Identification of Candidate Target Cyp Genes for microRNAs Whose Expression Is Altered by PCN and TCPOBOP, Representative Ligands of PXR and CAR

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MicroRNAs (miRNAs) are small non-coding RNAs that are involved in mRNA post-transcriptional regulation. The deregulation of miRNAs affects the expression of drug-metabolizing enzymes, drug transporters, and nuclear receptors, all of which are important in regulating drug metabolism. miRNA expression can be altered by several endogenous or exogenous agents, such as steroid hormones, carcinogens, and therapeutic drugs. However, it is unclear whether hepatic miRNA expression is regulated by nuclear receptors, such as pregnane X receptor (PXR) and constitutive androstan receptor (CAR), which are indispensable for the expression of the CYPs. Here we investigated the effects of the mouse PXR and CAR ligands pregnenolone-16α-carbonitrile (PCN) and 1,4-bis[(3,5-dichloropyridin-2-yl)oxy]benzene (TCPOBOP) on hepatic miRNA expression in mice. We found that the expression of 9 miRNAs was increased (>2-fold) and of 4 miRNAs was decreased (>50%) in response to PCN, while TCPOBOP treatment led to the up-regulation of 8 miRNAs and down-regulation of 6 miRNAs. Using several miRNA target prediction algorithms, we found that the predicted target genes included several lesser known Cyp genes (Cyp1a1, Cyp1b1, Cyp2b10, Cyp2c38, Cyp2u1, Cyp4a12a/b, Cyp4v3, Cyp17a1, Cyp39a1, and Cyp51). We analyzed the expression of these genes in response to PCN and TCPOBOP and found changes in their mRNA levels, some of which were negatively correlated with the expression of their corresponding miRNAs, suggesting that miRNAs may play a role in regulating Cyp enzyme expression. Further studies will be required to fully elucidate the miRNA regulatory mechanisms that contribute to modulating Cyp expression.

Key words microRNA; cytochrome P450; nuclear receptor; pregnane X receptor (PXR); constitutive androstan receptor (CAR)
involved in miRNA and target mRNA binding, 3) the ther-
sequences, 2) evolutionary conservation of the sequences
pairing between miRNAs and the 3
used by these programs are based on: 1) Watson–Crick base
which are available on the Internet. The prediction methods
microT, miRanda, miRWalk, RNAhybrid, and TargetScan),
monly used miRNA target prediction algorithms (DIANA-
calculated from the normalized data.
and PCN group, and the control and TCPOBOP group were
filter. The Log 2 ratios of the comparisons between the control
ning was performed using the SureScan Microarray Scanner
Microarray System (G4900DA) (Agilent). After scanning,
Processed according to the manufacturer’s instructions. Scan-
from mouse livers was isolated using miRNeasy kits (Qiagen)
used for total RNA isolation.
Animals Code,” approved by Mukogawa Women’s University.
PCN and TCPOBOP Administration PCN and
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Cyp3a11
GAGCTTTGCTTCACAATTT
Cyp2c38
CACCGCCCCATTTGTTATGTC
Cyp2a1
TCAAGGGTTTACCAATTTCC
Cyp3a11
GAGCTTTGCTTCACAATTT
Cyp4a12a/b
GGGGGATCAGACCCAAAAGC
TGGCTGTGGTTAGGGCTCA
AGCTTACGAGCTCGAA
Cyp17a1
GCCCAAGTCCAAGACACCTAA
Cyp39a1
AATGCGGCCGTTAATCTAGG
Cyp5l
TGGAGCGAAAAGTCCACCAC
Gapdh
AACCTTGGCATTTGGAAGG

