Nuclear receptor atlas of female mouse liver parenchymal, endothelial, and Kupffer cells

Zhaosha Li, J. Kar Kruijt, Ronald J. van der Sluis, Theo J. C. Van Berkel, and Menno Hoekstra

Division of Biopharmaceutics, Leiden Academic Centre for Drug Research, Leiden University, The Netherlands

Submitted 9 November 2012; accepted in final form 29 January 2013

Li Z, Kruijt JK, van der Sluis RJ, Van Berkel TJ, Hoekstra M. Nuclear receptor atlas of female mouse liver parenchymal, endothelial, and Kupffer cells. Physiol Genomics 45: 268–275, 2013. First published January 29, 2013; doi:10.1152/physiolgenomics.00151.2012.—The liver consists of different cell types that together synchronize crucial roles in liver homeostasis. Since nuclear receptors constitute an important class of drug targets that are involved in a wide variety of physiological processes, we have composed the hepatic cell type-specific expression profile of nuclear receptors to uncover the pharmacological potential of liver-enriched nuclear receptors. Parenchymal liver cells (hepatocytes) and liver endothelial and Kupffer cells were isolated from virgin female C57BL/6 wild-type mice using collagenase perfusion and counterflow centrifugal elutriation. The hepatic expression pattern of 49 nuclear receptors was generated by real-time quantitative PCR using the NUClear Receptor Signaling Atlas (NURSA) program resources. Thirty-six nuclear receptors were expressed in total liver. FXR-α, EAR2, LXR-α, HNF4-α, and CAR were the most abundantly expressed nuclear receptors in liver parenchymal cells. In contrast, NUR77, COUP-TFI, LXR-α/β, FXR-α, and EAR2 were the most highly expressed nuclear receptors in endothelial and Kupffer cells. Interestingly, members of orphan receptor COUP-TF family showed a distinct expression pattern. EAR2 was highly and exclusively expressed in parenchymal cells, while COUP-TFI was moderately and exclusively expressed in endothelial and Kupffer cells. Of interest, the orphan receptor TR4 showed a similar expression pattern as the established lipid sensor PPAR-γ. In conclusion, our study provides the most complete quantitative assessment of the nuclear receptor distribution in liver reported to date. Our gene expression catalog suggests that orphan nuclear receptors such as COUP-TFI, EAR2, and TR4 may be of significant importance as novel targets for pharmaceutical interventions in liver.

nuclear receptor; liver; parenchymal cell; endothelial cell; Kupffer cell; gene expression

THE NUCLEAR RECEPTOR SUPERFAMILY describes a related but diverse array of ligand-activated transcription factors that translate physiological signals into gene regulation involved in biological processes including metabolism. Liver is considered as the major organ with significant therapeutic importance for the maintenance of metabolic homeostasis. Liver utilizes many nuclear receptors for its functions, and nuclear receptors have been increasingly appreciated by researchers in the hepatic field (17). Toxin-activated nuclear receptors, such as constitutive androstane receptor (CAR) and pregnane X receptor (PXR), are key sensors to regulate xenobiotic clearance in the liver (9). Lipid-activated nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR), are attractive targets for therapeutic agents to regulate glucose metabolism, lipid metabolism, and inflammation (14). However, the function of orphan nuclear receptors whose endogenous and synthetic ligand(s) is unknown has not been fully exploited. It is thus of interest to quantitatively assess the expression and distribution of nuclear receptors in liver to discover the pharmacological potential of the remaining liver-enriched orphan nuclear receptors.

