結果、肝障害の発症時間に相関し、毒性発症が早いAPAPおよび四塩化炭素において投与後1時間において発現の増加が認められ、逆にそれ以外の比較的毒性発現が遅い化合物のBB, DMN, TAにおいては早い段階での抑制が認められた。これまでの報告では、miR-21の発現調節機構として、炎症性サイトカインIL-6による、転写因子Stat3を介した転写調節 (Loffler et al., 2007)が報告されている。急性炎症性肝障害におけるIL-6の応答は早く、mRNA levelにおいてはマウスにおいて投与後4時間の段階ですでに誘導が認められている (Masubuchi et al., 2003)。それ以前の時間で検討はなされていないが、投与後15分ですでに影響を受けている遺伝子も存在していることから (Ruepp et al., 2002)、ごく早期におけるIL-6の遊離が起こっている可能性も考えられる。

次に、miR-21の抑制状態における細胞障害性の影響について検討を行った。これまでの報告より、miR-21は広範な臓器に発現が認められ、miRNAMap (http://mirnamap.mbc.nctu.edu.tw/)のウェブサイトによると、ヒト正常組織では膀胱、腎、肺に多く、それ以外のほとんどの臓器にも発現している。特に、乳腺や膵臓、肝では正常組織と癌部を比較した結果、癌部において発現量が大きく増加することが知られており、膵臓癌においては過剰発現による肝への転移促進なども同時に報告されている (Iorio et al., 2005; Kutay et al., 2006; Roldo et al., 2006)。また、癌由来細胞株 (肝、乳腺、結腸、肺、膵臓、胃)でも増加が認められている (Meng et al., 2007; Volinia et al., 2006)。これまで報告は、発現の変化という事象を取り扱うものであり、その変化が具体的にどのような影響を及ぼしているのかについての言及はなかった。しかし前章で述べたとおり、現在多くの報告がなされている。その中において、二次元電気泳動による標的タンパクTropomyosin 1の発見 (Zhu et al., 2007)、ターゲット検索サイトを用いた標的タンパクPTENの同定 (Meng et al., 2007)および標的タンパクProgrammed cell death (PDCD4)の同定 (Asangani et al., 2007; Frankel et al., 2007)が報告された。特に最後の2つのグループは同一のタンパクをPDCD4の3'非翻訳領域の解析とMCF-7細胞に対するDNAマイクロアレイという全く異なった手法で同一の標的タンパクを同定している点は興味深い。また、間接的な作用による抗アポトーシス因子Bcl-2の発現抑制 (Ji et al., 2007; Si et al., 2007)などが報告されている。これらの報告から、癌由来細胞株には正常組織と比較して、かなりのmiR-21が発

現していることが示唆されており、本章ではできる限り発現量が低い HeLa 細胞を用いた。Mature miRNA は 18-22 塩基と非常に短いものである。そのため、標的とするには短すぎること、クラスターなどの転写直後の未成熟な miRNA を標的にすると他の miRNA を同時に抑制してしまう可能性があることから、RNAi 技術を適用することができない。そこで miRNA を抑制するために用いられる技術がアンチセンス法である。AsO の導入によって細胞内の miRNA は捕獲されて機能を停止する。当研究室の経験上、作用機序は定かではないが、AsO の導入によって標的 miRNA の分解を起こしている兆候が認められている (data not shown)。そのために、AsO 導入細胞において miR-21 総量の減少を確認することで AsO 導入の可否を確認した。

