gene expression is important to improve drug efficacy and toxicity, especially for the many drugs transported into and out of the liver. Drug transport into and out of the liver is controlled by two superfamilies of transporters, the solute carrier transporters (SLC) and the ATP Binding Cassette transporters (ABC). Among these transporters, 32 have currently been designated as clinically relevant drug transporters (pharmADME.org: International Transporter Consortium; International Transporter et al., 2010; Hillgren et al., 2013). For most of these transporters, gene expression has not been studied in primary human hepatocytes to assess the impact of rifampin.

Rifampin is commonly used to treat tuberculosis worldwide. It is well-known to induce the expression of many of the cytochrome P450 drug metabolism genes. It is also known to regulate the gene expression of several ABC drug transporters, but its effects on many of the transporters is yet unknown (Stapelbroek et al., 2010; Peters et al., 2011; Vilas-Boas et al., 2013; Weiss and Haefeli, 2013). Several clinically significant rifampin-induced drug-drug interactions have been documented (Niemi et al., 2003; Baciewicz et al., 2013). At least some of the rifampin effect is due to its binding to and activation of the pregnane X receptor (PXR). PXR is a transcription factor that regulates the expression of several of the ABC transporter genes (Mottino, 2008). In addition, rifampin can also regulate transporter expression indirectly by regulating microRNAs (miRNAs), which target the transporter miRNAs (Haenisch et al., 2011). miRNAs are short RNA sequences that regulate mRNA function by degrading the target miRNAs or blocking the mRNA translation. Rifampin induces hepatic expression of many miRNAs (Ramamoorthy et al., 2013), and miRNAs have been shown to regulate drug transporter expression and translation (Haenisch et al., 2011; Zhu et al., 2011; Bang and Thum, 2012), supporting the possibility that miRNAs mediate some of the effects of rifampin on transporter expression. In fact, this has been shown to be true where rifampin induces miRNA-379 expression in the HepG2 human liver cancer cell line and this miRNA subsequently down-regulates ABCC2 protein levels (Haenisch et al., 2011).

CONCLUSION: Rifampin alters the expression of many of the clinically relevant hepatic drug transporters, which may provide a rational basis for understanding rifampin-induced drug-drug interactions reported in vivo. The relevance of its effect on many other transporters remains to be studied.

Keywords: rifampin, drug transporter, gene expression, microRNA, human hepatocyte, ChIP-Seq, PXR binding site, RNA sequencing

METHODS

Human Hepatocytes, Drug Treatment, RNA Isolation, and miRNA Profiling

Our studies analyzed the RNA expression data collected by the studies done by Ramamoorthy et al. (2013). In those studies, “human hepatocytes from seven different subjects were obtained from CellzDirect (Durham, NC).” The primary human hepatocytes were grown on collagen coated plates and treated with rifampin (10 µM) or vehicle (methanol, 0.01%) for 24 h. Each culture from a different individual subject was treated as a biologic replicate (n = 7). In vitro studies were performed within 72–120 h from the time of hepatocyte isolation. Total RNA and miRNA were isolated with a miRNeasy kit (Qiagen, Valencia, CA). miRNAs (754) were analyzed using the Taqman OpenArray Human miRNA Panel in technical duplicates (each sample run on two OpenArrays). Global mRNA expression was measured by RNA-seq conducted on the SOLID4 system (Life Technologies, Inc, Carlsbad, CA). The hepatocyte tissues were purchased commercially, and were de-identified. These studies were not deemed human subject research. CellzDirect was purchased by another company, and specific demographic or clinical information on the donors was not transferred in the transition and the information could not be retrieved.

miRNA Bioinformatics

The miRNA bioinformatic analysis was re-performed from the original analysis done by Ramamoorthy et al. (2013). For the technical duplicate values, the C_T values were averaged. If a miRNA had no value for both technical replicates, then it was assigned the highest C_T value of 40. In rare cases, if one technical duplicate detected the miRNA and the other technical duplicate was undetectable, the detectable value was used. To determine which miRNAs to include in the study, we selected only those with at least four of the seven control samples or four of the seven rifampin treated samples with average C_T values < 35. All the
small nuclear RNA, U6 and RNUs, were removed. The remaining 333 miRNAs were Quantile normalized using PARTEK software. Quality control analysis after each normalization step included the visual evaluation of the histograms followed by principal component analysis (PCA) to identify systematic variations caused by various factors such as OpenArray runs. Normalized data were analyzed by ANOVA using a mixed effect model.

**RNA sequencing Analysis**

Our studies used the data processing, quality assessment, sequence alignment, and gene expression analysis that was previously performed and published by Ramamoorthy et al. (2013). As previously described for quality control, low quality. Briefly, RNA sequencing reads were removed including reads of less than 35 bases, or having a quality score of 20 or less. RNA was considered expressed if at least 10 reads were present after counts per million normalization.

Treatment and control groups were compared using the log₂ transformed fold-change values. Of note, six data points in the transporter gene mRNA expression data were undetectable. Since a zero cannot be used in the log₂ transformed fold-change calculations, the zero values were replaced with the minimum read numbers. The resultant p-values of the 410 transporters were corrected using a Benjamini and Hochberg’s false discovery rate correction. A fold-change of one represents no change; a two-fold change is a doubling of the expression level.

