DNA Methylation and Histone Modification Profiles of Mouse Organic Anion Transporting Polypeptides\textsuperscript{S}

Satoki Imai, Ryota Kikuchi, Hiroyuki Kusuhara, and Yuichi Sugiyama

Laboratory of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan (S.I., H.K., R.K.); Pharmacokinetics Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan (S.I.); and Sugiyama Laboratory, RIKEN Innovation Center, Research Cluster for Innovation, Riken, Kanagawa, Japan (Y.S.)

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ABSTRACT

Organic anion transporting polypeptides (rodents, Oatps; human, OATPs) are primarily involved in the transmembrane transportation of a wide range of endogenous and exogenous compounds. Multiple mouse Oatp1 isoforms are closely located on chromosome 6, where each isoform shows distinct tissue distribution; Oatp1b2, Oatp1a6, and Oatp1c1 are expressed exclusively in the liver, kidney, and cerebrum, respectively; Oatp1a1 in the liver and kidney; and Oatp1a4 in the liver and cerebrum. We have identified tissue-dependent differentially methylated region (T-DMR) around the transcriptional start site (TSS) of Oatp1b2, which correlates with its liver-specific expression. Bisulfite sequencing also demonstrated the presence of T-DMRs around the TSS in other Oatp1 genes: CpG dinucleotides at +149 relative to the TSS for Oatp1c1; −48, +101, and +356 for Oatp1a4; −572 and −550 for Oatp1a1; and −122 and +216 for Oatp1a6 were differentially methylated among the liver, kidney, and cerebrum. These methylation profiles were largely consistent with the tissue distribution of Oatp1 mRNAs. Chromatin immunoprecipitation assay revealed that the mRNA expression of Oatp1 genes was accompanied by acetylated histone H3. Human OATP1B1 and OATP1B3 are located on chromosome 12p12 in the OATP1 cluster; both show predominant expression in the liver. These genes also contained T-DMRs that were hypomethylated in the liver, compared with kidney cortex: −511, −411, and +92 relative to the TSS for OATP1B1 and −331, +70, and +73 for OATP1B3. These results suggest that the difference in epigenomic profiles comprising DNA methylation and histone acetylation determines the distinct tissue distribution of Oatp/OATP mRNAs.

Introduction

Organic anion transporting polypeptides (rodents, Oatps; human, OATPs) belong to SLC gene superfamily, which is composed of six subfamilies. Oatp isoforms classified into the Oatp1 subfamily mediate the sodium-independent transportation of various endogenous and exogenous compounds, and they play indispensable roles as xenobiotic detoxification system in the body (Hagenbuch and Gui, 2008). Mouse Oatp1 genes are located in chromosome 6, with each isoform exhibiting distinct expression pattern (Fig. 1). Oatp1b2, Oatp1a6, and Oatp1c1 are expressed exclusively in the liver, kidney, and cerebrum, respectively. Oatp1a1 is expressed in the liver and kidney. Oatp1a4 is expressed in the liver and cerebrum (Cheng et al., 2005). Oatp1a1, Oatp1a4, and Oatp1a6 show particularly high sequence homology at the amino acid level (>70%) and also show moderate sequence homology to Oatp1b2 and Oatp1c1 (>40%) (Hagenbuch and Meier, 2003), suggesting that these transporters are products of gene duplication from the same ancestor gene.

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we hypothesized that the epigenetic regulations generally govern the distinct tissue distribution of other isoforms in Oatp1 genes.

Human OATP1B1 and OATP1B3, the counterpart of rodent Oatp1b2, reside on chromosome 12p12 in close proximity to other OATP1 isoforms, OATP1C1 and OATP1A2. OATP1B1 and OATP1B3 are expressed exclusively in the liver. Cumulative clinical evidence shows their importance in the hepatic uptake of a variety of drugs, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, angiotensin-converting enzyme inhibitors, angiotensin receptor II antagonists, and cardiac glycosides, thereby affecting the pharmacological activity and/or adverse events (Giacomini et al., 2010; Hagenbuch and Gui, 2008; Maeda and Sugiyama, 2008). Previously, the promoter regions of OATP1B1 and OATP1B3 have been characterized to identify transcription factors responsible for their gene expression (Jung et al., 2001; Ohtsuka et al., 2006). However, the transcription factors, particularly hepatocyte nuclear factor (HNF) 1α, cannot solely account for the tissue-specific expression pattern of these transporters, because these transcription factors are also expressed in extrahepatic tissues, where their target genes OATP1B1 and OATP1B3 are not. We hypothesized that epigenetic systems underlie the tissue-specific expression of OATP1B1 and OATP1B3.

