Involvement of MicroRNAs in Dioxin-Induced Liver Damage in the Mouse

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MicroRNA (miRNA) is a class of small RNA that functions as a negative regulator of gene expression. Human and mouse genomes encode over 1400 and 700 miRNAs, respectively, and most of the cellular pathways are considered to be modulated by miRNAs. However, the pathophysiological role of miRNAs is still largely unknown. Thus, we investigated the possible involvement of miRNAs in the toxic responses to xenobiotic chemicals. Here, we searched for miRNAs responsible for inducing liver damage in mice exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and found that miR-101a and miR-122 are differentially downregulated by TCDD in a time-dependent manner. Because miRNA exerts multiple actions by repressing its target genes, we quantified the target genes of miR-101a, such as cyclooxygenase-2 (COX-2), enhancer of zeste homolog 2, and cFos, and found the upregulation of these genes, which suggests that miR-101a downregulates the expression of these genes in the mouse liver. A COX-2 selective inhibitor, NS-398, suppressed the onset of TCDD-induced liver damage. In conclusion, this study demonstrated that TCDD dysregulates the expression of miR101a and miR122 and that COX-2, a target gene of miR101a, plays a significant role in liver damage in mice exposed to TCDD.

Key Words: cyclooxygenase-2; liver damage; microRNA; 2,3,7,8-tetrachlorodibenzo-p-dioxin.
from dysregulation of glucose and lipid metabolism, liver damage, immunosuppression, neurobehavioral disorders, to reproductive toxicity, carcinogenicity, teratogenicity, and wasting syndrome that eventually leads to death (Pohjanvirta and Tuomistio, 1994). The majority of these toxicities are mediated by a transcription factor Ah receptor (AhR). The essentiality of AhR for dioxin toxicities was revealed by experiments using AhR-null mice, in which no such toxicities were produced (Gonzalez and Fernandez-Salgueiro, 1998; Mimura et al., 1997; Schmidt et al., 1996). Dioxin exposure elicits the upregulation of a large number of genes in an AhR-dependent manner (Boutros et al., 2009), and it is predicted that some of these AhR target genes are directly responsible for the induction of dioxin toxicities. A limited example is CYP1A1 that mediates 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced hypertension (Kopf et al., 2010). In addition, a few studies revealed the presence of genes responsible for the TCDD toxicities in a downstream of AhR signaling pathway; cyclooxygenase-2 (COX-2) is required for the onset of hydrenephrosis in rodents and pericardial edema in medaka, and Sox9b is responsible for blood circulation disorder in zebrafish (for a review, see Yoshioka et al., 2010). The precise molecular mechanisms that connect TCDD exposure and these genes remain to be elucidated, and the mechanisms of other TCDD toxicities are yet unknown.

Given that a large number of genes are regulated by miRNAs and that most of the biological processes including responses to xenobiotic chemicals are expected to be regulated by miRNAs, it is reasonable to hypothesize that there are certain types of miRNAs that regulate dioxin toxicities. Therefore, we searched for miRNAs that are dysregulated by dioxin exposure and that are involved in the dioxin toxicity. To this end, we employed a mouse model of dioxin-induced liver damage and identified dysregulated miRNAs and the associated molecular changes.

**MATERIALS AND METHODS**

**Animals and treatment.** Male C57BL/6f strain mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed in a room with temperature at 23 ± 1°C and humidity at 50 ± 10%, on a 12/12 h light:dark cycle. Laboratory rodent chow (Labo MR Stock, Nian, Yokohama, Japan) and distilled water were provided ad libitum. The experimental protocols of this study were approved by the Animal Care Committee of the Graduate School of Medicine, The University of Tokyo.

TCDD (purity > 99.5%; Cambridge Isotope Laboratory, Andover, MA) was used in this study. TCDD was dissolved in corn oil (Wako Pure Chemicals, Osaka, Japan). We conducted three sets of experiments. In all the experiments, 9-week-old male mice were injected intraperitoneally with a single dose of vehicle (control) or TCDD (50 μg/kg, 10 ml/kg body weight). The first experiment was a time course experiment to study the liver damage for 14-day experiment. Mice were intraperitoneally injected with NS-398 (Wako Pure Chemicals) at a dose of 20 or 40 mg/kg (10 ml/kg) body weight (n = 10 each for control groups and n = 5 each for NS-398 groups), 6 h prior to an intraperitoneal injection of TCDD. Then, they were administered NS-398 every 24 h for 14 days. We selected this dose according to a previous paper (Pyo et al., 2001). NS-398 was dissolved in saline containing 10% dimethyl sulfoxide (DMSO) and 1% Tween20. Control groups were injected with the vehicle containing DMSO and Tween20.