The liver consists of different types of cells, including parenchymal cells, namely hepatocytes, and a variety of nonparenchymal cells. Nonparenchymal cells mainly comprise liver sinusoidal endothelial cells and Kupffer cells. Liver endothelial cells form a continuous but fenestrated lining of the hepatic sinusoids, while Kupffer cells are found in the sinusoidal lumen on top of or between endothelial cells (40). Liver endothelial cells free the bloodstream from a variety of macromolecular waste products during inflammation (35). Kupffer cells are a population of hepatic resident macrophages. They constitute 80–90% of the tissue macrophages present in the body (6). Although nonparenchymal cells count for only 6.5% of the liver volume, they contain 55% of the lipid droplets and 43% of the lysosomes in the liver, and specific activities of enzymes are generally higher in nonparenchymal cells than in parenchymal cells (24, 38). Parenchymal and nonparenchymal cells synchronize crucial roles in liver metabolic homeostasis as well as inflammation. The majority of studies regarding nuclear receptors in liver has focused on the array of target genes and metabolic pathways within parenchymal cells (18, 23). However, nonparenchymal cells are also intimately involved in the pathogenesis of various liver metabolic diseases including steatohepatitis, nonalcoholic fatty liver disease, and liver fibrosis (20). Previous studies have shown that diet-induced hypercholesterolemia results in marked changes in the hepatic distribution of low-density lipoprotein (LDL) and significant accumulation of cholesteryl ester/lipid droplets in liver endothelial and Kupffer cells, suggesting a prominent role of liver nonparenchymal cells in removing modified LDL from blood (27, 28, 39). Other studies show that depletion of liver Kupffer cells and targeted inactivation of scavenger receptor A and CD36 expressed in Kupffer cells reduced hepatic inflammation and tissue destruction associated with diet-induced hepatic steatosis, indicating a role for liver macrophages in hepatic lipid metabolism and insulin sensitivity (1, 15). It has been shown that Kupffer cells and hepatocytes interact in regulating glycogenolysis (21) and hepatic lipid storage (36). It is thus of interest to further investigate the potential cross talk between nuclear receptors in nonparenchymal and parenchymal cells involved in hepatic metabolism regulation.

Nuclear receptors may have different distribution patterns in liver parenchymal and nonparenchymal cells. It has been shown that retinoid X receptor (RXR)-α and RXR-β expression levels are 5- to 10-fold higher in Kupffer cells than in other nonparenchymal cells, while all the subtypes of retinoid
acid receptor (RAR) family have similar expression level in both cell types (29). We have also demonstrated that for studies of certain nuclear receptors and their regulation in liver, their cellular localization should be taken into account, allowing proper interpretation of metabolic changes which are directly related to their intracellular expression level (12). Therefore, a systematic assessment of the nuclear receptor distribution in liver is necessary to determine their distinctive contributions in liver parenchymal, endothelial, and Kupffer cells.

To our knowledge, there has been no study illustrating the full expression pattern of the 49 murine nuclear receptors in different liver cell types. In the current study, mouse liver parenchymal and nonparenchymal cells were isolated by counterflow centrifugal elutriation, and gene expression profiling of nuclear receptors in liver parenchymal, endothelial, and Kupffer cells was performed to compose the hepatic expression pattern and identify novel targets for pharmaceutical interventions in the liver.

MATERIALS AND METHODS

Animals. Virgin female C57BL/6 mice 10–12 wk old were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in cages with a controlled 12 h light cycle for 2 wk and fed a regular chow diet containing 4.3% (wt/wt) fat (RM3; Special Diet Services, Witham, UK). Mice were fasted overnight before death and subsequent cell isolations. Animal care and procedures were performed in accordance with the national guidelines for animal experimentation. All protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Parenchymal and nonparenchymal cell isolation. Liver cells were isolated from mice at the same time of the day (10–11 AM, 3–4 h after lights on). Mice were anesthetized, liver tissue was dissociated, and parenchymal cells were isolated after collagenase perfusion, while nonparenchymal cells were collected as described previously (13).

Endothelial and Kupffer cell separation. The endothelial cells and Kupffer cells were further separated by counterflow centrifugal elutriation, which consists of a J2-MC centrifuge (Beckman) connected with a peristaltic pump (LKB, Bromma, Sweden). The elutriation was performed at 4°C at a speed of 3,250 rpm. Endothelial and Kupffer cells were separated at flow rate of 25 and 70 ml/min, respectively.

RNA isolation and gene expression analysis. Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. Quantitative real-time PCR was carried out using ABI Prism 7700 Sequence Detection system (Applied Biosystems) according to the manufacturer’s instructions using SYBR Green technology.