The present study was aimed at investigating the involvement of epigenetic systems in the tissue-specific expression of mouse and human OATP1 genes. We focused on mouse Oatp1, which are expressed in the liver, kidney, and cerebrum (Oatp1a1, Oatp1a4, Oatp1a6, Oatp1b2, and Oatp1c1), and human OATP1B1 and OATP1B3, which are expressed predominantly in the liver. Here, we provide clear evidence that the tissue-specific expression of these transporters is associated with epigenetic profiles comprising DNA methylation and histone acetylation around their TSS.

Materials and Methods

Reagents and Animals. All reagents were purchased from Wako Pure Chemicals (Osaka, Japan) unless stated otherwise. Male mice of C57BL/6NCrl strain were purchased from Charles River Japan (Kanagawa, Japan). All mice (11–14 weeks of age) used in this study were maintained under standard conditions, and food and water were available ad libitum.

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR). Mouse total RNA was isolated from the liver, kidney, and cerebrum with use of ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer’s instructions. The RNA was then treated with DNase I to remove the contaminated genomic DNA, followed by reverse transcription (RT) using a random nonamer primer (Takara, Shiga, Japan). The quantitative real-time PCR to detect the partial fragments of Oatp1a1, Oatp1a4, Oatp1a6, Oatp1b2, Oatp1c1, and β-actin cDNA was performed using a LightCycler and the appropriate software (version 3; Roche Diagnostics), according to the manufacturer’s instructions, with the primers shown in Supplemental Table 1. The protocol for PCR was as follows: 95°C for 30 seconds; 40 cycles of 95°C for 5 seconds, 58°C for 10 seconds, and 72°C for 15 seconds.

Preparation of Brain Capillary–Enriched Fraction. Mouse brain capillary–enriched fraction was prepared as reported previously (Ose et al., 2010). The mouse cerebrum was homogenized using a Polytron homogenizer in 20 volumes (v/w) of ice-cold 0.32 M sucrose (Sigma-Aldrich, St. Louis, MO), and the homogenate was centrifuged at 2200 g for 10 minutes. Ten milliliters of 25% bovine serum albumin was added to the pellet, and the tube was vortexed to suspend the pellet, followed by centrifugation at 2200 g for 10 minutes. The pellet was washed with 10 mM Tris-Cl and 0.5 mM dithiothreitol (pH, 7.6) three times and suspended in phosphate-buffered saline (PBS) containing 0.1% protease inhibitor.

Genomic DNA Extraction and Bisulfite Reaction. Human tissue specimens were obtained from nonprofit organization Human and Animal Bridging Research Organization (Tokyo, Japan). Human genomic DNA from three individual specimens and mouse genomic DNA from the liver, kidney, cerebrum, and brain capillary–enriched fraction were extracted using a Gen Pure DNA Kit (Dojindo Molecular Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Oatp1 mRNA expression levels are represented as a ratio to that of β-actin in each tissue. The results are presented as the mean value of two independent measurements for cDNAs derived from a single animal.

We obtained the mRNA expression profiles of the mouse Oatp transporter genes. (A) Gene loci of mouse Oatp1 transporters on chromosome 6. (B) mRNA expression profiles of the mouse Oatp genes. mRNA of Oatp1a1, Oatp1a4, Oatp1a6, Oatp1b2, and Oatp1c1 in mouse liver, kidney, and cerebrum was quantified by real-time PCR using specific primers (Supplemental Table 1) as described in Materials and Methods. The mRNA expression levels are represented as a ratio to that of β-actin in each tissue. The results are presented as the mean value of two independent measurements for cDNAs derived from a single animal.
between high- and low-expressing tissues were statistically analyzed using Fisher’s exact test, followed by Bonferroni adjustment.

