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Xenobiotic-Induced Fetal Hepatocyte Maturation

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Abstract

Introduction: Human fetal liver progenitor cells (hFLPCs) offer an emerging limitless source for generating mature hepatocytes that can be useful in cell therapies, as well as in pharmacological and toxicological studies. However, hFLPCs have very limited use over adult hepatocytes because of lower expression of drug-metabolizing enzymes. Here, we studied a novel method to achieve physiological maturity of fetal hepatocytes by exposing them to PXR and HNF4α agonist.
Materials and Methods: We investigated the expression of cytochrome P450s (*CYP3A4* and *CYP2B10*), glutathione *S*-transferase Mu 1 (*GSTM1*), UDP-glucuronosyltransferase 1-1 (*UGT1A1*), and drug transporters ATP-binding cassette subfamily C member (*ABCC2* and *ABCC3*) by exposing them to rifampicin (Rif, 10 and 30 μM), linoleic acid (50 and 100 μM), and 0.1% dimethyl sulfoxide (DMSO) for 4, 8, and 12 days. The real-time polymerase chain reaction and western blotting were used to determine mRNA and protein expression of *CYPs*.
Results: Increased *CYP* expression on the mRNA and protein level was detected in hFLPCs, which are exposed to DMSO- and Rif-treated cells that were much higher than in untreated. There was increase in levels of CYP2B10 protein with respect to time when hFLPCs exposed to DMSO.
Discussion and Conclusions: Thus, this is the first report on expression of few phase I, II, and III enzymes in hFLPCs challenged with PXR and HNF4α agonists and to generate hFLPCs expressing drug-metabolizing enzymes similar to that of primary human adult hepatocytes.

Keywords: Cytochrome P450s, drug-metabolizing enzymes, human fetal liver progenitor cells, PXR and HNF4α agonists

Introduction

Hepatocytes are being increasingly utilized for basic and clinical research. Clinical use of hepatocytes has gained interest in many clinicians in recent years. Bioartificial liver (BAL) support system has been carried out with porcine hepatocytes,[1] eternalized human cell lines,[2] and primary human hepatocytes for the treatment of inborn metabolic disorders.[3] Transplantation of hepatocytes is alternative to liver transplantation, but inadequate functional hepatocytes reduce their therapeutic potential. Also, primary hepatocytes lose their viability and function when cultured *in vitro*.[4] Many researchers concluded that human embryonic stem cells, bone marrow stem cells, and/or mesenchymal stem cells could be differentiated into hepatocyte-like cells.[5–7] These differentiated cells express albumin, cytokeratin 8, anti-alpha trypsin, glycogen, albumin, and urea. Although there is guaranteed transdifferentiation or dedifferentiation of such cells in BAL, their functional capability remains a major issue.

Hepatic stem cells from fetal livers are potential candidates in BAL modules. They possess high proliferative capacity as well as accessibility to differentiate into hepatocyte, cholangiocyte, and sinusoidal cells.[8,9] They can repopulate liver successfully and promote long-term engraftment.[8,9] In our earlier work,[10,11] we found that fetal hepatocytes (hFLPCs) have low metabolic capacities compared with adult hepatocytes. This could partly be due to the fact that a large part of hepatic differentiation takes place during postnatal period. With the physiological development, there is a rapid progression of normal hepatic function at the end of gestation. But ''physiological immaturity'' along with bile formation are there for a few months after birth.[12] This ''physiological immaturity'' leads to ineffective lipid digestion, late hepatic clearance, sluggish metabolism of xenobiotics (drugs), and endogenous substrate-like bile acids and bilirubin.[13,14]

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Thus, in this study, we propose to demonstrate the *in vitro* maturation of hFLPCs, by imitating the *in vivo* microenvironment. It will be accomplished by the addition of exogenous compounds. These compounds will incite normal metabolic processes such as oxidation, reduction, hydrolysis, and conjugations, inducing its capability to metabolize, detoxify, and excrete xenobiotics.