本研究における検討に用いた HeLa 細胞において、TA 処置による細胞毒性を引き起こすことができなかった。肝由来ではない上に、薬物代謝能が低下している培養系において毒性を引き起こすために必要な TA の濃度が非常に高くなってしまい、生存率の測定系にも影響が出たためである (data not shown)。それに加えて、miR-21 の標的蛋白質を複数の予測サイトによる検討の結果、PPAR-αを本検討における標的蛋白質として設定した。そこで、PPAR-αのアゴニストとして知られるフィブラート系薬剤との併用で、横紋筋融解症を引き起こすことが知られるスタチン系薬剤について検討した。脂溶性のシンバスタチン、ロバスタチンと、対照薬として水溶性のプラバスタチン、フルバスタチンについて検討を行ったところ、脂溶性スタチンにおいてのみ濃度依存的な細胞生存率の低下が認められた。過去の報告においても脂溶性スタチンによる骨肉腫細胞株、メサンギウム細胞を用いた *in vitro* における検討で細胞死が認められている (Fromigué et al., 2006; Heusinger-Ribeiro et al., 2004)。対照薬のプラバスタチン、フルバスタチンでは細胞生存率の若干の増加が見られたが、その原因は不明である。また、脂溶性スタチンの細胞毒性は AsO の導入によって有意に増強されたことから、miR-21 は脂溶性スタチンによる細胞毒性に対して保護的に働く因子を調節していることが示唆された。しかしながら、標的蛋白質の発現を検出することができず、AsO による蛋白質への影響を検討することはできなかった (data not shown)。前述の通り、miR-21 は現在急速に研究が進められている miRNA であるが、薬物誘導性細胞障害との関係を明らかにした報告は初めてである。一方、予備検討においてシンバスタチン処置前に

miR-21 precursor 導入について検討したが、細胞障害性への影響は認められなかった (data not shown)。影響しなかった理由として、HeLa 細胞には他の細胞株と比較して miR-21 が少ないとしても、正常組織より過剰な miR-21 が発現していたため影響を見ることができなかったと考えられる。

AsO を導入した群において SCR と比較して 50%程度の細胞生存率の減少が見られた (data not shown)。このこと癌由来細胞株に対して miR-21 のアンチセンスオリゴを導入した際の細胞生存率低下の報告 (Si et al., 2007, Meng et al., 2007)と一致していた。また、この検討に関連し、AsO の導入によって HeLa 細胞内で変化する蛋白質を二次元電気泳動によって検出する実験を試みたが、変化するスポットの数が非常に少なく、解析可能なスポットを得ることができなかった (data not shown)。

以上の結果から、見出された miR-21 の誘導は、薬物誘導性細胞障害に対して保護的な作用を示すことが示唆された。しかしながら、細胞障害に対する保護作用を示したと考えられる、HeLa 細胞における miR-21 の具体的な標的となる遺伝子を見出すには至らなかった。今後の検討課題として、*in vivo* における AsO の導入もしくは安定発現系を用い、生体内における miR-21 の抑制による薬物誘導性肝障害への影響の検討が考えられる。

E. 研究発表

1．論文発表　論文作成中

2．学会発表　該当無し

F. 知的財産権の出願・登録状況

該当なし

G. 参考文献

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分担研究報告書

マイクロRNAによるヒトPXRの発現制御とCYP3A4の個人差に関する検討

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研究要旨

プレグナンX受容体（Pregnane X receptor, PXR）は、チトクローム P450(CYP)3A4をはじめとする薬物代謝酵素やトランスポーターなどの誘導的な発現の制御に関わる主たる核内因子の1つであることが知られている。この研究で、我々は25名のヒト肝の試料について検討し、PXRmRNAの発現量がPXRたんぱく質の発現レベルと全く相関しないことを新たに見出した。このことはPXRが転写後に何らかの発現制御を受けていることを示唆している。さらに、このPXRの3'非翻訳領域にマイクロRNA(miR)-148aによって認識されると考えられる配列があることを見出した。ルシフェラーゼを用いたレポータージーンアッセイにより miR-148a は、PXR mRNA のmiR-148a認識様配列を確かに認識することを示した。細胞レベルでの検討において、PXRタンパク質はmiR-148aを過剰発現させると発現が減少し、miR-148aをアンチセンスオリゴヌクレオチドで阻害すると発現が増加した。また、miR-148a依存的なPXRの増減は、CYP3A4 mRNAの発現量に影響を及ぼしていた。さらに、PXRの翻訳効率の指標であるPXRタンパク質/PXR mRNA比は、miR-148aの発現量と逆の相関にあることを25名のヒト肝試料について明らかにした。このことは、miR-148aがヒト肝においてPXRの発現制御の役割をしていることを意味する。実際、ヒト肝におけるPXRタンパク質の発現量は、CYP3A4のmRNAおよびタンパク質の発現量を相関した。本研究で、我々はmiR-148aがヒトPXRの発現を転写後調節しており、これによりヒト肝における CYP3A4 の常在的および誘導的発現が制御されていることを見出した。この研究結果は、これまで解明されていなかったヒト CYP3A4 活性の大きな個人差の説明として新たな知見を提供するものである。