**Combined Bisulfite Restriction Analysis.** The DNA methylation profiles of the CpG dinucleotides at −572 bp, +101 bp, +216 bp, −175 bp, −411 bp, and +73 bp relative to the TSS of *Oatp1a1*, *Oatp1a4*, *Oatp1a6*, *Oatp1c1*, *OATP1B1*, and *OATP1B3*, respectively, were investigated using combined bisulfite restriction analysis (COBRA) (Xiong and Laird, 1997). The DNA fragments were amplified by PCR, as described above, using the following primers: *Oatp1a1*, −606-F to −305-R; *Oatp1a4*, −74-F to +261-R; *Oatp1a6*, −176-F to +363-R; *Oatp1c1*, −275-F to +179-R; *OATP1B1*, −619-F to −263-R; and *OATP1B3*, −22-F to +249-R (Supplemental Table 1). The PCR fragments were digested with HpyCH4IV (New England Biolabs, Beverly, MA), for *Oatp1a1*, *Oatp1a4*, *Oatp1a6*, *Oatp1c1*, and *OATP1B1*, or TaqI (Takara, Shiga, Japan), for *OATP1B3*. Because only unmethylated cytosine residues are converted to thymines by the bisulfite reaction, PCR fragments from unmethylated genomic DNA are resistant to restriction enzyme digestion, whereas those from methylated DNA are digested.

**Chromatin Immunoprecipitation Assay.** The chromatin immunoprecipitation (ChIP) assay was performed using anti-acetylated histone H3 antibody (Upstate Biotechnology, Lake Placid, NY) according to a procedure described previously (Imai et al., 2009). Normal rabbit IgG was used as a negative control to verify the immunoprecipitation specificity. In brief, 250 mg of liver, kidney, and cerebrum from 11-week-old male C57BL/6NcJ mice was prepared by homogenization, treatment with cell lysis buffer, and centrifugation to obtain small pieces and treated with 1% formaldehyde to form DNA-protein cross-links. The samples were then mixed with salmon sperm DNA/protein A-agarose suspension to reduce the nonspecific background, and the samples were preincubated with salmon sperm DNA/protein A-agarose suspension to reduce the nonspecific background, and the samples were incubated with an antibody against acetylated histone H3, normal rabbit IgG, or an equivalent amount of PBS as the sample at 4°C overnight. The samples were then mixed with salmon sperm DNA/protein A-agarose suspension, rotated for 60 minutes at 4°C, and centrifuged at 20,000 g for 1 minute at 4°C. DNA was purified from the precipitated immune complex after extensive washing and reversal of the DNA-protein cross-links. The supernatant from the no antibody sample was collected as the total input. Quantitative real-time PCR was performed using a LightCycler and the appropriate software (version 5: Roche Diagnostics), according to the manufacturer’s instructions, with the primers shown in Supplemental Table 1. The protocol for PCR was as follows: 95°C for 30 seconds and 40 cycles of 95°C for 5 seconds, 58°C for 10 seconds, and 72°C for 15 seconds. The enrichment of DNA in the immunoprecipitates was calculated relative to the 1:100 diluted total input samples. Statistical significance was assessed between tissues using one-way analysis of variance, followed by Tukey’s multiple comparison post hoc test. Significant differences (P < 0.05) between the tissues were denoted by asterisks.

**Results**

**mRNA Expression Profiles of the Mouse Oatps.** The mRNA expression of *Oatp1c1*, *Oatp1b2*, *Oatp1a4*, *Oatp1a1*, and *Oatp1a6* in the mouse liver, kidney, and cerebrum was quantified using real-time PCR (Fig. 1B). The results confirmed that *Oatp1b2*, *Oatp1a6*, and *Oatp1c1* are expressed predominantly in the liver, kidney, and cerebrum, respectively. *Oatp1a1* was expressed in the liver and kidney, whereas the expression in the cerebrum was negligible; *Oatp1a4* was expressed in the liver and cerebrum, and minimal expression was observed in the kidney. These distinct tissue distribution patterns of mouse Oatp mRNA are consistent with those reported previously (Cheng et al., 2005).

