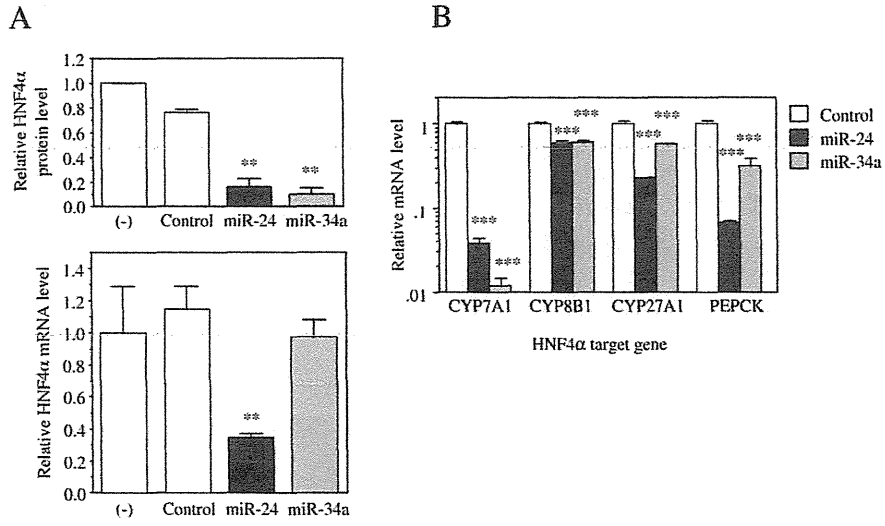


Fig. 13. Effects of miR-24 and miR-34a on the Human HNF4α Protein or mRNA Levels (A) and Its Downstream Genes (B)

The precursors for miR-24, miR-34a or control (50 nM) were transfected into HepG2 cells. After 48 h, total RNA and whole cell lysates were prepared. The HNF4α protein levels were determined by Western blot analysis and normalized with GAPDH protein level (A). The HNF4α mRNAs levels (A) and the CYP7A1, CYP8B1, CYP27A1, and PEPCK mRNA levels (B) were determined by real-time RT-PCR analysis and normalized with GAPDH mRNA level. Each column represents the mean±S.D. of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$.

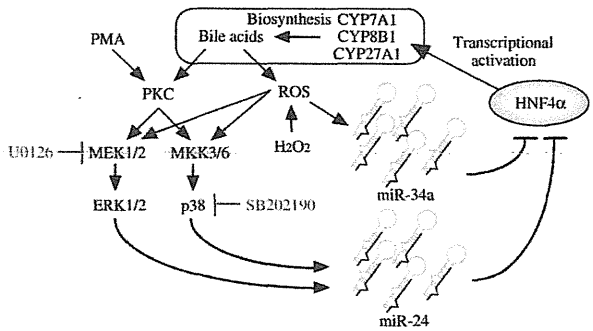


Fig. 14. The Regulatory Loop of miR-24, miR-34a and HNF4α in Bile Acid Synthesis

Bile acids are known to activate protein kinase C (PKC) and reactive oxygen species (ROS) generation, resulting in the activation of mitogen-activated protein kinase (MAPK) pathway. The expression of miR-24 and miR-34a is induced by MAPK-dependent and -independent pathways, respectively. In turn, miR-24 and miR-34a negatively regulate the HNF4α. The down-regulation of HNF4α decreases the expression of bile acid-synthesizing enzymes CYP7A1 and CYP8B1 resulting in the decrease of bile acids. ERK; extracellular signal-regulated kinase, MEK; MAPK/ERK kinase, MKK; mitogen-activated protein kinase kinase, PMA; phorbol 12-myristate 13-acetate.

ぼし，酵素の発現量に個人差をもたらす原因となっていることが報告された．mature miRNA 上に遺伝子多型がある場合も同様に発現制御機能に影響を及ぼす可能性もあり，pri-miRNA や pre-miRNA 上に遺伝子多型がある場合は mature miRNA の発現量の変動をもたらし，それが標的遺伝子の発現変動をもたらすこともある.[13] ファーマコジェネティクスの研究領域に miRNA を取り込むことで，これまで解明できなかった薬効・副作用の個人差が解明できる可能性があり，今後の研究の発展が望まれる．

miRNA の発現は様々な疾患において変動する.[13] また，薬物，毒物，発がん物質などの曝露や，ストレスに応答して miRNA の発現が変動することも示されている.[13] このような miRNA 発現の変動が，薬物の体内動態にどの程度影響を及ぼしているか解明することは今後の課題である．

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# Toxicological Implications of Modulation of Gene Expression by MicroRNAs

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**MicroRNAs (miRNAs) are a large family of non-coding RNAs that are evolutionarily conserved, endogenous, and 21–23 nucleotides in length. miRNAs regulate gene expression by targeting messenger RNAs (mRNAs) by binding to complementary regions of transcripts to repress their translation or mRNA degradation. miRNAs are encoded by the genome, and more than 1000 human miRNAs have been identified so far. miRNAs are predicted to target ~60% of human mRNAs and are expressed in all animal cells and have fundamental roles in cellular responses to xenobiotic stresses, which affect a large range of physiological processes such as development, immune responses, metabolism, tumor formation as well as toxicological outcomes. Recently, many reports concerning miRNAs related to cancer have been published; however, the miRNA research in the metabolism of xenobiotics and endobiotics and in toxicology has only recently been established. This review describes the current knowledge on the miRNA-dependent regulation of drug-metabolizing enzymes and nuclear receptors and its potential toxicological implications. In this review, miRNAs with reference to target prediction, potential modulation of toxicology-related changes of miRNA expression, role of miRNA in immune-mediated drug-induced liver injury, miRNA in plasma as potential toxicological biomarkers, and relevance of miRNA-related genetic polymorphisms are discussed.**

*Key Words:* microRNA; miRNA; P450; CYP; posttranscriptional regulation; toxicology; polymorphism.

MicroRNAs (miRNAs) are short (~22 to 25 nucleotides in length), single-stranded RNA genes possessing the reverse complement of the messenger RNA (mRNA) transcript of another protein-coding gene. miRNA was first found in *Caenorhabditis elegans* as RNA molecules that are complementary to the 3′ untranslated regions (UTRs) of the target transcript, such as *lin-4* (Lee *et al.*, 1993) and *let-7* (Lau *et al.*, 2001) genes. The development of the *C. elegans* was regulated by their respective targets. The miRNAs demonstrated diverse expression patterns during development and were found in diverse organisms, ranging from worms to humans (Lagos-Quintana *et al.*, 2003), suggesting that these molecules represent a gene family that has evolved from an ancient ancestral small RNA gene.

For nomenclature, miRNAs are assigned sequential numerical identifiers. The gene names are intended to convey limited information about functional relationships between mature miRNAs (Griffiths-Jones *et al.*, 2006). For example, has-miR-101 in human and mmu-miR-101 in mouse are orthologous, and more than half of the known miRNAs are conserved across vertebrate animals (Lagos-Quintana *et al.*, 2003). Paralogous sequences whose mature miRNAs differ at only one or two positions are given lettered suffixes, such as miR-10a and miR-10b. Distinct hairpin loci that give rise to identical mature miRNAs have numbered suffixes, such as miR-281-1 and miR-281-2. The passenger strand, named miRNA*, is usually degraded, although it is sometimes functional.

The single strand form of mature miRNA is selectively loaded onto the RNA-induced silencing complex, composed of RNase III Dicer, TAR RNA-binding protein, and Argonaute protein Ago2 and guides the complex to its mRNA targets with imperfect pairing causing cleavage or translational repression of targeted mRNAs resulting in gene silencing (Bartel, 2004). Mechanisms for gene regulation and biogenesis of miRNAs have been described in detail in other review articles (Chekulaeva and Filipowicz, 2009; Choudhuri 2010; Fabian *et al.*, 2010). *In silico* prediction estimates that ~60% of human mRNAs could be targets of miRNAs (Friedman *et al.*, 2009). Similar to mRNA, miRNA are expressed in a tissue- or cell-specific manner. miRNAs can potentially regulate every aspect of cellular processes such as differentiation, proliferation, apoptosis, and necrosis as well as a large range of physiological processes (Kloosterman and Plasterk, 2006).

There is a growing body of research on the role of miRNAs in toxicogenomics/toxicogenetics and the possibility of drug-

induced adverse effects. Because most drugs and chemical toxicants are biotransformed to exhibit their functions, the expression of drug- and xenobiotic-metabolizing enzymes and their regulation by miRNA is a potentially important determinant of the efficacy and toxicity. Although miRNA research in the metabolism of xenobiotics/endobiotics and in toxicology is still in its infancy, understanding of miRNAs is progressing rapidly. The purpose of this review is to summarize recent findings concerning the roles of miRNA in the regulation of cytochrome P450 (P450, CYP) and nuclear receptors and consider their potential relevance and application for toxicological studies.

## IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF miRNA TARGET GENE

Computational identification of miRNA target genes is challenging because miRNA bind to the target mRNA with partial complementarity over a short sequence. The 5′-region of miRNA of six to seven nucleotides is called the "seed sequence", and the 3′-mismatch is called the tolerant region (Mishra and Bertino, 2009). The seed sequence is critical and sometimes sufficient to repress the target translation (Lewis *et al.*, 2003). A number of freely accessible and useful miRNA database are available as summarized in Table 1. Several computational algorithms such as MiRanda (John *et al.*, 2004), TargetScan (Lewis *et al.*, 2003), and PicTar (Krek *et al.*, 2005) are also available to identify putative binding sites of miRNA to the target genes. A general algorithm to predict the target gene of miRNA has not been established, such that each *in silico* program can lead to different results due to the variable weight placed on the complementarity to the miRNA seed sequence, evolutionary conservation of the miRNA recognition element (MRE) of the target gene, free energy of the miRNA-mRNA duplex binding, and accessibility of the target site. Presently, the false-positive rate of the predicted candidate targets of a given miRNA is thought to be 30–50% (Alexiou *et al.*, 2009; Watanabe *et al.*, 2007), although additional enrichment analysis would help identify the most promising candidates (Hu *et al.*, 2007).