A. 研究目的

A key function of the liver is the metabolism and elimination of xenobiotics or endobiotics. The expression of genes involved in these processes is largely regulated by transcription factors belonging to the nuclear receptor family. Pregnane X receptor (PXR; alternate names SXR, PAR, NR1I2), a member of the nuclear receptor family, is a crucial regulator of drug metabolism and elimination. It is

predominantly expressed in liver and small intestine. PXR is activated by a broad spectrum of xenobiotics including antibiotics, antimycotics, and herbal components (1), dimerizes with retinoid X receptor α (RXRα) and binds to response elements of target genes including cytochrome P450s (CYPs), UDP-glucuronosyltransferases (UGTs), glutathione *S*-transferases (GSTs), sulfotransferases (SULTs), and various transporters such as multidrug resistance 1 (MDR1) and multidrug resistance associated protein 2 (MRP-2) to induce them (2). Thus, PXR is recognized as a xenosensor for the detoxification of foreign compounds. However, it also plays a role as a physiological sensor of bile acids to protect the body from toxicity by regulating the expression of target genes that decreases the synthesis and increases the elimination of bile acids (3, 4).

One of the best-known genes regulated by PXR is CYP3A4, the most abundant P450 in human liver that catalyze the metabolism of over 50% of current prescription drugs (5-7). A large interindividual difference (~50-fold) has been reported for the CYP3A4 level in the general population (8), which cannot be explained by genetic polymorphisms (9, 10). 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 (11). However, the cause of the large interindividual variability in CYP3A4 level is poorly understood, and is an urgent issue to be solved. The regulation by PXR may, in part, be responsible for such variability since PXR is activated by endogenous compounds such as steroid hormones and bile acids (1, 12),

PXR regulates many targets controlling pharmacokinetics, but its own regulation is not fully understood, with reports showing only that human PXR is induced by dexamethasone through glucocorticoid receptor (13) or by clofibrate through peroxisome proliferators-activated receptor α (14). Employing an on-line search using the miRBase Target database (15, http://microrna.sanger.ac.uk/), we found some potential recognition sites for microRNAs (miRNAs) in the 3' untranslated region (UTR) of the human PXR.

miRNAs are a recently discovered family of short non-coding RNA whose final product is an approximately 22 nucleotide functional RNA molecule (16). They play important roles in the regulation of target genes by binding to complementary regions of transcripts to repress their translation or regulate degradation. At present, more than 400 miRNAs have been identified in humans and miRNAs are predicted to control about 30% of the genes within the human genome (17, 18). The roles of miRNAs have received attention especially in the cancer field, but

hardly yet in the field of pharmacokinetics. In the present study, we investigated whether human PXR might be post-transcriptionally regulated by miRNA and its impact on the CYP3A4 expression.

B. 研究方法

Chemicals and reagents - Rifampicin was obtained from Wako Pure Chemicals (Osaka, Japan). The pGL3-promoter vector, pGL4.74-TK plasmid, Tfx-20 reagent and dual-luciferase reporter assay system were purchased from Promega (Madison, WI). Lipofectamine 2000, Lipofectamine RNAiMAX were from Invitrogen (Carlsbad, CA). Pre-miR miRNA Precursors for miR-148a and for the negative control were from Ambion (Austin, TX). Locked nucleic acid (LNA) modified antisense oligonucleotides (AsOs) for miR-148a (5'-ACAAAGTTCTGTAGTGCACTGA-3', LNA is indicated by underline) and for the negative control (5'-AGACUAGCGGUAUCUUAAACC-3') were commercially synthesized at Greiner Bio-One (Tokyo, Japan). All primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Goat anti-human PXR polyclonal antibodies (N-16), rabbit anti-human RXRα polyclonal antibodies (D-20), and goat anti-human HNF4α polyclonal antibodies (S-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-CYP3A4 polyclonal antibodies were from BD Gentest (Worburn, MA) and rabbit anti-human CAR polyclonal antibodies were from CHEMICON (Temecula, CA). Rabbit anti-human GAPDH polyclonal antibodies were from IMGENEX (San Diego, CA). Alexa Fluor 680 donkey anti-goat IgG was from Invitrogen. IRDye 680 goat anti-rabbit IgG was from LI-COR Biosciences (Lincoln, NE). All other chemicals and solvents were of the highest grade commercially available.