**Dioxin hepatotoxicity.** Blood samples were obtained from the tails of mice (n = 4 for control group and n = 3 for TCDD-exposed group) every other day, and the plasma aspartate aminotransferase (AST) activity was measured with Fuji Dri-Chem (Model 7000V, Fuji Film, Tokyo, Japan). Mice were sacrificed on day 2 and day 14 in the second and first experiments mentioned above, respectively. The liver specimens were fixed in 4% paraformaldehyde, cryoprotected in a solution of 20% sucrose, embedded in optimum cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan), snap-frozen in liquid nitrogen, and sliced to make 5 μm sections. The sections were stained with hematoxylin and eosin. Plasma obtained from the right ventricle was used for alanine aminotransferase (ALT) activity measurement with Fuji Dri-Chem.

**Quantitative reverse transcription-PCR.** Total RNA was isolated from the livers of mice using the miRNeasy Mini Kit (Qiagen, Tokyo, Japan). Complementary DNA synthesis of mRNA was carried out using oligo-dT$_{20}$ primer and Super Script III (Invitrogen, Carlsbad, CA). For poly-A polymerase (PAP) reverse transcription (RT)-PCR, small RNAs were subjected to polyadenylation and reverse transcription using PAP and modified Moloney murine leukemia virus reverse transcriptase (Mir-X miRNA First-Strand Synthesis Kit, Clontech, Mountain View, CA). For stem-loop RT-PCR, total RNA was reverse-transcribed using stem-loop primer (Chen et al., 2005) and Super Script III.

The quantitative detection of differentially expressed genes, including the internal standard gene cyclophilin B and U6 small nuclear RNA (snRNA), was performed using a Light Cycler instrument (Roche Molecular Biochemicals, Indianapolis, IN) and SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). Primers for each gene (Table 1) were designed on the basis of respective complementary DNA or mRNA sequences using the Primer3 program (Rozen et al., 2000). The sequences of primers used in this study are presented in Table 1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Target name</th>
<th>Primer sequences (5’ to 3’)</th>
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<tbody>
<tr>
<td>COX-2</td>
<td>TGTGAACACATCCAAACAAATTGATG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCCGAAATATCCACGCCCTAAGT</td>
</tr>
<tr>
<td>Cyclophilin B</td>
<td>TGGCGAAAGTTCTTAGGGGC</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCTGTCGGGAGTTGCTGCA</td>
</tr>
<tr>
<td>Ezh2</td>
<td>GAAAGGGACGAGCTTCTTAA</td>
</tr>
<tr>
<td>Reverse</td>
<td>CATGGACACTGTTGTGTTG</td>
</tr>
<tr>
<td>cFos</td>
<td>GAAGGGAACGGAATAAGATGG</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTTGTCCTGTTGAGAAGTTT</td>
</tr>
<tr>
<td>F4/80</td>
<td>TGCTACTAGCAATGGCAAGC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCTCCTTGATCCTATGGTAA</td>
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<tr>
<td>miR-101a</td>
<td>GGCTATTTCCAGTGCAAGCTC</td>
</tr>
<tr>
<td>RT</td>
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<tr>
<td>miR-122</td>
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<td>pri-miR-101a</td>
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<tr>
<td>Reverse</td>
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</tr>
<tr>
<td>pre-miR101a</td>
<td>CTGGCTCAATGCTAACCCTGAT</td>
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<tr>
<td>RT</td>
<td>CTTGCTCACTGTCAGTCTACACA</td>
</tr>
<tr>
<td>U6 snRNA</td>
<td>GTAGTGTCAGTTAG</td>
</tr>
<tr>
<td>RT</td>
<td>TATGGAAACGCTTCAGAATGTG</td>
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<tr>
<td>Forward</td>
<td>TTCTCCGCGACGCACTATCTAA</td>
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</table>