Materials and Methods

Fetal liver

For the use of human fetal liver tissue, institutional ethical committee approval was granted from Sahlgrenska University Hospital (Gothenburg, Sweden), in compliance with Swedish guidelines. Human fetal liver tissues were obtained from legally aborted first-trimester fetuses between 6 and 11 weeks of gestational age. A cell strainer (70 μm) was used for the preparation of a single-cell suspension. There was more than 90% ($n=5$) viability of the cells evaluated by trypan blue. Donors were screened serologically for syphilis, toxoplasmosis, rubella, cytomegalovirus, parvovirus, and herpes simplex virus types 1 and 2. Hepatitis B and C, and human immunodeficiency virus 1 and 2 were detected by quantitative polymerase chain reaction (PCR).

Isolation of hFLPCs

hFLPCs screened using CD117+, CD34+ lineage and were isolated by magnetic cell sorting.[9] hFLPCs from a mixed pool of 12 human donors (gestation week 6–11) were used. The cryopreserved cells were thawed and cultured in complete hepatocyte medium. hFLPCs cells were trypsinized when they were 80% confluent. hFLPCs from passages 5 to 10 were used for the current experiment.

Stimulating hepatic differentiation

Dimethyl sulfoxide (DMSO), rifampicin (Rif), and linoleic acid (LA) was purchased from Sigma (Sigma, Stockholm, Sweden). For the treatment, 10 and 30 μM Rif was dissolved in 100 μL DMSO, LA 50 and 100 μM was dissolved in 100 μL DMSO, and only DMSO (0.1%) used as vehicle control dissolved into complete fetal hepatocyte medium.

Cells were treated with test substances or vehicle control (0.1% DMSO) for 4, 8, and 12 days. Cells were passaged every third day, and new media were added every third day.

Fetal hepatocytes treated with Rif (10 and 30 μM) for 12 days and LA (50 and 100 μM) for 4 days samples were not analyzed because of low protein and RNA concentration.

Western blot analysis

Treated hFLPCs were bathed with phosphate-buffered saline two times, and then, 1 mL of the RIPA buffer was added to a T-75 size flask. The cells were scraped and collected in Eppendorf tubes and placed on ice for 10 minutes. The homogenate was centrifuged at 4500 *g* (10,000 rpm) for 10 minutes. The supernatant was collected and stored in −80°C for further studies. The protein concentration of the samples was evaluated by BCA protein assay kit (Pierce; Thermo Fisher Scientific). Thirteen micrograms of protein of each sample per lane on 12% Bis-Tris gel (Invitrogen) was transferred electrophoretically to a polyvinylidene fluoride membrane (Invitrogen) by semi-dry blotting for 1 hour (30 V for 1 hour, followed by 35 V for 30 minutes), and the membrane was stained with Ponceau S (Sigma) and digitally imaged. After that, the membrane was incubated for 1 hour in 5% skim milk and was incubated with CYP3A4 (Mouse Monoclonal) and CYP2B10 (Rabbit Polyclonal; Millipore) overnight at 4°C.

The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG/M at room temperature for 1 hour, followed by enhanced chemiluminescent (ECL) (Pierce, Thermo Scientific) detection. We used primary human hepatocyte cell lysate as the positive control. GAPDH was used as a loading control.

RNA isolation and cDNA synthesis

Total cellular RNA was isolated using a DNA/RNA/protein isolation kit (Norgen Biotek Corp., Thorold, Canada). The amount of RNA was quantified by measuring absorbance at 260 nm using a DU 730 spectrophotometer (Beckman Coulter, Fremont, CA). First-strand cDNA synthesis was performed. One microgram of total RNA was reverse-transcribed into cDNA at 50°C using an Advantage® first-strand synthesis kit (Clontech, Mountain View, CA). Quantification of cDNA was performed in the Applied Biosystems Prism 7300 real-time PCR. Samples were subjected to PCR amplification using specific primers (Table 1). PCR specificity was verified by ΔCt of the PCR products. 18S RNA levels were used for data normalization.

Statistical analysis

Data are represented as mean ± standard deviation. A paired Student's *t*-test was used to calculate statistically significant differences ($*p<0.05$).