**DNA Methylation Profiles Around the Transcriptional Start Site of Mouse Oatp1 Genes.** The DNA methylation profiles of the CpG dinucleotides from −600 to +440 relative to the TSS of mouse *Oatp1* genes were determined in the liver, kidney, and cerebrum (Fig. 2). The degree of DNA methylation in these regions was calculated and compared among the tissues (Table 1). The corresponding data for *Oatp1b2* were cited from our previous study (Imai et al., 2009) for comparison. In *Oatp1c1*, one CpG site located at +149 relative to the TSS was regarded as T-DMR, and the other 2 sites upstream of the TSS were similarly methylated among tissues (Fig. 2B). In *Oatp1a4*, two of six CpG sites downstream the TSS (+101 and +356) and one site upstream the TSS (−48) constitute the T-DMRs. *Oatp1a4* was largely and moderately methylated in the kidney and cerebrum, respectively, and only slightly methylated in the liver. In *Oatp1a1*, two CpG sites in the upstream of the TSS (−572 and −550) were identified as T-DMRs, and the degree of methylation at +59 CpG site was not statistically different between the tissues. *Oatp1a1* was moderately to largely methylated in the kidney and cerebrum and significantly hypomethylated in the liver. In *Oatp1a6*, both the upstream and the downstream CpG sites around the TSS (−122 and +216) were differentially methylated among tissues. *Oatp1a6* was moderately to largely hypermethylated in the liver and cerebrum and significantly hypomethylated in the kidney. The DNA methylation in the brain capillary–enriched fraction was also investigated for *Oatp1c1* and *Oatp1a4*, which are known to be localized in the mouse brain capillaries (Ose et al., 2010; Tohyama et al., 2004). Most of the CpG dinucleotides are relatively hypomethylated in the brain capillaries, being consistent with the mRNA expression of these transporters. The combined bisulfite restriction analysis corroborated the results of bisulfite sequencing for the DNA methylation status of CpG dinucleotides located at −572 bp, +101 bp, and −175 bp relative to the TSS of *Oatp1a1*, *Oatp1a4*, and *Oatp1c1*, respectively (Fig. 2C). Although the difference in DNA methylation was less apparent in COBRA than in bisulfite sequencing at +216 CpG dinucleotide in *Oatp1a6*, it was qualitatively similar where the site is relatively hypomethylated in the kidney, compared with the other two tissues.

**Histone Acetylation Profiles in the Promoter Region of Mouse Oatp Transporters.** ChIP assays were performed to examine the histone H3 acetylation profiles of mouse Oatp transporters (Fig. 3). The corresponding data for *Oatp1b2* were cited from our previous study (Imai et al., 2009) for comparison. The histone H3 around the TSS of *Oatp1c1* was hyperacetylated in the cerebrum, and the acetylation in the liver and kidney was minimal. Similarly, *Oatp1b2* and *Oatp1a1* were hyperacetylated only in the liver. *Oatp1a4* was hyperacetylated in the liver and cerebrum but not in the kidney. Acetylation of histone H3 was not observed for *Oatp1a6* in these tissues.

**DNA Methylation Profiles Around the Transcriptional Start Site of Human OATP1B1 and OATP1B3.** The DNA methylation profiles of CpG dinucleotides around the TSS of *OATP1B1* and *OATP1B3* were determined in three individual specimens of human liver and kidney cortex (Fig. 4). The CpG sites at −511, −411, and +92, relative to the TSS for *OATP1B1*, and those at −331, +70, and +73 for *OATP1B3* were significantly hypomethylated in the liver, compared with the kidney cortex, and regarded as T-DMRs. The degree of DNA methylation in these regions was calculated and compared among the tissues (Table 2). The CpG dinucleotides around the TSS of *OATP1B1* and *OATP1B3* were overall significantly hypomethylated in the liver, compared with the kidney cortex. The DNA methylation status examined by COBRA for the CpG dinucleotides at −411 bp and +73 bp, relative to the TSS of *OATP1B1* and *OATP1B3*, respectively, corroborated the results of bisulfite sequencing (Fig. 4C).

