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F. 健康危険情報

該当なし。

G. 研究発表

1. 論文発表

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H. 知的財産券の出願・登録

該当なし。

III. 研究成果の刊行に関する一覧表

別紙4
研究成果の刊行に関する一覧表

総説

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tsuyoshi Yokoi and Miki Nakajima	microRNAs as mediators of drug toxicity	Annu. Rev. Pharmacol. Toxicol	53	377-400	2013
Tatsuki Fukami and Tsuyoshi Yokoi	The emerging role of human esterases	Drug Metab. Pharmacokinet.	27	466-477	2012
中島　美紀	シトクロムP450と転写因子の microRNAによる発現制御	薬学雑誌	132	107-116	2012

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shingo Oda, Tatsuki Fukami, Tsuyoshi Yokoi, and Miki Nakajima	Epigenetic regulation is a crucial factor in the repression of UGT1A1 expression in human kidney	***Drug Metabolism and Disposition***		in press	2013
Masakazu Kakuni, Mayu Morita, Kentaro Matsuo, Yumiko Katoh, Miki Nakajima, Chise Tateno, and Tsuyoshi Yokoi	Chimeric mice with a humanized liver as an animal model of troglitazone-induced liver injury	***Toxicology Letters***	214	9-18	2012
Satonori Higuchi, Azusa Yano, Shohei Takai, Koichi Tsuneyama, Tatsuki Fukami, Miki Nakajima, and Tsuyoshi Yokoi	Metabolic activation and inflammation reactions involved in carbamazepine-induced liver injury	***Toxocological Sciences***	130	4-16	2012
Taishi Miyashita, Yasuyuki Toyoda, Koichi Tsuneyama, Tatsuki Fukami, Miki Nakajima, and Tsuyoshi Yokoi	Hepatoprotective effect of tamoxifen on steatosis and non-alcoholic steatohepatitis in mouse models	***Journal of Toxocological Sciences***	37	931-942	2012
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Shingo Oda, Miki Nakajima, Masahiko Hatakeyama, Tatsuki Fukami, and Tsuyoshi Yokoi	Preparation of a specific monoclonal antibody against human UGT1A9 and evaluation of UGT1A9 protein levels in human tissues	***Drug Metabolism and Disposition***	40	1620-1627	2012
Azusa Yano, Satonori Higuchi, Koichi Tsuneyama, Tatsuki Fukami, Miki Nakajima, and Tsuyoshi Yokoi.	Involvement of immune-related factors in diclofenac-induced acute liver injury in mice.	***Toxicology***	293	107-114	2012
Masanori Kobayashi, Satonori Higuchi, Mika Ide, Satomi Nishikawa, Tatsuki Fukami, Miki Nakajima, and Tsuyoshi Yokoi	Th2 cytokine-mediated methimazole-induced acute liver injury in mice.	***Journal of Applied Toxicology***	32	823-833	2012
Yu Yamaura, Miki Nakajima, Shingo Takagi, Tatsuki Fukami, Koichi Tsuneyama, and Tsuyoshi Yokoi	Plasma microRNA profiles in rat models of hepatocellular injury, cholestasis, and steatosis	***PLoS ONE***	7	e30250	2012
Yasuyuki Toyoda, Shinya Endo, Koichi Tsuneyama, Taishi Miyashita, Azusa Yano, Tatsuki Fukami, Miki Nakajima, and Tsuyoshi Yokoi	Mechanism of exacerbative effect of progesterone on drug-induced liver injury.	***Toxicological Sciences***	126	16-27	2012
Satonori Higuchi, Masanori Kobayashi, Azusa Yano, Koichi Tsuneyama, Tatsuki Fukami, Miki Nakajima, and Tsuyoshi Yokoi	Involvement of Th2 cytokines in the mouse model of flutamide-induced acute liver injury.	***Journal of Applied Toxicology***	32	815-822	2012

VI. 研究成果の刊行物・別刷

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microRNAs as Mediators of Drug Toxicity

Tsuyoshi Yokoi and Miki Nakajima

Department of Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920-1192, Japan; email: tyokoi@p.kanazawa-u.ac.jp, nmiki@p.kanazawa-u.ac.jp

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Keywords

P450, transcriptional factor, toxicology, polymorphism, posttranscriptional regulation