TABLE 1
*In Silico* Programs for the Prediction of miRNA Targets

| | |
|---|---|
| DIANA microT | http://diana.cslab.ece.ntua.gr/microT/ |
| EMBL-Target Gene Prediction | http://www.russelllab.org/miRNAs/ |
| MicroCosm | http://www.ebi.ac.uk/enrightsrv/microcosm/htdocs/ |
| MicroRNAdb | http://bioinfo.au.tsinghua.edu.cn/micrornadb/ |
| miRanda | http://www.microrna.org/microrna/home.do |
| miRBase | http://microrna.sanger.ac.uk/ |
| miRGator | http://genome.ewha.ac.kr/miRGator/ |
| mirnaviwer | http://cbio.mskcc.org/mirnaviewer/ |
| miRWalk | http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/ |
| PicTar | http://pictar.org/ |
| PITA | http://genie.weizmann.ac.il/pubs/mir07/index.html |
| RNA22 | http://cbcsrv.watson.ibm.com/rna22.html |
| RNAhybrid | http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/ |
| TargetRank | http://hollywood.mit.edu/targetrank/ |
| TargetScan | http://www.targetscan.org/ |

Confirmation and validation of the specific miRNA-mRNA interaction is most commonly addressed using luciferase reporter gene assays containing the MRE of the target downstream of the reporter gene. The constructs are cotransfected into cells with precursor miRNA (or the expression plasmid of miRNA) or antisense oligonucleotide for miRNA to overexpress or inhibit miRNA, and the reporter activity will establish whether the response is significantly decreased or increased compared with control. However, with reporter-based assays, it is necessary to examine whether the observed regulation would occur with the full-length UTR or whether other endogenous miRNAs regulate the candidate gene of interest. The overexpression and inhibition of miRNA are effective methods for determining the effects of miRNA on the target gene expression and understanding the biological function of miRNA. However, in overexpression experiments, a possible concern is that the concentration of miRNA may exceed physiological levels in the cells due to saturation of nuclear karyopherin exportin-5 resulting in potential aberrant cellular functions. The adverse effect of oversaturating endogenous small RNA pathways can be minimized by optimizing dose and sequence. In addition to the above direct methods, it is useful to determine the change of target mRNA or protein expression by microarray or proteome analysis after the overexpression and/or inhibition of miRNA (Beak *et al.*, 2008; Lim *et al.*, 2005). However, some caution is also required when using whole genomic or proteomic data because these platforms may also identify secondary targets, the expression of which may change as a result of changes in the expression of the primary targets.

In this review, we highlight the state of science regarding the potential roles for miRNAs in the regulation genes involved in xenobiotic metabolism, nuclear receptors, genetic polymorphisms, and broad toxicologic responses. The emerging field of miRNAs as potential biomarkers of toxicity is also presented. A summary of the miRNAs discussed herein is provided in Table 2.

## ROLE OF miRNAs IN REGULATING NUCLEAR RECEPTORS AND ENZYMES THAT METABOLIZE XENOBIOTICS/ENDOBIOTICS

The expression of drug- and xenobiotic-metabolizing enzymes and nuclear receptors and their regulation by miRNA could be important factors for the outcomes of toxicity.

TABLE 2
Nuclear Receptors and Drug Metabolizing Enzymes-Related MicroRNAs

| Target | miRNA | Reference |
|---|---|---|
| PXR | miR-148a | Takagi *et al.* (2008) |
| VDR | miR-125b | Mohri *et al.* (2009) |
| | miR-27b | Pan *et al.* (2009) |
| PPARα | miR-21, miR-27b | Kida *et al.* (2011) |
| PPARγ | miR-27a | Kim *et al.* (2010) |
| | | Lin *et al.* (2009) |
| | miR-27b | Karbiener *et al.* (2009) |
| | miR-130 | Lee *et al.* (2010) |
| RXRα (rat) | miR-27 | Ji *et al.* (2009) |
| HIF-1α | miR-17 | Taguchi *et al.* (2008) |
| HNF4α | miR-24a, miR-34 | Takagi *et al.* (2010) |
| ERα | miR-206 | Adams *et al.* (2007) |
| | miR-221/222 | Zhao *et al.* (2008) |
| | miR-22 | Xiong *et al.* (2010) |
| GR | miR-18, miR-124a | Vreugdenhil *et al.* (2009) |
| CYP1B1 | miR-27b | Tsuchiya *et al.* (2006) |
| CYP2A3 (rat) | miR-126* | Kalsheuer *et al.* (2008) |
| CYP2E1 | miR-378 | Mohri *et al.* (2010) |
| CYP3A4 | miR-27b | Pan *et al.* (2009a) |
| CYP24A1 | miR-125b | Komagata *et al.* (2009) |
| DHFR | miR-24 | Mishra *et al.* (2007) |
| SULT1A1 | miR-631 | Yu *et al.* (2010) |
| Thioredoxin reductase | miR–298, miR-370 | Fukushima *et al.* (2007) |
| Mitochondrial antioxidant enzymes | miR-17* | Xu *et al.* (2010) |

Members of the cytochrome P450 (P450, CYP) family are the most important enzymes catalyzing the metabolism of xenobiotics including drugs, environmental chemicals, and carcinogens. The different profiles of the expression of P450 isoenzymes determine the amount of reactive intermediates formed and the resulting toxic response. P450s are also known to bioactivate many procarcinogens to their ultimate carcinogens. Whereas the mechanisms of the transcriptional regulation of P450-related nuclear receptors have been well studied, the posttranscriptional regulation largely remains unknown. Recently, some P450s and nuclear receptors have been found to be posttranscriptionally regulated by miRNAs and are summarized herein.

*Human CYP1B1*

Human CYP1B1, expressed mainly in ovary, uterus, and breast (Shimada *et al.*, 1996; Sutter *et al.*, 1994), catalyzes the metabolic activation of a variety of procarcinogens and promutagens, including polycyclic aromatic hydrocarbons and aryl amines , and the metabolism of 17β-estradiol (Hayes *et al.*, 1996; Lee *et al.*, 2003; Spink *et al.*, 1997), which contributes to the growth and development of estrogen-dependent cancers such as breast and endometrial cancers (Henderson and Canellos, 1980). 4-Hydroxyestradiol, a catechol-type metabolite formed by CYP1B1, generates free radicals from reductive-oxidative cycling with the corresponding semiquinone and quinone forms, which cause DNA damage (Han and Liehr, 1994; Newbold and Liehr, 2000). It should be noted that there is no apparent difference in the CYP1B1 mRNA levels between tumor and normal tissues (Cheung *et al.*, 1999; Ragavan *et al.*, 2004), whereas the expression of CYP1B1 protein and its enzymatic activity is much higher in various types of malignant cancers compared with normal tissues (Murray *et al.*, 1997). Post-translational regulation of human CYP1B1 expression was suggested to result from polymorphism-dependent degradation of CYP1B1 protein by polyubiquitination but not phosphorylation (Bandiera *et al.*, 2005).

The first study to demonstrate that miRNA can regulate any CYP was reported for human CYP1B1. Specifically, human CYP1B1, which is highly expressed in estrogen target tissues (Tsuchiya *et al.*, 2004), is regulated by miR-27b in MCF-7 breast cancer cells (Tsuchiya *et al.*, 2006). Exogenously expressed miR-27b decreases the luciferase reporter activity in Jurkat cells (miR-27 negative). In MCF-7 cells (miR-27 positive), an antisense oligonucleotide to miR-27b restored the luciferase reporter activity and increased the protein level and enzymatic activity of endogenous CYP1B1 (Tsuchiya *et al.*, 2006). These lines of evidence strongly suggest that human CYP1B1 is posttranscriptionally regulated by miR-27b. Extending the work to breast cancer patients, the expression of miR-27b was decreased in most patients and that of CYP1B1 protein was increased in 24 cancerous tissues compared with noncancerous tissues ($p < 0.0005$) in each patient. Because miR-27b targets CYP1B1 mRNA, the decreased expression of miR-27b is one of the causes of the high expression of CYP1B1 protein. Furthermore, although 4-hydroxylation of estrogen by CYP1B1 decreases estrogenic activity, this metabolite is toxicologically active. Accordingly, miR-27b levels may contribute to estrogen-dependent molecular mechanisms of carcinogenesis.

*Human CYP3A4 and Pregnane X Receptor*

Human CYP3A4 is the most important CYP enzyme in facilitating the metabolism and elimination of a wide range of structurally different xenobiotics including more than 50% of all clinically relevant drugs (Bertz and Granneman, 1997). The CYP3A4 phenotype has been assessed using several substrates (e.g., midazolam and erythromycin), which revealed that there is at least sixfold interindividual variation of the activities in most populations (Floyd et al. 2003; Lin *et al.*, 2002; Rodriguez-Antona *et al.*, 2005), and this population variability cannot be explained solely by genetic polymorphisms (Floyd *et al.*, 2003; Lamba *et al.*, 2002). CYP3A4 expression is largely regulated at the transcriptional level by transcriptional factors, such as CCAAT/enhancer binding proteins, C/EBPα and C/EBPβ, and hepatocyte nuclear factors, hepatocyte nuclear factor 4α (HNF4α) and

HNF3γ, as well as constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Martinez-Jimenez *et al.*, 2007). Animal and human CYP3A enzymes are also involved in the metabolic activation of several drugs and xenobiotics to toxic metabolites (Thummel *et al.*, 1993). Human hepatocytes are ideal for *in vitro* cytotoxicity screening assays, but their overall utility is hampered by large interindividual variability of enzyme activity. A highly sensitive cell-based screening method for CYP3A4-dependent metabolic activation using HepG2 cells was demonstrated, and with this method, the cytotoxicity of drugs was efficiently evaluated (Hosomi *et al.*, 2010, 2011). Notably, aflatoxin B1 and G1 and benzo[*a*]pyrene (BaP) are known to be oxidized efficiently to genotoxic metabolite(s) by CYP3A (Forrester *et al.*, 1990; Shimada *et al.*, 1989).