**Discussion**

In the present study, we investigated the DNA methylation and histone acetylation profiles of mouse and human Oatp/OATP1 genes, which are localized on the same chromosome within close proximity, as a mechanism underlying their tissue-specific expression.
Fig. 2. DNA methylation profiles around the transcriptional start site of the mouse Oatp1 genes. Bisulfite genomic sequencing analysis and COBRA were performed for Oatp1c1, Oatp1a4, Oatp1a1, and Oatp1a6 with genomic DNA extracted from mouse liver, kidney, cerebrum, and brain capillary–enriched fraction as described in Materials and Methods. The data for Oatp1b2 obtained previously (Imai et al., 2009) was also presented for comparison. (A) Schematic diagrams of the genomic region around the transcriptional start site (TSS). The vertical lines and numbers indicate the positions of the cytosine residues of the CpG dinucleotides relative to the TSS (+1). (B) DNA methylation status of individual CpG dinucleotides. The open and closed circles represent unmethylated and methylated cytosines, respectively. Significant differences (P < 0.05) between the two samples: *liver versus kidney, †liver versus cerebrum, ‡kidney versus cerebrum. (C) Combined bisulfite restriction analysis. The bisulfite PCR products were digested with HpyCH4IV and electrophoresed on a 2.0% agarose gel to evaluate the methylation status of the CpG dinucleotides. The conditions of undigested (−) and digested (+) are indicated on the top of the gel.
Consistent with a previous report (Cheng et al., 2005), the Oatp1 isoforms showed distinct tissue distribution (Fig. 1). Bisulfite genomic sequencing located a number of CpG dinucleotides differentially methylated between tissues around the TSS of mouse Oatp1 genes (Fig. 2), where the difference in the DNA methylation status is in good agreement with the mRNA expression profiles of respective transporters. DNA methylation status of the entire region around the TSS was largely consistent with the mRNA expression of respective genes (Table 1). The CpG dinucleotides were hypomethylated (30% of methylation) in most of the transporter-expressing tissues (i.e., Oatp1b2, Oatp1a6, and Oatp1a1 in the liver; Oatp1a6 in the kidney; and Oatp1c1 and Oatp1a4 in the brain capillary–enriched fraction), whereas they were moderately (30%–70%) to largely (>70%) methylated in nonexpressing tissues (i.e., Oatp1c1 and Oatp1a6 in the liver; Oatp1c1, Oatp1b2, and Oatp1a4 in the kidney; and Oatp1b2, Oatp1a1, and Oatp1a6 in the cerebrum). There were some discrepancies between the mRNA expression and DNA methylation status: Oatp1a1 was moderately methylated (60%) in the kidney, and Oatp1c1 and Oatp1a4 were moderately methylated (37% and 51%, respectively) in the cerebrum. This could be ascribed to the regional expression of Oatp1a1 in the kidney and Oatp1c1 and Oatp1a4 in the cerebrum, where the expression is restricted to the proximal tubules (Oatp1a1) (Bergwerk et al., 1996) and brain capillaries (Oatp1c1 and Oatp1a4) (Ose et al., 2010; Tohyama et al., 2004). Indeed, the CpG dinucleotides around the TSS of Oatp1c1 and Oatp1a4 tend to be methylated to the lesser extent in the brain capillary–enriched fraction, compared with the cerebrum.

### Table 1

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<thead>
<tr>
<th>Tissue</th>
<th>Degree of DNA Methylation (%)</th>
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<tr>
<td></td>
<td>Oatp1c1</td>
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<tr>
<td>Liver</td>
<td>60</td>
</tr>
<tr>
<td>Kidney</td>
<td>50</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>37</td>
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<tr>
<td>Brain capillary–enriched fraction</td>
<td>20</td>
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* P < 0.05 between liver and kidney.
§ P < 0.05 between liver and cerebrum.
* P < 0.05 between kidney and cerebrum.

Fig. 3. Histone acetylation profiles around the transcriptional start site of the mouse Oatp1 genes. The histone H3 acetylation status of Oatp1c1, Oatp1a4, Oatp1a1, and Oatp1a6 around the transcriptional start site was determined by ChIP assay in mouse liver, kidney, and cerebrum as described in Materials and Methods. The data for Oatp1b2 and β-actin obtained in our previous study (Imai et al., 2009) was also presented for comparison. The closed columns represent the histone acetylation of each sample relative to its respective total input. Normal rabbit IgG was included as a negative control to verify the immunoprecipitation specificity (open columns). The results are presented as the mean ± S.D. of triplicate experiments. Significant differences (P < 0.05) between the tissues were denoted by asterisks.

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Epigenetic Profiles of Mouse Oatps

These results suggest that the tissue-specific expression of these transporters is regulated through DNA methylation-dependent gene silencing together with transcription factors. Generally, DNA methylation status is linked tightly with the histone acetylation status, and activated genes are associated with acetylated histone H3. Subsequent ChIP assays revealed that histone acetylation was associated with the expression of mouse Oatp1 genes except for Oatp1a1 and Oatp1a6, of which histone H3 around the TSS was not acetylated in the kidney regardless of their expression (Fig. 3). In the kidney, Oatp1a1 is exclusively expressed in proximal tubules (Bergwerk et al., 1996). It is possible that histone H3 around the TSS of Oatp1a1 is acetylated only in the proximal tubules, which could be masked when ChIP was performed using whole kidney. Indeed, Oatp1a1 expression was associated with acetylated histone H3 in the liver, which mainly consists of a single type of cells, hepatocyte. Regional expression of Oatp1a6 in the kidney has not been investigated. Nevertheless, the mRNA expression of mouse Oatp1 genes is associated with either DNA methylation or histone acetylation or both in most cases, indicating the role of epigenetic regulation in the distinct expression pattern of these genes.

