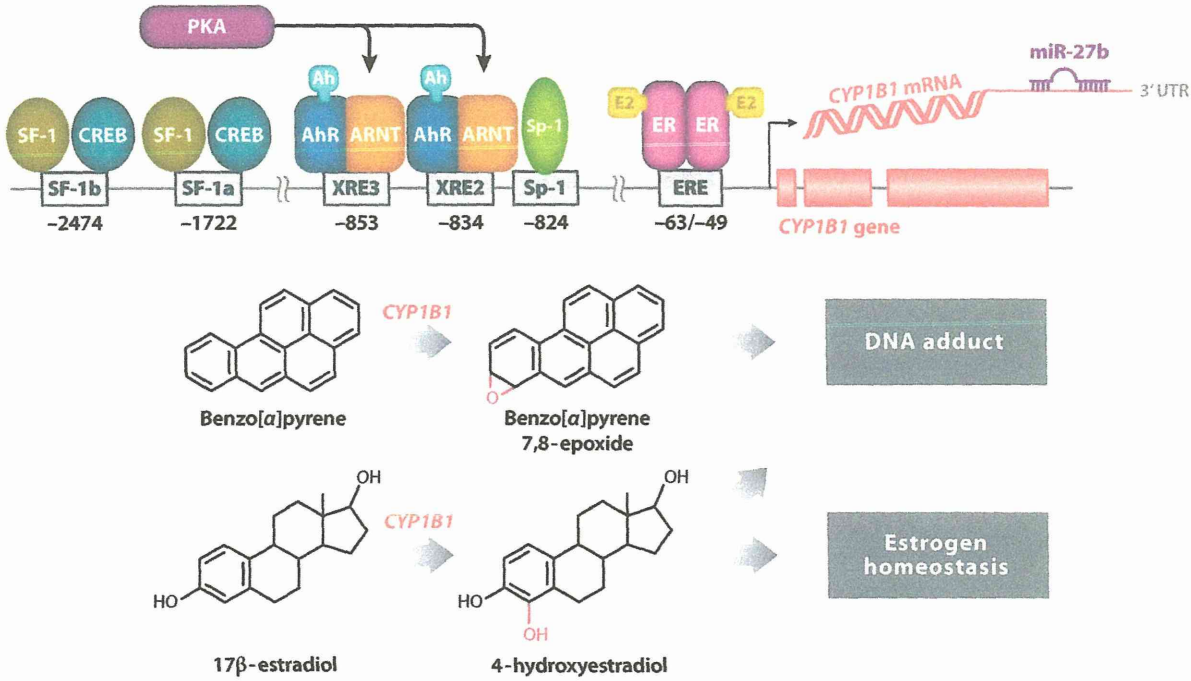

Figure 1

miR-27b-dependent posttranscriptional regulation of human CYP1B1. 4-Hydroxyestradiol, a catechol-type metabolite that is formed by CYP1B1, causes DNA damage. Abnormal CYP1B1 expression may be related to the development of estrogen-dependent cancer. Benzo[*a*]pyrene (BaP) 7,8-epoxide, an active BaP metabolite that is formed by CYP1B1, also causes DNA damage. The posttranscriptional regulation of CYP1B1 by miR-27b may serve as a possible mechanism for the high expression of CYP1B1 in cancerous tissues. Abbreviations: Ah, aryl hydrocarbon; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; PKA, protein kinase A.

N-nitrosopyrrolidine) (25). CYP2E1 is induced by its own substrates, such as isoniazid, ethanol, and acetone, and the subsequent enhanced CYP2E1 expression results in a positive feedback loop that thereby enhances substrate metabolism (26). Additionally, CYP2E1 is the most abundant isoform among all P450s in human liver (56% of total P450) at the mRNA level, followed by CYP2C and CYP3A4 (8–11% of total P450) (27). However, CYP2E1 is the fourth most abundant isoform (approximately 7% of total P450) at the protein level after CYP3A4 (30% of total P450), CYP2C (20% of total P450), and CYP1A2 (approximately 13% of total P450) (28). Therefore, posttranscriptional regulation may contribute to the constitutive and inducible expression of CYP2E1 in human liver. The potential for miRNAs to function in the posttranscriptional regulation of human CYP2E1 was studied after in silico analysis identified a possible MRE378 in the 3′ UTR of human CYP2E1 mRNA (29). Luciferase assays using HEK293 cells confirmed that miR-378 functionally recognized MRE378. When the precursor miR-378 was transfected into cells expressing human CYP2E1 that included the 3′ UTR, the CYP2E1 protein level and chlorzoxazone 6-hydroxylase activity (a CYP2E1 activity marker) were significantly decreased; this was not the case for cells expressing CYP2E1 that excluded the 3′ UTR. Unexpectedly, in both cell lines, the CYP2E1 mRNA levels were decreased by miR-378 overexpression, but miR-378 did not affect the stability of the CYP2E1 mRNA. Therefore, CYP2E1 downregulation by miR-378 appears to be caused by translational repression rather than mRNA degradation. In a panel of 25

BaP: benzo[*a*]pyrene

PXR: pregnane X receptor

CAR: constitutive androstane receptor

human livers, no positive correlation was observed between the CYP2E1 protein and CYP2E1 mRNA levels, supporting the idea that the protein expression is repressed posttranscriptionally. Consistent with this contention, the miR-378 levels were inversely correlated with CYP2E1 protein levels and the translational efficiency (protein/mRNA expression ratio) of human CYP2E1. The 3′ UTR of CYP2E1 is poorly conserved among human, rat, and mouse; thus, CYP2E1 regulation by miR-378 may be specific to humans.

In addition to playing a role in CYP2E1 expression, miR-378 promotes cell survival, tumor growth, and angiogenesis by repressing the expression of Sufu (suppressor of fused) and Fus-1 (one of the oxygen-binding functional units within keyhole limpet hemocyanin), which are tumor suppressors (30). Furthermore, miR-378 binds to the 3′ UTR of vascular endothelial growth factor (VEGF) and promotes VEGF expression (31). Thus, the involvement of miR-378 in CYP2E1 induction by chemicals/xenobiotics and its role in cell growth are likely to be toxicologically significant.