Human livers and cell culture conditions - Human liver samples from 25 donors were obtained from Human and Animal Bridging (HAB) Research Organization (Chiba, Japan). The human hepatocellular carcinoma cell lines HepG2 and HuH7 were obtained from Riken Gene Bank (Tsukuba, Japan), and HLE was from Japanese Collection of Research Bioresources (Tokyo, Japan). The human colon carcinoma cell lines LS180 and Caco-2, the human embryonic kidney cell line HEK293, and the human breast adenocarcinoma cell line MCF-7 were obtained from American Type Culture Collection (Rockville, MD). HepG2, HuH7, and HLE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). LS180, Caco-2, and MCF-7 cells were cultured in DMEM supplemented with 0.1 mM non-essential amino acid (Invitrogen) and 10% FBS.

Differentiated Caco-2 (Caco-2/D) cells were obtained by culture for three weeks post-confluence. HEK293 cells were cultured in DMEM supplemented with 4.5 g/L glucose, 10 mM HEPES, and 10% FBS. These cells were maintained at 37°C under an atmosphere of 5% CO_2-95% air.

Real-time RT-PCR for PXR and CYP3A4 - Total RNA was isolated from 25 human liver samples using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The cDNAs were synthesized from total RNA using ReverTra Ace (Toyobo, Osaka, Japan). The forward and reverse primers for CYP3A4 were 5'-CCAAGCTATGCTCTTCACCG-3' and 5'-TCAGGCTCCACTTACGGTGC-3', respectively. The forward and reverse primers for human PXR were 5'-TGCGAGATCACCCGGAAGAC -3' and 5'- ATGGGAGAAGGTAGTGTCAAAGG -3', respectively. The real-time PCR was performed using the Smart Cycler (Cepheid, Sunnyvale, CA) with Smart Cycler software (Ver. 1.2b) as follows: after an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 95°C for 6 s, annealing and extension at 68°C for 20 s for 40 cycles. The mRNA levels were normalized with GAPDH mRNA determined by real-time RT-PCR as described previously (19).

SDS-PAGE and Western blot analyses of PXR and CYP3A4 - Whole cell lysates were prepared from 25 human liver samples by homogenization with lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% NP-40) containing protease inhibitors [0.5 mM APMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin]. The protein concentrations were determined using Bradford protein assay reagent (Bio-Rad, Hercules, CA) with γ-globulin as a standard. The whole cell lysates (10-50 μg) were separated with 7.5% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA). The membranes were probed with goat anti-human PXR, rabbit anti-human CYP3A4, goat anti-human HNF4α, rabbit anti-human CAR, or rabbit anti-human GAPDH antibodies and the corresponding fluorescent dye-conjugated second antibody, and the band densities were quantified with Odyssey Infrared Imaging system (LI-COR Biosciences). Nuclear extracts (10 μg) from the HepG2 and LS-180 cells were also used to determine the PXR protein level.

Real-time RT-PCR for mature miR-148a - For the quantification of mature miR-148a, polyadenylation and reverse transcription were performed using the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. The forward primer for miR-148a was 5'- TCAGTGCACTACAGAACTTTGT

-3', and the reverse primer was the supplemented universal qPCR primer. The PCR analyses were performed as follows: after an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 95°C for 10 s, annealing and extension at 64°C for 10 s for 40 cycles. The mature miR-148a level was normalized with U6 snRNA determined by real-time RT-PCR as described previously (20).