The role of miRNA in the regulation of the expression of CYP3A4 has been reported (Takagi *et al.*, 2008). In a panel of 25 human livers, PXR mRNA level was not correlated with PXR protein, suggesting the involvement of posttranscriptional regulation. However, no involvement of miRNAs was suggested in CYP3A4 by the correlation analyses between the CYP3A4 mRNA level and CYP3A4 protein level. In contrast, a potential miR-148a recognition element was identified in the 3′-UTR of human PXR mRNA. A reporter assay revealed that miR-148a could recognize the miR-148a recognition element of PXR mRNA. Consequently, overexpression of miR-148a resulted in a reduction of the PXR protein, whereas inhibition of miR-148a by using antisense oligonucleotides increased the PXR protein level. The miR-148a-dependent decrease of PXR protein attenuated the induction and/or constitutive levels of CYP3A4 mRNA. Furthermore, the translational efficiency of PXR (PXR protein/PXR mRNA ratio) was inversely correlated with the expression levels of miR-148a in a panel of human livers. Actually, a potential miR-148a recognition element in the 3′-UTR of human CYP3A4 mRNA did not regulate CYP3A4 message level. There is one literature report that CYP3A4 protein in LS180 and human pancreas cancer-derived PANC1 cells was decreased by the overexpression of miR-27b, a result accompanied by a decrease of the CYP3A4 mRNA level (Pan *et al.*, 2009). In that report, only the overexpression of miR-27b was evaluated, and ideally, the results from inhibiting endogenous miRNA as well as a correlation analysis between the miRNA and target mRNA levels are necessary to fully evaluate the potential regulation of miRNAs. It has also been shown that PXR protein levels were not significantly correlated with CYP2B6 or MDR1 mRNA levels in the panel of human livers. Thus, it was speculated that the PXR level does not largely affect the constitutive expression of CYP2B6 and MDR1 in the liver. In an induction study, the induction of CYP2B6 (twofold) and MDR1 (fivefold) mRNA by rifampicin in LS180 cells was attenuated by the overexpression of miR-148a (Takagi *et al.*, 2008). Therefore, the new information was provided that the miR-148a posttranscriptionally regulated human PXR resulting in the modulation of the inducible and/or constitutive levels of CYP3A4 in human liver. This study suggested a new miRNA-dependent mechanism in the large interindividual variability of CYP3A4 expression *via* PXR expression in human.

*Human CYP2E1*

Human CYP2E1 is a pharmacologically and toxicologically important P450 isoform. Human CYP2E1 catalyzes the metabolism of numerous low molecular weight xenobiotics including drugs (e.g., acetaminophen, isoniazid, and brombenzene), organic solvents (e.g., ethanol, acetone, carbon tetrachloride, chloroform, vinyl chloride, glycerol, hexane, and toluene), and procarcinogens (e.g., N-nitrosodimethylamine, N-nitrosomethylethylamine, and N-nitrosopyrrolidine) (Lu and Cederbaum, 2008). CYP2E1 is induced by its own substrates such as isoniazid, ethanol, and acetone and enhances their metabolism (Bolt *et al.*, 2003). In addition, CYP2E1 is the most abundant isoform among all P450s in human liver (56% of total P450) at the mRNA level, followed by CYP2C9, CYP2C8, and CYP3A4 (8–11% of total P450) (Bieche *et al.*, 2007), whereas it is the fourth most abundant isoform (about 7% of total P450) at the protein level after CYP3A (30% of total P450), CYP2C (20% of total P450), and CYP1A2 (about 13% of total P450) (Shimada *et al.*, 1994). Collectively, 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 potential recognition element of miR-378 (MRE378) in the 3′-UTR of human CYP2E1 mRNA (Mohri *et al.*, 2010). Luciferase assays using HEK293 cells confirmed that miR-378 functionally recognized MRE378. Two HEK293 cell lines stably expressing human CYP2E1 including or excluding the 3′-UTR were established. When the precursor miR-378 was transfected into the cells expressing human CYP2E1 including 3′-UTR, the CYP2E1 protein level and chlorzoxazone 6-hydroxylase activity (marker activity of CYP2E1) were significantly decreased but not in the cells expressing CYP2E1 excluding 3′-UTR. Unexpectedly, in both cell lines, the CYP2E1 mRNA levels were decreased by overexpression of miR-378, but miR-378 did not affect the stability of CYP2E1 mRNA. Therefore, the down-regulation of CYP2E1 by miR-378 appears to be due to the 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 posttranscriptional repression. Interestingly, the miR-378 levels were inversely correlated with the CYP2E1 protein levels and the translational efficiency (protein/mRNA expression ratio) of human CYP2E1. It is important to note that the 3′-UTR of CYP2E1 is poorly conserved among human, rat, and mouse. As a result, the regulation of CYP2E1 by miR-378 may be specific to humans.

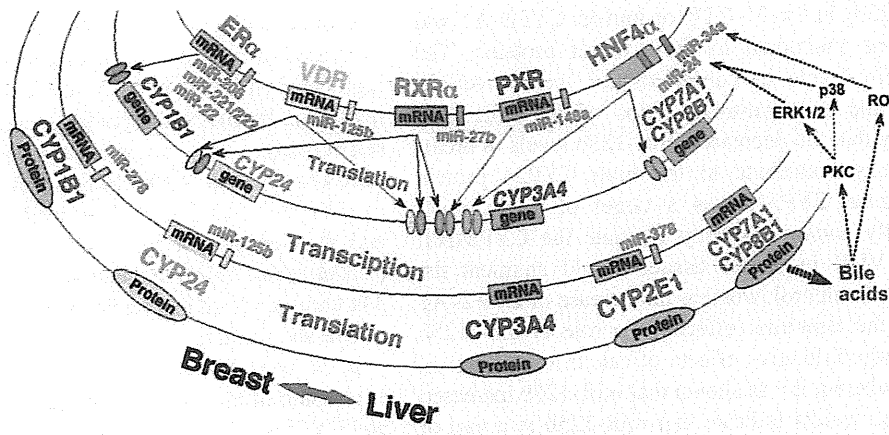


**FIG. 1.** miRNA-related networks of cytochrome P450 and nuclear receptors.

In addition to its 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 KLH), which are tumor suppressors (Lee *et al.*, 2007). Furthermore, miR-378 binds to the 3′-UTR of vascular endothelial growth factor (VEGF) and promotes expression of VEGF (Hua *et al.*, 2006). The expression of CYP2E1 is up-regulated in diabetes and obesity but down-regulated by insulin treatment (de Waziers *et al.*, 1995; Wang *et al.*, 2003; Woodcroft *et al.*, 2002). Thus, the involvement of miR-378 in the induction of CYP2E1 by chemicals/xenobiotics along with its role in cell growth and metabolic disease is likely to be toxicologically significant.

*Rat CYP2A3*

CYP2A3 has been isolated from a rat lung cDNA library and is expressed in lung but not in liver, kidney, or small intestines (Kimura *et al.*, 1989). 3-Methylcholanthrene increases lung CYP2A3 levels by ~threefold, whereas phenobarbital is not an inducer of CYP2A3 gene expression. In lung, CYP2A3 is a principal catalyst of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) α-hydroxylation, the primary bioactivation pathway for NNK (Jalas *et al.*, 2005). The chronic administration of NNK to F344 rats reduced the expression of several miRNAs including miR-126* and miR-34 in lung (Kalscheuer *et al.*, 2008). The passenger strand, named miRNA*, is usually degraded, although it is sometimes functional. It was found that CYP2A3 is regulated by the miR-126*. Because the reduced miR-126* expression was accompanied by increased CYP2A3 expression (both mRNA and protein levels) in the NNK-treated rats, these expression changes were thought to reinforce NNK genotoxicity. The reduced expression of miR-34 observed after NNK exposure is also noteworthy because it is reported to be involved in the regulation of a tumor suppressor p53 (Corney *et al.*, 2007; He *et al.*, 2007). However, p53 mRNA expression did not change in NNK-treated rats (Kalscheuer *et al.*, 2008).

## HUMAN CYP24A1 AND VITAMIN D RECEPTOR

Human CYP24A1 is an essential enzyme involved in the inactivation of 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$; calcitriol]. Calcitriol, a biologically active metabolite of vitamin D3, is typically considered a regulator of calcium homeostasis, but it has now received much interest for its antitumor activity (Deeb *et al.*, 2007: Nagpal *et al.*, 2005). CYP24A1 has been reported to be overexpressed in various tumor cells (Deeb *et al.*, 2007). Although there is some controversy about the expression of CYP24A1 mRNA and protein in cancer tissues compared with those in noncancerous tissue due to the heterogeneous background in different breast cancers (de Lyra *et al.*, 2006; Hicks *et al.*, 2006; Townsend *et al.*, 2005), the overexpression of CYP24A1 protein is not necessarily associated with the increased CYP24A1 mRNA level. Most of the biological effects of calcitrol are elicited by its binding to vitamin D receptor (VDR) (Carlberg and Polly, 1998). Calcitrol has been associated with the risk of cancer (Garland *et al.*, 2006). Because the vitamin D system has relevance for both the prevention and treatment of cancer (Holick, 2007), the development of a number of novel synthetic vitamin D analogues as a therapeutic agent for cancer has been attempted. It has been reported that, at the protein level, the VDR expression is higher in breast (Friedrich *et al.*, 2002) and thyroid (Khadzkou *et al.*, 2006) cancers than in normal tissues, but no difference was found between cancer and normal tissues at the mRNA level, suggesting the involvement of posttranscriptional regulation.