Rat CYP2A3

CYP2A3 is expressed in lung but not in liver, kidney, or small intestine tissues (32). In lung, CYP2A3 is a principal catalyst of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) α-hydroxylation, the primary bioactivation pathway for NNK (33). The chronic administration of NNK to Fisher 344 (F344) rats reduced the expression of several miRNAs, including miR-126* and miR-34 in the lung (34), and CYP2A3 was found to be regulated by miR-126*. Because reduced miR-126* expression was accompanied by increased CYP2A3 expression (at the mRNA and protein levels) in the NNK-treated rats, these expression changes were assumed to potentiate NNK genotoxicity. The reduced miR-34 expression observed after NNK exposure is also noteworthy because of its reported implication in regulating p53, a tumor suppressor gene (35, 36).

Human CYP3A4 and Pregnane X Receptor

Human CYP3A4 is the most important P450 enzyme, catalyzing the metabolism of more than 50% of all clinically relevant drugs (37). Animal and human CYP3A enzymes are also implicated in activating several drugs and xenobiotics into toxic metabolites. Notably, aflatoxins B1 and G1 and benzo[*a*]pyrene (BaP) are oxidized efficiently into genotoxic metabolite(s) by CYP3A4 (38, 39). There is at least a sixfold interindividual variation of CYP3A4 activity in most populations (40–42), and this interindividual variability cannot be explained solely by genetic polymorphisms (41, 43). CYP3A4 expression is predominantly regulated at the transcriptional level by several transcriptional factors including pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (44).

The role of miRNA in the regulation of CYP3A4 expression was first reported by Takagi et al. (45). In a panel of 25 human livers, the PXR mRNA level was not correlated with PXR protein levels, suggesting the involvement of posttranscriptional regulation. However, correlation analyses between CYP3A4 mRNA and protein levels suggest that miRNA is not likely to be involved. MRE148a was identified in the 3′ UTR of human PXR mRNA, as well as in the 3′ UTR of CYP3A4 mRNA. A reporter-gene assay revealed that miR-148a could recognize the MRE148a of the PXR mRNA; however, MRE148a of the CYP3A4 was not recognized. Consequently, miR-148a overexpression caused a reduction of the PXR protein, whereas when antisense oligonucleotides were used to inhibit miR-148a activity, the PXR protein level increased. The miR-148a-dependent decrease in PXR protein attenuated the induction and/or constitutive levels of CYP3A4 mRNA. Furthermore, the translational efficiency of PXR (ratio of PXR protein expression to PXR mRNA

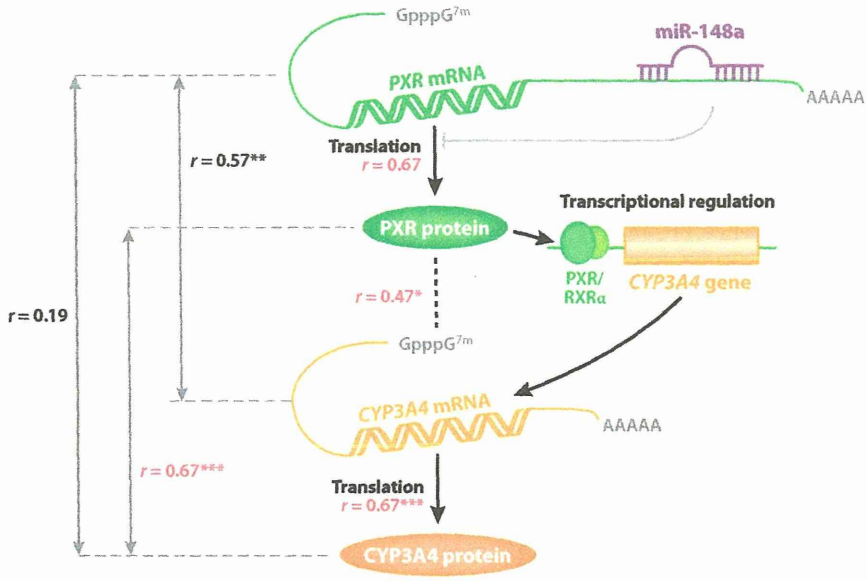

Figure 2

miR-148a-dependent posttranscriptional regulation of human PXR, which affects CYP3A4 expression levels in human liver. The CYP3A4 mRNA level was significantly correlated with CYP3A4 protein levels. The miR-148a level was inversely correlated with the translational efficiency of PXR (ratio of PXR protein expression to PXR mRNA expression). The PXR protein level was significantly correlated with CYP3A4 mRNA and CYP3A4 protein levels. The differences upon comparing each group were considered significant at $p < 0.05$ (single asterisk), $p < 0.01$ (double asterisk), and $p < 0.001$ (triple asterisk). Abbreviations: PXR, pregnane X receptor; RXR, retinoid X receptor.

expression) was inversely correlated with the expression levels of miR-148a in a panel of human livers.

One published report indicates that CYP3A4 protein in LS180 and human pancreatic cancer–derived PANC1 cells was decreased by miR-27b overexpression and that this decrease was accompanied by a decrease in the CYP3A4 mRNA level (46). In this study, only miR-27b overexpression was evaluated; to fully evaluate the potential regulation of miRNAs, additional experiments inhibiting endogenous miRNAs as well as a correlation analysis of the mRNA levels between the miRNA and target are necessary. Another study indicated that PXR protein levels were not significantly correlated with CYP2B6 or multidrug resistance protein 1 (MDR1) mRNA levels in the human liver panel. In an induction study (45), twofold CYP2B6 and fivefold MDR1 mRNA inductions by rifampicin in LS180 cells were attenuated by miR-148a overexpression. Therefore, new information indicates that miR-148a posttranscriptionally regulates human PXR, resulting in the modulation of inducible and/or constitutive CYP3A4 levels in human liver (**Figure 2**). This study suggested a new miRNA-dependent mechanism that explains the large interindividual variability of CYP3A4 expression via human PXR expression.