**References:**

RESULTS

Quantification of MiRNAs

MiRNAs are small RNAs of 19–25 nt without poly-A tails, which hinder the application of standard methods of quantitative RT-PCR. We isolated total RNA that included small RNAs, and compared the validity of the following two methods that are used to quantify miRNAs. In the PAP RT-PCR method, small RNAs were polyadenylated and reverse-transcribed using PAP and reverse-transcriptase (Fu et al., 2006). In the stem-loop RT-PCR method, small RNAs were transcribed using stem-loop primers specific to each of the small RNAs. Stem-loop primers are designed to recognize the length and sequence of the 3′ termini of the targeted small RNAs. The fidelity of the recognition precision was demonstrated by applying the stem-loop primers to let-7 miRNA family members that possess high similarity in their sequences (Chen et al., 2005).

Threshold cycles (C\textsubscript{T}) of detection in PCR were compared for miR-122 (Table 2), which is liver-specific and abundant in the mouse (Chang et al., 2004). The C\textsubscript{T} value, which is the number of cycles required for detection by PCR, was much less in the PAP RT-PCR method than in the stem-loop RT-PCR method, indicating that the former was more sensitive than the latter. Furthermore, the coefficient of variation (CV) of the calculated expression level was much smaller in the PAP RT-PCR method (7.6% for PAP RT-PCR vs. 46% for stem-loop RT-PCR), which demonstrated a higher precision of the PAP RT-PCR method than the stem-loop RT-PCR method. MiRNAs have sequence variations at their 3′ termini (Kuchenbauer et al., 2008), which complicated the design of the stem-loop primers. Owing to these advantages of the PAP RT-PCR method over the stem-loop RT-PCR method, at least under our experimental conditions, we adopted the PAP RT-PCR method to quantify miRNAs in our present study.

Time Course of Liver Damage

We examined the time course of liver damage manifestation, prior to the search for the endogenous biomolecules responsible for inducing it following TCDD exposure. Liver damage in TCDD-exposed mice was previously reported (Pande et al., 2005). They administered TCDD at a dose of 64 μg/kg body weight and found hepatic hydropic changes, infiltration of F4/80 positive cells, increased activity of hepatic enzyme in the serum, and apoptosis at 14-day postadministration. Using a similar dose of TCDD (50 μg/kg body weight), we studied whether and how miRNAs are involved in the liver damage. We found that a single intraperitoneal injection of...
TCDD increased plasma AST level at day 6 postadministration and thereafter (Fig. 1B). The body weight of the exposed group slightly decreased at day 14 postadministration (Fig. 1A). At this time, liver specimens were examined histologically. The livers of TCDD-exposed mice showed infiltration of inflammatory cells (Figs. 2C and 2D), which was confirmed by an increase in F4/80 mRNA, a marker for macrophage (Fig. 2E). The liver weight increased by 14% in response to TCDD (1.19 ± 0.03 g for control mice vs. 1.36 ± 0.04 g for exposed mice, \( p < 0.05 \)). These results indicated that the TCDD-exposed mice were free of overt abnormalities in the first 4 days, liver damage became apparent around day 6 and then progressed, and finally, body weight started to decline around day 14 when liver damage was clearly manifested.

**Early Changes in MiRNA Expression and Regulation by TCDD**

To find the miRNA responsible for inducing liver damage upon TCDD exposure, we screened miRNAs that were potentially involved in the onset of liver damage at day 2 postadministration. This time point is well before the onset of liver damage as supported by the plasma AST level (Fig. 1B), and no overt change or infiltration of macrophage was observed by histological examination (Figs. 2A and 2B) and the macrophage marker F4/80 mRNA (Fig. 2E). Therefore, it is plausible to exclude the possibility of detecting the altered miRNA expression as a result of liver damage. For the screening, we quantified miRNAs including miR-9, miR-16, miR-26b, miR-98, miR-101a, and miR-146 because they were reported to be involved in inflammation or predicted to have target genes related to apoptosis or inflammation. Among these miRNAs, we selected miR-101a for further analysis because its expression level was shown to be substantially decreased by an initial screening (\( n = 2 \), data not shown) and miR-101a was reported to target COX-2 (Chakrabarty et al., 2007; Strillacci et al., 2009; Tanaka et al., 2009).

