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シトクロム P450 と転写因子の microRNA による発現制御

中島美紀

Role of MicroRNAs in the Regulation of Cytochrome P450s and Transcriptional Factors

Miki Nakajima

Faculty of Pharmaceutical Sciences, Kanazawa University; Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan.

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MicroRNAs (miRNAs) are endogenous ~22-nucleotide non-coding RNAs that regulate gene expression through the translational repression or degradation of target mRNAs. The human genome contains over 1400 miRNAs and over 60% of human mRNAs are predicted to be targets of miRNAs. 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. 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 transcriptional factors by miRNAs, and its physiological and clinical significance, which were disclosed in our studies. 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. Utilizing miRNAs opens a new era in the fields of drug metabolism and pharmacokinetics as well as toxicology.

Key words—microRNA; cytochrome P450; nuclear receptor; post-transcriptional regulation

1. はじめに

薬の効果や副作用に認められる個人差は、薬の体内動態並びに薬物代謝酵素活性の個人差に起因することが多い。その個人差の理解のため、薬物代謝酵素の遺伝子多型や転写調節の研究が行われてきた。しかし、それでもなお薬物代謝能の個人差を説明できない事象が存在する。薬物代謝能を制御する可能性のある新たな因子として microRNA (miRNA) が考えられた。miRNA はタンパク質をコードしない 22 塩基程度の小さな RNA で、標的となる mRNA に結合して翻訳を抑制あるいは mRNA を分解することにより、タンパク質の発現を負に制御する機能を有している。1993 年線虫で最初に発見されたのち、2001 年にヒトにも存在することが明らかにな

り、以降その存在意義が解明され、発生、分化、増殖、アポトーシスなど、重要な生命現象に係わっていることがわかってきた。これまでにヒトでは 1400 種以上の miRNA が同定され、ヒト遺伝子産物の 60% 以上が、また二次的な影響を含めるとほぼすべての遺伝子産物が miRNA によって調節されていると考えられている。本稿では、miRNA の生合成と発現抑制機構について簡単に解説し、ある遺伝子の発現制御に係わる miRNA を同定するために用いられる一般的な方法について述べた後、筆者らの研究によって明らかになったヒトシトクロム P450 と転写調節因子の miRNA による発現制御とその意義について概説する。

2. miRNA の生合成と発現抑制機構

miRNA は、通常 RNA ポリメラーゼ II によってヘアピン構造を有する 200–5000 塩基ほどの転写産物 primary microRNA (pri-miRNA) として転写され、核内で Drosha などによりプロセッシングを受けて、ステムループ構造を持つ約 70 塩基の precu-

金沢大学医薬保健研究域薬学系 (〒920-1192 石川県金沢市角間町)

e-mail: nmiki@p.kanazawa-u.ac.jp

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sor microRNA (pre-miRNA) となる。その後、pre-miRNA は Exportin-5 を介して細胞質に輸送され、Dicer によってプロセッシングを受けて約 22 塩基の二本鎖 miRNA:miRNA* となり、Dicer, Ago2, tar-RNA-binding proteins (TRBP) などで構成される RNA-induced silencing complex (RISC) と複合体を形成する。そして、一本鎖化された mature miRNA がガイド鎖となり、標的 mRNA の主に 3'-非翻訳領域 (untranslated region, UTR) に存在する認識配列 (miRNA recognition element, MRE) に結合する。miRNA の標的 mRNA への結合は部分相補的であり、miRNA の 5'末端 2-7 塩基である seed 配列が相補的であることが重要とされている。RISC はリボソーム・サブユニット会合の阻害、リボソームの脱落、キャップ構造の脱離、脱アデニル化など、種々のメカニズムを介して翻訳を抑制又は mRNA を分解し、遺伝子サイレンシングを起こす。

3. 発現制御に係わる miRNA の同定

1つの miRNA は数百種類の mRNA を標的とする可能性があり、1つの mRNA は複数の miRNA によって認識されることもあるため、遺伝子の発現調節に係わっている miRNA を予測することは容易ではない。いくつかの予測プログラムが利用可能であるが、それぞれのプログラムで用いるアルゴリズムが異なっており、予測されてくる miRNA が異なることも多い。偽陽性の確率も高く、実際に標的となるかどうかは実験により確かめなければわからない。また、予測プログラムでは miRNA の発現量は考慮されていないため、当該遺伝子が発現している組織に予測された miRNA がどの程度発現しているかは別途考慮する必要がある。

遺伝子の発現制御に miRNA が係わっているか調べる一般的な方法は以下の通りである。まず、細胞に miRNA を過剰発現させ、当該タンパク質又は mRNA の発現量が低下するか調べる。miRNA の過剰発現による人為的な影響の結果である可能性を否定するためには、miRNA に対するアンチセンスオリゴヌクレオチド (AsO) を導入し、内因性の miRNA を抑制した際に、当該タンパク質又は mRNA の発現量が増加するか調べるのが肝要である。しかし、このような実験では、miRNA の作用が直接的なものか、別の標的遺伝子に作用した二

次的な結果なのか判断できない。そこで有用なのが 3'-UTR や MRE をルシフェラーゼ遺伝子の下流に組み込んだプラスミドを用いたルシフェラーゼアッセイである。miRNA の過剰発現又は AsO の導入によってルシフェラーゼ活性に変動が認められれば、直接的な発現制御を証明することができる。MRE への変異の導入や欠失、複数連結などによるルシフェラーゼ活性の変動を調べることで、MRE の機能性を確認することも重要である。細胞内の miRNA の発現量を人為的に変動させることなく、常在的な状態で miRNA の関与を提唱するには、当該タンパク質発現量と miRNA の発現量に負の相関関係が認められるか調べることも有用である。正常細胞とがん細胞の比較、複数の細胞株間での比較、複数の個人サンプル間の比較など、様々なパターンで適用できる。