Human CYP24A1 and Vitamin D Receptor

Human CYP24A1 is an enzyme that is essential for the inactivation of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$; calcitriol]. Calcitriol, a biologically active metabolite of vitamin D_3, is typically considered a regulator of calcium homeostasis, but it has recently received attention for its antitumor

MDR1: multidrug resistance protein 1

activity (47). CYP24A1 is overexpressed in various tumor cells (48), but CYP24A1 protein overexpression is not necessarily associated with an increased CYP24A1 mRNA level (49, 50). Most of the biological effects of calcitriol are elicited by its binding to vitamin D receptor (VDR) (51), whose system has relevance for cancer prevention and treatment (52). VDR expression at the protein level is higher in breast (53) and thyroid (54) cancers than in normal tissues, but no difference in VDR expression at the mRNA level is found between cancer and normal tissues, suggesting the involvement of posttranscriptional regulation.

VDR: vitamin D receptor

HNF4α: human nuclear factor 4α

ROS: reactive oxygen species

Both human CYP24A1 (55) and VDR (56) are posttranscriptionally regulated by miR-125b. A potential MRE125b in the 3′ UTRs of human CYP24A1 and VDR mRNAs was suggested to be involved in the expression of these proteins. The CYP24A1 protein levels in cancer tissues were inversely associated with the cancer/normal ratios of the miR-125b levels, suggesting that decreased miR-125b levels in breast cancer tissues may contribute to high CYP24 protein expression. Because CYP24A1 is a target of VDR, miR-125b may directly and/or indirectly regulate CYP24A1. Increased VDR levels in cancer tissues would augment the antitumor effects of calcitriol, whereas an increase in CYP24A1 would attenuate the antitumor effects. The role of miR-125b relative to the antiproliferative effects of calcitriol was studied in MCF-7 cells, and we found that miR-125b inhibited the effect of calcitriol, resulting in increased cell growth. These results indicate that miR-125b plays a role downstream of VDR activities.

miR-125b expression is differentially affected in various human tumors; for example, miR-125b is downregulated in breast, ovarian, and bladder cancers and upregulated in pancreas and stomach cancers (57). Thus, the functional effects of miR-125b differ among cancerous tissues. There is considerable interest in evaluating miR-125b as a potential biomarker of cancer-related outcomes, but additional research is needed.

Human Nuclear Factor 4α and CYP7A1

Human nuclear factor 4α (HNF4α), which belongs to the nuclear hormone receptor superfamily, is highly expressed in liver and to a lesser degree in kidney, small intestine, and colon. HNF4α regulates the expression of various genes implicated in the synthesis/metabolism of bile acid, fatty acid, cholesterol, glucose, and urea as well as in hepatocyte differentiation (58). It is well recognized that endobiotic- and xenobiotic-metabolizing enzymes—such as CYPs, UDP-glucuronosyltransferases (UGTs), and sulfotransferases—are under the control of HNF4α (59). HNF4α transactivates the expression of target genes not only via direct binding to their regulatory sequences but also through the regulation of other transcriptional factors, such as PXR and CAR, that regulate these target genes. HNF4α forms large transcriptional regulatory networks in the liver.

MRE24 was identified in the coding region and the 3′ UTR of HNF4α, whereas MRE34a was identified only in the 3′ UTR of HNF4α mRNA (60). HNF4α protein levels in HepG2 cells were markedly decreased by the overexpression of miR-24 and miR-34a, and HNF4α mRNA levels were significantly decreased by the overexpression of miR-24 but not miR-34a. The luciferase activity of a plasmid that contained the 3′ UTR of HNF4α was significantly decreased by miR-34a; similarly, the activity of a plasmid that contained the HNF4α coding region was significantly decreased by miR-24. Together, these findings suggest that MRE24 in the coding region and MRE34a in the 3′ UTR function in the downregulation of HNF4α by mRNA degradation and translational repression, respectively. The downregulation of HNF4α by these miRNAs caused the decrease in various target genes, such as CYP7A1 and CYP8B1, as well as morphological changes and the decrease in the S-phase population of HepG2 cells (60). Additionally, the expression of miR-24 and miR-34a was regulated by protein kinase C/mitogen-activated protein kinase and reactive oxygen species (ROS) pathways, respectively (**Figure 3**).

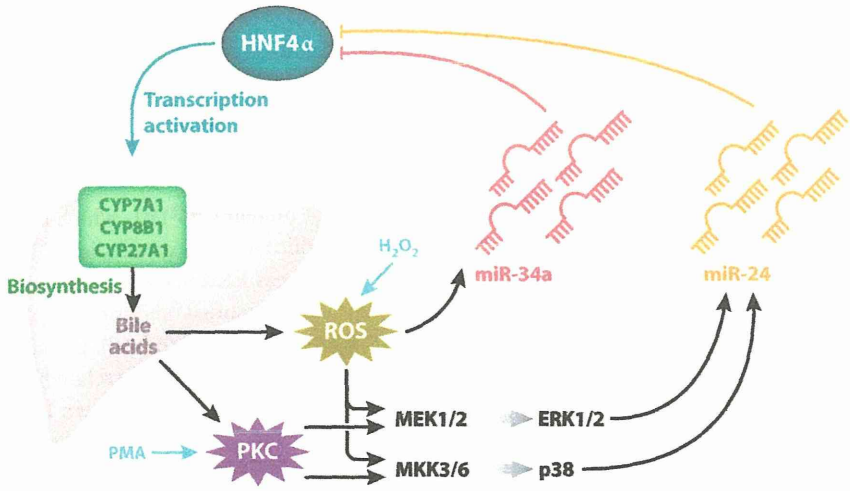

Figure 3

Regulatory loop of miR-24, miR-34a, and HNF4α in bile acid biosynthesis. Bile acids activate protein kinase C (PKC) and generate reactive oxygen species (ROS), both of which activate the mitogen-activated protein kinase (MAPK) pathway. The expression of miR-24 and miR-34a is induced by MAPK-dependent and MAPK-independent pathways, respectively. In turn, miR-24 and miR-34a downregulate HNF4α. The downregulation of HNF4α decreases the expression of the bile acid–synthesizing enzymes CYP7A1 and CYP8B1, resulting in a decline in bile acid biosynthesis. Abbreviations: ERK, extracellular signal-regulated kinase; HNF4α, human nuclear factor 4α; MEK, MAP/ERK kinase; MKK, mitogen-activated protein kinase kinase; PMA, phorbol 12-myristate 13-acetate.