Abstract

microRNAs (miRNAs) represent the most abundant class of gene expression regulators that bind complementarily to transcripts to repress their translation or mRNA degradation. These small (21–23 nucleotides in length) noncoding RNAs are derived through a multistep process by miRNA genes located in genomic DNA. Because miRNAs regulate fundamental cellular functions, their dysregulation affects a large range of physiological processes, such as development, immune responses, metabolism, and diseases as well as toxicological outcomes. Cancer-related miRNAs have been extensively studied; however, the roles of miRNAs in xenobiotic metabolism and in toxicology have only recently been explored. This review focuses on the current knowledge of miRNA-dependent regulation of drug-metabolizing enzymes and nuclear receptors and the associated potential toxicological implications. The potential modulation of toxicology-related changes in miRNA expression, the role of miRNA in immune-mediated drug-induced liver injuries, the use of circulating miRNAs in body fluids as potential toxicological biomarkers, and the link between miRNA-related pharmacogenomics and adverse drug reactions are highlighted.

INTRODUCTION

miRNA: microRNA

UTR: untranslated region

mRNA: messenger RNA

P450s, CYPs: cytochrome P450s

microRNAs (miRNAs) comprise a class of short noncoding single-stranded RNAs that regulate gene expression posttranscriptionally by binding to 3′ untranslated regions (UTRs), coding sequences, or 5′ UTRs of target messenger RNAs (mRNAs), leading to inhibition of translation or mRNA degradation. Currently, approximately 2,000 human miRNAs have been identified, and more than a half of the known miRNAs are conserved across vertebrate animals (1). Genes that encode for miRNAs are located in intergenic or intragenic (intronic or exonic) regions with both sense and antisense orientations. In humans, miRNAs are distributed as intergenic (52%), intronic (43%), and exonic (5%) (2). In silico prediction estimates that approximately 60% of human mRNAs could be targets of miRNA (3). Like mRNA, miRNAs are expressed in a tissue- or cell-specific manner. miRNAs are now clearly recognized to be involved in posttranscriptional gene regulation and contribute to cellular processes, such as development, differentiation, and cell signaling, as well as pathological and physiological processes, such as disease development, immune response, drug response, and carcinogenesis (4). The biogenesis of miRNA (i.e., how miRNAs are generated and processed, exported to the cytoplasm, and regulated) has been intensively investigated and well reviewed in previous publications (5–7). The development of miRNA-related experimental techniques—such as in silico target site predictions, precursor miRNA transfection or the use of miRNA oligonucleotides for overexpressing or inhibiting miRNA, and immunoprecipitation of RNA-induced silencing complex (RISC) followed by sequence analyses—has enabled us to identify miRNAs that are involved in numerous physiological and toxicological phenotypes.

In the fields of drug metabolism and toxicology, miRNA-related research is progressing. Because most drugs and chemical toxicants are biotransformed to exhibit their functions (i.e., forming detoxified metabolites or toxic metabolites), the expression of drug- or xenobiotic-metabolizing enzymes and their miRNA regulation are important for understanding toxicological phenotypes. The purpose of this review is to summarize the recent findings concerning the roles of miRNA in regulating cytochrome P450s (P450s, CYPs) and nuclear receptors as well as the toxicological significance of miRNAs by considering their potential use as biomarkers and/or in predictive toxicity.

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FUNCTIONAL CHARACTERIZATION OF IDENTIFIED TARGET GENES OF miRNAs

Gene names convey basic information concerning the functional relationships among mature miRNAs (8). For example, the human miRNA hsa-miR-22 and the mouse miRNA mmu-miR-22 are orthologous. Paralogous sequences, in which mature miRNAs differ at only one or two positions, are given lettered suffixes, such as miR-10a and miR-10b. Distinct hairpin loci that result in identical mature miRNAs have numbered suffixes, such as miR-281-1 and miR-281-2. The passenger strand, termed miRNA*, is usually degraded, although it may be functional.

Each miRNA may suppress multiple mRNA targets. Moreover, one mRNA can be targeted by multiple miRNAs, enabling the control of a wide range of cellular processes. Because miRNA binds to the target mRNA with partial complementarity over a short sequence, computational identification of the miRNA target gene is difficult. The 5′ end of the miRNA contains six to seven nucleotides known as a seed sequence, which is essential for the function of the miRNA. There are several free, accessible Internet sites that predict the miRNAs for target genes, such as miRanda (9; **http://www.microrna.org/**), TargetScan (10; **http://www.targetscan.org/**), and PicTar (11; **http://pictar.mdc-berlin.de/**). A general algorithm that predicts the precise target gene of each miRNA has not been established; thus, each program generates different results.