The primer sequences for all nuclear receptors assessed in the current study were obtained from literature (10) and available on the Nuclear Receptor Signaling Atlas (NURSA) website at http://www.nursa.org. Gene-specific primer sequences for housekeeping genes and cell characterization markers are listed in Table 2. β-Actin, hypoxanthine guanine phosphoribosyltransferase, acidic ribosomal phosphoprotein P0, and glyceraldehyde-3-phosphate dehydrogenase were used as internal housekeeping genes. The average Ct of four housekeeping genes was used to exclude that changes in the relative expression (RE) were caused by variations in the expression of separate housekeeping genes. RE levels of nuclear receptors as normalized against the housekeeping gene expression were calculated as ΔCt = Ct_housekeeping − Ct_nuclear_receptor. The respective average housekeeping Ct values used for normalization were 21.3 ± 0.6 for parenchymal cells, 24.1 ± 0.6 for endothelial cells, and 26.1 ± 0.8 for Kupffer cells. The numerical fold changes relative to housekeeping genes were calculated as 2−ΔCt. Transcripts with a Ct value >34 were considered not present/undetectable. Means and standard errors of the mean (SE) were calculated with the ΔCt formula.

Statistical analysis. Statistical analyses were performed by ANOVA for independent samples after confirmation of Gaussian distribution using the test of Golnogorov and Smirnov (Instat GraphPad software, San Diego, CA). Statistical significance was defined as P < 0.05. Data are expressed as means ± SE.

RESULTS AND DISCUSSION

Expression of nuclear receptors in liver. Real-time quantitative PCR is a standardized method in the nuclear receptor field to characterize the expression pattern of individual receptors in tissue or cells. It provides a simple but powerful way to obtain comprehensive understanding of the distribution and relational biological functions of nuclear receptors (4). Real-time quantitative PCR has been applied in numerous studies to profile the expression pattern of nuclear receptors in tissues representing diverse anatomical systems under various pharmacological conditions and genotypes (5, 42). For an overview of all murine nuclear receptors names and their gene symbols and classification see Table 1.

Our expression profiling using real-time quantitative PCR revealed that 36 (73%) of the in total 49 nuclear receptors known in mice are expressed in total liver of virgin female C57BL/6 mice. Similarly to previous findings by Bookout et al. (5), we could not detect (Ct >34) expression of the nuclear receptors DAX-1, ER-β, ERR-β, HNF4-γ, NORT1, NURR1, PNR, PR, ROR-β, SF-1, TLX, and VDR in whole liver specimens. In accordance with its crucial role in the control of hepatic bile acid metabolism (7), the nuclear bile acid receptor FXR-α was identified as the most abundantly expressed nuclear receptor in liver. The 10 highly expressed nuclear receptors (RE level >0.01) in liver were ranked in the following order: FXR-α > EAR2 > LXR-α > HNF4-α > CAR > LXR-β > NUR77 > RXR-α > SHP > PXR (Fig. 1). Seventeen nuclear receptors exhibited a moderate expression in total liver specimens (RE level between 0.001 and 0.01), including PPAR-α > ROR-γ > COUP-TFII > LXR-1 > REV-ERB-α > RXR-β > AR > ROR-γ > ERR-α > REV-ERB-β > ROR-α > GR > TR4 > TR-β > ER-α > PPAR-γ > TR-α (Fig. 1B). Our rank order of nuclear receptor expression differs somewhat from that previously published by Bookout et al. (5). This can probably be attributed to differences in sex (females vs. males) or the time (3–4 h vs. 0 h after lights on) and metabolic status (overnight fasting vs. ad libitum fed) upon tissue harvest. However, in agreement with the previous data from the NURSA real-time PCR platform, we did detect moderate to high expression of all nuclear receptors identified by Bookout et al. (5) to be highly expressed in tissues associated with metabolic functions such as the liver, i.e., COUP-TFI, EAR2, ERR-α, FXR-α, HNF4-α, LXR-β, RAR-α, ROR-γ, RXR-α, RXR-β, RXR-γ, and TR-α.

Interestingly, the second most highly expressed nuclear receptor in the liver was the orphan receptor EAR2, also known as COUP-TFI,II, with an mRNA level slightly higher than HNF4-α and LXR-α. Another member from this orphan receptor family, COUP-TFI, was moderately expressed in liver, with expression level similar to LXR-1 but about fivefold higher than PPAR-γ. In contrast, expression of COUP-TFI in liver was 32-fold and ~250-fold lower than that of COUP-TFI and EAR2.