HNF4α upregulates the expression of bile acid–synthesizing enzymes, such as CYP7A1 and CYP8B1. CYP7A1 catalyzes the first and rate-limiting step in the bile acid synthetic pathway (61). Therefore, the induction of miR-24 and miR-34a is expected to decrease bile acid synthesis via mainly CYP7A1, which is implicated in monitoring bile acid homeostasis in the human liver. Thus, miR-24 and miR-34a affect various hepatic functions through the negative regulation of HNF4α expression.

Human Aryl Hydrocarbon Receptor Nuclear Translocator

Aryl hydrocarbon receptor nuclear translocator (ARNT) forms a heterodimer with aryl hydrocarbon receptor (AhR) or hypoxia-inducible factor 1α (HIF-1α) to mediate biological responses to xenobiotic exposure and hypoxia (62). Earlier studies showed that the human ARNT protein level was decreased by hydrogen peroxide or ROS (63, 64). These stimuli increase the miR-24 level in various human cell lines. In silico analysis predicts that several miRNAs, including miR-16 and miR-23b, may bind to ARNT mRNA (65). Overexpression of miR-24 into HuH-7 and HepG2 cells significantly decreased the ARNT protein level but not the ARNT mRNA level, consistent with a mechanism that involves translational repression. However, overexpression of miR-16 or miR-23b caused no change in ARNT expression. The miR-24-dependent downregulation of ARNT decreased the expression of its downstream genes, such as CYP1A1 and carbonic anhydrase IX. The miR-24-binding element on ARNT mRNA predicted by in silico analysis was determined using luciferase reporter-gene assays. Additionally, the miR-24 levels in a panel of 26 human livers were inversely correlated with both the protein levels and the translational efficiency of ARNT. Taken together, these results demonstrate that miR-24 downregulates ARNT expression

ARNT: aryl hydrocarbon receptor nuclear translocator

AhR: aryl hydrocarbon receptor

in human livers and thus alters the expression of genes that are downstream of ARNT. This mechanism involving miR-24 could be one explanation for how ARNT protein is decreased by ROS (65).

PPARα: peroxisome proliferator-activated receptor α

IL: interleukin

RXR: retinoid X receptor

Human Estrogen Receptor α

Estrogen receptor α (ERα) regulates the expression of human CYP1B1, which catalyzes estradiol into the toxicologically active endogenous metabolite 4-hydroxyestradiol (24). It was first demonstrated that miR-206 regulates human ERα, whereas ERα activation decreases miR-206 expression, consistent with a regulatory mechanism involving mutual inhibition (66). miR-221 and miR-222 also inhibit human ERα expression at the translational level (67, 68). ERα is the primary target for miR-22, and expression of miR-22 and ERα protein are inversely related (69). miR-375 was identified as a potential target of dexamethasone-induced Ras-related protein 1 (RASD1), and studies to date indicate that RASD1 downregulates ERα expression (70). miR-27a indirectly regulates human ERα via ZBTB10, a specific protein repressor for Sp2, Sp3, and Sp4 (71). Multiple groups have suggested that a variety of miRNAs might be potential targets for antiestrogen therapy. However, the variations in experimental conditions, and model systems (cultured hepatoma cell lines and tumor tissues and/or human tissue samples) and the lack of quantitative analyses used in these studies underscore the need for further work in this area.

Peroxisome Proliferator-Activated Receptors α and γ

Peroxisome proliferator-activated receptor α (PPARα) regulates genes that encode endobiotic/xenobiotic enzymes (e.g., CYP4A11, UGT1A9, and UGT2B4) and lipid-metabolizing enzymes (e.g., acyl-CoA synthetase). The overexpression and inhibition of miR-21 or miR-27b in HuH7 cells significantly decreased and increased the PPARα protein level, respectively, but did not affect PPARα mRNA levels (72). These miRNAs downregulate PPARα expression in human liver, and because PPARα is an important regulator of fatty acid catabolism, miR-21 and miR-27b may contribute to the regulation of lipid metabolism.

miR-27a and miR-27b were also reported to be PPARγ targets (73–76). Notably, miR-27b inhibition, which was induced by lipopolysaccharide (LPS), reversed PPARγ mRNA degradation, whereas miR-27b overexpression decreased PPARγ mRNA, affecting the LPS-induced expression of proinflammatory cytokines, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (74). The expression levels of miR-27a and miR-27b were increased in the fat tissue of obese mice and were regulated by hypoxia (76). Overall, miR-27a, miR-27b, and miR-21 are of interest when PPARα- and PPARγ-related responses are considered.

Retinoid X Receptor

Retinoid X receptor α (RXRα) is a target of miR-27a and miR-27b in rat primary hepatic stellate cells (77). The MRE sequences on the RXRα mRNA are highly conserved among species, suggesting that human RXRα may also be regulated by miR-27a and miR-27b. RXRα, which is implicated in multiple signaling pathways that are associated with cell proliferation and differentiation, acts mainly as the heterodimeric partner of several nuclear receptors (78). Therefore, miR-27 appears to be implicated in the regulation of a wide variety of transcriptional factors that affect inter- and intraindividual differences in drug response, adverse reactions, and toxicity outcomes. As mentioned above, CYP1B1, PPARα, and PPARγ are direct targets of human miR-27b.

Glucocorticoid Receptor

Rat and human glucocorticoid receptors (GRs) are regulated by miR-18 and miR-124a, respectively, and the MRE sequences are well conserved among rat, mouse, and human. Whereas the expression of miR-124a is restricted to the brain, miR-18 is widely expressed throughout the body. Because GR is implicated in the regulation of CYP2B6, CYP2C9, CYP3A4, PXR, and CAR (79), additional studies are needed to determine whether the miRNA-dependent regulation of GR might affect drug metabolism and toxicological outcomes in vivo.