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Correlations of the rifampin effects on the expression of two transporter genes were performed using the Spearman rank correlations on the log₂ transformed fold-change values. Correlations of the rifampin effect on specific gene expression with specific miRNA expression were also performed using the Spearman rank correlations on the log₂ transformed fold-change using the delta Ct values for the miRNA expression.

**Selection of Transporters**

All human transporter genes were identified using the University of California Santa Cruz reference genome using the following inputs: http://genome.ucsc.edu/cgi-bin/hgTables, criteria- group: genes and gene prediction, track: RefSeq Genes, table: RefGene, assembly: feb. 2009 (GRch37/hg19), clade: mammal, genome: human, region: genome, output format: selected fields from primary and related tables, click get output button, then on the next screen, check both- Name [name of gen (usually transcript_id from GTF)], and name 2 [alternate name(e.g., gene_id from GTF)] and then click get output. Selected transporters evaluated in our study were queried from this UCSC gene list. These human transporters were selected using a combination of literature, pharmADME.org, and NIH NCBI Gene using the term transporter, and using terms transporter, influx transporter, efflux transporter. Transporter gene families were chosen based on possible relevance to drug metabolism or clinical relevance.

**Culture and Rifampin Treatment of Renal Cells**

Normal human proximal tubular kidney (NHPTK) cells were a gift from the Robert L. Bacallao Laboratory (Herbert et al., 2013). NHPTK cells are primary renal cells from a single donor immortalized with human telomerase plasmid transfection and maintained in REGM media (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (HyClone) to maintain their renal phenotype. NHPTK cells were diluted to 20–30% confluence three times a week and maintained at 37°C in 95% humidified atmosphere with 5% CO₂. All in vitro studies were performed on cells in passages 6 through 9 (corresponds to passage 3 through 6 following immortalization). Cultures from each passage were treated as biologic replicates (n = 4). NHPTK cells were treated for 24 h with rifampin (10 μM) or with the vehicle control of methanol (0.01%).
Statistical significance was assessed based on a student’s $t$-test between conditions.

**In silico ChIP-Seq PXR Binding Site Analysis**

A publically available chromosome immunoprecipitation DNA sequencing (ChIP-Seq) database available from Smith et al. (2014) was analyzed in silico for PXR binding sites within the 410 transporter genes in this study (Smith et al., 2014). This ChIP-Seq dataset analyzed the PXR binding in rifampin treated vs. DMSO vehicle treated HepG2 cells.

**Accession Numbers**

The raw RNA-seq data have been made publically available through NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE79933 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79933). The miRNA OpenArray data have been made publically available at http://compbio.iupui.edu/group/6/pages/rifampin.

**RESULTS**

**Regulation of Membrane Transporters by Rifampin**

A total of 410 transporter genes from 19 transporter gene families were selected for analysis in the primary hepatocytes. Two hundred sixty-seven of the genes were in the ABC and SLC superfamilies of transporters. Thirty-four of the 267 are drug superfamily transporter genes (Supplemental Table 3). Eleven of the 38 (32%) ATP-binding cassette (ABC) superfamily transporter genes were expressed in every donor sample; we also found six additional ABC genes and the expression of three transporters was reduced. The five SLC genes with the highest changes in expression levels are shown in Figures 1A,B. The expression of three out of the five (60%) detected genes of the SLCO family were also altered by rifampin (Supplemental Table 2). PXR has been shown to regulate SLCO2B1 and SLCO1B1, but the expression of only SLCO2B1 increased (1.73-fold) in our study. In addition, rifampin altered the expression of 12 of the 38 (32%) ATP-binding cassette (ABC) superfamily transporter genes (Supplemental Table 3). Eleven of these ABC genes were induced as shown in Figure 1C, and one (ABCA8) was reduced (Supplemental Table 2).

**Correlations of the Effect of Rifampin on Gene Expression**

We next tested whether there were correlations in the effect of rifampin on the expression of drug transporter and cytochrome P450 (CYP) genes. Since each hepatocyte culture replicate was from a different donor, we considered each replicate a separate individual. There were nine pairs of drug transporters that were positively correlated (FDR < 0.05; Figure 2; all CYP/Transporter gene expression correlations are found in Supplemental Table 3), i.e., each pair of genes was upregulated ($r>0.79$) upon rifampin treatment. We also correlated the rifampin effects on the drug transporters with rifampin effects on CYP gene expression. There were seven positive correlations (FDR < 0.05; Figure 3; for the correlations of all CYP/Transporter genes see Supplemental Table 4). Interestingly, four were correlations with CYP4F2 (SLCO2B1, SLC29A1, SLC47A1, and SLC04C1).