The hypothesis we formulated was that TCDD reduces the miR-101a expression level, which in turn activates the prostanoid signaling pathway catalyzed by COX-2, then the elevated levels of prostanoid cause the liver damage. To test this hypothesis, miR-101a and its target genes were analyzed by quantitative RT-PCR and immunoblotting. We found a significant decrease in the expression level of miR-101a (Fig. 3A), and a significant increase in that of COX-2 both at the mRNA (Fig. 4A) and protein (Fig. 4B) levels. The expression levels of EZH2 and cFos, other targets of miR-101a (Friedman et al., 2009; Li et al., 2009), were not significantly altered at the mRNA level (Figs. 4C and 4E) but substantially increased at the protein level (Figs. 4D and 4F). The increase in the protein amount without a significant change in the RNA amount indicated that EZH2 and cFos were regulated post-transcriptionally in the liver of the mice. No change in the expression level of miR-122 was found (Fig. 3B).

**Late Changes in MiRNA Expression, Regulation, and Associated Alterations by TCDD**

Because we observed signs of liver damage in TCDD-exposed mice at day 14 postadministration (Figs. 1B and 2), we quantified miRNAs and their target molecules in the same liver specimens and found that the expression level of miR-101a...
was reduced (Fig. 5A). As expected, the expression level of COX-2 was increased at both mRNA (Fig. 6A) and protein (Fig. 6B) levels. The increase of EZH2 and cFos were significant at the protein level (Figs. 6D and 6F). However, despite the increasing tendency, the changes in the mRNA level were not significant (Figs. 6C and 6E). These patterns of alterations nearly mirrored those of day 2 postadministration (Figs. 3 and 4), suggesting that the altered expression levels of miR-101a, COX-2, EZH2, and cFos had been sustained from day 2 through day 14 postadministration.

Because the elimination half-life of TCDD is 9–10 days in the C57BL/6 mouse strain used in this experiment (Birnbaum, 1986), TCDD retained in the liver is considered to induce the altered abundance of miR-101a and its targets.

To study how TCDD-induced COX-2 upregulation is related to the onset of hepatic damage, we utilized NS-398, a COX-2 selective inhibitor, and found that the elevated levels of plasma ALT and AST at day 14 postadministration of TCDD were suppressed by NS-398 in a dose-dependent manner (Figs. 7A and 7B). Administration of NS-398 did not affect the reduction of miR-101a by TCDD (Fig. 7C), which is in line with our hypothesis: TCDD downregulates the expression of miR-101a which then causes the upregulation of the COX-2 expression.

**Reduction in Abundance of MiR-101a Precursors**

A miRNA gene is transcribed to produce a pri-miRNA, trimmed into a pre-miRNA in the nucleus, exported to the cytoplasm, and trimmed again to form a mature miRNA (Krol et al., 2010). To determine whether any of the miRNA production steps was altered, we quantified the miR-101a precursors, that is, pri-miR-101a and pre-miR-101a, by quantitative RT-PCR. The amounts of these miR-101a precursors were found to be significantly reduced at day 2 (Figs. 8A and 8B) and day 14 (Figs. 8C and 8D) postadministration, indicating that TCDD exposure reduced the level of pri-miR-101a transcription, and the resulting pre-miR101a amount, and therefore that of miR-101a.

**FIG. 5.** MiRNAs expression levels in the liver of mice at day 14 postadministration of TCDD. Abundance of miR-101a (A) and miR-122 (B) was measured by quantitative RT-PCR and is shown here after normalization with U6 snRNA abundance. See the legend to Figure 2.

**FIG. 6.** The expression levels of target genes of miR-101a in the liver of mice at day 14 postadministration of TCDD. The mRNA abundance of COX-2 (A), EZH2 (C), and cFos (E) is shown after normalization with cyclophilin B abundance. The protein abundance of COX-2 (B), EZH2 (D), and cFos (F) were determined by immunoblotting and is shown after normalization with β-actin abundance. Representative immunoblotting data are shown in the insets. See the legend to Figure 2.