Construction of reporter plasmids - To construct luciferase reporter plasmids, various target fragments were inserted into the *Xba* I site downstream of the luciferase gene in the pGL3-promoter vector. The sequence from 3362 to 3383 in the human PXR mRNA (5'-ACAGACTCTTACGTGGAGAGTGCACT GA -3') was termed miR-148a recognition element (PXRMRE148). The fragment containing three copies of the PXRMRE148 (5'-CTAGAAGCCACAGACTCTTACGTGGA GAGTGCACTGACCTGTAGAAGCCACA GACTCTTACGTGGAGAGTGCACTGAC CTGTAGAAGCCACAGACTCTTACGTG GAGAGTGCACTGACCTGTAT -3', PXRMRE148 is underlined) was cloned into the pGL3-promoter vector (pGL3p/3xPXRMRE). The complementary sequence of three copies of the PXRMRE148 was also cloned into the pGL3-promoter plasmid (pGL3p/3xPXRMRE-Rev). A fragment containing the perfect matching sequence with the mature miR-148a, 5'-CTAGACAAAGTTCTGTAGTGCACTGA T -3' (the matching sequence of miR-148a is underlined) was cloned into the pGL3-promoter vector (pGL3p/c-148a). The nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analyses.

Luciferase assay - Various luciferase reporter plasmids (pGL3p) were transiently transfected with pGL4.74-TK plasmid into HEK293 or HepG2 cells. Briefly, the day before transfection, the cells were seeded into 24-well plates. After 24 h, 70 ng of pGL3p plasmid, 30 ng of pGL4.74-TK plasmid and 4 pmol of the precursors for miR-148a or control were transfected into HEK293 cells using Lipofectamine 2000. For HepG2 cells, 80 ng of pGL3p plasmid, 20 ng of pGL4.74-TK plasmid and 10 pmol of the AsOs for miR-148a or control were transfected using Tfx-20 reagent. After incubation for 48 h, the cells were resuspended in passive lysis buffer, and then the luciferase activity was measured with a luminometer (Wallac, Turku, Finland) using the dual-luciferase reporter assay system.

Transfection of precursor or antisense for miR-148a into HepG2 and LS-180 cells and isolation of nuclear extract and total RNA - To investigate the effect of miR-148a on the expression of PXR protein, 50 nM precursor

or 50 nM AsO for miR-148a or control were transfected into HepG2 cells using Lipofectamine RNAiMAX. After 72 h, total RNA was isolated using ISOGEN and the mature miR-148a levels were determined by Northern blotting as described above. Nuclear extract was isolated using the NE-PER Nuclear and Cytoplasmic extraction reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. LS180 cells were transfected with 50 nM precursor for miR-148a or control using Lipofectamine RNAiMAX. After 72 h, the cells were treated with 50 μM rifampicin or 0.1% (v/v) DMSO for 24 h. Then, total RNA and nuclear extract were isolated.

Evaluation of the expression level of PXR in HepG2 using reporter construct containing PXR responsive element - The reporter construct pCYP3A4-362-7.7K contains the promoter region (-362 to +11) including the ER6 (everted repeat separated by six nucleotides) motif and the distal enhancer region (-7836 to -7200) including the DR3 (direct repeat separated by three nucleotides) motif of the *CYP3A4* gene, to which PXR binds (21). The day before transfection, HepG2 cells were seeded into 24-well plates. After 24 h, 180 ng of pCYP3A4-362-7.7K, 20 ng of pGL4.74-TK plasmid and various doses of the precursors and AsOs for miR-148a or control were transfected using Tfx-20 reagent. After incubation for 48 h, the cells were treated with 10 μM rifampicin or 0.1% DMSO for 24 h, and then the luciferase activity was measured.

Statistical analyses - Statistical significance was determined by analysis of variance followed by Dunnett multiple comparisons test or Tukey method test. Comparison of two groups was made with an unpaired, two-tailed student's *t* test. Correlation analyses were performed by Spearman rank method. A value of $P < 0.05$ was considered statistically significant.

C. 研究結果

PXR protein level is not associated with PXR mRNA level in human livers - We first examined the PXR mRNA level in a panel of 25 human livers by real-time RT-PCR assay and investigated the relationship with the PXR protein level. As shown in Fig. 1, no statistically significant correlation was observed between the PXR mRNA and protein levels (Rs = 0.10), indicating the involvement of post-transcriptional regulation of human PXR. To uncover the molecular mechanism of the post-transcriptional regulation, we sought to examine the involvement of miRNA-mediated regulation. Employing an on-line search using the miRBase Target database (15, http://microrna.sanger.ac.uk/), potential recognition elements for 16 kinds of miRNA such as miR-148a, miR-192, miR-560 were found in the 3' UTR in

human PXR. Among them, we focused on miR-148a because it is selectively and abundantly expressed in liver (22) and has high complementarity in the 5' end at miRNA-mRNA duplexes including the seed sequence. The potential miR-148a target site is approximately 200 bases downstream of the stop codon of the human PXR mRNA. The alignment of hsa-miR-148a with the 3' UTR of human PXR mRNA (Fig. 2*A*) was drawn using RNAhybrid (23, http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). We investigated whether this region termed the miR-148a recognition element (PXRMRE148) might be involved in the regulation of PXR by miR-148a.