**Correlation between miRNA Induction and Changes of Clinically Relevant Drug Transporter Expression**

We have previously shown that rifampin induces the expression of several miRNAs in hepatocytes (Ramamoorthy et al., 2013). To identify drug transporters that may be modulated by these miRNAs, we determined the correlations between rifampin induction of miRNAs and the rifampin effect on drug transporter gene expression. The direct effects of the miRNAs on gene expression would be expected to downregulate transporter expression; however, since miRNAs could also have indirect effects and induce the expression of the drug transporter genes, we searched for both positive and negative correlations. We

**TABLE 1** Clinically important drug transporters.

| Influx Gene name | Efflux Gene name | Influx/Efflux Gene name |
|------------------|------------------|------------------------|
| *MCT1* SLC16A1   | *MDR1* ABCB1     | *ENT1* SLC29A1         |
| *PEPT1* SLC15A1  | *BCRP* ABCG2     | *ENT2* SLC29A2         |
| *OCT1* SLC22A1   | *MRP2* ABCG2     | *OSTalpha* SLC51A      |
| *OAT2* SLC22A7   | *MRP3* ABCG2     | *OSTbeta* SLC51B       |
| *OATP4C1* SLC04C1| *MATE1* SLC47A1  | *OCTN2* SLC22A5        |
| *OATP2B1* SLC02B1| *MRP4* ABCG2     |                       |
| *NTCP* SLC10A1   | *MRP6* ABCG2     | OAT4 SLC22A11          |
| *OATP1B3* SLC01B3| *BSEP* ABCB1     | OCTN1 SLC22A4          |
| *OATP1B1* SLC01B1| *MRP5* ABCG2     |                       |
| *OAT7* SLC22A9   | MATE2-K SLC47A2  |                       |

Clinical importance of drug transporters as determined by the International Transporter Consortium, and pharmADME.org. (Bold): The 24 genes with detectable expression in the primary human hepatocytes.
identified 14 highly correlated negative associations (Table 3; for the correlations of the all transporter/miRNAs gene expression see Supplemental Table 5). Three miRNAs, miR-320, miR-616, and miR-95, each correlated with two different drug transporters. Three drug transporters, ABCB11, ABCC4, and ABCC5 were negatively correlated with two different miRNAs each. Based on the bioinformatics tools we employed, none of these were predicted to be direct effects. Only three rifampin-induced miRNAs were predicted to bind to drug transporters. Target Scan predicted miRNA binding of miR-335 to SLC29A2, miR-200b# to SLC29A2, SLCO1B1, and miR-616 to SLCO1B1. However, neither of these transporters genes expression were altered by rifampin.

We identified 18 highly positive correlations between the rifampin effects on miRNAs and its effects on drug transporter expression levels (Table 3; Supplemental Table 5). miR-660 positively correlated with seven different transporters, miR-766 positively correlated with three transporters, and miR-886-3p and let-7g positively correlated with two transporters. None of the rifampin-induced miRNAs were predicted to bind to any of the rifampin-regulated drug transporters.

**Rifampin's Modulation of Drug Transporter Gene Expression is Tissue Specific**

Many of the drug transporters that we detected in the hepatocytes are also expressed in other tissues, including the kidney (Hillgren et al., 2013). Thus, we determined if rifampin demonstrated any tissue specificity by regulating the same transporters in human kidney cells. Normal human proximal tubular kidney (NHPTK) cells were treated with rifampin analogously to primary hepatocytes, and gene expression was measured. Seven transporters were selected based on their differential expression in hepatocytes and their renal proximal tubular relevance. Expression of SLC22A1, SLC22A5, SLC15A1, SLC29A1, SLCO4C1, MRP2, and MRP4 was detected, but did not change significantly following rifampin treatment (Supplemental Figure 1A; statistics are provided in Supplemental Table 6). CYP3A4 expression was low in NHPTK cells with raw Ct values between 35 and 37. Expression was not significantly elevated following rifampin treatment (Supplemental Figure 1B). As CYP3A4 is PXR dependent, gene expression of NR1I2 (PXR) was also measured in NHPTK cells and compared to NR1I2

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### Table 2 | Rifampin-induced changes in gene expression of clinically relevant drug transporters.