4. miR-27b によるヒト CYP1B1 の発現制御¹⁾

CYP1B1 は多環芳香族炭化水素や芳香族アミンの代謝的活性化を触媒し、またエストロゲンを DNA 損傷性の代謝物に変換することから、発がんに関与している分子種である。CYP1B1 は卵巣、子宮、乳腺などの組織において mRNA レベルでは高く発現しているものの、タンパク質レベルではほとんど検出できないことから、転写後調節の寄与が示唆され、miRNA による発現制御の可能性を検討した。CYP1B1 mRNA の長さは約 5.2 kb であり、そのうち 3'-UTR は約 3.1 kb と半分以上を占める。CYP1B1 mRNA の配列をヒト、マウス、ラットで比較すると翻訳領域の相同性は 80% 以上と高いのに対し、3'-UTR 全体の相同性は 30% ほどしかない (Fig. 1)。ところが、ポリ A に近い領域に 86% と高い相同性を示す領域が 44 bp ほど存在し、その中に miR-27b との結合が予想される配列が存在していた (Fig. 1)。miRNA の配列は種を超えて保存されていることが多く、MRE の配列も種で保存されているほど、その miRNA によって制御されることに意義があるものと推定される。ルシフェラーゼ遺伝子の下流に MRE や 3'-UTR を組み込んだプラスミドを Jurkat 細胞に pre-miR-27b とともに導入するとルシフェラーゼ活性の低下が認められた (Fig. 2)。一方、miR-27b の発現量の高い MCF-7 細胞に導入した際、MRE や 3'-UTR を組み込んだプラスミドでコントロールプラスミドと比べてルシフェ

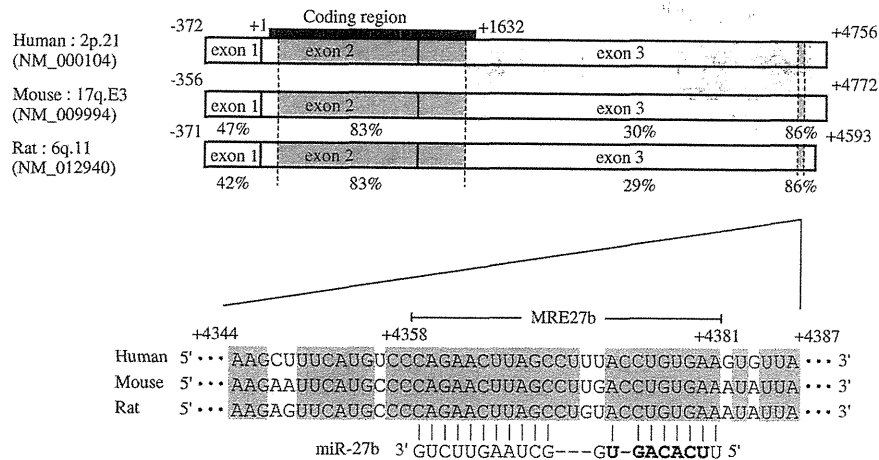


Fig. 1. Homology between Human, Mouse, and Rat CYP1B1 mRNAs and the Predicted Target Sequences of miR-27b
 CYP1B1 mRNAs in human, mouse, and rat are ~5 kb in length and consist of three exons. The numbering refers to the translation start site as 1. The sequence of MRE27b is located on +4358 to +4381 in the 3'-UTR of human CYP1B1. Highly conserved regions are shown in gray color. Bold letters: seed sequence.

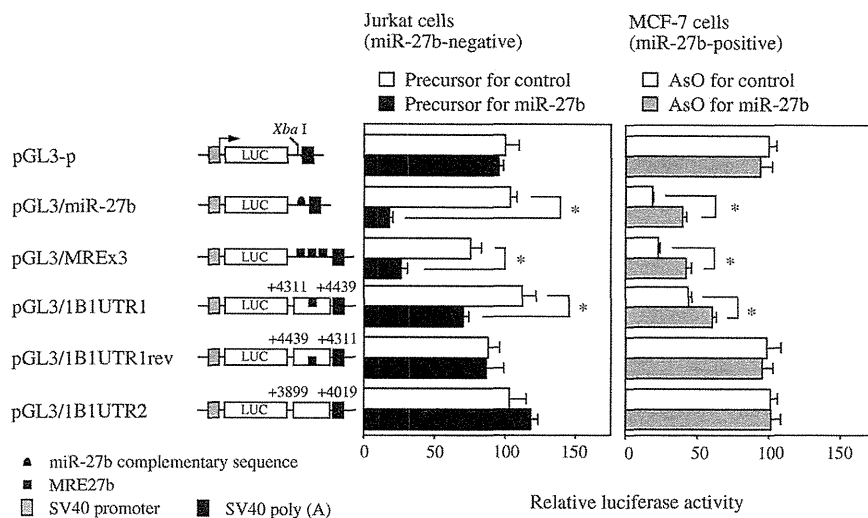


Fig. 2. Luciferase Assay with Reporter Constructs Containing MRE27b or 3'-UTR of Human CYP1B1 in Jurkat or MCF-7 Cells
 A series of reporter constructs was transfected into Jurkat cells with precursor for miR-27b or control, or into MCF-7 cells with AsO for miR-27b or control. Values are expressed as percentages of the relative luciferase activity of pGL3 promoter plasmid. Each column represents the mean \pm S.D. of three independent experiments. * $p < 0.05$.

ラーゼ活性は低値を示したが、miR-27b に対する AsO の導入により活性の回復が認められたことから、予測された MRE に miR-27b が結合し、発現を抑制していることが示された。また、miR-27b に対する AsO の導入により、MCF-7 細胞における内因性 CYP1B1 の発現量の増大が認められ、CYP1B1 が miR-27b によって制御されていることが明らかになった。この発現制御機構の生体内における意義を解明するにあたり、CYP1B1 タンパク質発現量は正常組織よりもがん組織で多いことに注目し、そ

の現象に miR-27b が関わっている可能性を考慮した。乳がん組織とその周辺の非がん部における miR-27b の発現量を調べたところ、がん部では発現量が少ないことが明らかになり [Fig. 3(A)], がん部における miR-27b の発現量と CYP1B1 タンパク質発現量との間に負の相関関係が認められた [Fig. 3(B)]. したがって、正常組織中では miR-27b が CYP1B1 の発現を抑制的に制御しており、がんでは CYP1B1 が高発現している理由の 1 つとして miR-27b の低下が挙げられることを明らかにした。これ