It was reported that both human CYP24A1 (Komagata *et al.*, 2009) and VDR (Mohri *et al.*, 2009) are posttranscriptionally regulated by miR-125b. A potential miR-125b recognition

element (MRE125b) in the 3′-UTR of human CYP24A1 and VDR mRNA was functional to these target proteins. The CYP24A1 protein levels in cancer tissues were inversely associated with the cancer/normal ratios of the miR-125b levels, suggesting that the decreased miR-125b levels in breast cancer tissues may contribute to the high CYP24 protein expression. Because CYP24A1 is a target of VDR, miR-125b may directly and/or indirectly regulate the CYP24A1. An increase of VDR in cancer tissues would augment the antitumor effects of calcitrol, whereas an increase of CYP24A1 would attenuate the antitumor effects. The role of miR-125b relative to the antiproliferative effects of calcitrol was studied in MCF-7 cells, wherein it was shown that miR-125b increased cell growth. These results indicate that miR-125b is a part of VDR downstream activities.

In other studies, miR-125b inhibited proliferation of human hepatocellular carcinoma cells (Li *et al.*, 2008), thyroid carcinoma cells (Visone *et al.*, 2007), and human breast cancer cells (Scott *et al.*, 2007). In contrast, inhibition of miR-125b decreased growth of human prostate cancer cells (Lee *et al.*, 2005). Importantly, miR-125b expression is differentially affected in various human tumors, with evidence for being down-regulated in breast, ovarian, and bladder cancers, whereas its expression is up-regulated in pancreas and stomach cancers (Volinia *et al.*, 2006). Thus, the functional effects of miR-125b differ in each cancerous tissue. There is presently considerable interest in evaluating miR-125b as a potential biomarker of cancer-related outcomes, but more research is needed.

*Human HNF4α and Bile Acid Toxicity*

Human 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 and regulates the expression of various genes involved in the synthesis/metabolism of bile acid, fatty acid, cholesterol, glucose, and urea as well as hepatocyte differentiation (Gonzalez, 2008). It is well recognized that endo/xenobiotic-metabolizing enzymes such as CYPs, UGTs, and sulfotransferase are under the control of HNF4α (Kamiyama *et al.*, 2007). 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, which regulate these target genes. HNF4α forms large transcriptional regulatory networks in the liver.

Potential recognition elements for miR-24 (MRE24) were identified in the coding region and the 3′-UTR of HNF4α, whereas miR-34a (MRE34a) recognition elements were identified in only the 3′-UTR of HNF4α mRNA (Takagi *et al.*, 2010). HNF4α protein levels in HepG2 cells were markedly decreased by overexpression of miR-24 and miR-34a, and HNF4α mRNA levels were significantly decreased by the overexpression of miR-24 but not by miR-34a. The luciferase reporter activity of plasmid containing the 3′-UTR of HNF4α was significantly decreased by miR-34a and that of plasmid containing the HNF4α coding region was significantly decreased by miR-24, suggesting that the MRE24 in the coding region and MRE34a in the 3′-UTR is functional in the negative regulation by mRNA degradation and translational repression, respectively. The down-regulation of HNF4α by these miRNAs resulted in the decrease of various target genes such as CYP7A1 and CYP8B1 as well as morphological changes and the decrease of the S phase population in HepG2 cells (Takagi *et al.*, 2010). In addition, expression of miR-24 and miR-34a were regulated by protein kinase C/mitogen-activated protein kinase and reactive oxygen species pathways, respectively.

It is well known that HNF4α positively regulates 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 (Pikuleva, 2006). Therefore, induction of miR-24 and miR-34a is expected to decrease bile acid synthesis *via* mainly CYP7A1. In addition, the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) was also down-regulated by the decrease of HNF4α expression by miRNAs. Thus, miR-24 and miR-34a affect the various hepatic functions through the negative regulation of HNF4α expression (Takagi *et al.*, 2010).

## ROLE OF miRNAs IN REGULATING OTHER NUCLEAR RECEPTORS

*Human Estrogen Receptor α*

Estrogen receptor α (ERα) is an important marker for the prognosis and is predictive of the response to endocrine therapy in breast cancer patients. Up to one third of the patients with breast cancer lack ERα at the time of diagnosis. It was previously reported that ERα regulates the expression of human CYP1B1, which catalyzes the metabolism of estradiol to the toxicologically active endogenous metabolite, 4-hydroxyestradiol (Tsuchiya *et al.*, 2004). It was first demonstrated by Adams *et al.* (2007) that human ERα is regulated by miR-206, whereas the activation of ERα results in the decreased expression of miR-206, showing mutually inhibitory regulation. miR-221 and miR-222 also inhibit human ERα expression at the translational level (Xiong *et al.*, 2010; Zhao *et al.*, 2008). Expression of miR-22 and ERα protein were inversely associated and ERα is the primary target (Pandey and Picard, 2009). miR-375 was identified as a potential target of dexamethasone-induced Ras-related protein 1 (RASD1), and it was found that RASD1 negatively regulates ERα expression (Simonini *et al.*, 2010). miR-27a indirectly regulates human ERα *via* ZBTB10, a specific protein repressor for Sp2, Sp3, and Sp4 (Li *et al.*, 2010). From these lines of data, the authors suggested that a variety of miRNAs may be potential targets for anti-estrogen therapy. However, it should be noted that study-specific experimental conditions varied considerably as they were derived from cultured hepatoma cell lines, established cells from tumor tissues and/or

tissue samples, and lacked quantitative consideration of the data. Thus, although these data offer the potential for potential treatment possibilities, more research is needed to establish causal relationships in this area.

## PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

Peroxisome proliferator-activated receptor (PPAR) α is an important transcriptional factor that regulates genes encoding endo/xenobiotic enzymes and lipid-metabolizing enzymes. The overexpression and inhibition of miR-21 or miR-27b in HuH7 cells significantly decreased and increased the PPARα protein level, respectively, but not PPARα mRNA level (Kida *et al.*, 2011). These miRNAs negatively regulate the expression of PPARα 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.

In spite of the different experimental conditions in each research group, miR-27a and miR-27b were reported to be targets of PPARγ (Jennewein *et al.*, 2010; Karbiener *et al.*, 2009; Kim *et al.*, 2010; Lin *et al.*, 2009). Interestingly, the inhibition of miR-27b, induced by lipopolysaccharide (LPS), reversed PPARγ mRNA degradation. Whereas miR-27b overexpression decreased PPARγ mRNA, affecting the LPS-induced expression of the pro-inflammatory cytokines, tumor necrosis factor α and interleukin-6 (IL-6; Jennewein *et al.*, 2010). The expression of miR-27a and miR-27b increased in fat tissue of obese mice and was regulated by hypoxia (Lin *et al.*, 2009). miR-130 potently repressed PPARγ expression by targeting both the PPARγ mRNA coding and 3′-UTR regions, thereby controlling adipocyte gene expression programs (Lee *et al.*, 2010).

### *Retinoid X Receptor*

Retinoid X receptor (RXR) α has been shown to be a target of miR-27a and miR-27b in rat primary hepatic stellate cells (Ji *et al.*, 2009). The sequences of MRE on the RXRα mRNA are highly conserved among species, suggesting that human RXRα may also be regulated by miR-27. RXRα is involved in multiple signaling pathways related to cell proliferation and differentiation, mainly as the heterodimeric partner of several nuclear receptors (Imai *et al.*, 2001). Therefore, miR-27 seems to be involved in the regulation of a wide variety of transcriptional factors affecting inter- and intraindvidual differences in drug response, adverse reactions, and toxicity outcomes. In addition, as mentioned above, CYP1B1 is a direct target of human miR-27b (Tsuchiya *et al.*, 2006).

The review of miRNA regulation of CYP and nuclear receptor regulation demonstrates that miRNA regulatory 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 different kinds of miRNA (Fig. 1). As we learn more about this important research field, it is anticipated that miRNAs will be shown to contribute broadly to understanding mechanisms of toxicity and for predicting the risk susceptibility for drugs, chemical toxicants, and environmental pollutants.

## *IN VITRO* AND *IN VIVO* MODULATION OF TOXICOLOGY-RELATED miRNA EXPRESSION

Although the precise roles of miRNA in the response to xenobiotics, drugs and chemical toxicants, remain to be established, there is little doubt that miRNAs are important in the cellular and *in vivo* responses to xenobiotics (Taylor and Gant, 2008). However, the regulatory networks of miRNAs are complex, and decreased expression of miRNAs will generally lead to high expression of the target proteins. In contrast, increased expression of miRNAs is less likely to be associated with toxicological phenomena. Comprehensive studies using miRNA arrays as well as DNA microarrays and proteomics analyses are powerful tools to investigate the mechanisms of individual susceptibility to toxicants and adverse drug reactions. Recently, a large number of studies on the roles of miRNAs in cancer have been investigated, but few studies have reported the altered expression profiles of miRNA in drug-related adverse reactions and in toxicology-related outcomes.