Expression levels of miR-148a in human cancer cell lines – Real-time RT-PCR analysis using NCode miRNA First-Strand cDNA Synthesis Kit was performed to determine the expression levels of mature miR-148a in 8 kinds of human cancer cell lines (Fig. 2*B*). The mature miR-148a was detected in all cell lines tested in this study, with large variability among cell lines (37-fold). The U6 snRNA levels, which were used for normalization, varied < 3-fold in the experiment; much less than the miR-148a levels. The mature miR-148a was higher in HepG2 and differentiated Caco-2 cells than the other cell lines. It was also interesting that, in Caco-2 cells, the expression level of mature miR-148a was increased with differentiation. Thus, the expression levels of mature miR-148a were highly variable among the human cancer cell lines.

Repressive regulation of PXR by miR-148a in human cell lines - To investigate whether PXRMRE148 is functional in the regulation by miR-148a, luciferase assays were performed with HEK293 cells (Fig. 2*C*). We first confirmed that the luciferase activity of the pGL3p/c-148a plasmid, in which the miR-148a complementary sequence was inserted downstream of the *luciferase* gene, was significantly ($P < 0.01$) decreased by the co-transfection with the precursor for miR-148a. The luciferase activity of pGL3p/3xPXRMRE plasmid, in which three copies of the potential miR-148a recognition site were inserted downstream of the *luciferase* gene, was also significantly ($P < 0.01$) decreased by co-transfection with the precursor for miR-148a (33% of control), while that of pGL3p/3xPXRMRE-Rev plasmid with the inverted recognition site was not affected. In HepG2 cells, which showed the highest expression of miR-148a (Fig. 2*B*), the luciferase activities of pGL3p/c-148a and pGL3p/3xPXRMRE plasmid were significantly ($P < 0.01$) lower than those of the control pGL3p plasmid (Fig. 2*D*). These activities were significantly ($P < 0.01$) restored by the transfection of AsO for miR-148a. These results underscore that miR-148a functionally recognizes

PXRMRE148 to decrease the expression.

Effects of overexpression or inhibition of miR-148a on the PXR protein level in a human cell line - We next examined the change in endogenous PXR protein expression by the overexpression or inhibition of miR-148a. By the transfection of the precursor for miR-148a into HepG2 cells that harbor the increased level of mature miR-148a, the PXR protein level was significantly ($P < 0.05$) decreased compared with the control (Fig. 3*A*). Conversely, by the transfection of the AsO for miR-148a into HepG2 cells, where the expression of mature miR-148a was extinguished, the PXR protein level was significantly ($P < 0.05$) increased compared with the control (Fig. 3*B*). Meanwhile, the expression level of RXRα protein, a heterodimer partner of PXR, was not affected by the overexpression or inhibition of miR-148a. It is well known that ligand-activated PXR activates the transcription of targets by binding to the responsive element. Using the pCYP3A4-362-7.7K plasmid containing the PXR responsive element as a reporter construct, the changes in the PXR protein levels were monitored with the reporter activity (Fig. 3*C* and 3*D*). The luciferase activity of pCYP3A4-362-7.7K plasmid was prominently (5.3-fold) increased by the treatment with rifampicin in HepG2 cells (Fig. *3E)*. Transfection of the precursor for miR-148a significantly decreased both the rifampicin-induced and basal transcriptional activities, resulting in a dose-dependent decrease of the induction. In contrast, the transfection of antisense for miR-148a significantly increased both the rifampicin-induced and basal transcriptional activity, resulting in a dose-dependent increase of the induction (Fig. 3*F*). These results suggest that miR-148a negatively regulates the expression of PXR protein and, subsequently, the induction of its targets.