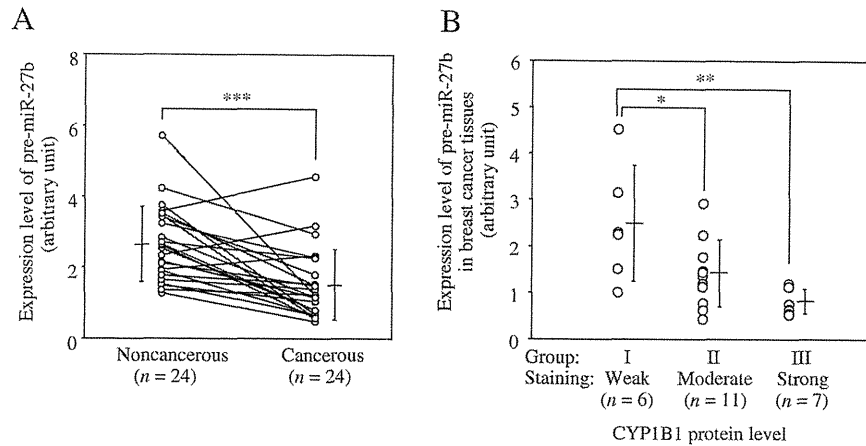


Fig. 3. Expression of miR-27b in Human Breast Cancerous and Adjacent Noncancerous Tissues (A) and the Relationship between the Expression Levels of miR-27b and CYP1B1 Protein Level in Human Breast Cancer (B)

The expression levels of pre-miR-27b and CYP1B1 protein were determined by real-time RT-PCR and immunostaining, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Human VDR mRNA

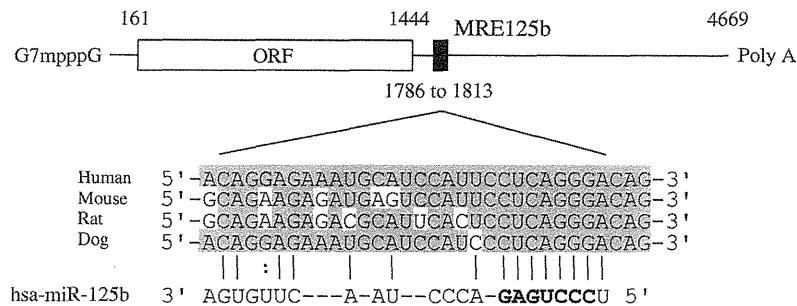


Fig. 4. Schematic Representation of Human VDR mRNA and the Predicted Target Sequence of miR-125b

The numbering refers to the 5'-end of mRNA as 1. The sequence of MRE125b is located on +1786 to +1813 in the 3'-UTR of human VDR. Highly conserved sequence is shown in gray color. Bold letters: seed sequence.

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は miRNA が薬物代謝酵素の発現制御に係わっていることを示した世界初の研究成果である。¹⁾

5. miR-125b によるビタミン D 受容体 (VDR)²⁾ と CYP24³⁾ の発現制御

ビタミン D₃ は血中カルシウム濃度の恒常性維持や骨代謝に重要な役割を担う一方、細胞増殖抑制作用や分化誘導及びアポトーシス誘導作用を有しており、抗がん薬としての可能性が期待されている。ビタミン D₃ の作用は VDR を介して発揮される。VDR は、mRNA 発現量としてはがん部位と正常部位で差が認められないものの、タンパク質発現量としては正常部位に比べがん部位で高いことが報告されており、転写後調節の関与が示唆された。VDR mRNA に結合する可能性のある miRNA を探索し

たところ、いくつかの miRNA が予測されたが、中でも miR-125b の認識部位 MRE125b の配列は種を超えて高く保存されていた (Fig. 4) ことから、miR-125b が VDR の発現を制御している可能性を検討した。ルシフェラーゼアッセイにより MRE125b が機能的に働いていることが示された。miR-125b が VDR のタンパク質発現量を抑制しているか、ヒトがん由来細胞株における VDR 発現量をウェスタンブロットで解析したが、市販の抗体では非特異的なバンドが多く、検出が困難であった。そこで、ゲルシフトアッセイを利用した検出を試みた。VDR は活性化されるとレチノイド X 受容体 α (RXRα) とヘテロダイマーを形成して、標的遺伝子の応答配列に結合して転写を活性化する。MCF-7 細胞に

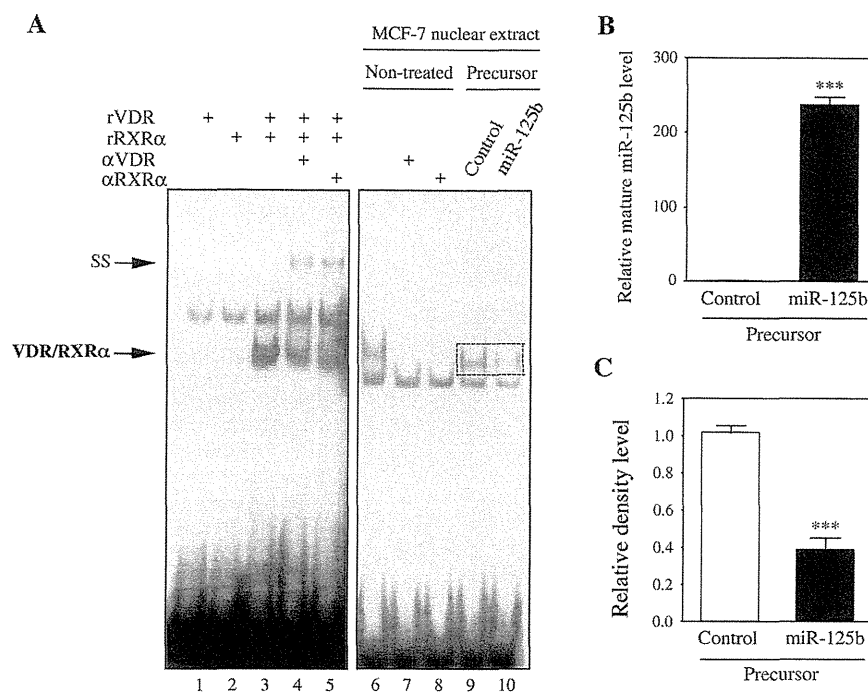


Fig. 5. Electrophoretic Mobility Shift Assay to Evaluate the Endogenous VDR Protein Level

The ^{32}P labeled probe containing the VDRE in human CYP24 promoter was incubated with *in vitro*-synthesized VDR (rVDR) and RXR α (rRXR α) or the nuclear extract prepared from the precursors for miR-125b or control-transfected MCF-7 cells (A). The mature miR-125b level was determined by real-time RT-PCR analysis (B). The relative density of the shifted band including VDR/RXR α complex was shown as the mean \pm S.D. of three independent experiments (C). *** $p < 0.001$.