There are numerous examples of the potential roles whereby miRNAs can influence toxic responses. Many of these have only been evaluated *in vitro* but are relevant to toxicology and disease development. For example, human miR-222 regulates matrix metalloproteinase 1 (MMP1) expression level through both direct cis-regulatory mechanisms (targeting MMP1 mRNA) and direct trans-regulatory mechanisms (indirect controlling of MMP1 gene expression by targeting superoxide dismutase-2 (SOD2) (Liu *et al.*, 2009). SOD2-dependent up-regulation of MMPs may, at least in part, contribute to the increased invasion and metastatic capacity of tumors displaying elevated SOD2 levels. A significant observation from both *in vitro* and *in vivo* studies is that cigarette smoking causes the down-regulation of many miRNAs in the lungs of both mice and rats (Izzotti *et al.*, 2011) as well as in human airway epithelial cells (Schembri *et al.*, 2009). Finally, arsenite, which is known to activate nuclear factor-erythroid 2-related factor 2 (Nrf2) (Aono *et al.*, 2003), affects miRNA expression in human lymphoblastoid TH-6 cells (Marsit *et al.* 2006), although the impact of such changes on toxicity is not yet known. It has also been demonstrated that miR-17* suppresses the primary mitochondrial antioxidant enzymes, such as SOD2, glutathione peroxidase-2 (GPX2), and thioredoxin reductase-2 (RXR2), in prostate cancer PC-3 cells (Xu *et al.*, 2010). The luciferase reporter activities were suppressed by the overexpression of miR-17* and disulfiram, a dithiocarbamate drug, induced the expression level of mature miR-17*. It was previously reported that miR-17 is able to silence

hypoxia-inducible factor-1α expression (Taguchi *et al.*, 2008). From these reports, it is conceivable that the miR-17 and miR-17* might involve in maintaining the homeostasis against cellular redox stress.

Several studies employing toxicogenomics have been carried out to evaluate the responses of miRNAs in rodent liver with the goal of identifying potential biomarker(s) for toxicological risk assessment. It was reported that single administration of acetaminophen or carbon tetrachloride to rats resulted in different expression profiles of miRNA in the liver (Fukushima *et al.*, 2007). Changes in miRNA-298 and miR-370, which presumably target oxidative stress-related enzymes including thioredoxin reductases were noted. In this early work, the sample size was very small and no statistical analyses were conducted. However, miRNA suppression occurred as early as 6 h later, which coincided with the early phase toxicity, prior to cellular necrosis.

It has also been demonstrated that let-7C, a miRNA important in cell growth, was inhibited with the potent PPARα agonist WY-14,643 in wild-type mice (Shah *et al.*, 2007). In addition, let-7C was also shown to target c-myc *via* direct interaction with the 3′-untranslated region of c-myc, which subsequently increasing the expression of the oncogenic miR-17-92. Thus, a let-7C signaling cascade appears to be critical for PPARα agonist-induced liver proliferation.

In interesting *in vivo* finding is that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) did not cause any potent changes in hepatic miRNA expression in TCDD-resistant H/W rats and TCDD-sensitive L-E rats (Moffat *et al.*, 2007), and similar results were obtained in mice. The authors concluded that down-regulation of hepatic miRNA by TCDD is unlikely to play a significant role in TCDD toxicity in adult rodent liver. In addition, BaP3 (daily doses of 150 mg/kg) caused widespread changes in gene expression (>400 genes), but almost no changes in miRNA expression when evaluated by microarray analyses. Collectively, although miRNA expression would be co-ordinately regulated with the mRNA transcript, it is interesting that hepatic miRNA *in vivo* is not directly responsive to aryl hydrocarbon receptor (AHR)-agonists such as TCDD and BaP *in vivo* in rodents (Yauk *et al.*, 2011).

Concerning *in vitro* studies, cells treated with γ-irradiation showed no alteration in miRNA expression (Marsit *et al.*, 2006). In contrast, when the human lymphoblast cell line IM9 was treated with γ-irradiation, various changes in miRNAs in a dose-dependent manner were noted (Cha *et al.*, 2009). The conflicting data for γ-irradiation may be a function of cell types or experimental conditions used in these studies and need to be clarified. From these data, it is conceivable that there are different responses in miRNA changes between *in vitro* and *in vivo*, and an *in vitro* study alone may not able to predict the *in vivo* responses of miRNA for these kinds of toxic chemicals.

Finally, chronic exposure to toxic chemicals in rodents can produce different results in miRNA expression profiles compared with acute toxicity studies. For example, it was reported that tamoxifen, a potent hepatocarcinogen in rats, caused statistically significant differential expression of 33 hepatic miRNAs (20 genes up-regulated; 13 genes down-regulated) when administered to Fisher 344 rats for 24 weeks (Pogribny *et al.*, 2007). A significant up-regulation of oncogenic miRNAs, such as the miR-17-92 cluster, miR-106a, and miR-34, was observed. A number of miRNAs, including miR-152 and miR-195, were down-regulated in the livers of tamoxifen-treated rats. These miRNAs are frequently down-regulated in solid tumors (Murakami *et al.*, 2006). In addition, the differential expression of 55 miRNAs (31 genes up-regulated; 25 genes down-regulated) in mice fed a diet containing hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a common environmental contaminant, at 5 mg/kg for 28 days (Zhang and Pan, 2009). A significant up-regulation of oncogenic miRNAs and a significant down-regulation of tumor-suppressing miRNAs, such as let-7, miR-17-92 cluster, miR-10b, miR-15, miR-16, miR-26, and miR-181, were observed. Thus, chronic administration of toxic chemicals will affect the changes of miRNA expression in the liver *in vivo*, which are different compared with those of acute administration.

## ROLE OF miRNA IN IMMUNE-MEDIATED DRUG-INDUCED LIVER INJURY

Cytokines are recognized to decrease CYP-associated drug metabolism in humans during inflammation and infection (Abdel-Razzak *et al.*, 1993), which influences the susceptibility to various drugs and toxic chemicals. IL-6 was demonstrated to decrease both the rifampicin- and phenobarbital-mediated induction of CYP2B6, CYP2C8, CYP2C9, and CYP3A4, whereas, the transcriptional activity of PXR and CAR is not affected by IL-6 (Pascussi *et al.*, 2000).

With respect to cytokines and drug-induced liver injury (DILI), halothane- and α-naphthylisothiocyanate-induced liver injury is reported to be mediated by IL-17 (Kobayashi *et al.*, 2009, 2010), whereas IL-4 mediates dicloxacillin- and flutamide-induced liver injury in mice (Higuchi *et al.*, 2011). Furthermore, IL-6 and IL-4 are essential for the differentiation of Th17 and Th2 cells, respectively, from naïve T cells. The generation of reactive metabolites by the administered drugs is catalyzed by P450s, which are suggested to be a major causal factor for the initiation of DILI, and thereafter, the exacerbation of DILI will be affected by ILs.

In recent years, many studies have highlighted the fact that miRNAs play a critical role in the differentiation and function of the adaptive and innate immune system (Carissimi *et al.*, 2009). Indeed, several studies demonstrated the involvement of ILs in relation to miRNA-related diseases and cancer as follows. The expression of miR-148a, miR-152, and miR-301 was decreased in IL-6-overexpressing malignant cholangocytes.

IL-6 can increase the exprssion of DNA methyltransferase-1, which is a target of miR-148a and miR-152 (Braconi *et al.*, 2010). The expression of bone morphogenic protein receptor type II (BMPR2) through an miR-17/92 pathway is modulated by IL-6. Because IL-6 signaling is mainly mediated by STAT3, the expression of STAT3 was knocked down by small interfering RNA, which abolished the IL-6-mediated expression of miR-17/92 (Brock *et al.*, 2009). miR-21 contributes to the oncogenic potential of Stat 3 in multiple myeloma cells. miR-21 induction by IL-6 was strictly Stat 3 dependent through a highly conserved enhancer (Loffler *et al.*, 2007). Six miRNAs, let-7a, miR-26, miR-146a/b, miR-150, and miR-155 were significantly up-regulated in the IL-17 producing T cells. miR-146a is associated with IL-17 expression in the peripheral blood mononuclear cells in rheumatoid arthritis patients (Niimoto *et al.*, 2010). Interestingly, microRNA expression analysis during the tolerized state of THP-1 cells showed only miR-146a overexpression, suggesting its important role in LPS tolerance. Transfection of miR-146a into THP-1 cells mimicked LPS priming, whereas transfection of miR-146a inhibitor largely abolished LPS tolerance (Nahid *et al.*, 2009).

## RELEVANCE OF miRNA-RELATED GENETIC POLYMORPHISM TO PHARMACOGENETICS/GENOMICS STUDIES

The human genome contains about 3 billion base pairs, and single-base variations (called SNPs, single-nucleotide polymorphisms) are on the average as 1/1000 bases. Thus, the SNPs may affect either the expression or activities of various enzymes and are associated with the differences in drug responses and adverse effects of drugs and toxic chemicals. SNPs are present not only in the mRNA but also in mature miRNA sequences. SNPs in primary (pri)-miRNAs, precursor (pre)-miRNAs, or mature miRNA could modify various biological processes by influencing the processing or target selection of miRNAs (Duan *et al.*, 2007; Iwai and Naraba, 2005). SNPs in pri- or pre-miRNA are relatively rare. Only ~10% of human pre-miRNAs have documented SNPs and <1% of miRNAs have SNPs in the functional seed sequence region (Saunders *et al.*, 2007). Although seed region variations in miRNA seem to be very rare, they have the potential to influence the expression of hundreds of genes and related pathways. An interesting study regarding the correlation between SNPs in the miRNA sequence and the clinical drug therapy was published by Boni *et al.* (2010). An SNP (rs7372209) in the pri-miR26a gene and an SNP (rs1834306) in the pri-miR-100 gene were significantly associated with the tumor response or time to progression in 61 metastatic colorectal cancer patients treated with 5-fluorouracil and CPT-11 (Boni *et al.*, 2010). This is the first report to suggest a relationship between the clinical outcome of drug therapy and SNPs in the miRNA-biogenesis machinery, in both primary and precursor miRNAs, but the molecular mechanisms by which these polymorphisms act have not yet been clarified (Shomron, 2010).