| Common name | Gene name | Fold change | p-value | FDR   | Example of substrates* |
|-------------|-----------|-------------|---------|-------|------------------------|
| OStbeta     | SLC51B    | 12.67       | 1.20E-113 | 4.90E-111 | Bile acids, conjugated steroids |
| MRP2        | ABCC2     | 1.85        | 3.56E-23  | 2.92E-21  | Chemotherapy, statin, antiepileptics, tamoxifen, DMARDs, antidepressants, clopidogrel, anti-retroviral |
| MDR1        | ABCB1     | 1.72        | 6.63E-18  | 3.02E-16  | Chemotherapy, statin, antiepileptics, tamoxifen, DMARDs, antidepressants, clopidogrel, anti-retroviral |
| OATP2B1     | SLCO2B1   | 1.73        | 2.34E-16  | 8.73E-15  | Fleroxidase, montelukast, grapefruit, orange juice, apple juice, endogenous steroids |
| MRP6        | ABCG6     | 1.65        | 5.62E-14  | 1.84E-12  | Thalidomide, docetaxel |
| OATP1B3     | SLCO1B3   | −1.57       | 2.15E-11  | 5.18E-10  | Irinotecan, docetax, myophenolate, statin, methotrexate |
| OATP4C1     | SLCO4C1   | 1.46        | 2.84E-07  | 3.88E-06  | Estrone 3-sulfate |
| MATE1       | SLC47A1   | 1.37        | 2.64E-06  | 2.84E-05  | Meformin, trimethoprim |
| ENT1        | SLC29A1   | 1.38        | 4.68E-06  | 4.80E-05  | Gemcitabine, pacitax, gemcitabine, peginterferon, ribavirin |
| OAT7        | SLC22A9   | −1.34       | 9.76E-05  | 0.0007    | Methotrexate |
| PEPT1       | SLC15A1   | −1.27       | 5.01E-04  | 0.0031    | Statin, onsetamivir |
| MRP3        | ABC2C     | 1.18        | 0.0098    | 0.038     | Cisplatin, MTX, doxorbuicn, cyclophosphamide, clopidogrel, coidiene, morphine, etoposide |
| OStalpha    | SLC51A    | 1.21        | 0.014     | 0.050     | Bile acids, conjugated steroids |
| BCRP        | ABCG2     | 1.18        | 0.015     | 0.054     | Antiepileptics, MTX, statin, anti-retrovirals, tectans, anti-neoplastics (irinotecan, platnum, anthracycline, taxane) acetaminophen, uric acid |
| OCT1        | SLC22A1   | −1.16       | 0.019     | 0.060     | Morphine, metformin, tramadol, antilnetics, dopamine agonist, antiretrovirals, soralinb |
| OAT2        | SLC22A7   | −1.15       | 0.040     | 0.11      | Fluoropyrimidine, zidovudine |
| MRP5        | ABCC5     | 1.17        | 0.066     | 0.16      | Antiepileptics, irinotecan |
| MRP4        | ABCC4     | 1.17        | 0.090     | 0.21      | cisplatin, antiretroiral, bisphosphonates, MTX, acetaminophen, azathioprine, mercaptopurine |
| NTCP        | SLC10A1   | −1.08       | 0.21      | 0.39      | Bile acids |
| OCTN2       | SLC22A5   | −1.08       | 0.30      | 0.51      | Imatinib, oxaliplatin |
| BSEP        | ABCB11    | −1.07       | 0.32      | 0.55      | Statin |
| OATP1B1     | SLCO1B1   | 1.04        | 0.57      | 0.74      | Simvastatin, statin, rifampin, irinotecan, methotrexate, thyroid, estrostrate, sulfase, angiotensin receptor blocker, MTX, caspofungin, ace inhibitor |
| MCT1        | SLC16A1   | 1.03        | 0.61      | 0.78      | Lactate |
| ENT2        | SLC29A2   | −1.03       | 0.89      | 0.94      | Gemcitabine, thiolpurine |

Primary human hepatocytes were treated with 10 µM Rifampin or vehicle for 24 h. Fold-change is the ratio of gene expression treatment/control. A fold-change equal 1 is defined as no change, and a fold change of 2 is defined as 2x the expression. FDR, False Discovery Rate. *Cited from PharmGKB.org.
expression in a positive control cell line, HepG2 cells. Since we expected NR1I2 expression to be much lower than hepatocytes, we used HepG2 as a positive control, which is known to have relatively low NR1I2 expression levels (Gerets et al., 2012). The expression of NR1I2 was much lower in NHPTK cells when compared to HepG2 (Supplemental Figure 1C).

### 38 Transporters have Rifampin Induced PXR Binding Sites

Utilizing a publically available ChIP-Seq database from Smith et al. (2014), Figure 4 illustrates our in silico analysis of this dataset. The 410 transporter genes were mapped to Ensembl annotation. The transcription start site (TSS) was located for each gene and a promoter region was defined as ± 2 kilobases from TSS of each gene. From the ChIP-Seq dataset, 6302 PXR peaks were identified in the rifampin treated HepG2 cells and 1157 PXR peaks in DMSO treated HepG2. PXR peaks were extracted only if uniquely found in rifampin treated HepG2 vs. DMSO vehicle. The remaining 6063 unique rifampin peaks were compared for overlap with the 410 promoter regions of the transporters. 58 of the 410 transporter genes’ promoter regions were identified to have PXR binding sites. Table 4 illustrates the PXR binding site for one isoform of each gene transcript, while Supplemental Table 7 provides a list of the PXR binding site analysis for all gene transcript isoforms. Thirty three members of the SLC (SLC10A7, SLC16A3, SLC17A5, SLC1A4, SLC20A1, SLC20A2, SLC22A15, SLC22A3, SLC23A2, SLC25A1, SLC25A10, SLC25A11, SLC25A2, SLC25A4, SLC25A42, SLC25A5, SLC26A2, SLC29A1, SLC29A2, SLC30A10, SLC37A3, SLC39A7, SLC39A8, SLC3A2, SLC41A2, SLC43A1, SLC47A1, SLC5A11, SLC5A3, SLC5A9, SLC7A5, and SLC2B1), and 7 members of the ABC (ABCA2, ABCA3, ABCA9, ABCB4, ABCC10, ABCC2, and ABCC3) superfamily of transporters have PXR binding sites. Five of the clinically relevant drug transporter genes, ABCC2, ABCC3, SLC29A1, SLC47A1, and SLCO2B1 were found to have PXR peaks within their promoter regions.