Studies to confirm the specific miRNA-mRNA interactions, such as luciferase reporter-gene assays that contain the miRNA recognition element (MRE) of the target, are commonly conducted. The reporter gene is cotransfected into cells with a precursor miRNA or with an expression plasmid that encodes the miRNA or antisense oligonucleotides complementary to the miRNA; this precursor miRNA may also be transfected to overexpress or inhibit miRNA. The miRNA-mRNA interactions are determined on the basis of whether the luciferase activity significantly decreases or increases compared with the activity in the control. When reporter-gene assays are utilized, the methodology should evaluate the effects on the candidate gene of the full length of the 3′ UTR or whether other endogenous miRNAs affect the candidate gene. The overexpression of miRNAs is a useful method to elucidate the effects of a miRNA on gene expression targets. However, aberrant cellular functions become apparent when miRNAs exceed physiological levels within cells. Therefore, the use of normal physiological miRNA concentrations in vivo is preferred. Furthermore, determining target mRNA or protein expression changes by microarray or proteome analysis after the overexpression and/or inhibition of miRNA is useful (12, 13). However, these assays are likely to show effects that are caused by miRNAs acting on secondary targets. Immunoprecipitation of RISC and sequence analyses are also informative methods (14).

MRE: miRNA recognition element

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THE ROLE OF miRNAs IN REGULATING NUCLEAR RECEPTORS AND ENZYMES THAT METABOLIZE XENOBIOTICS

P450s are important enzymes that catalyze the detoxification of xenobiotics, such as drugs, environmental chemicals, and carcinogens. P450s also bioactivate many drugs and procarcinogens to their toxic metabolites in a process termed metabolic activation. The different P450 isozyme expression profiles determine the amount of reactive intermediates formed and the resulting toxic response. The regulation of enzyme expression is important in the individual variability of P450 activities. Whereas mechanisms of P450 transcriptional regulation by nuclear receptors have been well studied, posttranscriptional regulation largely remains unknown. Recently, many P450s and nuclear receptors (**Table 1**) have been found to be regulated posttranscriptionally by miRNAs, thus establishing an additional means for modulating detoxification mechanisms and drug and chemical metabolic activation.

Human CYP1B1

Human CYP1B1, expressed mainly in ovarian, uterine, and breast tissues (15, 16), catalyzes the metabolic activation of a variety of procarcinogens and promutagens, including polycyclic aromatic hydrocarbons and aryl amines (16), and 17β-estradiol metabolism (17), which contributes to the growth and development of estrogen-dependent cancers (18). 4-Hydroxyestradiol, a catechol-type metabolite formed by CYP1B1, generates free radicals from reductive-oxidative cycling with the semiquinone and quinone forms, which cause DNA damage (19, 20). There is no apparent difference in the CYP1B1 mRNA levels between tumor and normal tissues (21), whereas the expression of CYP1B1 protein and its enzymatic activity are much higher in various types of malignant cancers than they are in normal tissues (22). Posttranscriptional regulation has been suggested as a mechanism for this difference.

The first study (23) to demonstrate that miRNA can regulate any CYP was conducted on human CYP1B1. Specifically, human CYP1B1 is regulated by miR-27b (24). In Jurkat (miR-27-negative) cells, exogenously expressed miR-27b decreased luciferase activity; in MCF-7 (miR-27-positive) cells, an antisense oligonucleotide to miR-27b restored the luciferase activity and increased the protein level and enzymatic activity of endogenous CYP1B1 (23). These findings strongly suggest

Table 1 Drug-metabolizing enzymes and nuclear receptors that are regulated by microRNAs