Expression of nuclear receptors in liver parenchymal, endothelial, and Kupffer cells. Liver is a highly differentiated organ composed of parenchymal cells and nonparenchymal cells. They play independent but also co-operative roles in health and disease. Thus, separation of liver cells is essential to discover the pharma-
Table 1. *Gene symbols, official abbreviations, and full names of the 49 murine nuclear receptors*

<table>
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<th>Symbol</th>
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<th>Name</th>
<th>Symbol</th>
<th>Classification</th>
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<td>AR</td>
<td>NR3C4</td>
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<td>NR2F1</td>
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<td>DAX-1</td>
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<td>Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1</td>
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<td>SF-1</td>
<td>NR5A1</td>
<td>Steroidogenic factor 1</td>
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<td>NR5A2</td>
<td>Liver receptor homolog-1</td>
<td>SHP</td>
<td>NR0B2</td>
<td>Small heterodimer partner</td>
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<td>NR1H3</td>
<td>Liver X receptor-α</td>
<td>TLX</td>
<td>NR2F1</td>
<td>Homolog of the Drosophila tailless gene</td>
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<td>NR1H2</td>
<td>Liver X receptor-β</td>
<td>TR-α</td>
<td>NR1A1</td>
<td>Thyroid hormone receptor-α</td>
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<td>MR</td>
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<td>Mineralocorticoid receptor</td>
<td>TR-β</td>
<td>NR1A2</td>
<td>Thyroid hormone receptor-β</td>
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<td>NR4A3</td>
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<td>TR2</td>
<td>NR2C1</td>
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<td>NR4A2</td>
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<td>VDR</td>
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<td>PNR</td>
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<td>Photoreceptor cell-specific nuclear receptor</td>
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Fig. 1. Expression profile of nuclear receptors present in total liver specimens of female C57BL/6 mice as determined by real-time quantitative PCR. Nuclear receptors are ranked in decreasing order according to their expression level. Bar values are relative to housekeeping gene expression and represent means ± SE of 4 mice per group. Numbers above the bars indicate the measured average Ct value.
consisted solely of hepatocytes (data not shown). All isolated cells subsequently used for RNA isolation were viable as noted from negative trypan blue staining. The cell separation was confirmed by real-time quantitative PCR characterization of specific gene markers. Expression of the hepatocyte marker CYP7A1 was detected only in the liver parenchymal cell fraction (Fig. 2), indicating the success of the parenchymal cell separation and no contamination of parenchymal cells in nonparenchymal cell fractions. Similarly, expression of the endothelial cell marker PECAM-1 and macrophage marker CD68 was only detected in nonparenchymal cell fractions and significantly higher in endothelial \((P < 0.001)\) or Kupffer \((P < 0.01)\) cell fractions, respectively (Fig. 2), indicating the efficiency of the endothelial and Kupffer cell separation.

Among the 36 nuclear receptors expressed in liver, 33 (92%) of them were expressed in parenchymal cells. The same 10 nuclear receptors were most highly expressed in parenchymal cells as those in whole liver (Fig. 3). The expression profiles of the 16 nuclear receptors that are moderately expressed in parenchymal cells are also shown in Fig. 3. In nonparenchymal cells, 29 (80%) of the liver-expressed nuclear receptors were detected. NUR77, COUP-TFI, LXR-\(\alpha\), LXR-\(\beta\), FXR-\(\alpha\), and EAR2 were identified as the most abundantly expressed nuclear receptors in both endothelial and Kupffer cells.

**Comparison of nuclear receptor expression in liver parenchymal and nonparenchymal cells.** To further characterize the potential relationship between nuclear receptors, the liver-expressed nuclear receptors were grouped into three clusters.
according to the substantial differences in their distribution patterns over parenchymal and nonparenchymal cells.