The high sequence identity of mouse Oatp1 genes suggests that they are the products of genetic duplication from the same ancestor gene. Gene duplication is regarded as a key mechanism for the evolution of new genes (Ohno, 1970). The connection between gene duplication and epigenetic modifications has not been well established (Zheng, 2008), and to our knowledge, there are few systematic studies investigating the epigenetic regulation of duplicated genes. Rodin and Riggs proposed an epigenetic complementation model in which duplicated genes are epigenetically silenced in a tissue-complementary manner, exposing each of the duplicates to negative selection, thus protecting from pseudogenization (Rodin and Riggs, 2003). Recently, DNA methylation status of the mouse prolactin superfamily was analyzed, and it was suggested that the methylation is involved in the tissue-specific expression and sequence diversity during evolution (Hayakawa et al., 2012), which is similar to our present findings. Taken together, it is plausible that the epigenetic profiles differentiate the mouse Oatp1 genes in terms of their tissue distributions during the gene duplication events.

We and others have revealed the involvement of DNA methylation-dependent gene silencing in the tissue-specific expression of transporters in humans and mice (Douet et al., 2007; Imai et al., 2009; Jin et al., 2012; Kikuchi et al., 2007; Kikuchi et al., 2010). Both human and mouse OAT1/Oat1, which is hypomethylated in the kidney and hypermethylated in the liver around the TSS (Jin et al., 2012). On the other hand, most of the CpGs in the 5′-flanking region of human OAT3 is hypermethylated in the liver and hypomethylated in the kidney, and T-DMR is not prevalent in mouse Oat3, and vice versa for human and mouse URAT1 genes; mouse but not human Urat1 genes contained a T-DMR associated with the tissue-specific mRNA expression. This motivated us to investigate the DNA methylation profile in human counterparts of mouse Oatp1 genes. Consistent with Oatp1b2 in mouse, both human OATP1B1 and OATP1B3 genes contained T-DMRs, which were hypomethylated in the liver but hypermethylated in the kidney (Fig. 4 and Table 2). Because the promoters of human OATP1B1 and OATP1B3 and mouse Oatp1b2 are activated by the same transcription factor, HNF1α (Jung et al., 2001; Maher et al., 2006), it is most likely that DNA demethylation of these genes in the liver evokes the acetylation of histone H3, which allows HNF1α to bind to its recognition sites and activate the promoters. The same regulatory cascade has been proposed for the kidney-specific.
expression of organic anion transporters and amino acid transporters (Jin et al., 2012; Kikuchi et al., 2010). As reported previously for OAT4 and URAT1 promotors (Jin et al., 2012), the methylation profiles of OATP1B1 and OATP1B3 in the liver had a moderate batch difference, hypomethylated in liver-1 and moderately methylated in liver-2 and -3 (Fig. 4B). It is possible that this leads to the inter-individual differences in the mRNA expression and, consequently, to the in vivo transportation activities. Additional studies are necessary to elucidate the association and its impact on the pharmacokinetics of drugs.

In conclusion, we have demonstrated a clear association between the tissue-specific expression and epigenetic profiles comprising DNA methylation and histone acetylation for Oatpl1a1, Oatpl1a4, Oatpl1a6, Oatplb1b2, and Oatplc1 in mice and OATP1B1 and OATP1B3 in humans. It is likely that the difference in the epigenetic profiles underlies the distinct tissue distribution of each of the Oatp/OATP family transporters that seem to be evolved from the same ancestor genes.

Authorship Contributions

Participated in research design: Imai, Kikuchi, Kusuhara, Sugiyama.

Conducted experiments: Imai.

Performed data analysis: Imai, Kikuchi, Kusuhara.

Wrote or contributed to the writing of the manuscript: Imai, Kikuchi, Kusuhara, Sugiyama.

References