Table 1. Real-Time PCR Primers

| Gene   | Forward (5’ to 3’)                        | Reverse (5’ to 3’)   |
|--------|------------------------------------------|---------------------|
| Cyp1a1 | CAGATGATAAAGGTCATCAGCA                  | TGGGGATATAGAAGCCATTC|
| Cyp1b1 | CAGTCTGCGGTCTGGTCATC                    | GCTCGGTGGATCGGAA    |
| Cyp2b10| ACCCCACGTTCCTCTTCCA                     | CAGCAGGGCCGAAAGACTGA|
| Cyp2c38| CACCGCCCCATTTGTTATGTC                   | TGGATGTTAACAGCTTGTCTT|
| Cyp2a1 | TCAAGGGTTTACCAATTTCC                    | CGATGAGGACAGAAGTCTCGT|
| Cyp3a11| GAGCTTTGCTTCACAATTT                      | TCAAACACCCCACTGTITT|
| Cyp4a12a/b | GGGGAGATCAGACCCAAAAGC               | ATTTGCTGGTGCTGAACCAT|
| Cyp4v3 | TGGCTGTGGTTAGGGCTCA                     | AGCTTACGAGCTCGAAAGA|
| Cyp17a1| GCCCAAGTCCAAGACACCTTA                   | GTACCCAGGGCAGAAGATAGA|
| Cyp39a1| AATGCGGCCGTTAATCTAGG                    | TGTTCCTTTGCGTGAGGAAGA|
| Cyp5l  | TGGAGCGAAAAGTCCACCAC                    | TGCATCACTCCCCAGAAGGTA|
| Gapdh  | AACCTTGGCATTTGGAAGG                     | GGATGCAGGGTGATGTTC|

water ad libitum. All animals were treated in accordance with
the Guiding Principles of the “Care and Use of Laboratory
Animals Code,” approved by Mukogawa Women’s University.

nuclear receptor PXR, while
the binding site accessibility in the secondary structure of the
target mRNA. Since each program has its own characteristics,
we cross-validated the outputs of the five algorithms to iden-
tify true positive target miRNAs more accurately. Finally,
we extracted the common target Cyp genes predicted by all
of the algorithms.

Reverse Transcription and Real-Time PCR cDNA
synthesis and real-time PCR were performed as previously
described. Briefly, cDNAs were synthesized from the total
RNA of each group using the PrimeScript RT reagent Kit
(TaKaRa Bio), according to the manufacturer’s protocol. Real-
time PCR was conducted using gene-specific primers (Table
1). We calculated mRNA expression values using the delta-
delta Ct method and normalized them to that of Gapdh in the
same sample.

Statistical Analysis All results are presented as the
mean±standard error (S.E.) The results from the various exper-
imental groups and their corresponding controls were com-
pared using unpaired t-test. Differences with p-values <0.05
were considered significant.

RESULTS AND DISCUSSION

Effects of PCN and TCPOBOP on Hepatic miRNA
Expression The expression of Cyp3a11 is regulated by the
nuclear receptor PXR, while Cyp2b10 expression is regulated
by CAR. Using our in vivo experimental conditions, we recon-
firmed the increased expression of the Cyp3a11 (6-fold) and
Cyp2b10 (90-fold) miRNAs in PCN- and TCPOBOP-treated
mice, respectively (data not shown). Next, we examined the
regulation of miRNAs in the livers of mice treated with these
ligands using a miRNA microarray system (miRCURY LNA™
microRNA Array). We observed the expression of 366 and
399 out of 1119 miRNAs in the comparisons between the
control and PCN group, and the control vs. TCPOBOP group,
respectively. We found that the expression levels of 9 miRNAs
were increased >2-fold, and that the levels of 4 miRNAs were
decreased >50% in PCN-treated mice, while TCPOBOP-
treated mice exhibited 8 up-regulated and 6 down-regulated
miRNAs (Table 2). The observed miRNA expression levels
in this study were similar to other studies; namely, 1.3-fold
by benzo(a)pyrene which is potent aryl hydrocarbon receptor-
agonist in mice, 2.3-fold increase by rifampicin in human
hepatocyte.15)
Closely inspection of the results revealed that the expression levels of miR-1249-3p, miR-483-3p, miR-26b-3p, miR-673-3p, miR-532-3p, and miR-1894-5p were increased in both the PCN and TCPOBOP treatment groups and that miR-666-5p and miR-760-5p were down-regulated in both groups (Fig. 1). These results suggested that PCN and TCPOBOP treatment probably induce common transcriptional programs, consistent with the findings of Moore et al., who showed that PXR and CAR can recognize each other's cis-elements and are likely to share target genes. 16) Thus, we speculated that the miRNAs observed in both groups could be regulated by both PXR and CAR.