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pre-miR-125b を導入して核抽出液を調製し、VDR の標的遺伝子の1つであるCYP24の応答配列をプローブとしてゲルシフトアッセイを行ったところ、VDR/RXR α ヘテロダイマーの結合量が低下し、VDR発現量の低下が示された (Fig. 5). また、VDRのリガンドである1 α ,25-ジヒドロキシビタミンD $_3$ の処置により、標的遺伝子であるCYP24 mRNAは顕著に誘導されるが、その誘導能はmiR-125bにより有意に抑制された (Fig. 6). 以上より、ヒトVDRがmiR-125bによって発現制御されていることが明らかになった.²⁾

また興味深いことに、CYP24の発現もmiR-125bで制御されていることを、過剰発現又は阻害実験及びルシフェラーゼアッセイなどの手法を用いて明らかにした (Fig. 7).³⁾つまり、CYP24はmiR-125bによって直接的に、及びVDRの発現抑制を介して間接的に発現抑制されていることになる。CYP24も正常組織に比べてがん組織で高発現しており、がん組織におけるmiR-125bの発現低下が原因であることも示された。様々ながん組織において多くの

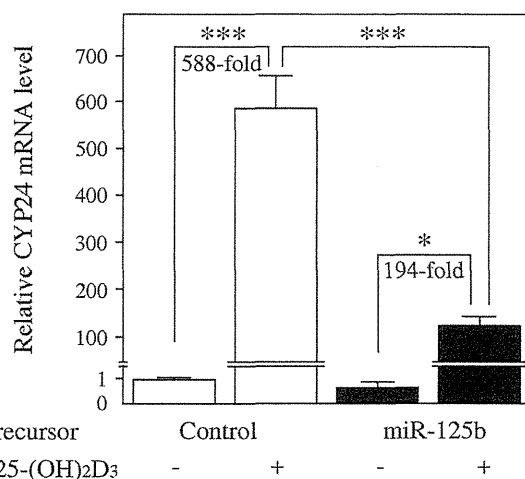


Fig. 6. Induction of CYP24 mRNA in MCF-7 cells by 1 α ,25-dihydroxyvitamin D $_3$

The precursors for miR-125b or control (50 nM) were transfected into MCF-7 cells. After 72 h, the cells were treated with 100 nM 1 α ,25-dihydroxyvitamin D $_3$ or 0.1% ethanol (vehicle) for 24 h and then CYP24 mRNA levels were determined by real-time RT-PCR and normalized with the GAPDH mRNA level. Each column represents the mean \pm S.D. of three independent experiments. * $p < 0.05$, *** $p < 0.001$.

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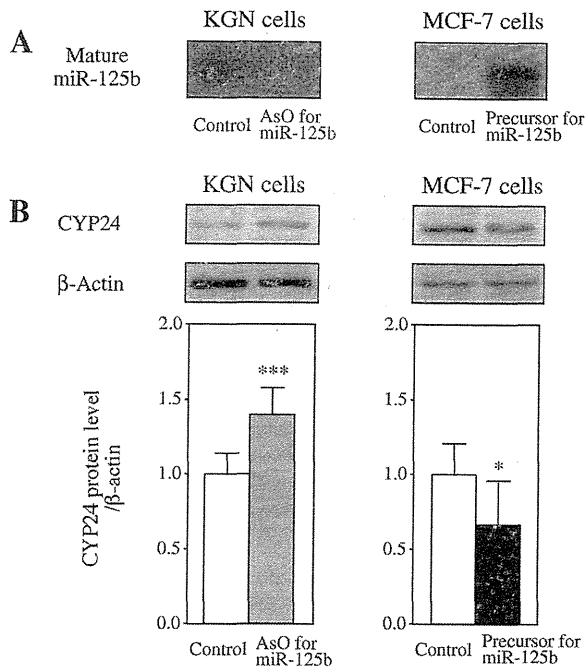


Fig. 7. Effects of miR-125b on the Endogenous CYP24 Protein Level in KGN or MCF-7 Cells

AsO for miR-125b or control (2.5 pmol/4×10⁵ cells) were transfected into KGN cells and precursors for miR-125b or control (84 pmol/1.68×10⁵ cells) were transfected into MCF-7 cells. After 72 h, total RNA and whole cell lysate were prepared. The expression levels of mature miR-125b were determined by Northern blot analysis (A). The expression levels of CYP24 protein were determined by Western blot analysis and normalized with β -actin protein level (B). Each column represents the mean \pm S.D. of three independent experiments. **p*<0.05, ****p*<0.005

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miRNA の発現が大きく変動していることが示されている。⁴⁾ その原因として、転写活性の変化、エピジェネティック発現調節の変動、遺伝子変異、DNA コピー数の異常など、いくつかの理由が挙げられるが、miRNA をコードする遺伝子の半数以上はがんに関連する fragile site 上に存在することから、DNA コピー数の異常が主な原因と考えられている。⁵⁾ miR-125b は pre-miR-125b-1 と pre-miR-125b-2 の 2 つの前駆体から生成されるが、それらをコードする遺伝子はそれぞれ 11q24.1 と 21q11.2 にある。11q23-24 は乳がん、卵巣がん、肺がんなどで欠失し易く、^{6,7)} 21q11-21 は乳がん、食道がん、胃がん、卵巣がん、肺がんなどで欠失し易い領域である。⁸⁾ そのため miR-125b の発現量ががん組織で低下しているものと考えられる。

CYP24 は活性型ビタミン D₃ の 24 位水酸化反応を触媒し、不活性化する酵素である。活性型ビタミン

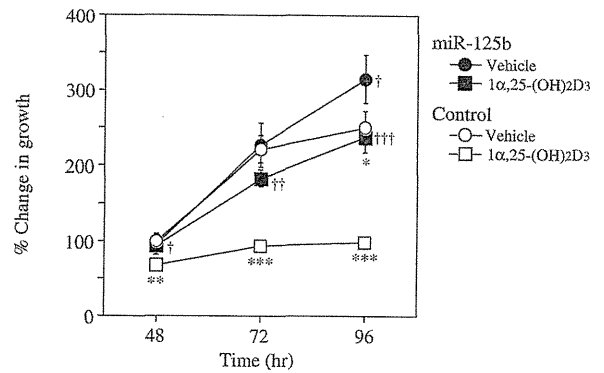


Fig. 8. Antiproliferative Effects of 1 α ,25-dihydroxyvitamin D₃ in MCF-7 Cells

The precursors for miR-125b or control (20 nM) were transfected into MCF-7 cells. After 24 h, the cells were treated with 1 μ M 1 α ,25-dihydroxyvitamin D₃ or 0.1% ethanol (vehicle) for 48–96 h and then crystal violet assays were performed. Values are expressed as percentages change in growth relative to the cell viability in the precursor for control-transfected cells in the absence of 1 α ,25-dihydroxyvitamin D₃ after 48 h incubation. Each point represents the mean \pm S.D. of three independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001, compared with the vehicle. †*p*<0.05, ††*p*<0.01, †††*p*<0.001, compared with the precursor for control.