Results

Analysis of cytochrome P450 protein expression

The expression of CYP protein in primary adult hepatocytes, fetal hepatocytes, and hFLPCs challenged with Rif (10 and 30 μM) and 0.1% DMSO (vehicle control) was estimated by western blotting. There was presence of CYP3A4

Table 1. Primer Sequences

CYP3A4	F	5′-ACC AGT GGA AAA CTC AAG GAG-3′
	R	5′-TGA TCA CAT CCA TGC TGT AGG-3′
GSTM1	F	5′-CTC CAC CGT ATA TTT GAG CCC-3′
	R	5′-AGC CAT CTT TGA GAA CAC AGG-3′
GSTM1	F	5′-AGG AAA AGA AGT ACA CGA TGG G-3′
	R	5′-TTG CTC TGG GTG ATC TTG TG-3′
UGT1A1	F	5′-TCA CCA AAA TCC ACT ATC CCA G-3′
	R	5′-GCC CAA AGC ATC AGC AAT TG-3′
ABCC2	F	5′-TCA TCG TCA TTC CTC TTG GC-3′
	R	5′-ACG GAT AAC TGG CAA ACC TG-3′
ABCC3	F	5′-ACC TGT CCA AGC TCA AGA TG-3′
	R	5′-GGG TGA CAA AGA AAA CAG GG-3′

F, forward; R, reverse.

and CYP2B10 protein in hFLPCs challenged with DMSO and Rif (10 and 30 μM) treatment after 4, 8, and 12 days. hFLPCs and fetal hepatocytes challenged with the 30 μM Rif for 8 days showed slightly different molecular weight than other cells. There was highest CYP3A4 protein concentration in hFLPCs treated with DMSO for 12 days, which was slightly higher than primary adult hepatocytes. hFLPCs treated with DMSO for 12 days and Rif (30 μM) for 4 and 8 days showed slightly increased CYP3A4, but this was at a lesser level as that of primary adult hepatocytes (Figs. 1 and 2).

The expression of CYP2B10 protein was increased when hFLPCs were exposed to DMSO for 4, 8, and 12 days. DMSO treatment for 4, 8, and 12 days resulted in 5.7-, 8.9-, and 10.6-fold increase, respectively, compared with fetal hepatocytes. Rif (10 μM) treatment for 4 days showed a ninefold increase over untreated hFLPCs.

Analysis of gene expressions by real-time PCR

CYP3A4. Primary adult hepatocyte CYP3A4 mRNA expression was slightly higher than untreated hFLPCs (Fig. 3). DMSO treatment for 8 days resulted in 470-fold increase in mRNA expression compared with untreated hFLPCs. Rif (10 μM) treatment for 4 days resulted in 19-fold increased expression. Other doses of Rif and LA show marginal levels of mRNA expression.

GSTM1. Two different sets of primers were used to detect GSTM1 (glutathione *S*-transferase Mu 1) expression with real-time PCR. Treatment of DMSO did not show increased GSTM1 expression compared with primary adult hepatocytes. Treatment of 30 μM Rif for 4 days and 100 μM of LA for 12 days showed slightly increased concentration of GSTM1 compared with hFLPCs (Fig. 3).

UGT1A1. The amount of UGT1A1 (UDP-glucuronosyltransferase 1-1) mRNA was higher in primary adult hepatocytes compared with untreated hFLPCs. UGT1A1 mRNA levels were also higher in DMSO-treated cells for 12 days and Rif (30 μM) for 8 days compared with untreated hFLPCs. Moreover, high UGT1A1 mRNA was detected in hFLPCs treated with the 30 μM Rif for 4 days. The expression level for UGT1A1 mRNA in other groups was low compared with primary adult hepatocytes (Fig. 4).

ABCC2. The expression level of ABCC2 (ATP-binding cassette subfamily C member 2) was very low in the untreated hFLPCs compared with primary adult hepatocytes.