Target	miRNA	Reference
CYP1B1	miR-27b	23
CYP2A3 (rat)	miR-126*	34
CYP2E1	miR-378	29
CYP3A4	miR-27b	46
CYP24A1	miR-125b	55
ARNT	miR-24	65
ERα	miR-206	66
	miR-221/222	67
	miR-22	68
GR	miR-18, miR-124a	138
HIF-1α	miR-17	82
HNF4α	miR-24a, miR-34	60
PPARα	miR-21, miR-27b	72
PPARγ	miR-27a	75
		76
	miR-27b	73
		74
PXR	miR-148a	45
RXRα (rat)	miR-27	77
VDR	miR-125b	56
	miR-27b	46
DHFR	miR-24	126
SULT1A1	miR-631	124
Nrf2	miR-28	139
Keap1	miR-200a	140
MMP1	miR-222	80
Thioredoxin reductase	miR-298, miR-370	86
Mitochondrial antioxidant enzymes	miR-17*	81

that human CYP1B1 is posttranscriptionally regulated by miR-27b. Extending the work to breast cancer patients revealed decreased miR-27b expression and increased CYP1B1 protein levels in 24 cancerous tissues compared with noncancerous tissues ($p < 0.0005$) in each patient. Because miR-27b targets CYP1B1 mRNA, the decreased miR-27b expression is one of the causes of high expression of CYP1B1 protein. Furthermore, although CYP1B1-mediated 4-hydroxylation of estrogen decreases estrogenic activity, the metabolite (4-hydroxyestradiol) is toxicologically active. Accordingly, miR-27b levels may contribute to estrogen-dependent molecular mechanisms of carcinogenesis (**Figure 1**).

Human CYP2E1

Human CYP2E1 catalyzes the metabolism of numerous low-molecular-weight xenobiotics, including drugs (e.g., acetaminophen, isoniazid, and bromobenzene), organic solvents (e.g., ethanol, acetone, carbon tetrachloride, chloroform, vinyl chloride, glycerol, hexane, and toluene), and procarcinogens (e.g., *N*-nitrosodimethylamine, *N*-nitrosomethylethylamine, and

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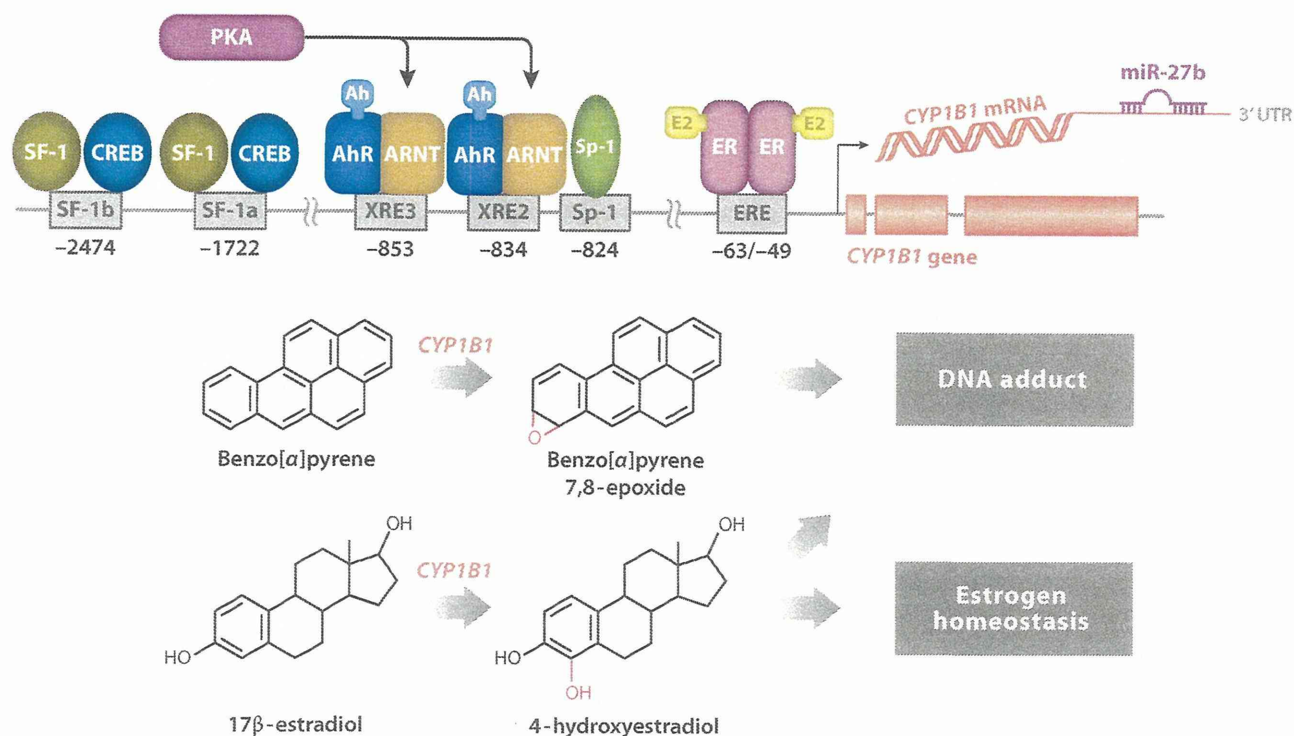

Figure 1

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.