Role of miR-148a-dependent PXR regulation in the induction of endogenous CYP3A4 mRNA in a human cell line - We next sought to examine whether the miR-148a-dependent change of the PXR protein level affects the CYP3A4 induction in human cells. LS-180 cells were used because this cell line expressed relatively higher CYP3A4 mRNA than the other cell lines (data not shown). By the transfection of the precursor for miR-148a into the LS-180 cells, the PXR protein level was significantly ($P < 0.01$) decreased (Fig. 4*A*), concomitant with dramatic increase of the mature miR-148a level, while the PXR mRNA level was not decreased at any time after the transfection (Fig. 4*B*). The RXRα protein level was not affected by the overexpression of miR-148a (Fig. 4*A*). As shown in Fig. 4*C*, the CYP3A4 mRNA level was significantly increased by the treatment with rifampicin (5.0-fold). However, this induction was diminished by the overexpression of

miR-148a, although the basal CYP3A4 mRNA level was not affected. These results suggest that the miR-148a-dependent regulation of PXR affects the induction of CYP3A4.

The miR-148a-dependent PXR regulation may control CYP3A4 expression in human liver tissue - To further investigate the effects of the miR-148a-dependent regulation of PXR in human liver tissue, the relationships between the expression levels of miR-148a, PXR, and CYP3A4 were investigated using a panel of 25 human livers (Supplemental Table 1). The expression levels of miR-148a were variable (95-fold) in the panel of human livers. The miR-148a level in liver sample No. 18 was comparable to that in HepG2 cells. The PXR mRNA (75-fold) and CYP3A4 mRNA (363-fold) were also variable. As shown in Fig. 1, the PXR mRNA level was not correlated with the PXR protein level. In contrast, the CYP3A4 mRNA level was significantly correlated (Rs = 0.67, $P < 0.001$) with the CYP3A4 protein level (Fig. 5*A*). When the PXR protein/PXR mRNA ratios were calculated as an index of the translational efficiency of PXR, they were inversely correlated with the miR-148a level (Rs = -0.41, $P < 0.05$, Fig. 5*B*), suggesting that PXR is negatively regulated by miR-148a in human liver. The PXR protein level was significantly correlated with both the CYP3A4 mRNA level (Rs = 0.47, $P < 0.05$, Fig. 5*C*) and the CYP3A4 protein level (Rs = 0.67, $P < 0.001$, Fig. 5*D*). As summarized in Fig. 5*E*, the post-transcriptional regulation of PXR by miR-148a appeared to have substantial impact on the CYP3A4 level in human livers.

D. 考察

PXR regulates at least 40 genes encoding proteins responsible for the metabolism and elimination of drugs (14). The study of PXR regulation assists in the understanding of the inter- and intra-individual variability in the pharmacokinetics of drugs. Although many research groups have found variability in the PXR mRNA levels in human liver samples, the correlation with its protein level has not fully been investigated. In this study, we first demonstrated that there was no significant correlation between them in human livers. For human PXR, two splicing variants, including exon 1b (PXR.2) or deleting the 5'-end of the exon 5 (PXR.3), have been reported (24, 25), which cannot be distinguished with our PCR primers. However, since the expression levels of these splicing variants were extremely low in our analysis (data not shown), consistent with a report by Lamba et al. (26), the dissociation of the PXR protein level with its mRNA level is not an artificial phenomenon. Identification of the miRNA recognition element in the human *PXR* gene suggested the involvement of miRNA in the regulation

of PXR.

The luciferase assays showed that the endogenous and exogenous miR-148a negatively regulated the activity through PXRMRE148. In addition, the endogenous PXR protein level was diminished by the overexpression of miR-148a and elevated by its inhibition. These results clearly indicated that human PXR is post-transcriptionally regulated by miR-148a. The miR-148a-dependent changes of PXR protein affected the induction of CYP3A4 in LS180 cells. To further investigate whether the miR-148a affects the induction of other targets of PXR, we determined the expression levels of MDR1 and CYP2B6 in LS180 cells (data not shown). Rifampicin induced the MDR1 (5-fold) and CYP2B6 (2-fold) mRNAs, known targets of PXR (27, 28), and the induction was attenuated by the overexpression of miR-148a. Thus, the miR-148a-dependent regulation of PXR appeared to affect its target genes in common.