Compared with the low level of variation in the functional regions of miRNAs, a considerable level of variation at the target sites is conceivable. Many miRNA target-related polymorphisms are reported to be associated with the phenotypes of diseases because a gain-of-function or loss-of-function would result in changes in the expression of target mRNAs (Sethupathy and Collins, 2008). However, actually, there are few examples regarding miRNA-related polymorphisms affecting the drug response, adverse reactions, and toxicological outcomes, such as human dehydrofolate reductase (DHFR) and sulfotransferase isoform 1A1 (SULT1A1) as mentioned later.

Recent genome-wide analyses of human SNPs have revealed that many polymorphisms exist in the miRNA binding sites. Approximately 400 SNPs were found at verified target sites or predicted target sites, and about 250 SNPs potentially create novel target sites for miRNAs in humans (Saunders *et al.*, 2007). More recently, roughly 20,0000 miRNA target-related polymorphisms were systematically searched using Patrocles (http://www.patrocles.org/Patrocles.htm) and PolymiRTS (http://compbio.uthsc.edu/miRSNP/). Those *in silico*-predicted database should be carefully validated by functional studies in the future.

Concerning an miRNA target polymorphism in relation to drug-metabolizing enzymes, it was demonstrated that a C829T SNP, a naturally occurring SNP, at the miR-24 recognition site in the 3′-UTR of human DHFR leads to DHFR overexpression and methotrexate resistance (Mishra *et al.*, 2007). Cells with the mutant 3′-UTR had a twofold increase in DHFR mRNA half-life, expressed higher levels of DHFR mRNA and DHFR protein, and were fourfold more resistant to methotrexate as compared with wild-type cells. In a case-controlled study of childhood leukemia patients, those possessing C to T SNP occurred with 14.2% allelic frequency in the Japanese population. The T allele of the SNP resulted in the loss of the miR-24-mediated regulation of DHFR, high DHFR protein levels and methotrexate resistance. This finding may be useful in predicting the clinical efficacy of methotrexate treatment (Mishra *et al.*, 2007; Mishra and Bertino, 2009).

Sulfotransferase isoform 1A1 (SULT1A1) is one of the essential enzymes in the metabolism of endo- and exobiotics and dietary and environmental procarcinogen/promutagen activation and/or detoxification. SULT1A1 activity shows high interindividual variability in the expression of the protein (Jones *et al.*, 1993) and a genetic polymorphism was suggested, although not fully accounted for by the variation of SULT1A1 activity. An SNP in the 3′-UTR (972C>T [rs1042157]) is significantly associated with the SULT1A1 mRNA level ($p = 0.029$) and enzymatic activity ($p = 0.012$) (Yu *et al.*, 2010). From subsequent functional analyses, it was found that miR-631 directly regulates SULT1A1 expression in an allele-specific manner of the 3′-UTR (SNP of 972 C > T), which

provides new insight into the mechanism of SULT1A1 regulation and new information for molecular epidemiology and risk assessment studies of heterocyclic amines such as N-hydroxyarylamines, N-hydroxy-heterocyclic amines, and arylhydroxamic acids.

Drug responses or susceptibility to xenobiotic toxicity will be predicted by miRNA expression profiles. Interindividual variability in adverse drug responses and toxicity will be predicted partly by using miRNA-related polymorphisms. This new class of miRNA-related polymorphisms may contribute to the interindividual differences in drug responses and toxicant-induced adverse events.

## miRNAs IN PLASMA AS POTENTIAL TOXICOLOGICAL BIOMARKERS

Circulating miRNA in plasma was first demonstrated as diagnostic biomarkers of lung (Chen *et al.*, 2008), colorectal (Chen *et al.*, 2008), and prostate cancers (Mitchell *et al.*, 2008). The dynamic changes of circulating miRNAs in the plasma resulting from drug exposure was first demonstrated by Wang *et al.* (2009). In mice, acetaminophen increased miR-122 and miR-192 in liver and plasma in a dose- and exposure-duration manner that paralleled ALT and histopathological changes in the liver. The changes of miRNAs were earlier than those of ALT. This discovery of plasma miRNA opens up the great possibility of using miRNAs as a sensitive, informative, and non-invasive potential biomarker for drug-induced liver injury and toxicological outcomes. Many studies have already been conducted regarding circulating miRNA and the clinical diagnosis and prognosis of cancer, but few papers have been published in relation to toxicological studies. It was reported that increased plasma concentrations of miR-122, miR-133a, and miR-124 corresponded to injuries in liver, muscle, and brain, respectively, although each of these is the abundant and specific miRNA in each organ (Laterza *et al.*, 2009). The miR-122 concentration in plasma increased earlier than the increase of aminotransferase activities in the blood (Zhang *et al.*, 2010). This change was more specific for viral-, alcohol-, and chemical-related liver injury than other organ damage and was a more stable and reliable biomarker (Zhang *et al.*, 2010). Although several challenges remain to be addressed, circulating miRNAs have great potential in toxicological studies, e.g., as a novel, noninvasive method for the extrapolation of the toxicity data from animal to human.

## CONCLUSIONS

This review has described several examples of miRNAs and their relationship to drug-metabolizing enzymes, their interactions with nuclear receptors as well as implications for toxicological studies. Although the field of miRNA-related drug metabolism and toxicology studies is still in its infancy, we are now entering an interesting period in which the contribution of miRNAs in controlling various pharmacological and toxicological outcome will become more clear. In the near future, miRNA profiling may lead to the discovery of novel miRNA biomarkers that might improve the prediction of metabolic activation and detoxification of drugs *in vivo* in human. As the methodologies for miRNA studies are now becoming more widespread, comprehensive understanding of miRNAs is expected to progress in toxicology.

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# MicroRNAs from biology to future pharmacotherapy: Regulation of cytochrome P450s and nuclear receptors

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ABSTRACT

MicroRNAs (miRNAs) are a family of short, non-coding RNAs whose final product is a 22-nucleotide functional RNA molecule. They regulate the expression of target genes by binding to complementary regions of transcripts to repress their translation or promote mRNA degradation. Since miRNAs regulate every aspect of cellular function, their dysregulation is associated with a variety of diseases including cancer, diabetes, and cardiovascular diseases. Therefore, miRNAs are now considered new therapeutic targets. However, the roles of miRNAs in the metabolism of xenobiotics and endobiotics have only recently been revealed. This review describes the current knowledge on the regulation of cytochrome P450s and nuclear receptors by miRNAs, the physiological and clinical significance. The miRNA expression is readily altered by chemicals, carcinogens, drugs, hormones, stress, or diseases, and the dysregulation of specific miRNAs might lead to changes in the drug metabolism potency or pharmacokinetics as well as pathophysiological changes. In the field of pharmacogenomics, the evaluation of miRNA-related polymorphisms would provide useful information for personalized medicine. Utilizing miRNAs opens a new era in the fields of drug metabolism and pharmacokinetics as well as toxicology.



**Contents**



## 1. Introduction

MicroRNAs (miRNAs) are short (~22-nucleotide), endogenous non-coding RNAs that lead to gene silencing through translational repression or mRNA degradation (Bartel, 2004). They are now recognized as critically important regulators of gene expression. So far, more than 1000 human miRNAs have been identified (miRBase, release 17, http://www.mirbase.org/). Computational prediction estimates that 60% of human mRNAs could be targets of miRNAs (Friedman et al., 2009). The miRNAs have roles in fine-tuning the expression of their target genes forming intricate networks. Research on miRNA is growing exponentially, and it is now clear that miRNAs can potentially regulate every aspect of cellular processes such as differentiation, proliferation and apoptosis as well as a large range of physiological processes such as development, immune response, metabolism, tumor formation, and disease development (Kloosterman & Plasterk, 2006). Since 30% of miRNA is expressed in a tissue- or cell-specific manner, the expression

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patterns are associated with the physiological functions of miRNAs. Dysregulation of the miRNA function may lead to certain diseases.

In the fields of drug metabolism and pharmacokinetics (DMPK), the roles of miRNAs have only recently started to become clear. The purpose of this review is to summarize recent findings concerning the roles of miRNA in the regulation of cytochrome P450s (P450, CYP) and nuclear receptors and their clinical significance with consideration of their potential application for pharmacotherapy.

## 2. Biogenesis of miRNAs and gene regulation by miRNAs

Genes encoding miRNA are located in intergenic or intragenic (intronic or exonic) regions with both sense and antisense orientations (Lagos-Quintana et al., 2001). In human, the distribution of miRNA is as follows: 52% intergenic miRNAs, 43% intronic miRNAs, and 5% exonic miRNAs (Hinske et al., 2010). The miRNAs are first transcribed as long primary transcripts, generating a stem-loop containing primary miRNA (pri-miRNA). The pri-miRNA is processed to a 70- to 100-nucleotide hairpin-shaped precursor miRNA (pre-miRNA) by a microprocessor complex including Drosha and DiGeorge syndrome critical region 8 protein (DGCR8) in the nucleus. The pre-miRNA is exported into the cytoplasm by Exportin 5, processed to miRNA duplex of about 22 nucleotides by Dicer or Ago2, and then unwound into the single strand form of mature miRNAs. The functional guide strand is loaded onto the RNA-induced silencing complex (RISC), composed of Dicer, TAR RNA binding protein (TRBP) and Argonaute protein Ago2, and guides the complex to its mRNA targets with imperfect pairing causing translational repression or mRNA degradation (Krol et al., 2010). Target sites of animal and human mRNAs are usually at the 3′-untranslated region (UTR), although there are examples of target sites in the coding region as well (Duursma et al., 2008). The passenger strand, named miRNA*, is usually degraded, although it is sometimes functional. Gene silencing by miRNAs includes multiple mechanisms and the details can be found in other review articles (Chekulaeva & Filipowicz, 2009; Fabian et al., 2010).