When we compared RE levels of the nuclear receptors as normalized against housekeeping gene expression levels, 20 showed dominant expression in parenchymal cells. In line with the distinct localization of HNF4-α protein in hepatocytes within the liver of mice (16), HNF4-α mRNA expression was solely detected in liver parenchymal cells (Fig. 3). The hepatic detoxification mediators CAR and PXR as well as SHP, PPAR-α, ROR-γ, ER-α, and TR-β were also exclusively expressed in parenchymal cells (Fig. 3). FXR-α, EAR2, and RXR-α showed significantly dominant (20- to 10-fold higher) expression in liver parenchymal cells compared with nonparenchymal cells (P < 0.001, Fig. 3), which corresponds to previous findings of Higashiyama et al. (11) that showed extensive immunolabeling of FXR protein in mouse liver in hepatocytes with a weak expression in endothelial cells. Furthermore, AR, ERR-γ, RXR-α, GR, LRH-1, REV-ERB-α, RXR-β, RXR-γ, and TR2 showed an average fivefold higher expression in liver parenchymal than in endothelial and Kupffer cells (P < 0.01, Fig. 3).

Expression of seven nuclear receptors, including COUP-TFI, COUP-TFII, and the three RAR family members, was low in liver parenchymal cells but significantly higher in nonparenchymal cells, namely in both endothelial and Kupffer cells. In contrast to the exclusive expression of EAR2 in parenchymal cells, COUP-TFI was exclusively expressed in endothelial and Kupffer cells. Furthermore, COUP-TFII showed approximately fivefold higher expression in nonparenchymal cells than in parenchymal cells (Fig. 4A). Importantly, immunohistochemical staining for COUP-TFII performed by Suzuki et al. (37) on human liver tissue also revealed expression of the COUP-TFII protein specifically in sinusoidal endothelium and not in hepatocytes. Despite the moderate to high expression level in the liver, the physiological functions of COUP-TFs have not been fully explored, and the ligand for COUP-TFs remains to be identified. Our data suggest the physiological importance of EAR2 in hepatocytes and that of COUP-TFII in endothelial cells and macrophages. Previous studies have shown that the activity of COUP-TFs is associated with the transcriptional regulations of a number of genes expressed mainly in the liver (22). COUP-TFII and EAR2 have been generally considered to be repressors or regulators for transcription of nuclear receptors such as RARs, TRs, PPARs, and HNF4-α (30). The distribution pattern from the present study further suggests that EAR2, given its high expression in liver parenchymal cells, may have a potential role in hepatic lipid and xenobiotic metabolism regulation through cross talk with other liver-enriched nuclear receptors. In accordance with the expression of COUP-TFII specifically in endothelial cells, a role of COUP-TFII in angiogenesis and generation of hematopoietic cell clusters has been described (45). In addition, COUP-TFII was shown to have a potential role in regulation of cholesterol homeostasis (25). The observation from the current study that expression of COUP-TFII is relatively high in
Kupffer cells raises interest to further investigate whether COUP-TFII is involved in lipid metabolism in macrophages.

In contrast to the nuclear receptors discussed above, which were mainly characterized by significantly high expression in either parenchymal or nonparenchymal cells, several nuclear receptors were identified as being ubiquitously expressed in all three cell types, including LXR-β and NUR77, which are highly expressed in liver, and PPAR-γ, TR-α, and TR4, which show a moderate expression level. For these nuclear receptors, there was no significant difference in expression level between liver parenchymal, endothelial, and Kupffer cells.

LXR-α has emerged as an important drug target for metabolic regulation of cholesterol efflux based upon its high expression in macrophages. Total expression of LXR-β in liver was about twofold lower than that of LXR-α. However, expression of LXR-β was comparable to LXR-α in Kupffer cells and threefold higher than LXR-α in endothelial cells (Fig. 4F), which is in accordance with published data implying similar efficacy of the two LXR subtypes in stimulating macrophage cholesterol efflux (32). In addition, our observation that LXR-β is twofold less expressed in parenchymal cells compared with LXR-α supports the hypothesis that LXR-α is the primary LXR subtype responsible for the undesirable effects of LXR pan-agonists via activating SREBP-1c in parenchymal cells (34), and LXR-β-specific agonists may preferentially activate macrophage cholesterol efflux without or to a lesser extent causing adverse hypertriglyceridemia.