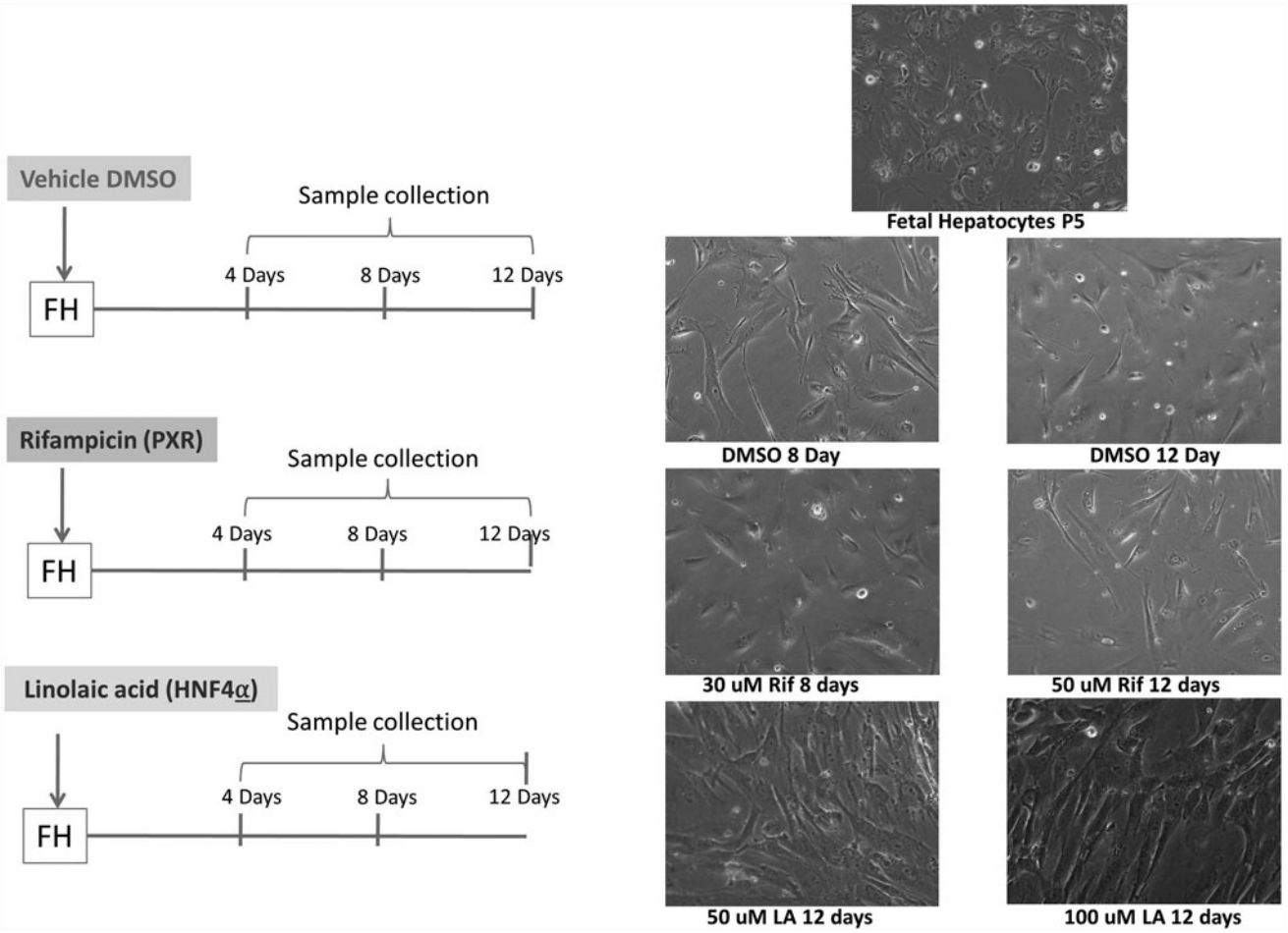

FIG. 1. Simulating hepatic differentiation using nuclear receptor agonists.