**FIG. 4.** The expression levels of target genes of miR-101a in the liver of mice at day 2 postadministration of TCDD. The mRNA abundance of COX-2 (A), EZH2 (C), and cFos (E) is shown after normalization with cyclophilin B abundance. The protein abundance of COX-2 (B), EZH2 (D), and cFos (F) were determined by immunoblotting and are shown after normalization with β-actin abundance. Representative immunoblotting data are shown in the insets. See the legend to Figure 2.

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DISCUSSION

Altered Regulation of MiRNAs Triggered by Dioxin Exposure

We found significant changes in the expression levels of miR-101a and miR-122 in the liver of TCDD-exposed mice. Reduction in the expression level of miR-101a was evident as early as day 2 and leveled off at least until day 14 postadministration, whereas the reduction in the expression level of miR-122 was detected only at day 14. The expression levels of these miRNAs fluctuated minimally within each group (Table 3). In control groups, CV values of the expression levels of miR-101a and miR-122 ranged from 5.9 to 12.9%, and in TCDD-exposed groups, the miRNA levels had CV values from 9.8 to 10.0%. Such small variations observed in this study demonstrated that the expression levels of the miRNAs are strictly regulated under nonstimulated and TCDD-exposed conditions, which was confirmed by the reliable measurement of miRNAs by the PAP RT-PCR method. This finding indicated that the amounts of miRNAs are precisely tuned to regulate biological pathways. The difference in the time points at which the expression levels of miR-101a (day 2) and miR-122 (day 14) were reduced in response to TCDD suggested that there are yet unidentified multiple mechanisms of miRNA regulation in the liver of TCDD-exposed mice.

A previous report by Moffat et al. (2007) concluded that miRNAs in adult rodent liver are refractory to dioxin treatment. They administered a high dose of TCDD to mice and rats and analyzed the expression of miRNAs. In contrast to our study, in which the expression levels of miR-101a and miR-122 were reduced by TCDD exposure, they did not find such conclusive changes induced by TCDD exposure. There are at least two possible explanations for this apparent discrepancy between these two studies. First, the difference in time point may explain the discrepancy; we analyzed the liver at day 2 (or hour 48) postadministration whereas they did so at hour 19. Such a time difference might be critical for the reduction of the miR-101a expression level. Second, the difference in methods might cause this discrepancy in the miRNA expression level; we analyzed miRNAs by the PAP RT-PCR method, whereas they did so by the stem-loop RT-PCR method. As shown in this paper, the PAP RT-PCR method had significant advantages over the stem-loop RT-PCR method in terms of both sensitivity and precision (Table 2), at least under our experimental conditions. We consider that the PAP RT-PCR method provides more reliable data than the stem-loop RT-PCR method.
Reduction of MiR-101a Expression Level and Associated Changes in the Liver

TCDD administration significantly reduced the miR-101a expression level before the manifestation of liver damage. This finding is thought to exclude the possibility that such an early reduction in the miR-101a level is the consequence of liver damage, and to propose the possibility that it was rather the cause of the upregulation of its target genes and the subsequent downstream phenomena. This possibility was supported by the following two points. First, COX-2 is a well-established target of miR-101a (Chakrabarty et al., 2007; Strillaccis et al., 2009; Tanaka et al., 2009). 3’ UTR of COX-2 mRNA has a binding sequence of miR-101a, and a reporter gene having this binding sequence is downregulated in response to the overexpression of miR-101a in a binding-sequence specific manner. In addition, studies using several cell lines showed that the knockdown of miR-101a results in the upregulation of COX-2 protein expression, and the overexpression of miR-101a results in the downregulation of COX-2 protein expression (Chakrabarty et al., 2007; Strillaccis et al., 2009; Tanaka et al., 2009). Second, the protein amounts of EZH2 and cFos, the targets of miR-101a (Friedman et al., 2009; Li et al., 2009), were also increased simultaneously as that of COX-2. The fact that COX-2, EZH2, and cFos proteins were all increased at the same time suggested the existence of a common upregulation mechanism for these three genes. On the other hand, the increase of COX-2 mRNA alone could either be the consequence of variation in their binding sequence of miR-101a, or effects of other mechanisms involved in COX-2 gene regulation, such as nongenomic action of AhR that exerts calcium-dependent rapid mechanism in the cytoplasm to upregulate COX-2 mRNA (Li et al., 2010). The reduced expression level of miR-101a by TCDD is considered to account for the common upregulation of these target genes. Taken together, these points strongly support the existence of post-transcriptional regulation of COX-2, EZH2, and cFos mediated by miR-101a in the mouse liver exposed to TCDD.