Regulatory miRNA networks are complex because a single miRNA can target numerous mRNAs, often in combination with other miRNAs and nuclear receptors, and a single target can be regulated by various types of miRNAs. Although this research field is nascent, miRNAs associated with drug metabolism and toxicology could contribute broadly to understanding the mechanisms of toxicity and to predicting the risk susceptibility of drugs, chemical toxicants, and environmental pollutants. Data from several recent reports have suggested that responses to changes in miRNA levels may differ between in vivo and in vitro studies. Thus, considerable attention should be paid to the experimental conditions when evaluating data.

POTENTIAL MODULATION OF TOXICOLOGY-RELATED miRNA EXPRESSION BY DRUGS, CHEMICALS, AND ENVIRONMENTAL TOXICANTS

The precise roles of miRNA in response to xenobiotics, drugs, and chemical toxicants remain to be established; however, the complexity of the regulatory miRNA networks complicates this type of research. Generally, decreased miRNA expression causes target protein expression. However, changes in miRNA expression are not always likely to be associated with toxicological phenomena because various targets are affected by miRNA. Comprehensive studies that utilize miRNA arrays, DNA microarrays, and proteomics analyses are powerful tools for investigating individual susceptibility to toxicants and adverse drug reactions. Recently, a large number of studies on the roles of miRNAs in cancer have been conducted, but few miRNA studies have investigated drug-related adverse reactions and toxicology-related in vivo outcomes.

In Vitro and In Vivo Evaluation of the Toxicology-Related Functions of miRNAs

Several examples demonstrate how miRNAs may influence toxic responses. Although many of these examples have been in vitro–only evaluations, they are relevant to studying toxicity mechanisms. For example, human miR-222 regulates matrix metalloproteinase 1 (MMP1) expression through both *cis*-regulatory mechanisms (targeting MMP1 mRNA) and *trans*-regulatory mechanisms [indirectly controlling MMP1 gene expression by targeting superoxide dismutase-2 (SOD2)] (80). In addition, miR-17* suppresses primary mitochondrial antioxidant enzymes—such as SOD2, glutathione peroxidase-2, and thioredoxin reductase-2—in prostate cancer PC-3 cells (81). Luciferase activities were suppressed by the overexpression of miR-17*. The dithiocarbamate drug disulfiram induced the miR-17* expression level. Furthermore, miR-17 was reported to silence HIF-1α expression (82). These in vitro experimental reports indicate that miR-222, miR-17, and miR-17* would be involved in maintaining homeostasis against cellular redox stress.

It has also been demonstrated in vivo and in vitro that let-7c, an important miRNA for cell growth, was inhibited with a potent PPARα agonist, WY-14,643, in mice (83). The in vitro study showed that let-7c targets c-Myc via a direct interaction with the 3′ UTR of c-Myc, subsequently increasing the expression of the oncogenic miRNA miR-17-92. Thus, the let-7c signaling cascade

may be critical for PPARα agonist–induced liver proliferation and carcinogenesis. Arsenite, which activates nuclear factor-erythroid 2-related factor 2 (Nrf2) (84), affects miRNA expression in human lymphoblastoid TH-6 cells (85), although the impact of such changes on toxicity outcomes in vivo is unknown.

Several experimental reports suggest that there may be large, differential responses in miRNA expression changes among in vitro and in vivo studies. Regulatory networks between miRNAs and targets are complex, and in vitro studies alone may be inappropriate to predict miRNA responses in vivo for these types of toxic drugs and chemicals.

Expression Profiles of miRNA in Toxicogenomics Studies

miRNA regulatory networks are complex because a single miRNA can target numerous mRNAs, often in combination with other miRNAs; thus, an understanding of miRNA roles in toxicological processes requires an overall toxicogenomics approach. Studies that employ toxicogenomics have been performed to evaluate the miRNA responses in rodent livers in order to identify potential biomarker(s) for toxicological risk assessment. A single administration of acetaminophen or carbon tetrachloride to rats has been reported to cause different miRNA expression profiles in the liver (86), specifically changes in miR-298 and miR-370 levels, which presumably target oxidative stress-related enzymes such as thioredoxin reductases. In this early work, the sample size was small, and no statistical analyses were conducted. However, significant miRNA suppression occurred as early as 6 h after exposure to the drug or chemical (3 h was not investigated in this study), which coincided with early-phase toxicity, prior to cellular necrosis. Subsequently, many studies were extensively conducted using rats or mice. For example, a significant observation from in vivo studies is that cigarette smoking causes the downregulation of many miRNAs in the lungs of both mice and rats (87), and similar results were obtained in human airway epithelial cells (88). Rats or mice were exposed to BaP or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and their miRNA expression profiles were analyzed (see below) (89–94). If studies of miRNA expression profiles can predict effective drug-target interactions, they may also be useful in predicting environmental toxicant–target interactions and defining individual toxicant susceptibility.

Changes in miRNA Expression Affected by Environmental Pollutants and Mutagens

Dioxin toxicities range from the dysregulation of glucose and lipid metabolism, liver damage, immunosuppression, and neurobehavioral disorders to reproductive toxicity, carcinogenicity, and teratogenicity (95). In vivo, TCDD did not cause any potent hepatic miRNA expression changes in a TCDD-resistant strain of Han/Wister rats and a TCDD-sensitive strain of Long-Evans rats (89), and similar results were obtained in mice. Moffat et al. (89) concluded that downregulation of hepatic miRNAs by TCDD is unlikely to play a significant role in TCDD toxicity in adult rodent liver. Investigations focusing on particular miRNAs have demonstrated that TCDD dysregulates the expression of miR-101a and miR-122 and that cyclooxygenase-2 (COX-2), a target of miR-101a, plays a significant role in liver damage in TCDD-exposed mice (90). COX-2, which is downstream of the AhR signaling pathway, is required for the onset of hydronephrosis in rodents. Comprehensive analyses are required to reveal why hepatic expression changes in miRNA and mRNA levels were uncorrelated and to lead to an understanding of the role of miRNA in severe TCDD-induced toxicity.