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ン D₃ の効果発揮に必要な VDR と不活性化に係わる CYP24 がともに miR-125b により制御されていることが明らかになり、それがビタミン D₃ による細胞増殖抑制作用にどのような結果をもたらすかが疑問に残った。そこで、MCF-7 細胞の増殖能を評価することで検討したところ、細胞増殖能は 1 α ,25-ジヒドロキシビタミン D₃ により有意に抑制され、その抑制効果は pre-miR-125b の導入により低下した (Fig. 8)。したがって、この実験条件下では miR-125b は VDR に対する抑制効果を優先的に示すことが明らかになった。すなわちがん組織では miR-125b の発現が低下しており、VDR を介した抗腫瘍作用を増大させる生体防御機構が働いている可能性が考えられた。

6. miR-148a によるプレグナン X 受容体 (PXR) の発現制御と CYP3A4 発現量への影響⁹⁾

ヒト CYP3A4 は肝臓及び小腸に高く発現し、医薬品代謝の約 50% に関与する最も重要な薬物代謝酵素である。CYP3A4 の発現量や酵素活性には 100 倍ほどの大きな個人差が認められるが、遺伝子多型でも個人差を説明できない。筆者らは CYP3A4 とその発現に重要な役割を果たすプレグナン X 受容体 (pregnane X receptor, PXR) の 3'-UTR に共通して miR-148a 認識配列が存在することを見出し

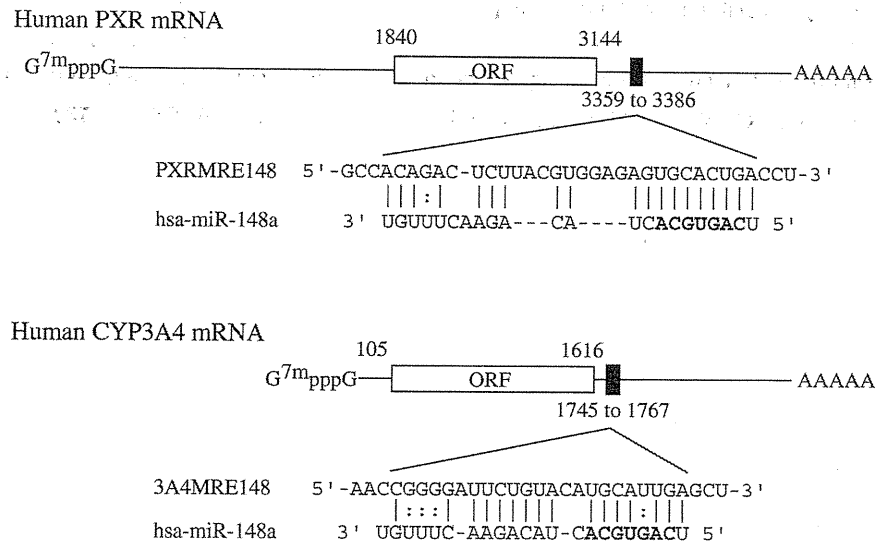


Fig. 9. Schematic Representation of Human PXR and CYP3A4 mRNAs and the Predicted Target Sequence of miR-148a
 The numbering refers to the 5'-end of mRNA as 1. The sequence of MRE148a is located on +3359 to +3386 in the 3'-UTR of human PXR mRNA and +1745 to +1767 in the 3'-UTR of human CYP3A4 mRNA. Bold letters: seed sequence.

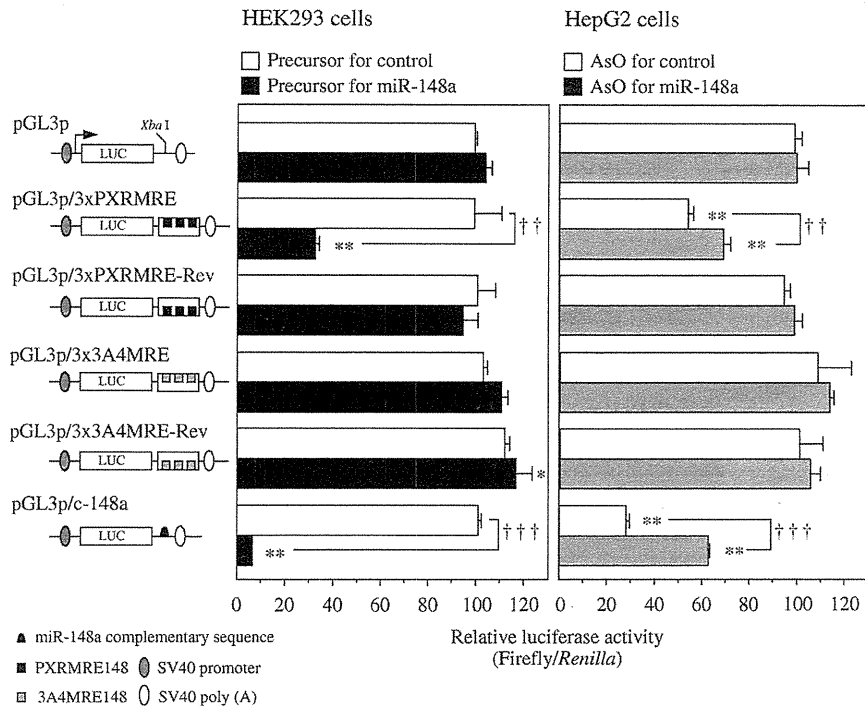


Fig. 10. Luciferase Assay with Reporter Constructs Containing MRE148a in the 3'-UTR of PXR or CYP3A4 in HEK293 or HepG2 Cells

A series of reporter constructs was transfected into HEK293 cells with precursor for miR-148a or control, or into HepG2 cells with AsO for miR-148a or control. Values are expressed as percentages of the relative luciferase activity of pGL-3 promoter plasmid. Each column represents the mean \pm S.D. of the independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with pGL3, † $p < 0.01$, †† $p < 0.001$ compared with precursor or AsO for control.