COX-2 is the inducible form of COX that converts arachidonic acid into prostaglandin (PG)H2. PG synthases in turn metabolize PGH2 to prostanoids, including PGE2. COX-2 is induced at sites of inflammation in response to inflammatory stimuli and one of its major products, PGE2, plays multiple roles in inducing inflammation in a tissue- or cell-specific manner. The central role of COX-2 in inflammation is clear from the fact that it is the target molecule of anti-inflammatory agents. Importantly, the upregulation of COX-2 preceded the onset of liver damage in mice exposed to TCDD. This early upregulation and role of PG production indicate that COX-2 plays a critical role in TCDD-induced liver damage, which was confirmed by the inhibition experiment. That is, a COX-2 selective inhibitor suppressed the signs of liver damage in the TCDD-exposed mouse liver in a dose-dependent manner (Fig. 7). Besides PGs, inflammatory cytokines were reported to be involved in the onset of TCDD-induced liver damage in the study using the triple knock-out mouse that lacked receptors for TNF-α and β, and IL-1α and β (Pande et al., 2005). The signs of hepatic damage, including increases in a numbers of F4/80-positive inflammatory cells and TUNEL-positive cells and serum ALT level, were reversed by the absence of these receptors. Elucidation of the relationship of PGs and inflammatory cytokines in the liver exposed to TCDD will deepen our understanding of liver damage induced by dioxin.

EZH2, the protein level of which was significantly increased by TCDD exposure (Figs. 4D and 6D), is a methyltransferase that transfers methyl groups to lysine 27 of histone H3 (H3K27). The resulting H3K27 trimethylation leads to the transcriptional repression of target genes, including several genes of the Wnt family (Wang et al., 2010). Some of the dioxin-induced toxicities, such as abnormal prostate development in mice and hampered fin regeneration in zebrafish, were reported to be induced by the dysregulation of Wnt signaling (Allgeier et al., 2008; Mathew et al., 2008). Thus, EZH2 could be an important molecule that links AhR and Wnt in the toxicity of dioxin. A plausible scenario is that the upregulation of EZH2 by TCDD leads to the downregulation of Wnt genes, which in turn elicits dioxin toxicities in developmental stage-specific and tissue-dependent manners.

Reduction of MiR-122 Expression Level

TCDD administration reduced the miR-122 expression level at day 14 postadministration, but not at day 2, which indicated that the reduction of the miR-122 expression level is a secondary effect, because other physiological changes such as the induction of CYP1A1, a prototypical indicator of AhR action, occurs within 1-day postadministration. The reduction of miR-122 expression level in the liver could be a consequence of TCDD-induced liver damage or other cellular events. Concerning the relationship between miR-122 and liver damage, Wang et al. (2009) found that the miR-122 expression level was decreased in the acetaminophen-damaged liver.

MiR-122 is the most abundant liver-specific miRNA (Chang et al., 2004; Cheung et al., 2008) and has been known to play a regulatory role in lipid metabolism. Inhibition of miR-122 expression with antisense oligonucleotides was reported to change the composition of fatty acids in the liver (Gaffield et al., 2009), to lessen the severity of liver steatosis (Esau et al., 2006) and to reduce the plasma cholesterol level (Esau et al., 2006). It was also reported that the miR-122 expression level is reduced in the liver of humans with nonalcoholic fatty liver disease (Cheung et al., 2008). These studies raise the possibility that the reduction of the miR-122 expression level by TCDD exposure is involved in the dysregulation of lipid metabolism elicited by dioxin (Pohjanvirta and Tuomisto, 1994).

In conclusion, we found specific miRNAs, miR-101a and miR-122, which were differentially regulated in the course of
dioxin-induced changes. Because a single miRNA has multiple targets through the binding sequences just as a single transcription factor does, the alterations in the expression levels of miRNAs we found could affect multiple biological pathways through their target genes. Among these target genes, COX-2 was demonstrated to be significantly involved in pathways through their targets genes. Among these target transcription factor does, the alterations in the expression targets through the binding sequences just as a single dioxin-induced changes. Because a single miRNA has multiple

**REFERENCES**


