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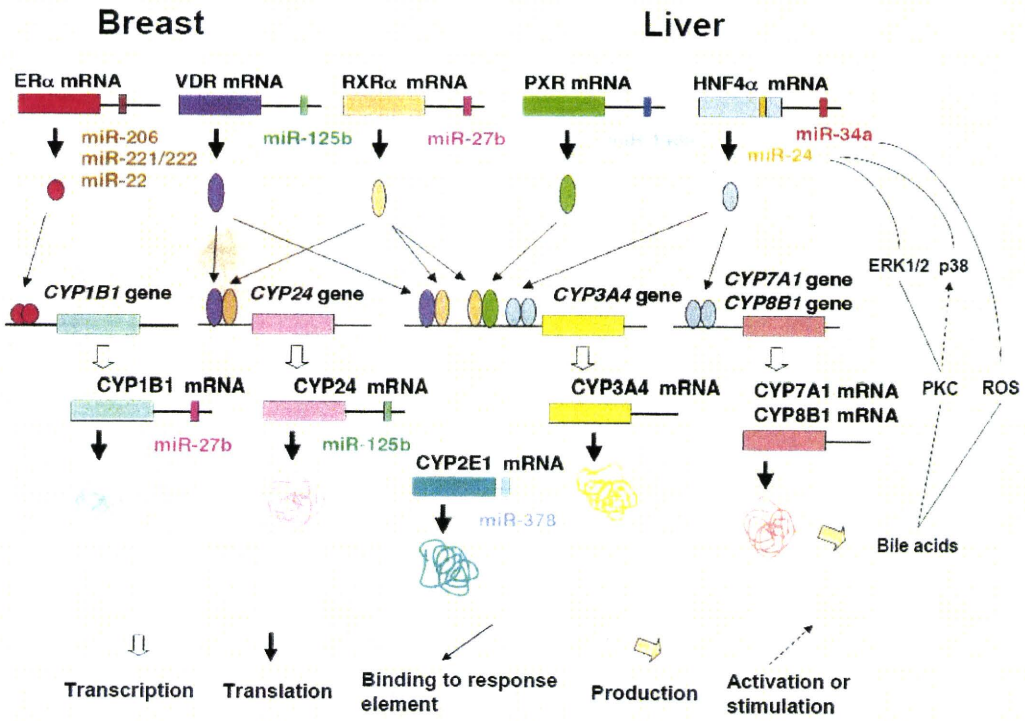
Table 1. Cytochrome P450s and nuclear receptors that are regulated by miRNAs.

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	Kim et al., 2010, Lin et al., 2009
	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.

Figure legends

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.



Toxicological implications of modulation of gene expression by microRNAs

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Abstract

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 1,000 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 were discussed.

Keywords: microRNA, miRNA, P450, CYP, post-transcriptional regulation, toxicology, polymorphism

Introduction

MicroRNAs (miRNAs) are short (~22-25 nucleotides in length), single stranded RNA genes possessing the reverse complement of the mRNA transcript of another protein-coding gene. MiRNA was first found in *C. elegans* as RNA molecules that are complementary to the 3' untranslated regions (UTR) 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. 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. More than half of those are conserved of the sequence between other vertebrate animals (Lagos-Quintana et al., 2003). Paralogous sequences whose mature niRNAs 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 (RISC), composed of RNase III Dicer, TAR RNA binding protein (TRBP) 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; Fabian *et al.*, 2010; Choudhuri, 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 has been exciting research on the role of miRNAs in toxicogenomics/toxicogenetics and the possibility of drug-induced adverse effects. Since most drugs and chemical toxicants are biotransformed to exhibit their functions, the expression of drug- and xenobiotic-metabolizing enzymes and their regulation by miRNA would be 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 functionalization of target genes of miRNAs.

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 6-7 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), PicTar (Krek *et al.*, 2005) are available to find the putative binding sites of miRNA to the target genes. A general algorithm to predict the target gene of miRNA has not been established, thus 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. 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)

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 co-transfected into the cells with precursor miRNA (or the expression plasmid of miRNA) or antisense oligonucleotide for miRNA to overexpress or inhibit miRNA. It can be established whether the reporter activity is significantly decreased or increased compared to a control. However, with the reporter-based assays, it should be examined 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 would be effective methods for determining the

effects of miRNA on the target gene expression and understanding the biological function of miRNA. In the overexpression experiments, a possible concern is that the concentration of miRNA may exceed the physiological levels in the cells due to saturation of nuclear karyopherin exportin-5 causing aberrant cellular functions. The adverse effect of oversaturating endogenous small RNA pathway can be minimized by optimizing dose and sequence. 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 and inhibition of miRNA to determine comprehensively the targets of a given miRNA (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.