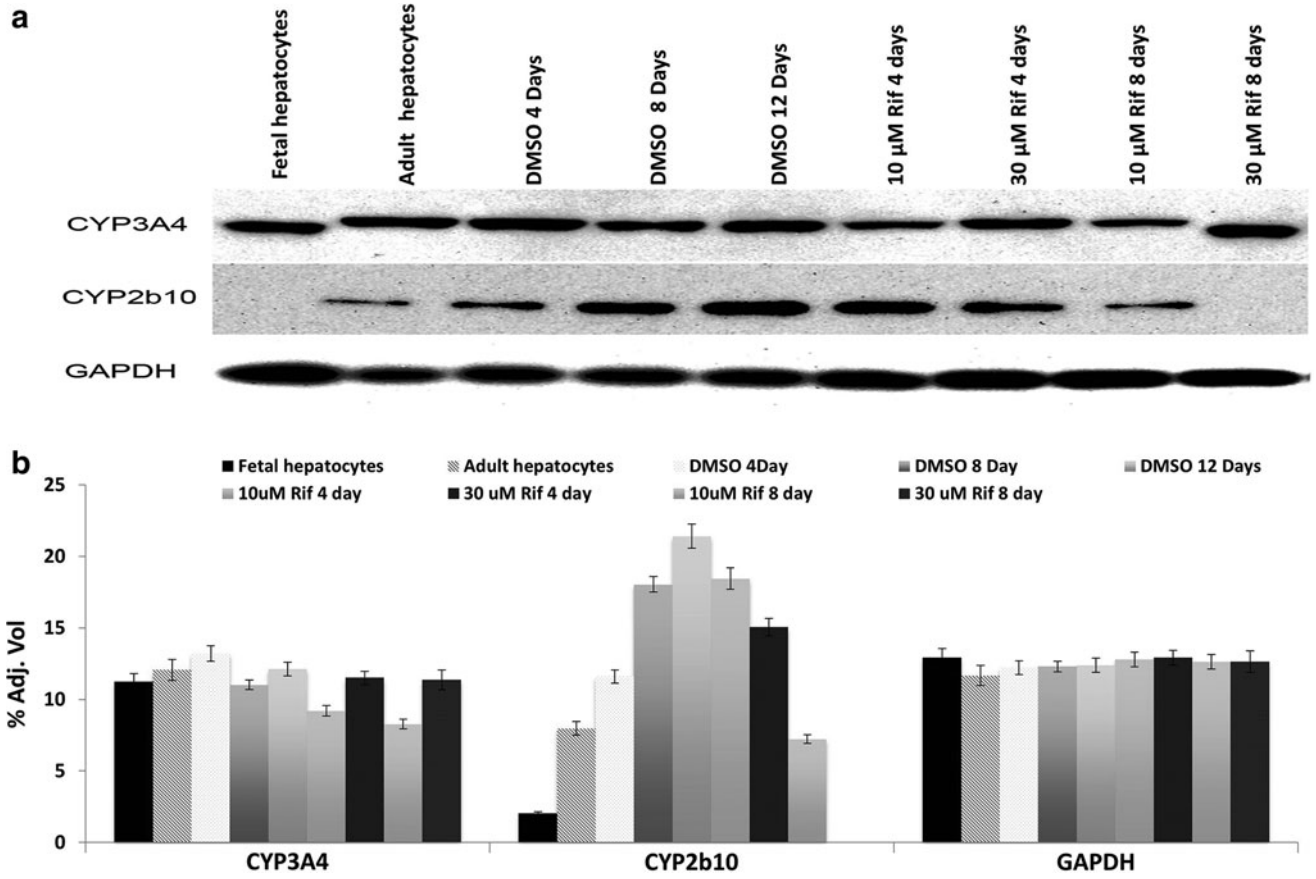

FIG. 2. Western blot of CYP3A4 and CYP2B10 **(a)** analysis, **(b)** densitometry analysis.

The relative expression of ABCC2 in the hFLPCs treated with the 10 μM Rif was slightly higher than untreated hFLPCs (Fig. 4).