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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

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.

BaP: benzo[*a*]pyrene

PXR: pregnane X receptor

CAR: constitutive androstane receptor

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

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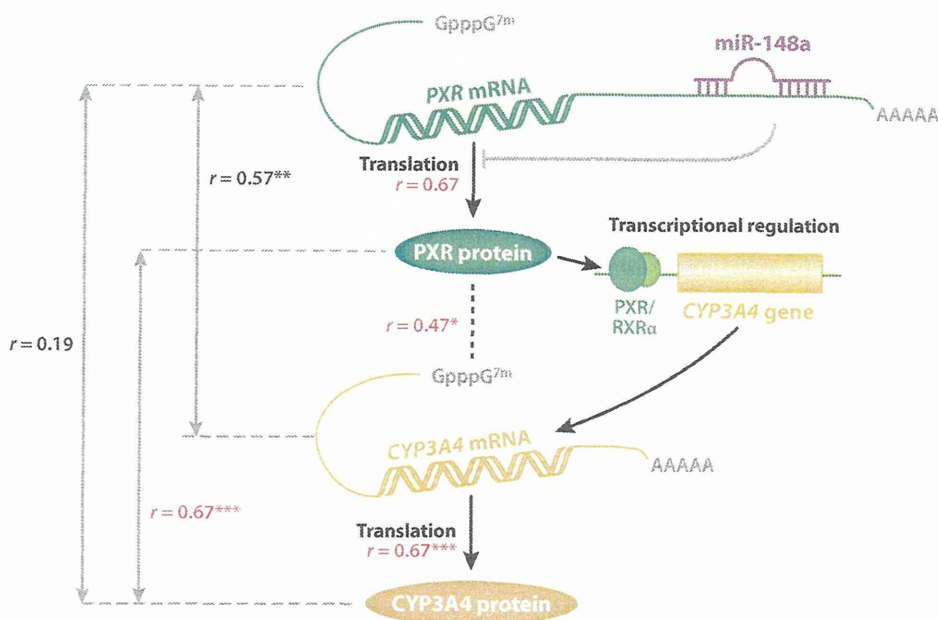

Figure 2

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

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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.

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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**).

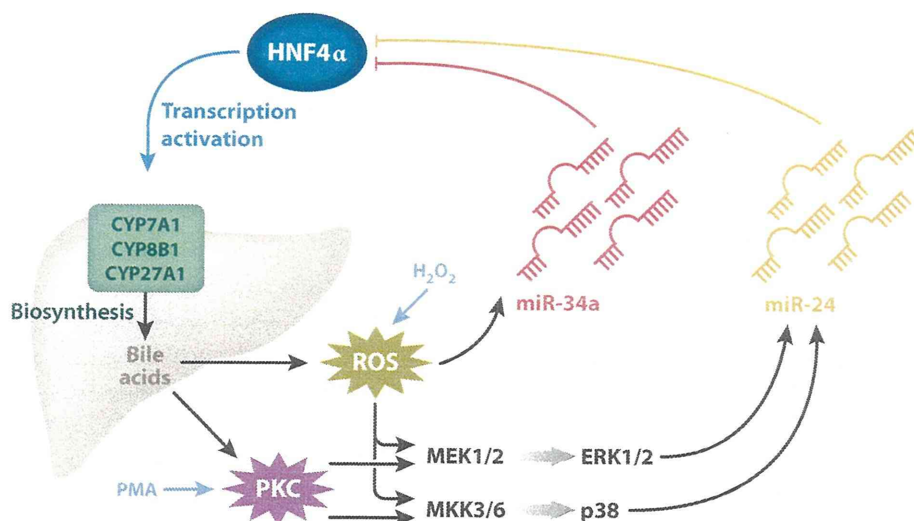

Figure 3

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.

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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.

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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.

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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

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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.

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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