NUR77, also known as NGFI-B, is highly and ubiquitously expressed in parenchymal and nonparenchymal cells (Fig. 4C). In hepatocytes, NUR77 modulates lipid metabolism by decreasing the activity of the lipogenic transcription factor SREBP-1c (31). In addition, it has been revealed that NUR77 is highly expressed in vascular endothelial cells and plays a role in angiogenesis and vascular inflammation by negatively regulating endothelial cell activation (44). NUR77 also regulates lipid metabolism and the inflammatory response in macrophages (3). The ubiquitously high expression of NUR77 over the different liver cell types supports further investigation of NUR77 in both hepatic lipid metabolic process and vascular modulation in multiple tissues.

Interestingly, two orphan receptors that were not highly expressed in whole liver showed considerable expression in nonparenchymal cells. The expression of COUP-TFII in liver endothelial and Kupffer cells was similar to that of NUR77 (Fig. 4C), despite a threefold lower total expression in liver than NUR77. In addition, TR4 was twofold more highly expressed in endothelial and Kupffer cells than PPAR-γ (Fig. 4D), although the total expression of TR4 in liver was only slightly higher than PPAR-γ. Similarly to the COUP-TFs, the orphan nuclear receptor TR4 employs repression and gene-silencing events to control basal activities or hormonal responsiveness of numerous target genes (46). Since TR4 and PPAR-γ show a highly similar expression pattern, it can be suggested that TR4 may function as a lipid sensor as PPAR-γ. Previous findings have uncovered an important signaling pathway where TR4 modulates CD36 expression in macrophages and controls CD36-mediated foam cell formation (41). CD36 is an established target gene of PPAR-γ. Interestingly, in parallel with the established role for PPAR-γ in adipogenesis (19), TR4 has recently been shown to facilitate lipid accumulation in adipocytes (8). Given the comparable expression patterns of TR4 and PPAR-γ, it is of interest to investigate the potential regulatory cross talk between these two nuclear receptors in liver and macrophages.

Two different transcript isoforms of PPAR-γ exist, i.e., PPAR-γ1 and PPAR-γ2, which may execute different effects on hepatic steatosis and could therefore also show distinct expression profiles in liver. Since the NURSA PPAR-γ primer combination used cannot differentiate between the two isoforms, we designed new primer sets (for primer sequences see Table 2) to measure separately the RE level of each PPAR-γ transcript in the different liver cells. PPAR-γ1 exhibits a significantly higher expression level in liver parenchymal cells compared with both types of nonparenchymal cells (P < 0.05). In contrast, PPAR-γ2 expression is relatively low in parenchymal cells, while PPAR-γ2 levels in liver endothelial and Kupffer cells resemble those observed for PPAR-γ1 (Fig. 4E). Of interest, the cellular expression profile of the highly sensitive PPAR-γ target gene CD36 more closely resembles that of PPAR-γ2, i.e., predominant expression in nonparenchymal cells (Fig. 4F). This suggests that PPAR-γ2, and not PPAR-γ1, is primarily responsible for CD36 transcription, at least in healthy (nonfatty) liver.

In conclusion, we have composed the cell type-specific expression pattern of 49 nuclear receptors in liver parenchymal, endothelial, and Kupffer cells of female C57BL/6 mice. This study provides the most complete quantitative assessment of nuclear receptor distribution in the liver reported to date. Our data suggest that certain orphan nuclear receptors such as COUP-TFII, EAR2, and TR4, which are highly expressed in a specific liver cell type, may be of significant importance in hepatic function and metabolism. Further investigation into the biology of these nuclear receptors and the possible impact of sex differences will be necessary before we can understand the functional implications of their hepatic expression patterns.

### Table 2. Primers used for quantitative real-time PCR analysis

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<td>TCACGCCGAGCATATCCGA</td>
<td>NM_008084</td>
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<td>HPRT</td>
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<td>PECAM-1</td>
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<tr>
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</tr>
</tbody>
</table>
Ultimately, cell-specific targeting systems have been developed for liver (2, 33), and the identification of hepatic cell-specific nuclear receptors may lead to the development of cell-specific therapeutic molecules to reduce off-target side effects.

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