ABCC3. Expression of ABCC3 (ATP-binding cassette subfamily C member 3) was low in untreated hFLPCs compared with primary adult hepatocytes. DMSO treatment for 4 and 12 days resulted in 2.6-fold and slightly increased gene expression compared with untreated hFLPCs, respectively. Rif treatment (10 μM for 8 days) resulted in 2.03-fold increased gene expression compared with untreated hFLPCs. LA treatment (100 μM) for 8 and 12 days resulted in slightly

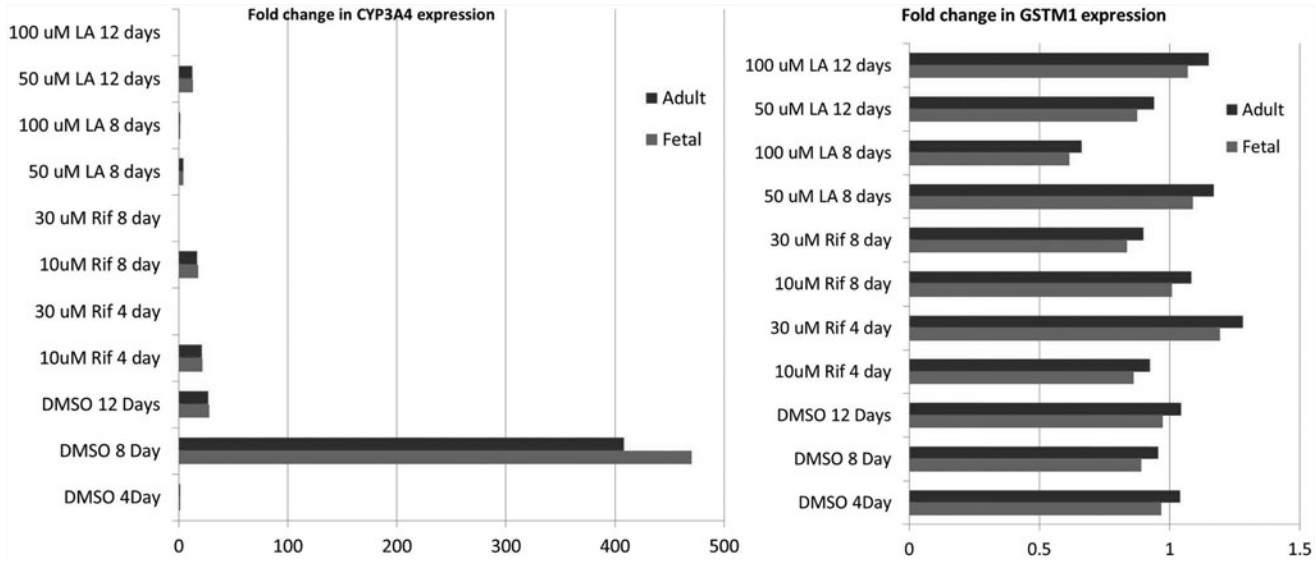

FIG. 3. Fold change in CYP3A4 and GSTM1 gene expression by real-time PCR. PCR, polymerase chain reaction.