(Fig. 9), 解析したところ, miR-148a は CYP3A4 には直接作用しないが, PXR の発現を抑制的に制御し (Fig. 10), CYP3A4 の発現量に影響を与えて

いることを明らかにした (Fig. 11). タンパク質は DNA から転写された mRNA に基づいて合成されるため, かならず転写レベルでの調節を受けている。

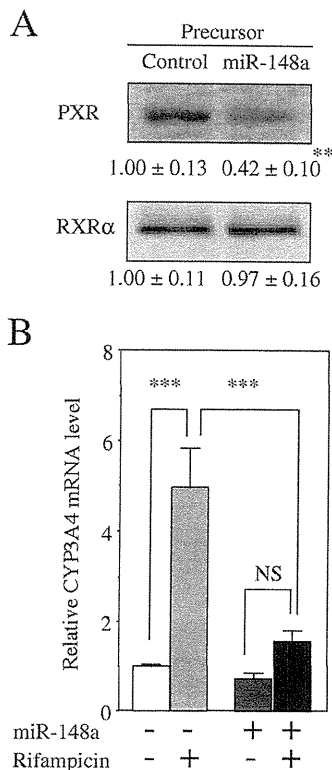


Fig. 11. Effects of Overexpression of miR-148a on the Endogenous PXR Level and the Induction of CYP3A4 mRNA in LS180 Cells

The precursors for miR-148a or control (50 nm) were transfected into LS180 cells. After 72 h, the cells were harvested and nuclear extracts were isolated. The PXR and RXR α protein levels were determined by Western blot analysis (A). The precursor-transfected LS180 cells were treated with 50 μ M rifampicin or 0.1% DMSO for 24 h and the CYP3A4 mRNA levels were determined by real-time RT-PCR and normalized with the GAPDH mRNA level. Data are the mean \pm S.D. of three independent experiments. ^{**} $p < 0.01$, ^{***} $p < 0.001$; NS: Not significant.

25 検体のヒト肝試料を用いた検討において、PXR では mRNA 発現量とタンパク質発現量との間に正の相関関係が認められず ($r = 0.1$)、転写後調節が大きく寄与していることが示唆された (Fig. 12)。一方、CYP3A4 では mRNA 発現量とタンパク質発現量との間に有意な正の相関関係が認められた ($r = 0.67$, $p < 0.001$) ことから、転写調節が主要であり、miRNA による転写後調節の寄与は大きくないことが示された。

7. miR-24 と miR-34a による hepatocyte nuclear factor 4 α (HNF4 α) の発現制御と胆汁酸合成への影響¹⁰⁾

肝臓や腎臓、腸管などに発現しており、非常に多くの遺伝子発現を制御することからマスターレギュレーターとよばれる肝細胞核因子 4 α (hepatocyte

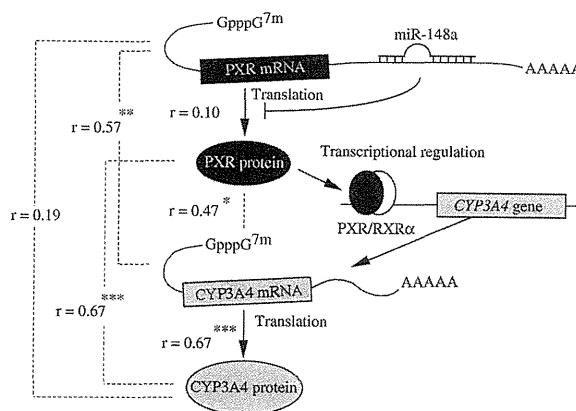


Fig. 12. Schematic Representation of miR-148a-dependent Post-transcriptional Regulation of Human PXR Affecting the Expression Level of CYP3A4 in Human Livers
^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$.

nuclear factor 4 α , HNF4 α) が miRNA で制御されている可能性を検討した。興味深いことに、miR-34a は 3'-UTR に結合して翻訳を抑制し、miR-24 は翻訳領域に結合して mRNA の分解を介して発現を抑制することを明らかにした [Fig. 13(A)]。この発現制御は肝臓中において HNF4 α の下流遺伝子である CYP7A や CYP8B などの胆汁酸合成酵素の発現低下を招くことが示された [Fig. 13(B)]。胆汁酸は HNF4 α の発現を低下させ、胆汁酸合成を抑制する、というネガティブフィードバック機構が存在することが報告されていたが、そのメカニズムは不明であった。本研究では、胆汁酸によるプロテインキナーゼ C の活性化や活性酸素種の産生を介したシグナル伝達経路の活性化が miR-24 及び miR-34a の発現を増加させ、それが HNF4 α の発現低下をもたらしていることを示し、メカニズムの一因に miRNA が関わっていることを明らかにした (Fig. 14)。

8. おわりに

上述の研究に加え筆者らは、ヒト CYP2E1 が miR-378 で制御されていること¹¹⁾ ヒト PPAR α が miR-21 及び miR-27b で制御されていること¹²⁾ も最近明らかにしており、薬物・異物代謝における microRNA の役割についてかなり情報が蓄積されてきた。¹³⁾ 興味深いことに、ジヒドロ葉酸還元酵素 (dihydrofolate reductase, DHFR)¹⁴⁾ や硫酸転移酵素 (sulfotransferase, SULF) 1A1¹⁵⁾ の 3'-UTR に存在する一塩基多型 (single nucleotide polymorphism, SNP) が、miRNA による結合・制御能に影響を及