### DISCUSSION

Our study has several important first-time characterizations of rifampin’s direct and indirect regulation of membrane transporters. First, rifampin regulates the gene expression of many members of the two major superfamilies (ABC and SLC) involved with clinically relevant drug transport. Second, the modulation of rifampin of transporter gene expression is associated with changes in miRNA expression. Third, we
FIGURE 2 | The significant correlations between the expression of clinically relevant transporter genes that were regulated by rifampin. The expression (represented by log2 fold change [Log2 FC]) after rifampin treatment) was correlated for all 410 transporter genes. Only the significant correlation ($p < 0.05$ by Spearman correlation) are shown for transporters regulated by rifampin with an FDR $<0.05$. Spearman correlation was performed because an assumption of normal distribution could not be made.

demonstrated that the effect of rifampin on transporter gene expression is tissue-specific. To the best of our knowledge, this study provides the first global characterization of rifampin induction of drug transporter gene expression.

Our studies demonstrate that rifampin treatment induces the gene expression of many members of the SLCO, SLC, and ABC transporter families. Twelve ABC transporter genes were regulated either directly or indirectly by rifampin; of these 12 ABC transporters, five of have previously been shown to be regulated by rifampin (Nishimura et al., 2002; Teng et al., 2003; Stapelbroek et al., 2010; Vilas-Boas et al., 2013; Weiss and Haefeli, 2013; Williamson et al., 2013) and two of the three ABC genes with the smallest $p$-values in our data set were drug transporters (ABCC2, and ABCB1). ABCC2 and ABCB1 have been shown to be induced by the nuclear receptor, PXR (Geick et al., 2001; Kast et al., 2002) and our in silico ChIP-Seq analysis. PXR mediates at least part of the rifampin effects through direct binding to rifampin and nuclear localization (Stapelbroek et al., 2010; Honorat et al., 2011; Roques et al., 2013; Vilas-Boas et al., 2013; Weiss and Haefeli, 2013; Williamson et al., 2013). We likely did not identify the ABCB1 PXR binding site within the promoter region due to defining the promoter region using the common convention of $±$ 2 kilobases around the TSS. In Kast et al. (2002), the ABCB1 PXR binding site was over 7 kilobases from the TSS.

In the SLC family, the expression of 44 transporters were induced by rifampin. The most highly induced SLC transporter gene was the clinically relevant drug transporter SLC51B (OSTbeta; >12-fold), which is a newly recognized transporter that heterodimerizes with OST alpha and transports bile acids and conjugated steroids (Ballatori et al., 2013). For comparison, CYP3A4 was induced 22-fold (Ramamoorthy et al., 2013). We used a similar method of analyzing the data as Ramamoorthy et al., and with the same RNA-seq data set, CYP3A4 was induced
FIGURE 3 | The significant correlations between the expression of drug metabolism genes and clinically relevant transporter genes that are regulated by rifampin. The expression [represented by log2fold change (Log2FC)] after rifampin treatment was correlated between all CYP and 410 transporter genes. The figure illustrates, the seven significant positive and one significant negative correlations between the expression of CYP and drug transporter genes that were regulated by rifampin with an FDR $< 0.05$. These were the only correlations with a $p < 0.05$ by Spearman correlation. Spearman correlation was performed because an assumption of normal distribution could not be made.

22-fold in our analysis as well. None of the SLC drug transporter genes (excluding SLCOs) have previously been shown to be induced by PXR or rifampin (Dixit et al., 2007). Our ChIP-Seq analysis identified that 33 SLC genes (Table 4 and Supplemental Table 7) had PXR binding sites. The two clinically relevant drug transporter SLC genes with a PXR binding peak identified in their promoter region were SLC29A1, and SLC47A1. The genes were induced by rifampin 1.38- and 1.37-fold ($p < 0.05$) respectively. SLC31B was not one of them. Although PXR and rifampin regulation of SLC transporters was mentioned in a review article (Ihunnah et al., 2011), we have been unable to find any published data supporting their statement for rifampin or PXR, but our ChIP-Seq analysis suggests that the rifampin induction of much of the SLC gene expression may be through other transcription factors (Rae et al., 2001; Hagenbuch and Meier, 2004).

In the SLCO family, two transporter genes (SLCO4C1 and SLCO2B1) were induced by rifampin; both are clinically relevant drug transporter genes. Neither of these SLCOs expressions have been previously shown to be altered by rifampin or PXR. Based on our ChIP-Seq data, SLCO2B1 has a PXR binding site in their promoter.

Overall, five of the 12 rifampin induced clinically relevant transporters have PXR binding sites by ChIP-Seq analysis. With an additional sixth gene, ABCB1, having a published report of a PXR binding site, half of the rifampin induction of the clinical relevant transporter gene expression in this study was likely in part due to PXR activation. Our data suggest that a smaller portion of the induction of all ABCs, SLCs, and SLCOs gene expressions by rifampin is through PXR.