## 3. Prediction/identification of miRNA(s) for a given target gene

Identification of miRNA(s) for a target gene is a challenging task because miRNAs bind to their target by partial complementarity over a short sequence. The nucleotides 2–8 at the 5′-end of the miRNA, called the seed sequence, are critical and sometimes sufficient to repress the target translation (Lewis et al., 2005). To predict the miRNAs for the target genes, several computational programs such as MicroCosm Targets (John et al., 2004), TargetScan (Lewis et al., 2003, 2005), Pictar (Krek et al., 2005), and microrna.org (Betel et al., 2008) are available. In most cases, many kinds of miRNAs are predicted. The predicted miRNAs vary significantly depending on the different algorithms used in the programs, because these algorithms place variable weight on complementarity to the miRNA seed sequence, evolutionary conservation of the microRNA recognition element (MRE) of the target gene, free energy of the miRNA-mRNA duplex, and accessibility of the target site. The false positive rate of the predicted candidate targets of a given miRNA is thought to be 30–50% (Watanabe et al., 2007; Alexiou et al., 2009). Confirming experimentally the validity of all of them is a time-, money, and energy-consuming process. We consider the following points to choose miRNAs that are to be experimentally verified: 1) convergence of algorithms and 2) conservation of the target site sequence among species, although these points are neither a necessity nor a guarantee of function, 3) accessibility to target: it seems to be a critical feature of miRNA target recognition, and 4) whether the miRNA is substantially expressed in the tissue where the target mRNA is expressed.

To validate specific miRNA:mRNA interaction, several lines of experiments can be used. The most commonly employed technology is the use of luciferase assays using reporter constructs containing the MRE of the target mRNA downstream of the reporter gene. The constructs are co-transfected into the cells with precursor miRNA (or the expression plasmid of miRNA) or antisense oligonucleotide (AsO) for miRNA to overexpress or inhibit miRNA. Evidence can be established whether the reporter activity is significantly decreased or increased. The overexpression or inhibition of miRNA is an effective method to determine potential miRNA:mRNA interactions. In the overexpression experiments, a possible concern is that the decrease of target expression might be an artifact of overexpression. From this point, experiments to inhibit endogenous miRNA are valuable. Furthermore, an inverse relationship between the protein and miRNA levels is expected, for example, cancer versus normal tissues, or among individuals or cell lines.

In addition to the above direct methods, it is useful to determine the change of mRNA or protein expression by microarray or proteome analysis after the overexpression or inhibition of miRNA to determine the targets of a given miRNA comprehensively (Lim et al., 2005; Baek et al., 2008). It should be noted that these include secondary targets, the expression of which may change owing to the expression changes of the primary targets. We should also pay attention to the fact that different proteins have different turnover rates. This means that the level of protein with high turnover will change rapidly, whereas stable proteins will be affected later. In addition, if one determines only the changes in mRNA levels, some targets whose expression repression is caused by translational repression could be missed. Thus, for comprehensive analysis, both mRNA and protein analyses should be considered. Another method is immunoprecipitation of RISC and sequence analysis of mRNAs in the immunoprecipitant (Beitzinger et al., 2007). In such an experiment, we don't know which mRNAs are targets of which miRNA. To overcome this problem, the overexpression or inhibition of miRNA before the immunoprecipitation and comparison with control by microarray analysis will be useful (Karginov et al., 2007; Chi et al., 2009).

## 4. miRNAs that regulate P450s and nuclear receptors

P450s are important enzymes that catalyze the metabolism of xenobiotics including drugs, environmental chemicals, and carcinogens as well as endobiotics such as steroids, bile acids, and fatty acids. Most P450s are transcriptionally regulated by nuclear receptors. The understanding of the mechanisms of the transcriptional regulation of P450s has progressed greatly, but post-transcriptional regulation largely remains to be clarified. Recently, some P450s and nuclear receptors have been found to be post-transcriptionally regulated by miRNAs (Table 1, Fig. 1). We introduce the examples that have been

**Table 1**
Cytochrome P450s and nuclear receptors that are regulated by miRNAs.

| Target | miRNA | Reference |
|---|---|---|
| CYP1B1 | miR-27b | Tsuchiya et al., 2006 |
| CYP2A3 (rat) | miR-126* | Kalscheuer et al., 2008 |
| CYP2E1 | miR-378 | Mohri et al., 2010 |
| CYP3A4 | miR-27b | Pan et al., 2009a |
| CYP24A1 | miR-125b | Komagata et al., 2009 |
| PXR | miR-148a | Takagi et al., 2008 |
| VDR | miR-125b | Mohri et al., 2009 |
| PPARγ | miR-27a | Lin et al., 2009; Kim et al., 2010 |
| | miR-27b | Karbiener et al., 2009; Jennewein et al., 2010 |
| | miR-130 | Lee et al., 2010 |
| RXRα (rat) | miR-27 | Ji et al., 2009 |
| HNF4α | miR-24a, miR-34 | Takagi et al., 2010 |
| ERα | miR-206 | Adams et al., 2007 |
| | miR-221/222 | Zhao et al., 2008 |
| | miR-22 | Xiong et al., 2010 |
| GR | miR-18, miR-124a | Vreugdenhil et al., 2009 |

If not specified, the targets mean human mRNAs.

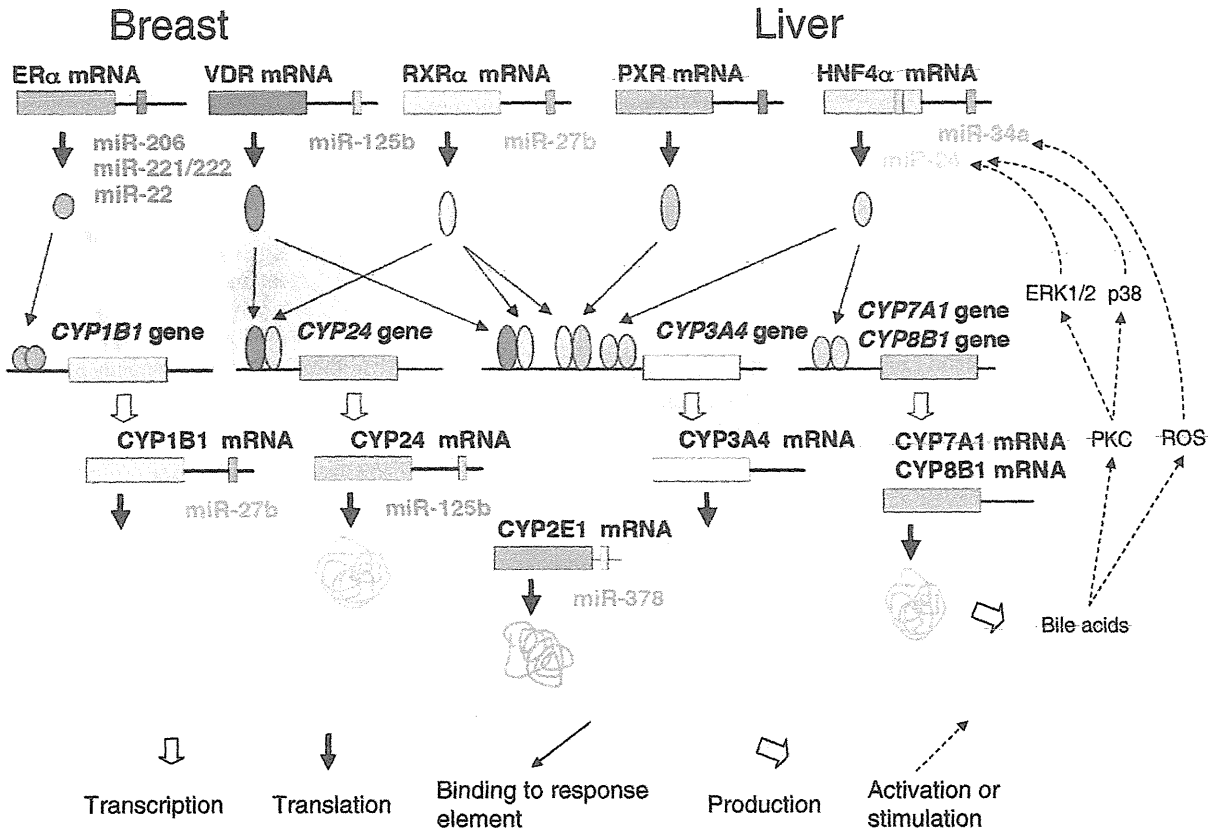


**Fig. 1.** Cytochrome P450s and nuclear receptors that are regulated by miRNAs. The miRNAs regulate targets forming an intricate network with transcriptional factors and signal transduction pathways.

confirmed as targets of miRNA by direct experimental methods as described above, and the clinical significance.

### 4.1. Relationship to cancer

miRNA studies have achieved much progress in the field of cancer. There are many publications concerning miRNAs related to cancer. The miRNA expression in most types of cancer cells is quite different from that in normal cells. Certain miRNAs have been found to serve as oncogenes or tumor suppressor genes, being associated with cancer initiation and/or progression. P450s are also one of the factors associated with the initiation or progression of cancer. In cancer tissues, some P450 expressions are modulated, and that would lead to an imbalance in the metabolism of exo/endobiotics. Some P450s and nuclear receptors related to cancer have been found to be regulated by miRNA as follows.