MiRNA in 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. Members of the cytochrome P450 (P450, CYP) family are the most important enzymes catalizing 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. P450 has also been implicated in the bioactivation of procarcinogens to their ultimate carcinogens. The mechanisms of the transcriptional regulation of P450-related nuclear receptors have been considerably clarified, but the post-transcriptional regulation largely remains to be elucidated. Recently, some P450s and nuclear receptors have been found to be post-transcriptionally regulated by miRNAs. In

this paragraph, recently elucidated miRNA functions in relation to CYP and CYP-related representative nuclear receptors were focused.

【*Human CYP1B1*】

Human CYP1B1, expressed mainly in ovary, uterus and breast (Sutter *et al.*, 1994; Shimada *et al.*, 1996), catalyzes the metabolic activation of a variety of procarcinogens and promutagens, including polycyclic aromatic hydrocarbons and aryl amines (Shimada *et al.*, 1996), and the metabolism of 17β-estradiol (Hayes *et al.*, 1996; Spink *et al.*, 1997; Lee *et al.*, 2003), which contributes to the growth and development of estrogen-dependent cancers such as breast and endometrial cancers (Henderson and Canellos., 1980). 4-Hydroxyestradiol, a cathchol-type metabolite formed by CYP1B1, generates free radicals from reductive-oxidative cycling with the corresponding semiquinone and quinone forms, which cause DNA damege (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 due to polymorphism-dependent degradation of CYP1B1 protein by polyubiquitination but notphosphorylation (Bandiera *et al.*, 2005). Although there is no direct evidence to lack of association between mRNA and protein of CYP1B1 in panel of human tissues, the phenomena are reminiscent of novel post-transcriptional regulation mechanism.

It has been reported that human CYP1B1, which is highly expressed in estrogen target tissues (Tsuchiya *et al.*, 2004), could be regulated by miR-27b in MCF-7 breast cancer cells (Tsuchiya *et al.*, 2006). Exogenously expressed miR-27b could decrease the luciferase reporter activity in Jurkat cells (miR-27 negative). In MCF-7 cells (miR-27 positive), the antisense origonucleotide for 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 suggested that human CYP1B1 is post-transcriptionally 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 to 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. Because 4-hydroxylation of estrogen by CYP1B1 leads to decrease of the estrogenic activity but the produced metabolite is toxicologically active, it suggests a toxicological significance in the estrogen-dependent molecular mechanism for the estrogen-dependent carcinogenesis. This is the first study to demonstrate that miRNA regulates CYP.

【*Human CYP3A4 and PXR (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., midazoram, and erythromycin), which revealed that

there is at least 6-fold interindividual variation of the activities in most populations (Lin *et al.*, 2002: Floyd *et al.*, 2003: Rodriguez-Antona *et al.*, 2005), which cannot be explained solely by genetic polymorphisms (Lamba *et al.*, 2002; Floyd *et al.*, 2003). The 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, HNF4α and HNF3γ, as well as constitutive androstane receptor (CAR) and PXR (Martinez-Jimenez *et al.*, 2007). However, the cause of the large interindividual variability in the CYP3A4 level is poorly understood and is an urgent issue to be solved.

Animal and human CYP3A enzymes are involved in the metabolic activation of several drugs and xenobiotics to toxic metabolites(Thummel *et al.*, 1993). The amount of CYP3A enzymes in the liver is much higher than other CYP isoforms, especially in conditions induced by co-administered drugs. Human hepatocytes are generally used as in vitro cytotoxicity screening assay, however large interindividual variability of the enzyme activity makes the assay difficult. 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 were efficiently evaluated (Hosomi *et al*, 2010 and 2011). Notably, aflatoxin B_1 and G_1 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). The PXR mRNA level was not correlated with the PXR protein level in a panel of 25 human livers, suggesting the involvement of post-transcriptional regulation. However, no involvement on miRNA was suggested in CYP3A4 by the correlation analyses

between the CYP3A4 mRNA level and CYP3A4 protein level. 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, the 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 liver. Actually, a potential miR-148a recognition element in the 3'-UTR of human CYP3A4 mRNA did not regulate CYP3A4 message level. It was 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 (Pan *et al.*, 2009). In that report, the result was based on only an overexpression study, but an inhibition study of endogenous miRNA as well as a correlation analysis between the miRNA and target mRNA levels is necessary. If miRNA could not overcome the strong transcriptional regulation of the target gene, the involvement of miRNA may be invisible.