Furthermore, rifampin also reduced the gene expression of three clinically relevant drug transporters (SLC15A1, SLC22A9, and SLC01B3). Of these transporter, only SLC01B3 expression has studies evaluating rifampin regulation of gene expression. SLC01B3 gene expression was shown not to change in primary hepatocytes when subjected to similar conditions (24 h 10 µM rifampin treatment; Williamson et al., 2013). Hafner et al.
TABLE 3 | All clinically relevant drug transporters correlated with miRNA induced by rifampin with a p < 0.05.

| miRNA       | Gene     | Spearman correlation | p-value | miRNA       | Gene     | Spearman correlation | p-value |
|------------|----------|----------------------|---------|------------|----------|----------------------|---------|
|            |          | Positive correlation |         |            |          | Negative correlation |
| hsa-miR-200b# | SLC01B1 | 0.96                 | 0.0028  | hsa-miR-107 | SLC16A1 | −0.96                | 0.0028  |
| hsa-miR-766 | ABCG2    | 0.96                 | 0.0028  | hsa-miR-202 | SLC22A9 | −0.93                | 0.0067  |
| hsa-miR-660 | ABCB11   | 0.96                 | 0.0028  | hsa-miR-616 | SLC22A5 | −0.93                | 0.0067  |
| hsa-miR-660 | ABCC6    | 0.93                 | 0.0067  | hsa-miR-95  | ABCB11  | −0.93                | 0.0067  |
| hsa-miR-616 | ABCC5    | 0.89                 | 0.012   | hsa-miR-335 | ABCC4   | −0.93                | 0.0067  |
| hsa-miR-886-3p | SLC51B | 0.89                 | 0.012   | hsa-miR-320 | ABCB5   | −0.89                | 0.012   |
| hsa-miR-886-3p | SLC51A | 0.89                 | 0.012   | hsa-miR-638 | ABCB5   | −0.86                | 0.024   |
| hsa-miR-766 | SLC01B1  | 0.86                 | 0.024   | hsa-miR-186 | SLC29A2 | −0.86                | 0.024   |
| hsa-miR-660 | SLC47A1  | 0.86                 | 0.024   | hsa-miR-92a | ABCC4   | −0.83                | 0.021   |
| hsa-miR-335 | SLC29A2  | 0.86                 | 0.024   | hsa-miR-320 | ABCB11  | −0.82                | 0.034   |
| hsa-miR-107 | SLC22A7  | 0.82                 | 0.034   | hsa-miR-616 | SLC01B1 | −0.79                | 0.048   |
| hsa-miR-660 | SLC02B1  | 0.82                 | 0.034   | hsa-miR-30d# | SLC29A1 | −0.79                | 0.048   |
| hsa-miR-660 | SLC04C1  | 0.82                 | 0.034   | hsa-miR-95  | SLC47A1 | −0.79                | 0.048   |
| hsa-miR-660 | SLC51A   | 0.82                 | 0.034   | hsa-miR-92a | SLC01B3 | −0.76                | 0.049   |
| hsa-miR-766 | SLC02B1  | 0.79                 | 0.048   |             |          |                      |         |
| hsa-miR-660 | ABCC2    | 0.79                 | 0.048   |             |          |                      |         |
| hsa-miR-660 | SLC22A1  | 0.79                 | 0.048   |             |          |                      |         |

Correlation was assessed for all miRNA tested and the 410 transporters expressed in hepatocytes. Shown in this table are the all the clinically relevant drug transporters regardless of rifampin regulation with the miRNAs induced by rifampin with a p < 0.05. In bold, the subset of the only clinically relevant transporters whose expression was regulated by rifampin with an FDR < 0.05 and correlated with rifampin induced miRNAs. Spearman correlation selected as samples because assumption of normal distribution could not be made.

MicroRNAs are potentially involved in the effect rifampin exerts on the expression of clinically relevant drug transporter genes. For example, four transporters genes were negatively correlated with rifampin-induced miRNA expression: SLC47A1/miR-95, SLC29A1/miR-30d#, SLC22A9/miR-202, and SLC01B3/miR-92a (Table 3). Only SLC01B3 and SLC22A9 gene expression were reduced after rifampin treatment. This was surprising because no seed binding sites were predicted for the rifampin-induced miRNAs in the 3′ UTRs of these transporter genes (data not shown). Possibly, gene reduction may occur through less well understood miRNA binding in other mRNA regions (e.g., coding and 5′ UTR regions; Clark et al., 2014).

Rifampin’s ability to alter gene expression patterns also demonstrated tissue specificity. Since the kidney proximal
TABLE 4 | The 58 transporters with ChIP-Seq determined PXR binding sites.