BaP (150 mg kg^{-1} for 3 days) caused widespread changes in gene expression (in more than 400 genes investigated using DNA microarray analyses) in mice, but miRNA microarray analyses

showed almost no hepatic changes in miRNA expression (91). Similarly, chronic exposure to 50 mg kg^{-1} and or 75 mg kg^{-1} BaP for 28 days demonstrated significant elevation of the miR-34a expression only in the mouse liver (92). This BaP exposure triggers a DNA damage response and cell cycle effects. Thus, although miRNA expression would be coordinately regulated with the mRNA transcript, hepatic miRNA is not directly responsive to AhR agonists, such as BaP and TCDD, administered in vivo to rodents. BaP exposure does cause lung cancer; thus, miRNAs in the lung are more responsive to BaP than are those in the liver, suggesting that the organ-specific expression of miRNAs may be a possible explanation for the selective effects of BaP. Supporting these toxicological outcomes, it was reported that BaP (oral doses of 150 mg kg^{-1} or 300 mg kg^{-1} for 3 days) caused downregulation of miR-150, miR-241-5p, and miR-122 and upregulation of miR-34c, miR-34b-5p, and miR-29b in mouse lung (93). These miRNAs are implicated in a variety of biological processes, including immune responses, cell proliferation, and the cell cycle, which are the main pathways affected at the mRNA level. In additional experiments, miR-638 expression was increased in 68% (34 out of 50 samples) of primary human non-small-cell lung cancer tissues; this finding also suggests the possible involvement of this miRNA in BaP-induced carcinogenesis (94). Presently, miRNAs are expected to be sensitive biomarkers for evaluating environmental chemical exposure, and they may be applicable for human risk assessment.

The tumor suppressor p53 cascade has been extensively studied due to its important physiological role. Loss of p53 function is the most frequent genetic alteration in various types of human cancer, as the transcriptional activity of p53 is critical to its function as a tumor suppressor. Recently it was reported that p53 regulates transcriptional expression and the maturation of a group of miRNAs—in particular, the miR-34 family. Conversely, miR-34 can regulate p53 activity and function by directly repressing p53 or p53 regulators in cells (96). The p53 hot spot, located at codon 249 of exon 7, provides a molecular signature for aflatoxin exposure (97). Ingested aflatoxin B_1, which is metabolized in the liver, can bind to DNA, where it may cause a characteristic inactivation mutation of codon 249 of the p53 gene. This mutation is found in 30–60% of hepatocellular carcinomas in aflatoxin-endemic areas (98). Until now, no reports on the effect of aflatoxins on miRNA expression have been published, but it is plausible that aflatoxins could modulate p53-induced miRNAs.

Effects of Chronic Administration of Drugs or Chemicals on miRNA Expression

Chronic exposure to toxic chemicals in rodents (diet containing 420 ppm tamoxifen for 6, 12, 18, and 24 weeks) can induce alterations in miRNA expression profiles that differ from the alterations induced by acute exposure to such chemicals. For example, tamoxifen, a potent hepatocarcinogen in rats, caused significant differential expression of 33 hepatic miRNAs (20 genes upregulated; 13 genes downregulated) when administered to F344 rats for 24 weeks (99). A significant upregulation of oncogenic miRNAs—such as the miR-17-92 cluster, miR-106a, and miR-34—was observed. Numerous miRNAs, including miR-152 and miR-195, were downregulated in the livers of rats that underwent chronic treatment with tamoxifen. These miRNAs are frequently downregulated in solid tumors (100). Another study (101) demonstrated differential expression of 56 miRNAs (31 genes upregulated; 25 genes downregulated) in mice fed a diet that contained the environmental contaminant RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), at 5 mg kg^{-1} for 28 days. Also observed were a significant upregulation of oncogenic miRNAs and a significant downregulation of tumor-suppressing miRNAs, such as let-7, the miR-17-92 cluster, miR-10b, miR-15, miR-16, miR-26, and miR-181. These findings show that chronic administration of toxic chemicals affects miRNA expression changes in the target tissues in vivo and that these effects differ from those seen with acute administration.

Involvement of miRNAs in Epigenetic Modifications

Epigenetic factors should be considered for a comprehensive understanding of environmental effects on the expression changes at the genome-wide level. There is a growing awareness that histone modification influences pre-mRNA splicing and that pre-mRNA splicing itself influences chromatin organization. In contrast, miRNA can be epigenetically regulated by DNA methylation or specific histone modification. Furthermore, miRNAs can themselves repress enzymes of DNA methylation and histone deacetylation (102). Recently, it was reported that tumor-associated aberrations in the miRNA and epigenetic machineries are widely distributed in human cancer. A meta-analysis of 45 published studies reported that 122 miRNAs were epigenetically regulated in 23 cancer types (102). Several cancer therapeutic strategies have been proposed on the basis of synthetic analogs of miRNAs; however, this field is nascent. At this time, no specific studies on the effects of drugs, chemicals, or environmental pollutants on epigenetics-related enzymes via miRNAs have been performed.

REGULATION OF IMMUNE-RELATED FACTORS BY miRNA AND THE ROLE OF miRNA IN INFLAMMATION- AND IMMUNE-MEDIATED DRUG-INDUCED LIVER INJURY

Recent studies have demonstrated that miRNAs play a crucial role in the development of immune cells and in the function of the immune system, including the differentiation and survival of immune cells, antibody production, and the inflammatory mediator release in the innate and adaptive immune responses. In fact, many studies have demonstrated the involvement of interleukins (ILs) in relation to miRNA-related diseases, such as inflammatory disorders and cancer. For example, miR-125a-5p, which was identified as a target for vascular inflammation, mediates lipid uptake and decreases the secretion of several inflammatory cytokines (e.g., IL-2, IL-6, and TNF-α) in monocyte-derived macrophages (103). The expression of miR-148a, miR-152, and miR-301 decreased in IL-6-overexpressing malignant cholangiocytes. IL-6 can increase the expression of DNA methyltransferase-1, which is a target of miR-148a and miR-152 (104). Because IL-6 signaling is mediated mainly by STAT3 (signal transducer and activator of transcription 3), the expression of STAT3 was knocked down by small interfering RNA, which decreased the IL-6-mediated expression of miR-17-92 (105). miR-21 contributes to the oncogenic potential of STAT3 in multiple myeloma cells. miR-21 induction by IL-6 was strictly STAT3 dependent through a highly conserved enhancer (106). IL-6 and STAT3 are essential cytokine and transcriptional factor molecules, respectively, for the differentiation of Th17 cells from naive T cells. This signaling pathway is essential for the inflammatory immune response as well as cancer susceptibility.