**Table 2. PCN- or TCPOBOP-Regulated miRNAs in Mouse Liver**

| MiRNA         | Log2 ratio | PCN  | TCPOBOP |
|---------------|------------|------|---------|
| Up-regulated  |            |      |         |
| mmu-miR-1249-3p | 2.06       | 2.05 |
| mmu-miR-483-3p | 1.64       | 1.50 |
| mmu-miR-3473c  | 1.31       | 0.98 |
| mmu-miR-1894-5p| 1.30       | 1.25 |
| mmu-miR-532-3p | 1.21       | 1.07 |
| mmu-miR-26b-3p | 1.13       | 1.24 |
| mmu-miR-673-3p | 1.04       | 1.24 |
| mmu-miR-345-3p | 1.01       | 0.96 |
| mmu-miR-99b-3p | 1.01       | 0.81 |
| mmu-miR-695    | 0.92       | 1.04 |
| mmu-miR-1188-3p| ND         | 1.11 |
| mmu-miR-466h-3p| ND         | −1.05|
| mmu-miR-5120   | ND         | −1.29|
| mmu-miR-344e-3p| −0.92      | −1.08|
| mmu-miR-5114   | −0.99      | −1.11|
| mmu-miR-28b    | −1.04      | ND   |
| mmu-miR-5621-3p| −1.08      | −0.92|
| mmu-miR-760-5p | −1.15      | −1.24|
| mmu-miR-666-5p | −1.39      | −1.46|

| Down-regulated | | | |
|----------------| | | |
| mmu-miR-466h-3p| ND         | −1.05|
| mmu-miR-5120   | ND         | −1.29|
| mmu-miR-344e-3p| −0.92      | −1.08|
| mmu-miR-5114   | −0.99      | −1.11|
| mmu-miR-28b    | −1.04      | ND   |
| mmu-miR-5621-3p| −1.08      | −0.92|
| mmu-miR-760-5p | −1.15      | −1.24|
| mmu-miR-666-5p | −1.39      | −1.46|

ND: not detected.

A) **Up-regulated miRNAs**

B) **Down-regulated miRNAs**

**Table 3. Number of Predicted miRNA Target Genes in PCN- or TCPOBOP-Treated Mice**

| Ligand      | Differentially expressed miRNAs | Number of target genes | Cyp genes | Liver (+)* |
|-------------|---------------------------------|------------------------|-----------|------------|
| PCN         | 13                              | 6638                   | 9         | 5          |
| TCPOBOP     | 14                              | 6616                   | 16        | 8          |

a) Number of Cyp genes selected from total target genes. b) Number of Cyp genes expressed in mouse liver.

**Table 4. miRNA/target Gene Pairs Whose PCN or TCPOBOP-Induced Changes in Expression Are Negatively Correlated**

| Ligands      | Change (up: ⬆, down: ⬇) | Target Cyp genes | Change (up: ⬆, down: ⬇, no change: −) | Negative correlation |
|--------------|--------------------------|------------------|---------------------------------------|---------------------|
| PCN          |                          |                  |                                       |                     |
| MiR-532-3p   | ⬆                         | Cyp1a1           | ⬇                                     | ⚩                    |
| MiR-26b-3p   | ⬆                         |                  | ⬆                                     | ⚩                    |
| MiR-5621     | ⬇                         | Cyp1b1           | ⬆                                     | ⚩                    |
| MiR-483-3p   | ⬆                         | Cyp39a1          | ⬇                                     | ⚩                    |
| MiR-3473c    | ⬇                         |                  | ⬇                                     | ⚩                    |
| MiR-3473c    | ⬇                         | Cyp51            | ⬇                                     | ⚩                    |
| TCPOBOP      |                          |                  |                                       |                     |
| MiR-532-3p   | ⬆                         | Cyp1a1           | ⬆                                     | ⚩                    |
| MiR-26b-3p   | ⬆                         |                  | ⬆                                     | ⚩                    |
| MiR-5114     | ⬇                         | Cyp2b10          | ⬆                                     | ⚩                    |
| MiR-28b-3p   | ⬆                         | Cyp2c38          | ⬇                                     | ⚩                    |
| MiR-344e-3p  | ⬇                         | Cyp2u1           | ⬆                                     | ⚩                    |
| MiR-695      | ⬆                         | Cyp4a12a/b       | ⬇                                     | ⚩                    |
| MiR-5114     | ⬇                         |                  | ⬇                                     | ⚩                    |
| MiR-483-3p   | ⬇                         | Cyp17a1          | ⬇                                     | ⚩                    |
| MiR-5114     | ⬇                         |                  | ⬇                                     | ⚩                    |