| Gene          | Transcript ID (Isoform) | Chromosome | Start of transcription start site (TSS) | Strand | Distance from the center of the PXR binding peak to the TSS |
|--------------|-------------------------|------------|----------------------------------------|--------|---------------------------------------------------------|
| SLC2A1       | ENST000000415851        | chr1       | 43424501                               | −      | 228.5                                                   |
| SLC5A9       | ENST000000425816        | chr1       | 46868357                               | +      | −1875.5                                                 |
| ABCD3        | ENST000000394233        | chr1       | 94883933                               | +      | −233                                                    |
| SLC22A15     | ENST000000369503        | chr1       | 116519119                              | +      | 884.5                                                   |
| ANX9         | ENST000000368947        | chr1       | 150964493                              | +      | 97.5                                                    |
| ATP1B1       | ENST000000367816        | chr1       | 169074935                              | +      | 22.5                                                    |
| SLC30A10     | ENST000000366926        | chr1       | 220101945                              | −      | 2161.5                                                  |
| ANXA11       | ENST000000481805        | chr10      | 81928844                               | −      | −190.5                                                  |
| ABCC2        | ENST000000370449        | chr10      | 101542489                              | +      | −195                                                    |
| SLC25A22     | ENST000000524891        | chr11      | 795007                                 | −      | −46.5                                                   |
| PEX16        | ENST000000527371        | chr11      | 45937095                               | −      | −2271.5                                                 |
| SLC43A1      | ENST000000533515        | chr11      | 57267151                               | −      | 2075                                                   |
| SLC3A2       | ENST000000644377        | chr11      | 62647894                               | +      | 470.5                                                   |
| SLC2B1       | ENST000000526839        | chr12      | 74876919                               | +      | −884                                                    |
| CACNA2D4     | ENST000000588896        | chr12      | 1905116                                | −      | −248.5                                                  |
| SCN8A        | ENST000000546961        | chr12      | 51984050                               | +      | 424                                                     |
| ATP2B1       | ENST000000428670        | chr12      | 90102609                               | −      | −682.5                                                  |
| SLC41A2      | ENST000000432951        | chr12      | 105352067                              | −      | −234.5                                                  |
| SCAR81       | ENST000000541205        | chr12      | 125324218                              | −      | 1402                                                   |
| ATP11A       | ENST000000487903        | chr13      | 113346443                              | +      | −604.5                                                  |
| ABCA3        | ENST000000666823        | chr16      | 2390599                                | −      | −384.5                                                  |
| SLC5A11      | ENST000000347898        | chr16      | 24857162                               | +      | −1215.5                                                 |
| SLC7A5       | ENST0000005663489       | chr16      | 8780412                                | −      | −1384.5                                                 |
| SLC25A11     | ENST000000570543        | chr17      | 4841853                                | −      | −1323.5                                                 |
| SLC47A1      | ENST000000571335        | chr17      | 19436775                               | +      | −62                                                     |
| ABCC3        | ENST000000503304        | chr17      | 48745791                               | +      | −353.5                                                  |
| ABCA9        | ENST000000461623        | chr17      | 67045499                               | −      | 1577.5                                                  |
| KCNJ2        | ENST000000535240        | chr17      | 66164814                               | +      | 1032                                                   |
| ATP5H        | ENST000000538432        | chr17      | 73043046                               | −      | 190                                                     |
| MFSD11       | ENST0000005690070       | chr17      | 74769179                               | +      | 2135                                                   |
| SLC25A10     | ENST0000005641223       | chr17      | 79670404                               | +      | 87.5                                                    |
| SLC16A3      | ENST0000005682744       | chr17      | 80186908                               | +      | 2067.5                                                  |
| ATP9B        | ENST0000005690608       | chr18      | 76886267                               | +      | −1304                                                   |
| SLC25A42     | ENST000000318596        | chr19      | 19174808                               | −      | −476                                                    |
| SLC14A4      | ENST000000493121        | chr2       | 65215611                               | +      | 1472                                                   |
| SLC20A1      | ENST000000272542        | chr2       | 113403434                              | −      | −488                                                    |
| ATP603       | ENST000000392541        | chr2       | 176046392                              | −      | −105.5                                                  |
| SLC22A2      | ENST000000423430        | chr20      | 4880294                                | −      | 887.5                                                   |
| SLC2A10      | ENST000000359271        | chr20      | 45358126                               | +      | 124.5                                                   |
| SLC5A3       | ENST000000381151        | chr21      | 35445870                               | +      | 34.5                                                    |
| PEX26        | ENST0000003296272       | chr22      | 18560869                               | +      | −1844.5                                                 |
| SLC25A1      | ENST000000461267        | chr22      | 19165994                               | −      | −31.5                                                    |
| SCARB2       | ENST000000511129        | chr4       | 77088018                               | −      | 949                                                     |
| SLC39A8      | ENST000000394833        | chr4       | 103266297                              | −      | 412                                                     |
| SLC10A7      | ENST000000507020        | chr4       | 147442870                              | −      | −390                                                    |
| SLC25A4      | ENST000000281456        | chr4       | 186064395                              | +      | 15                                                      |
| SLC26A2      | ENST000000286298        | chr5       | 149340300                              | +      | −390                                                    |
| ANXA6        | ENST000000522664        | chr5       | 150484834                              | −      | 319.5                                                   |
| SLC39A7      | ENST000000444757        | chr6       | 33168222                               | +      | −418.5                                                  |
tubules express transporters that may contribute to plasma drug concentrations, we chose to study a proximal kidney cell line to explore the effects of rifampin on clinically relevant drug transporter gene expression. We used human proximal tubular epithelial cells, NHPTK, because of the known role of the proximal tubules in organic cation, anion, and drug transport (Giacomini et al., 2010; Kido et al., 2011). This cell line closely resembles primary proximal tubular cells with a renal phenotype that includes, parathyroid hormone responsiveness, tight junction formation, and transporter expression and function (Wieser et al., 2008; Herbert et al., 2013). High levels of drug transporter mRNA expression was detected, but rifampin did not induce or reduce transporter expression in a significant manner. This finding is in agreement with prior studies evaluating rifampin effects on individual renal drug transporters expression. For example, rifampin did not lead to alterations in SLC22A2 expression in MDCK cells (Shu et al., 2001). Renal gene expression of NR1I2 (PXR) is minimal in contrast to levels observed in liver and intestine (Cheng and Klaassen, 2006). Although renal transporter expression did not increase with rifampin treatment, this is likely secondary to low intracellular PXR protein concentrations. PXR appears to be a major determinant for rifampin’s tissue selective regulation of drug transporters.