Cytokine production by dendritic cells (DCs) plays a critical role in responding to LPSs. miRNA expression analysis during the tolerized state of THP-1 cells demonstrated only miR-146a overexpression, which suggests the important role of miR-146a in LPS tolerance (107). Reduced inflammatory responses to Toll-like receptor 4 (TLR4), TLR2, and TLR5 ligands were caused by the knockdown of miR-146a, which targets IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6. This result suggests a regulatory effect of miR-146a on the signaling of these TLRs. Transfection of miR-146a into THP-1 cells mimicked LPS priming, which resulted in reduced TNF-α production, whereas transfection of the miR-146a inhibitor largely decreased LPS tolerance (108). Additionally, experiments in murine DCs demonstrated that miR-142-3p was among the most highly expressed endogenous miRNAs, whereas IL-6 was among the most highly expressed mRNAs after LPS stimulation (109). The 3′ UTR of mouse IL-6 was predicted and confirmed to be a target of miR-142-3p by a luciferase reporter-gene assay and in vitro and in vivo knockdown assays. Overall, these results demonstrated that targeting

miRNAs (miR-146a and/or miR-142-3p) was a novel strategy for the treatment of endotoxin-induced adverse effects and mortality. Regarding drug-induced liver injury, halothane-induced, α-naphthylisothiocyanate-induced, and diclofenac-induced liver injuries in mice are mediated by ILs (110–112); thus, involvement of these miRNAs could be of interest.

Cytokines decrease CYP-associated drug metabolism during inflammation and infection (113), which influences susceptibility to various drugs and toxic chemicals. IL-6 decreases both the rifampicin- and phenobarbital-mediated induction of CYP2B6, CYP2C8, CYP2C9, and CYP3A4, whereas the transcriptional activities of PXR and CAR are unaffected by IL-6 (114). The generation of reactive metabolites of drugs and chemicals that are catalyzed by P450s is suggested to be a major factor in initiating drug-induced liver injury. Subsequently, the exacerbation of drug-induced liver injury is affected by increased IL expression. With respect to cytokines and drug-induced liver injury, IL-4 mediates dicloxacillin- and flutamide-induced liver injury in mice (115, 116). Th17 cell differentiation is affected by IL-6 and STAT3. IL-4, IL-5, and STAT6 are essential factors for the differentiation of Th2 cells from naive T cells. Accordingly, immune- and inflammation-mediated drug-induced liver injury might be regulated by miRNAs. We hope that the currently unreported mechanism of idiosyncratic drug-induced liver injury, in which immune- and inflammation-mediated factors are essential, will be clarified by progress in miRNA research as well as by studies of metabolic activation reactions that are catalyzed by P450s.

SNP: single-nucleotide polymorphism

pri-miRNA: primary miRNA

pre-miRNA: precursor miRNA

miRNA-RELATED PHARMACOGENOMICS

The majority of pharmacogenomics research has focused on the role of single-nucleotide polymorphisms (SNPs). Recent large-scale studies have identified 15 million SNPs in the human genome, many of which affect gene function in promoters or in *cis*- or *trans*-regulatory elements by modifying sequences of proteins (117). Therefore, SNPs may affect activities of various enzymes and are associated with differences in drug response as well as the adverse effects of drugs and toxic chemicals. SNPs in primary miRNAs (pri-miRNAs), precursor miRNAs (pre-miRNAs), or mature miRNAs could modify various biological processes by influencing the processing or target selection of miRNAs (118, 119). SNPs in pri-miRNA or pre-miRNA are relatively rare. Only approximately 10% of human pre-miRNAs have documented SNPs, and less than 1% of miRNAs have SNPs in the functional seed sequence region (120). Although SNPs in the seed sequence region of miRNAs may be rare, these SNPs may influence the expression of hundreds of genes and related pathways. An interesting study regarding the association between SNPs in the miRNA sequence and clinical drug therapy was published by Boni et al. (121). A SNP (rs7372209) in the pri-miR-26a gene and a SNP (rs1834306) in the pri-miR-100 gene were significantly associated with the tumor response or time to progression in 61 patients with metastatic colorectal cancer who were treated with 5-fluorouracil and CPT-11 (121). This study is the first to suggest a relationship between the clinical outcome of drug therapy and SNPs in both pri-miRNAs and pre-miRNAs.

The frequency of SNPs in the MRE is low, but MRE-site polymorphisms, which affect the expression levels of relevant genes, may have a relatively higher frequency. Thus, early studies concerning miRNA in pharmacogenomics investigated SNPs in MREs on mRNAs. Many MRE-site polymorphisms are thought to be associated with phenotypes of various types of diseases because a gain of function or loss of function results in changes in the expression of target mRNAs (122). Many correlation analyses between SNPs in MREs and diseases in vitro or in silico were conducted, but only a little functional evidence has been confirmed (122). Human dihydrofolate reductase (DHFR) (123) and sulfotransferase isoform 1A1 (SULT1A1) (124) have been associated with miRNA-related polymorphisms that affect the drug response, adverse reactions, and toxicological outcomes (see below).

Approximately 20,000 MRE-site polymorphisms in miRNAs were systematically searched using Patrocles (**http://www.patrocles.org/Patrocles.htm**) and PolymiRTS (**http://compbio.uthsc.edu/miRSNP/**), databases that allow in silico predictions. However, these predictions should be carefully validated by functional studies. Recent genome-wide association studies (GWAS) performed a genome-wide scan of human variation within validated and predicted miRNA binding target sites. The genetic variants at putative miRNA binding sites are possibly functional and may contribute to phenotypic variation in drug/chemical responses and adverse reactions. From computational analyses using human whole-genome and SNP databases, 87 SNPs were identified in miRNA seed sequences, which may represent functional variants of identified GWAS SNPs (125). Furthermore, miRNAs encoding these 39 seed-site polymorphisms have been reported to be coexpressed with their predicted mRNA targets; however, functional analyses are required.