#### 4.1.1. CYP1B1

CYP1B1 catalyzes the metabolic activation of a variety of procarcinogens and promutagens, including polycyclic aromatic hydrocarbons and aryl amines (Shimada et al., 1996). In addition, CYP1B1 metabolizes 17β-estradiol to form a catechol metabolite that cause DNA damage (Han & Liehr, 1994). It has been demonstrated that the expression level of CYP1B1 protein is higher in various types of cancer compared with normal tissue (Murray et al., 1997), whereas there is no difference in the CYP1B1 mRNA levels between cancerous and normal tissues (Cheung et al., 1999) implying post-transcriptional regulation. This background prompted us to investigate the possibility that human CYP1B1 might be regulated by miRNA, and we found that it is negatively regulated by miR-27b via translational repression (Tsuchiya et al., 2006). Interestingly, the expression level of miR-27b was lower in breast cancer tissues than in adjacent normal tissues. A significant inverse association was observed in the expression levels of miR-27b and CYP1B1 protein between breast cancer and normal tissues. The decreased expression of miR-27b would be one of the causes of the high expression of CYP1B1 protein in cancer tissues. The study by Tsuchiya et al. (2006) is the first to reveal the regulation of P450 by miRNA.

#### 4.1.2. CYP2A3

Kalscheuer et al. (2008) have reported that chronic administration of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to F344 rats reduced the expression of several miRNAs including miR-126* in the lungs. They found that CYP2A3, which catalyzes the metabolic activation of NNK, is regulated by miR-126*. Since the reduced miR-126* expression was accompanied by increased CYP2A3 expression (at both mRNA and protein levels) in the NNK-treated rats, they concluded that the expression changes would reinforce NNK genotoxicity.

#### 4.1.3. CYP24 and vitamin D receptor (VDR)

1,25-Dihydroxyvitamin $D_3$ (calcitriol) has now received much interest for its anti-tumor activity (Deeb et al., 2007), although it is well known as a regulator of calcium and bone homeostasis. Most of the biological effects of calcitriol are elicited by the binding to VDR (Carlberg & Polly, 1998). Calcitriol is then inactivated by CYP24, a key metabolic enzyme, the expression of which is regulated by VDR (Chen & DeLuca, 1995). It has been reported that CYP24 (Deeb et al., 2007) and VDR (Friedrich et al., 2002; Khadzkou et al., 2006) are overexpressed in various cancers. We found that both human CYP24 (Komagata et al., 2009) and VDR (Mohri et al., 2009) are post-transcriptionally regulated

by miR-125b. It was demonstrated that the miR-125b expression is decreased in breast cancer tissues, being a causal factor of the overexpression of CYP24 and VDR. Since CYP24 is a target of VDR, the miR-125b would directly and indirectly regulate CYP24. The increase of VDR in cancer tissues would augment the anti-tumor effects of calcitriol, whereas the increase of CYP24 would attenuate the anti-tumor effects. When we investigated the effects of miR-125b on the anti-proliferative effects of calcitriol using human breast cancer-derived MCF-7 cells, the overexpression of miR-125b restored the cell growth suppressed by calcitriol, indicating that miR-125b had a great impact on the VDR function in this cell system.

#### 4.1.4. Estrogen receptor α (ERα)

Three fourths of diagnosed breast cancers express ERα, which is a target of estrogen, exerting proliferative effects. ERα is an important marker for the prognosis and is predictive of the response to endocrine therapy in breast cancer patients. ERα regulates the expression of human CYP1B1, which catalyzes the metabolism of estradiol to a carcinogenic metabolite, 4-hydroxyestradiol (Tsuchiya et al., 2004). It has been demonstrated that human ERα is regulated by miR-206, whereas the activation of ERα results in the decreased expression of miR-206, showing mutually inhibitory regulation (Adams et al., 2007). After this report, it was reported that miR-221, miR-222 (Zhao et al., 2008), and miR-22 (Xiong et al., 2010) also regulate human ERα expression. The authors suggested the possibility that these miRNAs may be potential targets for anti-estrogen therapy.

### 4.2. Relationship to metabolism of xenobiotics/endobiotics

#### 4.2.1. CYP2E1

CYP2E1 catalyzes the metabolism of a variety of low molecular-weight xenobiotics including drugs, organic solvent, and procarcinogens. CYP2E1 is inducible by some compounds through post-transcriptional or post-translational mechanisms. In a panel of human liver samples, no positive correlation between the CYP2E1 activity and CYP2E1 mRNA levels was observed (Sumida et al., 1999), indicating the post-transcriptional regulation for the constitutive expression. As for the molecular mechanism, we found that human CYP2E1 is regulated by miR-378 (Mohri et al., 2010). The significance of miR-378 in the regulation of CYP2E1 was supported by the finding of an inverse correlation between the miR-378 level and CYP2E1 protein level in a panel of human liver samples. This study could provide a new insight into the unsolved mechanism of the post-transcriptional regulation of CYP2E1.

#### 4.2.2. CYP3A4 and pregnane X receptor (PXR)

CYP3A4 is the most abundant P450 in human liver, and it is responsible for the metabolism of more than one half of prescription drugs and endogenous compounds such as steroids. There are large interindividual differences (~50 fold) in the CYP3A4 expression level in human livers. CYP3A4 is inducible by a wide variety of exogenous and endogenous compounds through the activation of PXR, possibly being responsible for the variability in the expression levels. We studied whether CYP3A4 and PXR may be regulated by miRNA focusing on miR-148a among the various predicted miRNAs, because miR-148a was commonly predicted and is substantially expressed in the liver. Another reason was that miRNA tends to regulate genes in the same pathway to exert its biological function (i.e., miR-148a repression of both PXR and CYP3A4 would result in both direct and indirect mechanisms to regulate CYP3A4).

Our data demonstrated that the miR-148a regulates PXR but not CYP3A4 (Takagi et al., 2008). Interestingly, the miR-148-dependent regulation of PXR affected the induction of endogenous CYP3A4 in human colon carcinoma-derived LS180 cells. Correlation analysis using human liver samples showed that the PXR protein level was not positively correlated with PXR mRNA supporting the post-transcriptional regulation. In contrast, the CYP3A4 protein level showed a significantly positive correlation with the CYP3A4 mRNA level indicating that the transcriptional regulation mainly contributes to the expression. Although we didn't examine CYP3A4 further, Pan et al. (2009a) subsequently reported that CYP3A4 protein in LS180 and human pancreas cancer-derived PANC1 cells was decreased by the overexpression of miR-27b, accompanied by a decrease of the CYP3A4 mRNA level. A limitation of their study may be that the conclusion was drawn from only an overexpression study. Again, to determine the impact of miRNAs on their targets, an inhibition study of endogenous miRNA as well as correlation analysis between the miRNA and target mRNA levels would be necessary. All proteins are produced by mRNA, which means that transcriptional regulation certainly participates. The balance between the transcriptional and post-transcriptional regulation is critical. If miRNA cannot overcome the strong transcriptional regulation of a target gene, the involvement of miRNA may be invisible. We think that in CYP3A4 this might be the case.

In our study, since the translational efficiency of PXR was inversely correlated with the miR-148a levels, it is suggested miR-148a would actually be involved in the regulation of PXR in human livers. In addition, the positive correlation between the PXR protein level and CYP3A4 at both the mRNA and protein levels suggests that the miR-148a-dependent PXR regulation would have great impact on the basal expression of CYP3A4 in human livers.

PXR regulates a variety of drug-metabolizing enzymes and transporters. We found that the induction of CYP2B6 (2-fold) and multi-drug resistance 1 (MDR1)/P-glycoprotein (5-fold) mRNA by rifampicin in LS180 cells was also attenuated by the overexpression of miR-148a. Thus, the miR-148a-dependent regulation of PXR appeared to affect target genes in common.

#### 4.2.3. Hepatocyte nuclear factor 4α (HNF4α) and CYP7A

HNF4α is a master regulator of drug-metabolizing enzymes, drug transporters, and genes involved in the synthesis/metabolism of bile acids, fatty acids, cholesterol, glucose, and urea (Gonzalez, 2008). HNF4α regulates gene expression not only via direct binding to the gene's regulatory sequences but also through the regulation of other transcriptional factors such as PXR and constitutive androstane receptor (CAR). HNF4α forms large transcriptional regulatory networks in the liver. Therefore, it is believed that the change of HNF4α expression has a great impact upon the function of the liver. We found that human HNF4α is regulated by miR-24 and miR-34a (Takagi et al., 2010). Interestingly, miR-34a recognizes MRE in the 3′-UTR and causes translational repression, whereas miR-24 recognizes MRE in the coding region and causes mRNA degradation. The down-regulation of HNF4α by these miRNAs resulted in the decrease of various target genes such as CYP7A1, CYP8B1 (bile acid-synthesizing enzymes), CYP27A1 (a cholesterol-metabolizing enzyme), and phosphoenolpyruvate carboxykinase (PEPCK, a gluconeogenetic enzyme). We could also provide a novel feedback loop for bile acid synthesis as follows: bile acids activate protein kinase C (PKC)/mitogen-activated protein kinase (MAPK) and reactive oxygen species (ROS) pathways. The PKC/MAPK and ROS pathways increase miR-24 and miR-34a expression, respectively. These miRNAs down-regulate the HNF4α expression resulting in the expression of bile acid-synthesizing enzymes. Thus, the involvement of miRNAs in the fine-tuning of bile acid synthesis was demonstrated.

### 4.3. Others

Several research groups (Karbiener et al., 2009; Lin et al., 2009; Jennewein et al., 2010; Kim et al., 2010) have reported that miR-27a and miR-27b repress the expression of peroxisome proliferator-activated receptor γ (PPARγ) suggesting an association with the change in adipocyte differentiation and anti-inflammatory effects. In addition, a recent study reported that adipogenesis-regulated miR-