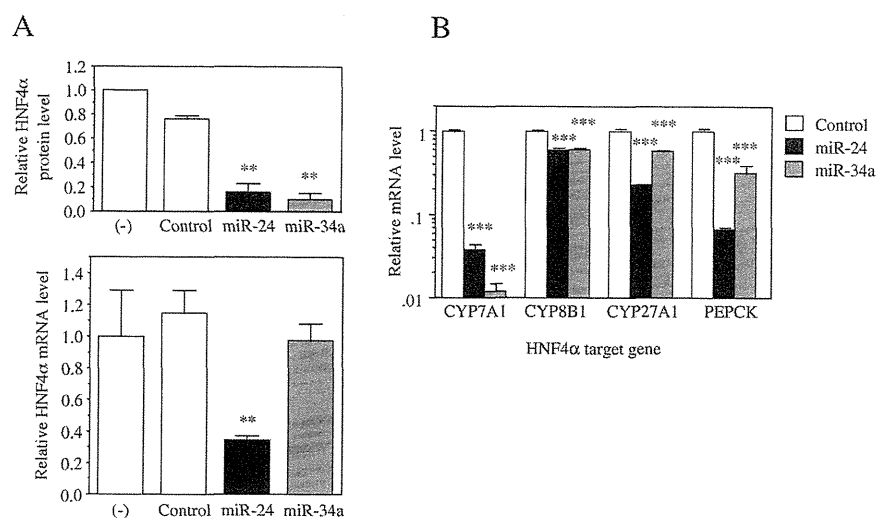


Fig. 13. Effects of miR-24 and miR-34a on the Human HNF4 α Protein or mRNA Levels (A) and Its Downstream Genes (B)

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 HNF4 α protein levels were determined by Western blot analysis and normalized with GAPDH protein level (A). The HNF4 α 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 \pm S.D. of three independent experiments. ** p <0.01, *** p <0.001.

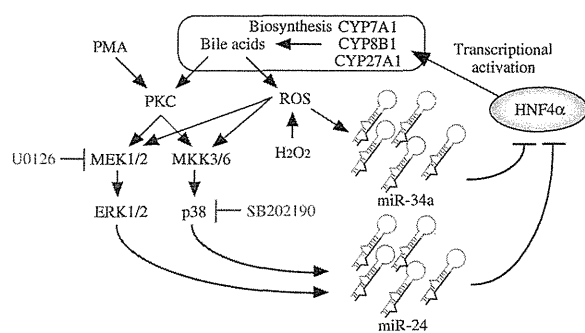


Fig. 14. The Regulatory Loop of miR-24, miR-34a and HNF4 α in Bile Acid Synthesis

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 HNF4 α . The down-regulation of HNF4 α 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.

ばし、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す、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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Epigenetic regulation is a crucial factor in the repression of UGT1A1 expression in the human kidney

Shingo Oda, Tatsuki Fukami, Tsuyoshi Yokoi, and Miki Nakajima

Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan.

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Running title: Epigenetic control of tissue-specific expression of UGT1A1

To whom all correspondence should be addressed:

Miki Nakajima, Ph.D.

Drug Metabolism and Toxicology

Faculty of Pharmaceutical Sciences

Kanazawa University

Kakuma-machi

Kanazawa 920-1192, Japan

Tel / Fax +81-76-234-4407

E-mail: nmiki@p.kanazawa-u.ac.jp

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ABBREVIATIONS: 5-Aza-dC, 5-aza-2'-deoxycytidine; ChIP, chromatin

immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNF, hepatocyte

nuclear factor; PCR, polymerase chain reaction; RT, reverse transcription; TSA, trichostatin A;

UGT, UDP-glucuronosyltransferase.

Abstract

Human UDP-glucuronosyltransferase (UGT) 1A1 catalyzes the metabolism of numerous clinically and pharmacologically important compounds such as bilirubin and SN-38. UGT1A1 is predominantly expressed in the liver and intestine, but not in the kidney. The purpose of this study was to uncover the mechanism of the tissue-specific expression of UGT1A1, focusing on its epigenetic regulation. Bisulfite sequence analysis revealed that the CpG-rich region near the *UGT1A1* promoter (-85 to +40) was hypermethylated (83%) in the kidney, whereas it was hypomethylated (37%) in the liver. A chromatin immunoprecipitation assay demonstrated that histone H3 near the promoter was hypoacetylated in the kidney but was hyperacetylated in the liver; this hyperacetylation was accompanied by the recruitment of HNF1 α to the promoter. The *UGT1A1* promoter in human kidney-derived HK-2 cells that do not express UGT1A1 was fully methylated, but was relatively unmethylated in human liver-derived HuH-7 cells that express UGT1A1. Treatment with 5-aza-2'-deoxycytidine (5-Aza-dC), an inhibitor of DNA methylation, resulted in an increase of UGT1A1 mRNA expression in both cell types, but the increase was much larger in HK-2 cells than in HuH-7 cells. The transfection of an HNF1 α expression plasmid into the HK-2 cells resulted in an increase of UGT1A1 mRNA only in the presence of 5-Aza-dC. In summary, we found that DNA hypermethylation along with histone hypoacetylation interferes with the binding of HNF1 α , resulting in the defective expression of UGT1A1 in the human kidney. Thus, epigenetic regulation is a crucial determinant of tissue-specific expression of UGT1A1.

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Introduction

UDP-glucuronosyltransferases (UGTs) catalyze the glucuronidation of a large number of endogenous and exogenous compounds. In humans, there are 19 functional UGT enzymes; these enzymes are classified into three subfamilies, UGT1A, UGT2A, and UGT2B (Mackenzie et al., 2005). The *UGT1A* genes, located on chromosome 2q37, contain multiple unique first exons and common exons 2 to 5 and encode nine kinds of functional UGT1A enzymes (Ritter et al., 1992). The *UGT2* genes, located on chromosome 4q13, comprise six exons that are not shared between the UGT2 family members, with the exception of UGT2A1 and UGT2A2, which are formed by exon sharing as in UGT1A. The *UGT2* genes encode three UGT2A and seven UGT2B functional enzymes.

Human UGTs show tissue-specific expression. Although most UGTs are predominantly expressed in the liver, UGT1A7, UGT1A8, and UGT1A10 are exclusively expressed in the gastrointestinal tract (Strassburg et al., 1997 and 1998). UGT1A1 is expressed in the liver, small intestine and colon, but not in the kidney (Nakamura et al., 2008; Ohno and Nakajin, 2009; Court et al., 2012). The expression of UGT2A1 and 2A2 are limited to the olfactory epithelium (Court et al., 2012). UGT2B7 is abundantly expressed in the liver, kidney, small intestine, and colon, whereas UGT2B10 is expressed only in the liver (Court et al., 2012). To understand the underlying mechanisms of the tissue specific-expression of UGTs, some studies were conducted with a focus on transcriptional regulation (Gardner-Stephen and Mackenzie, 2008; Mackenzie et al., 2010). It has been demonstrated that the intestine-specific transcription factor, caudal-type homeobox protein 2 (Cdx2), Sp1 and hepatocyte nuclear factor (HNF) 1 α regulate UGT1A8 and 1A10 expression in the intestine (Gregory et al., 2004). HNF1 α and Cdx2 cooperatively regulate UGT2B7 expression in the intestine, whereas HNF1 α and octamer transcription factor-1 cooperatively regulate its expression in the liver and kidney (Gregory et al., 2006). HNF1 α is also involved in the regulation of UGT1A1 expression in the liver

(Bernard et al., 1999). Thus, knowledge of the transcriptional regulation of the tissue-specific expression of the UGTs is accumulating.