Fig. 1. Venn Diagram Illustrating the Results of the Mouse microRNA Array Experiment

A) Mouse miRNAs whose expression was either up-regulated (>2-fold) (solid circles), or B) down-regulated (>50%) (solid circles) by PCN or TCPOBOP treatment.

**Target Prediction and Screening of Target Cyp Genes**

To predict the target genes of the differentially expressed miRNAs, we used five different miRNA target prediction algorithms. We cross-evaluated the output from these algorithms to more accurately identify target mRNAs. 17) The bioinformatics programs identified 6638 mRNAs as predicted target genes for the 13 independent PCN-regulated miRNAs, and 6616 mRNAs as predicted target genes for the 14 TCPOBOP-regulated miRNAs (Table 3). Next we searched for genes belonging to the Cyp gene superfamily. Among the ca. 6500 predicted target genes, 9 Cyp genes were identified as candidate targets of the PCN-regulated miRNAs, and 16 Cyp genes were identified as candidate targets of the TCPOBOP-regulated miRNAs. The identified Cyp genes were further screened based on their localization to ensure that they were expressed in the liver. For this information, we referred to Hrycay and Bandiera. 18) Finally, we identified 5 Cyp genes (Cyp1a1, Cyp1b1, Cyp2c38, Cyp39a1, and Cyp51) as candidate targets of the PCN-regulated miRNAs and 8 Cyp genes...
(Cyp1a1, Cyp2b10, Cyp2c38, Cyp2u1, Cyp4a12a/b, Cyp4v3, Cyp17a1, and Cyp39a1) as candidate targets of the TCPOBOP-regulated miRNAs.

The miRNAs and their candidate Cyp target genes are shown in Table 4. Interestingly, the Cyp genes identified as miRNA target genes do not encode well-known Cyp isoforms, suggesting that the miRNAs might target minor isoforms rather than major ones. Notably, some of the Cyp genes were predicted to be candidate targets of more than one of the ligand-regulated miRNAs.

Experimental Confirmation of the Expression of Cyp Genes Predicted to be miRNA Targets

In general, miRNAs negatively affect their target’s expression by either mRNA degradation or translational repression; thus, the up-regulation of a miRNA’s expression is usually associated with the down-regulation of its target’s expression, and vice versa. As a result, the expression levels of miRNAs are usually negatively correlated with that of their targets.

To determine whether the expression of the PCN-regulated miRNAs was negatively correlated with that of their predicted targets, we quantified the expression levels of the 5 Cyp target genes in response to PCN using real-time PCR. As shown in Fig. 2A, the relative expression levels of Cyp1a1, Cyp2c38, Cyp39a1, and Cyp51 were significantly decreased after PCN treatment. These Cyp genes were the predicted targets of the up-regulated miRNAs, indicating that their expression levels were negatively correlated with those of their associated miRNAs (Table 4). While Cyp1b1 was the only mRNA

Fig. 2. Effects of PCN and TCPOBOP on the Expression of Cyp Genes Predicted To Be miRNA Targets