Our study has several strengths. First, primary human hepatocytes serve as an in-vitro system to predict changes in gene expression patterns in the human liver. Second, RNA sequencing allowed us to characterize all of the clinically relevant drug transporter genes that are expressed in human liver. Third, this approach allowed us to determine relationships between the gene expression patterns of the clinically relevant drug transporters, drug metabolizing enzymes, and miRNAs. Lastly, our findings provide a blue print for future investigations exploring rifampin’s regulation of the transporter function.

There are a few secondary issues of some importance worth addressing. This study also produced a wealth of data on transporters that are not yet known to be clinically relevant for drug transport. In fact, the vast majority of ABC and SLC family members have not yet been shown to be clinically relevant to drug transport. The SLC superfamily is of particular interest in drug therapeutics as it includes transporters for e.g., serotonin, norepinephrine, and glucose that are drug targets (Getcher et al., 2006; David-Silva et al., 2013; Felts et al., 2014). SLC5A12 for instance is a sodium-coupled monocarboxylate transporter (SMCT-2) that regulates the lactic acid levels in the blood and has been linked to nicotine uptake (Ohkubo et al., 2012; Garnett et al., 2013); this gene was found in our study to be highly induced (>7-fold increase) by rifampin. Hence, our systematic characterization of transporter gene expression provides a more complete picture of the effects of rifampin on transporters at large. This information can inform the selection of gene targets for future mechanistic studies of transporter function.

Our study also has some limitations. Hepatocytes from only 7 different individuals is not sufficient to determine the population variability of the effects of rifampin. Using hepatocytes from seven donors for this study should identify the major transporters that are commonly regulated by rifampin (Kato et al., 2005); however, there may be some transporters that were not statistically significantly regulated by rifampin due to the low sample number, but are in fact regulated in some subpopulations of individuals. Another limitation is that the impact of genetic variation on expression levels was not taken into account. We also acknowledge that the fold-changes for the clinically relevant drug transporter gene expression were 1.18 to 1.85-fold. However, the levels of transporter mRNA expression have been highly correlated with their protein expression (when protein levels were analyzed by mass spectrometry; Ohtsuki et al., 2012; Lee et al., 2013). Also, these changes in expression levels represent a single time point, i.e., 24 h after rifampin treatment. Many of the correlations may not be direct, and represent inhibition of repressors or induction of co-activators. We also note that the ChIP-Seq dataset from HepG2 cells may not represent all of the PXR induction seen in primary hepatocytes, and limiting the promoter region to ± 2 kilobases around the TSS may have limited the detection of all PXR binding sites.

In conclusion we identified clinically relevant drug transporters exhibiting measurable changes that occur within a relatively short time period after treatment with rifampin. The coordinated changes of transporter expression by rifampin, and

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**TABLE 4 | Continued**

| Gene | Transcript ID (Isoform) | Chromosome | Start of transcription start site (TSS) | Strand | Distance from the center of the PXR binding peak to the TSS |
|------|------------------------|------------|----------------------------------------|--------|--------------------------------------------------------|
| ABCC10 | ENST00000372515 chr6 | 43395104 | + | 618.5 |
| SLC22A1 | ENST00000393844 chr6 | 44187242 | + | 1915 |
| SLC17A5 | ENST00000393019 chr6 | 74386967 | − | 92.5 |
| SLC22A3 | ENST00000275300 chr6 | 160769000 | + | 1939.5 |
| ABCB4 | ENST00000453509 chr7 | 87104782 | − | −315.5 |
| SLC37A3 | ENST00000340308 chr7 | 140096258 | − | −58 |
| SLC20A2 | ENST00000161817 chr8 | 42396198 | − | 194.5 |
| ABCA2 | ENST00000492260 chr9 | 139926244 | − | 1220.5 |
| SLC25A5 | ENST00000317881 chrX | 118602363 | + | 714.5 |

In silico analysis utilizing a publicly available HepG2 ChIP-Seq database to test the 410 transporter genes for PXR binding after rifampin treatment. Of the 410 transporter genes analyzed, 58 transporters were found to have PXR binding sites.

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other potential inducing/repressing factors, may contribute to transporter-mediated drug-drug interactions. The rifampin-regulated transporters that we identified would be candidates for further investigations to understand the mechanisms (e.g., direct promoter regulation by PXR, indirect effects through miRNAs) involved in the up- and down-regulation of these transporters. Rifampin may serve as a representative ligand of many other PXR ligands (e.g., carbamazepine, St. John’s Wort) may also cause effects on transporter expression similar to that of rifampin.

**AUTHOR CONTRIBUTIONS**

Participated in research design: EB, TS, ME, ZD. Conducted experiments: EB, ME. Performed data analysis: EB, YL, HI, KB, MS, TS. Wrote or contributed to the writing of the manuscript: EB, ME, TS, ZD, AG, YL, KB, MS.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2016.00117

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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