However, a question that has yet to be answered is why UGT1A1 is not expressed in the kidney, even though HNF1 α is expressed in this tissue (Rey-Campos et al., 1991). In this study, we sought to clarify the mechanisms underlying the defective expression of UGT1A1, with a focus on epigenetic regulation. It is known that epigenetic changes including DNA methylation and histone modification are key regulators of tissue-dependent gene expression (Shiota, 2004; Ohgane et al., 2008). Supporting our hypothesis, a previous study found that the DNA methylation status of the proximal promoter region of the *UGT1A1* gene affects UGT1A1 expression in colon cancer cell lines (Gagnon et al., 2006). We investigated whether DNA methylation of the promoter and histone modification might be determinants of the tissue-specific expression of human UGT1A1.

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Materials and Methods

Materials. 5-Aza-2'-deoxycytidine (5-Aza-dC) and trichostatin A (TSA) were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-human HNF1 α polyclonal antibody (C-19), mouse anti- β -actin monoclonal antibody (C-14), and control rabbit and goat IgGs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human acetyl histone H3 polyclonal antibody was purchased from Millipore (Billerica, MA). Primers were commercially synthesized at Hokkaido System Science (Sapporo, Japan). All other chemicals and solvents were of the highest grade commercially available.

Human tissues. Human liver and kidney samples from five Japanese donors (donor 1, an 80-year-old female; donor 2, a 54-year-old male; donor 3, a 39-year-old female; donor 4, a 13-year-old male; donor 5, a 40-year-old male) were obtained from autopsy materials that were discarded after pathological investigation. The use of the human livers and kidneys was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan) and Iwate Medical University (Morioka, Japan).

Cell culture. Human kidney tubular epithelial cell line HK-2 and human hepatocellular carcinoma cell line HuH-7 were obtained from the American Type Culture Collection (Manassas, VA) and the RIKEN BioResource Center (Ibaraki, Japan), respectively. These cells were cultured as previously described (Nakamura et al., 2008).

RNA isolation and real-time reverse transcription (RT)-polymerase chain reaction (PCR).

Total RNA was isolated from human liver and kidney samples using RNAiso (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. The cDNA was synthesized from the total

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RNA using ReverTraAce (Toyobo, Osaka, Japan). The UGT1A1 mRNA levels were determined by real-time RT-PCR and normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as described previously (Izukawa et al., 2009).

Genomic DNA extraction and bisulfite reaction. Genomic DNA samples were prepared from human liver (donor 3) and kidney (donor 1) samples, cell lines, or human hepatocytes (HH268, a 54-year-old Caucasian female, Tissue Transformation Technologies, Edison, NJ) with a Genra Puregene Tissue kit (Qiagen, Valencia, CA). Five hundred nanograms of genomic DNA digested with *EcoR* I was treated with bisulfite using the EZ DNA Methylation kit (Zymo Research, Orange, CA). The DNA fragment near the transcription start site of the *UGT1A1* gene was amplified by PCR using the primer pair shown in Table 1. The PCR products were cloned into the pT7Blue T-Vector (Novagen, Madison, WI), and randomly picked clones were sequenced. The DNA methylation status of the sequence was analyzed using the web-based tool QUMA (Kumaki et al., 2008).

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed using the ChIP assay kit (Millipore) with slight modifications. Approximately 200 mg of frozen human liver (donor 3) or kidney (donor 1) was minced on ice and suspended in 1% (v/v) formaldehyde to cross-link proteins to DNA. After centrifugation, the precipitate was resuspended in cell lysis buffer and homogenized using a Dounce homogenizer. After centrifugation, the precipitate was resuspended in nuclei lysis buffer and sonicated to shear the genomic DNA. After centrifugation, the supernatant (100 μ L) was diluted ten-fold with IP dilution buffer and incubated with Dynabeads protein G (Life Technologies, Gaithersburg, MD) conjugated to antibodies against acetylated histone H3 (5 μ g) or HNF1 α . A proportion of the diluted supernatant was kept as an input. The Dynabeads protein G was precipitated and was washed

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sequentially one time each with a low-salt immune complex wash buffer, a high-salt immune complex buffer, and a LiCl immune complex buffer. The DNA-protein complex was eluted with elution buffer twice, and the cross-links were reversed by adding NaCl. DNA was extracted by phenol-chloroform extraction and ethanol precipitation. The -118 to +91 region of the *UGT1A1* gene was amplified by real-time PCR with the primers shown in Table 1. The protocol for the PCR was as follows: 95°C for 30 s followed by 45 cycles of 94°C for 4 s and 62°C for 20 s. DNA extraction and real-time PCR were also performed for the input samples, and the data were used as a control to evaluate the enrichment of DNA in the immunoprecipitates.

Construction of an HNF1 α expression plasmid. Human HNF1 α cDNA was amplified by PCR using the primer pair shown in Table 1 and human liver cDNA as a template. The PCR product was subcloned into the pTARGET vector (Promega, Madison, MI). The nucleotide sequence was confirmed by DNA sequencing analysis.

Chemical treatment and transfection of expression plasmid into the cells. HK-2 and HuH-7 cells were seeded onto a 12-well plate at 0.5×10^5 cells/well and incubated for 24 h. For dose response experiments, the cells were treated with 0.01, 0.1, 1 or 10 μ M 5-Aza-dC for 120 h or treated with 50, 100, or 300 nM TSA for 24 h and then subjected to RNA isolation. For the overexpression of HNF1 α , the cells were transiently transfected with 0.5 μ g of an HNF1 α expression plasmid or an empty pTARGET plasmid using the X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Indianapolis, IN). After 12 h, the cells were treated with 0.1 μ M 5-Aza-dC for 96 h, followed by treatment with TSA for an additional 24 h. The *UGT1A1* mRNA levels were determined as described above.