(A, B) Mice were injected with corn oil, PCN, or TCPOBOP once a day, for 2 d. The livers were excised and total RNA was extracted 3 h after the second administration. Real-time PCR was used to quantify the mRNA expression of Cyp1a1, Cyp1b1, Cyp2c38, Cyp39a1, and Cyp51 in PCN-treated mice (A), and the expression of Cyp1a1, Cyp2b10, Cyp2c38, Cyp2u1, Cyp4a12a/b, Cyp4v3, Cyp4v3, and Cyp39a1 in TCPOBOP-treated mice (B). Each mRNA measurement was normalized to that of Gapdh in the same sample. The results were expressed as relative fold-changes in response to ligand. Values represent the mean±S.E. for each group (n=3). *p<0.05, **p<0.01, ***p<0.001, compared with the control group.
whose expression showed a 1.6-fold increase in response to PCN treatment, miR-5621-3p, which is the only miRNA that targeted Cyp1b1, was one of the miRNAs whose expression was down-regulated by PCN (Fig. 1), indicating that the miR-5621-3p expression was negatively correlated with that of its target, Cyp1b1. Thus, negative correlations between PCN-regulated miRNAs and their target Cyp genes were observed consistently.

We also quantified the expression of the 8 Cyp genes predicted to be targets of the TCPOBOP-regulated mRNAs. We observed statistically significant changes in the expression of almost all of the Cyp mRNAs in response to TCPOBOP treatment, except for Cyp4a12a/b (Fig. 2B), and found negative correlations for Cyp2b10, Cyp2c38, and Cyp17a1 and their associated miRNAs (Table 3). Here, we must pay attention to the direct transcriptional induction by TCPOBOP. We observed the negative correlation between Cyp2b10 and miR-5114, but Cyp2b10 gene is well-known to be directly transcribed by TCPOBOP via activation of CAR. We assume that the TCPOBOP-mediated reduction in miR-5114 expression can play a little role in the up-regulation of Cyp2b10.

In contrast, we did not observe negative correlations between Cyp1a1, Cyp2a1, Cyp4a12a/b, Cyp4v3, or Cyp39a1 and their miRNAs (Table 4). Various explanations may account for these findings. For example, since the expression of Cyp1a1 is up-regulated by CAR activation, the TCPOBOP-mediated induction of Cyp1a1 may have masked the inhibitory effects of miR-532-3p and miR-26b-3p on Cyp1a1 expression. Cyp4a12a/b’s expression remained unchanged by TCPOBOP treatment. However, negative correlations between miRNAs and their targets are not always observed, since in some cases miRNAs suppress the translation of their targets without affecting their mRNA expression. Further studies quantifying the protein level and/or enzymatic activity of Cyp4a12a/b should clarify this issue. In addition, we unexpectedly found positive miRNA-target correlations for Cyp2u1, Cyp4v3, and Cyp39a1. Since several transcription factors (e.g., other nuclear receptors, such as HNFs) are involved in the regulation of Cyp genes, it is possible that other miRNAs whose targets include transcriptional factors indirectly influence the expression of these Cyp genes.

Among our results, the negative correlations of two miRNA-target pairs stood out. The negative correlation of the miR-26b-3p/Cyp2c38 miRNA-target pair was notable because it was observed in response to both PCN and TCPOBOP. Our study demonstrated for the first time that Cyp2c38 is down-regulated by PCN or TCPOBOP treatment and suggests that miR-26b-3p may play a role in Cyp2c38’s regulation. In addition, our results showed that the mRNA expression of Cyp1b1, which is known to metabolize several pro-carcinogens, was increased in response to PCN. This result is consistent with recent reports from two separate groups indicating that PXR activation up-regulates Cyp1b1’s expression in vascular endothelial cells and skin, however, the induction mechanism was unclear. Our results suggest that the PCN-mediated reduction in miR-5621 expression could contribute to the up-regulation of Cyp1b1. Further experiments using miRNA mimics and antagonists should clarify the role of miR-5621 in regulating Cyp1b1 expression. However, we do acknowledge one limitation to this study, namely, we only analyzed mRNA level of all Cyp genes. miRNAs can alter mRNA translation without altering the transcript levels, so proteomic analysis are needed to further elucidate the effects of the miRNAs on the Cyp gene expression.

CONCLUSION
We found that treating mice with nuclear receptor ligands led to altered hepatic miRNA expression. The computational identification of several Cyp genes as miRNA gene targets, followed by their quantification in response to ligand treatment, suggested that some of these novel miRNA/Cyp gene pairs may be physiologically relevant. Further studies are required to elucidate the roles that miRNAs play in regulating the expression of CYP metabolic enzymes.

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Conflict of Interest The authors declare no conflict of interest.

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