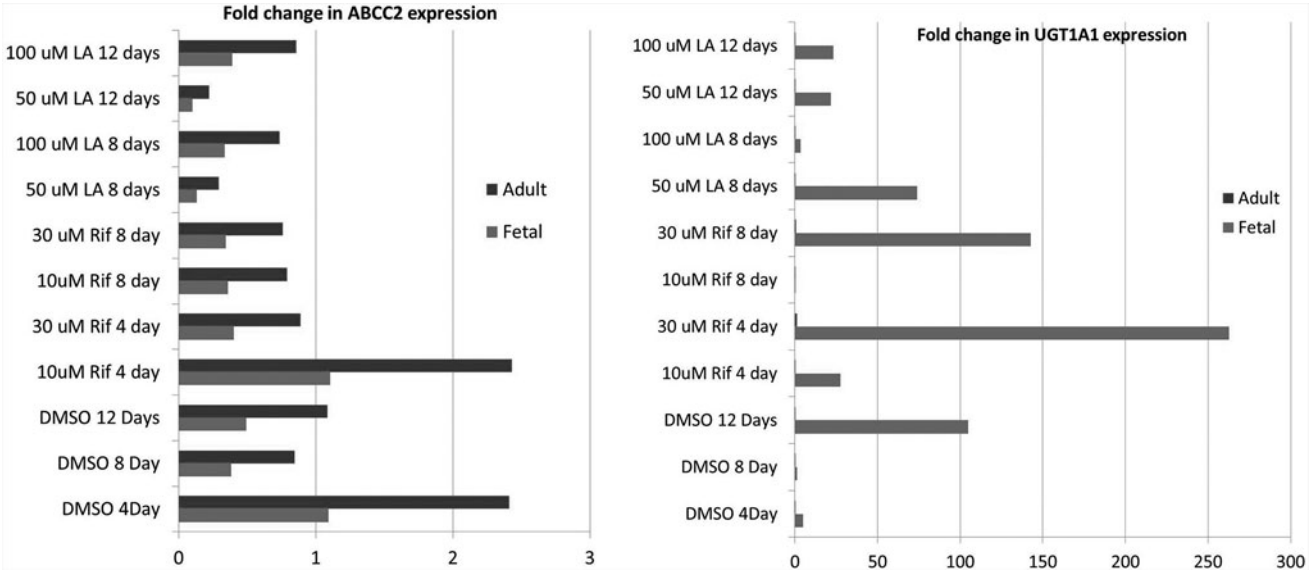

FIG. 4. Fold change in ABCC2 and UGT1A1 gene expression by real-time PCR.

increased ABCC3 concentration, respectively, compared with untreated hFLPCs (Fig. 5).

Discussion

Here, we show that PXR and HNF4α agonist induces differentiation in hFLPCs. Since DMSO is widely used as a vehicle for the delivery of various molecules in cultured cells, in the present study, we have studied the potential impact of DMSO as a vehicle on hFLPCs. Morley and Whitfield suggested that DMSO-induced Ca^{2+} release from intracellular stores may play a role in the induction of cell differentiation.[15] Moskot et al. studied the role of DMSO in gene modulation and glycosaminoglycan metabolism in lysosomal storage disorders.[16] Similarly, there is a distinct variation in expression patterns when hFLPCs were exposed to Rif and DMSO. We also show limited capacity of LA to induce differentiation in hFLPCs. Therefore, liver-specific genes such as *CYP3A4* were only detected in hFLPCs exposed to Rif and DMSO, which denotes a distinction in the scale of differentiation.

The human body gets exposure to xenobiotics constantly; thus, biotransformation system evolves to detoxify and eliminate different substances over time. In fetal life, exposure