A report concerning a MRE-site polymorphism in relation to drug-metabolizing enzymes and adverse reactions has demonstrated that a C829T SNP, which occurs naturally at the MRE of miR-24 in the 3′ UTR of human DHFR, causes DHFR overexpression and consequent methotrexate resistance (126). Compared with wild-type cells, cells with the mutant 3′ UTR showed a twofold longer half-life of the DHFR mRNA, showed higher DHFR mRNA and DHFR protein expression levels, and were fourfold more resistant to methotrexate. In a case-control study of childhood leukemia patients, the allelic frequency of C-to-T SNPs is 14.2% in the Japanese population (123).

SULT1A1 is one of the essential enzymes in the activation or detoxification of endo- and xenobiotics as well as of dietary and environmental procarcinogens/promutagens. SULT1A1 activity showed large interindividual variability (9), which may be caused by genetic polymorphism, although the latter would not fully account for such a variation. A SNP in the 3′ UTR (972C>T [rs1042157]) is significantly decreased with the SULT1A1 mRNA level ($p = 0.029$) and with enzymatic activity ($p = 0.012$) (124), demonstrating that miR-631 directly regulates SULT1A1 expression of the 3′ UTR (SNP of 972C>T) in an allele-specific manner. This finding provides new information for risk assessment studies of heterocyclic amines such as *N*-hydroxyarylamines, *N*-hydroxy-heterocyclic amines, and arylhydroxamic acids.

UGT2 families are important enzymes that are involved in the metabolism of multiple endobiotics and xenobiotics. Sun et al. (128) identified a SNP, c.1761T>C (rs3100), in 3′ UTR of the UGT2B15 isoform; this SNP affects the expression of the UGT2B15 mRNA in HepG2, MCF-7, and Caco-2 cell lines. The location of the SNP may create or diminish an unknown miRNA binding site. Another SNP, C3435T, in exon 26 of the MDR1 gene, caused no amino acid change and was associated with the significantly lower duodenal expression of MDR1 and thereby the increased plasma concentrations of digoxin after oral administration (129). Because the full miRNA profile is unlikely to be known, an unknown miRNA binding site may be created or diminished by the SNP. Further experimental evidence is required.

In the near future, drug responses or susceptibility to xenobiotic toxicity will be predicted by large-scale GWAS and follow-up functional experiments. Data indicating which miRNAs are relevant in various phenotypic outcomes will be gathered. This new class of miRNA-related polymorphisms may contribute to the interindividual variability in adverse drug responses and toxicant-induced adverse events.

CIRCULATING miRNA IN BODY FLUIDS AS POTENTIAL TOXICOLOGICAL BIOMARKERS

The presence of stable circulating miRNAs in serum and their potential as cancer markers was first reported by Lawrie et al. (130), and, subsequently, circulating miRNAs have been extensively

studied mainly for the diagnosis and prognosis of cancer diseases, especially at an early stage (131). However, these early studies have been limited by their lack of constant standards in quantification and by inconsistencies in methodologies. The first study of a plasma miRNA profile in liver injury was conducted by Wang et al. (132). The authors reported that acetaminophen increased the levels of miR-122 and miR-192 in mouse plasma in a dose-dependent and exposure-duration-dependent manner that paralleled that of alanine aminotransferase (ALT) and caused histopathological changes in the liver. Subsequently, increased plasma concentrations of miR-122, miR-133a, and miR-124 were reported to correspond to injuries in liver, muscle, and brain tissues, respectively, as each of these is the abundant and specific miRNA in each organ (133). The miR-122 plasma concentration increased more quickly and dramatically than did the aminotransferase activities in the blood samples of liver-injured mice, reflecting the extent of hepatocellular injury (134). This change was more specific for viral-, alcohol-, and chemical-induced liver injury than for other organ damage and was a more stable and reliable biomarker (134). The plasma miRNA profiles in the rat liver injury model could be useful in identifying specific and sensitive biomarkers for differentiating pathogenesis of acute and chronic liver injury, as well as hepatocellular injury, cholestasis, steatosis, steatohepatitis, and fibrosis (135).

These lines of data suggest that plasma miRNAs may be superior noninvasive human biomarkers in distinguishing the different types of liver injury compared with conventional biomarkers, such as ALT and alkaline phosphatase (ALP), although detailed evaluations remain to be performed. Plasma miRNAs might be used to determine the types of liver injury in order to decide on the appropriate therapy, or they might be used to clinically evaluate the progress of liver function recovery following liver injury. Urinary miRNA profiles could diagnose liver injury induced by hepatotoxicants (acetaminophen or carbon tetrachloride) in rats (136). Discerning miRNA-related associations with disease development (137) and conducting comparative miRNA expression profile studies across models will be important for a comprehensive understanding of the physiological implications of miRNA changes. Moreover, can circulating miRNAs affect other signaling pathways? Nevertheless, circulating miRNAs in biological fluids have great potential in toxicological studies, e.g., as a novel, informative, and noninvasive method for extrapolating animal toxicity data to humans.

CONCLUSIONS

The importance of understanding the critical role of miRNAs, their involvement with drug-metabolizing enzymes and nuclear receptors, and implications for toxicological studies is recognized. Increasing interest in miRNA research creates new areas in toxicology: the effects of physiological conditions such as alcohol, diet, smoking, stress, supplements, hormones, medication, or diseases on miRNA expression; the effects of inter- and intraindividual differences in miRNA expression on drug response or susceptibility to xenobiotic toxicants; the effects of miRNA and/or target mRNA genetic polymorphisms on drug response, pharmacokinetics, and pharmacodynamics; and the identification of new noninvasive biomarkers to extrapolate animal-derived toxicology data to humans. miRNAs will contribute to the comprehensive understanding of drug- or chemical-induced toxicity, which will facilitate drug development and clinical pharmacotherapy.

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