The PXR protein level was not significantly correlated with the 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 (2-fold) and MDR1 (5-fold) 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 post-transcriptionally

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 one of the pharmacologically and toxicologically important P450 isoforms. 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 resulting in the enhancement of 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, post-transcriptional regulation could be suggested for not only the inducible but also the constitutive expression of CYP2E1 in human liver. However, the molecular basis of the human CYP2E1 regulation largely remains unknown.

MicroRNA may be involved in the post-transcriptional regulation of human CYP2E1 was studied (Mohri *et al.*, 2010). *In silico* analysis identified a potential recognition element of miR-378 (MRE378) in the 3'-UTR of human CYP2E1 mRNA. Luciferase assays using HEK293 cells confirmed that miR-378 functionally recognized MRE378. Two HEK293 cell lines stably expressing human CYP2E1 including or excluding 3'-UTR was establised. 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 the CYP2E1 mRNA. Therefore, the down-regulation of CYP2E1 by miR-378 would mainly be due to the translational repression, not the mRNA degradation. In a panel of 25 human livers, no positive correlation was observed between the CYP2E1 protein and CYP2E1 mRNA levels, supporting the post-transcriptional 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. This study could provide new understanding forward solving the mechanism of the post-transcriptional regulation of CYP2E1. The 3'-UTR of CYP2E1 is poorly conserved among human, rat, and mouse. The regulation of CYP2E1 by miR-378 would be specific in human.

The miR-37 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 the expression of VEGF (Hua *et al.*, 2006). The expression of CYP2E1 is up-regulated in diabetes and obesity, but down-regulated by insulin treatment (Wang *et al.*, 2003; de Waziers *et al.*, 1995; Woodcroft *et al.*, 2002). The involvement of miR-378 in the induction of CYP2E1 by chemicals/xenobiotics would be of toxicologically interest.

【*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 treatment of rats increases lung CYP2A3 levels by ~3-fold, whereas phenobarbital is not a 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 (JaJas *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*. Since 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. MiR-34 has been reported to be involved in the regulation of a tumor suppressor p53 (Corney *et al.*, 2007; He *et al.*, 2007), but p53 mRNA expression did not change in NNK-treated rats (Kascheuer *et al.*, 2008). Changed miR-34 expression would affect the p53-related cell proliferation and cell death.

【*Human CYP24A1 and VDR (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 to those in noncancerous tissue due to the heterogeneous background in different breast cancers (Hicks *et al.*, 2006; de Lyra *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 VDR (Carlberg and Polly, 1998). Calcitrol is recently found to be associated with the risk of cancer (Garland *et al*, 2006). Since 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 post-transcriptional regulation.

It was reported that both human CYP24A1 (Komagata *et al.*, 2009) and VDR (Mohri *et al.*, 2009) are post-transcriptionally 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, indicating that the decreased miR-125b levels in breast cancer tissues would be one of the causes of the high CYP24 protein expression. Since CYP24A1 is a target of VDR, miR-125b would directly and/or indirectly regulate the CYP24A1. The increase of VDR in cancer tissues would augment the anti-tumor effects of calcitrol, whereas the increase of CYP24A1 would attenuate the anti-tumor effects. The effect of miR-125b on the antiproliferative effects of calcitrol using human breast cancer-derived MCF-7 cells was investigated. Then, it was clarified that miR-125b increased cell growth indicating that miR-125b is a part of VDR downstream activities.

MiR-125b inhibited the cell proliferation of human hepatocellular carcinoma cells (Li *et al.*, 2008), and thyroid carcinoma cells (Visone *et al.*, 2007) as well as 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). Because the miR-125b expression differentially changes in human tumors and down-regulated in breast, ovarian, and bladder cancers but is up-regulated in pancreas and stomach cancers (Volinia *et al.*, 2006), functional effects of miR-125b differ in each cancerous tissue, and miR-125b might be a potential biomarker of cancer-related outcomes.

【*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, 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, and those for miR-34a (MRE34a) were identified in only the 3'-UTR in HNF4α mRNA (Takagi *et al.*, 2010). The HNF4 α protein level in HepG2 cells was markedly decreased by the overexpression of miR-24 and miR-34a. The HNF4α mRNA level was 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, which 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, the expressions of miR-24 and miR-34a were regulated by protein kinase C/mitogen-activated protein kinase and reactive oxygen species (ROS) pathways, respectively.