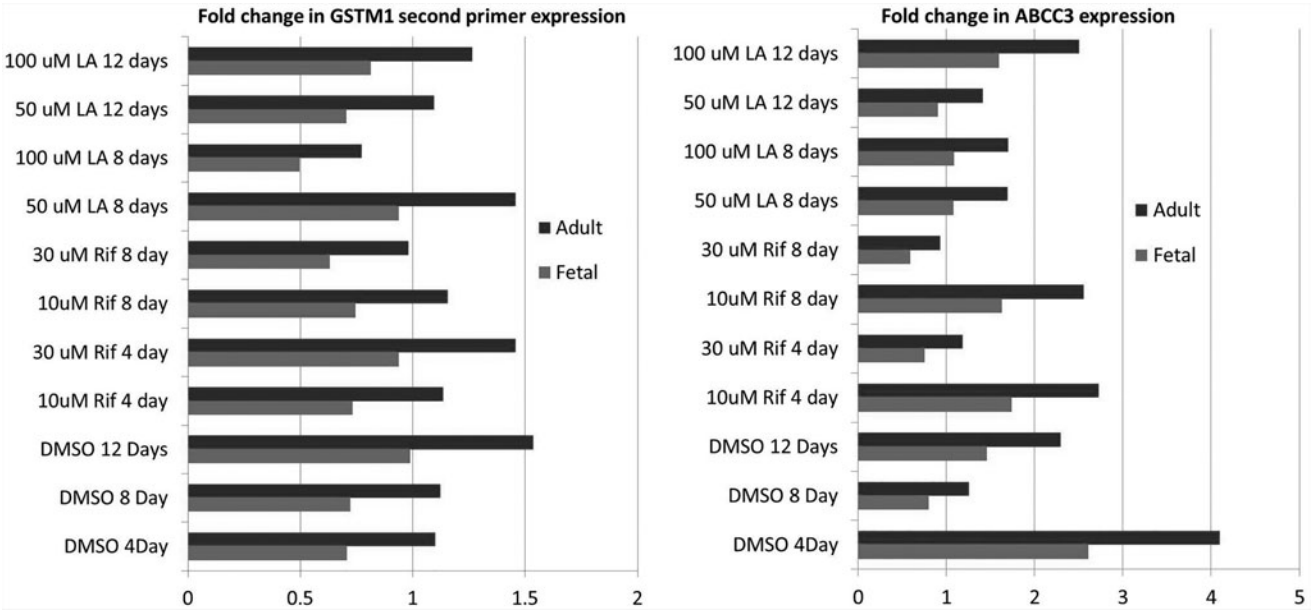

FIG. 5. Fold change in GSTM1 and ABCC3 gene expression by real-time PCR.

to different xenobiotics is limited, and thus, hFLPCs have limited biotransformation function. Isoforms of cytochrome P450s (*CYPs*) display differential expression levels during developmental phases of the liver. Human fetal liver mainly expresses *CYP3A7*, whereas *CYP3A4* is predominantly expressed in adult hepatocytes.[17] Biotransformation of the newborn is starting with ingestion of breast milk. The biotransformation of any xenobiotics resulted in elimination that was initially catalyzed by phase I metabolizing enzymes such as *CYPs*, which acts as monoxygenases. Furthermore, phase II metabolizing enzymes use hydrophilic groups transferred by phase I enzymes to conjugate or glucuronidate xenobiotics, followed by export transportation carried out by phase III protein transporters.[18] In our preliminary study, we investigate *CYP* expression along with phase II enzymes and transporters. Our results indicate that hFLPCs exposed to Rif and DMSO expressed phase I (*CYP3A4* and *CYP2B10*) and II enzymes (UGT1A1 and GSTM1), as well as phase III proteins (ABCC2 and ABCC3). Nuclear receptors have an important function in regulating drug-metabolizing enzymes as well as transporter proteins.[19] Thus, PXR induces expression of *CYP2A, 2B, 2C, 3A, 7A, UGT1A1, SULT1A1, GST-A2* and *GST, MDR1, MRP 2* and *3, OATP2,* and *OCT1*.[19,20]

Considering the effects of PXR, we used PXR agonist Rif to induce differentiation of hFLPCs. These results showed that 10 and 30 μM Rif induces expression of important drug-metabolizing enzyme genes. An interesting finding of our study is that we could get differentiation using DMSO alone. LA-induced differentiation is restricted to *GSTM1, ABCC2,* and *ABCC3*.

Up to the present time, human adult hepatocytes are considered to be the most appropriate *in vitro* model for biotransformation in human liver, and it is used for toxicological and pharmaceutical studies. Human adult hepatocytes are difficult to isolate and they fail to maintain their metabolic activity after few weeks. A disadvantage of hFLPCs is lack of biotransformation, but they are easier to isolate and cryopreserve an ample number of cells for study. Our results show that hFLPCs can be a valuable *in vitro* model to study toxicology if they are primed for biotransformation using Rif and LA.

Conclusions

The preliminary results demonstrate that Rif- and DMSO-challenged hFLPCs showed expression of *CYP3A4* and *CYP2B10*, but LA-challenged hFLPCs showed no or very low expression of *CYPs*. Importantly, phase I and II enzyme induction being observed when cells are exposed to Rif, LA, and DMSO. In this study, we conclude a simple and relatively cost-effective protocol for induction of biotransformation in hFLPCs. This will open up new avenues for drug metabolism, hepatotoxicity, and cell therapy studies using primary fetal hepatocytes.

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Author Disclosure Statement

No competing financial interests exist.

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