Interestingly, the miR-148a recognition element is also present in the 3'-UTR of CYP3A4 mRNA. The complementarity of CYP3A4 with miR-148 (score 15.48, energy -24.27) was higher than that of human PXR (score 15.14, energy -19.52). To investigate whether CYP3A4 is directly regulated by miR-148a, luciferase assays were performed using a plasmid containing three copies of the miR-148a recognition element in the *CYP3A4* gene. However, unlike the PXRMRE, the element in CYP3A4 did not respond to miR-148a (Supplemental figure 1), indicating that CYP3A4 is not directly regulated by miR-148a.

In the panel of human livers, the expression level of miR-148a was inversely correlated with the translational efficiency of PXR, supporting the role of miR-148a in the regulation of PXR in liver. The significant correlation between the CYP3A4 mRNA and the CYP3A4 protein level in human livers in this study, in accordance with previous studies (29, 30), supported the finding that miRNA did not directly regulate the CYP3A4 expression. The PXR protein level was correlated with the CYP3A4 mRNA level in human liver, indicating that miR-148a affects the CYP3A4 expression through modulating PXR expression. The PXR protein level was not correlated with the CYP2B6 (Rs = 0.31, $P > 0.1$) or MDR1 (Rs = -0.20, $P > 0.3$) mRNA levels in the panel of human livers (data not shown), in contrast to CYP3A4. Thus, we speculate that the PXR does not largely affect the constitutive expression of CYP2B6 and MDR1 in liver. In our panel of human livers, the CYP3A4 mRNA level was not correlated with the HNF4α protein level (Rs = -0.14, $P > 0.5$) or CAR protein level (Rs = 0.12, $P > 0.5$), indicating a significant contribution of PXR to the constitutive CYP3A4 level.

Most of the genes in the vertebrate nuclear receptor superfamily are strongly conserved between species. The

ligand-binding domain of PXR shares amino acid identity of 75% between human and rodent, which can explain why the key ligands for PXR vary across species. Meanwhile, the DNA-binding domain of PXR shares more than 95% amino acid identity (1) inducing a similar set of genes. Most miRNAs are evolutionally conserved, which suggests that the miRNA-mediated regulation of certain genes would be common among species. The MRE148 is also identified in the 3' UTR in mouse PXR (score 16.63, energy -21.4) and rat PXR (score and energy are not calculated at miRBase, but has only a 1-base difference with the corresponding mouse sequence) at ~670-bp downstream of the stop codon, although the 3' UTR of PXR is poorly conserved between human and rodent. It is therefore possible that rodent PXR may also be regulated by miR-148a, suggesting that rodent might be a useful model animal to investigate the role of miR-148a in drug metabolism and elimination *in vivo*.

In conclusion, we found that human PXR is post-transcriptionally regulated by miR-148a affecting the CYP3A4 level in human liver. This study would provide new insight into the unsolved mechanism of the large interindividual variability of CYP3A4 expression.

G. 研究発表

1. 学会発表

miR-148a によるヒト pregnane X receptor (PXR) の発現制御と CYP3A4 の発現に及ぼす影響、第 17 回アンチセンスシンポジウム　2007. 12. 3-4　金沢

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2. 論文発表

Shingo Takagi, Miki Nakajima, Takuya Mohri, and Tsuyoshi Yokoi. Post-transcriptional regulation of human pregnane X receptor by microRNA affects the expression of cytochrome P450 3A4. *J. Biol. Chem.*, in press.

H. 知的財産権の出願・登録状況

該当なし。

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FIGURE LEGENDS

FIGURE 1. **Correlation between the PXR mRNA and protein levels in 25 human livers.** The PXR mRNA level was determined by real-time RT-PCR and normalized with the GAPDH mRNA level. The PXR protein level was determined by Western blot analysis and normalized with the GAPDH protein level.

FIGURE 2. **Repressive regulation of human PXR by miR-148a.** ***A***, Complementarity of miR-148a to the predicted target sequence of human PXR. The potential miR-148a recognition element (PXRMRE148) is located on +3359 to +3386 in the 3' UTR of human PXR mRNA, where the numbering refers to the 5' end of mRNA as 1. ***B***, The mature miR-148a levels in HepG2, HuH7, HLE, Caco-2, Caco-2/D (differentiated), LS180, HEK293, MCF-7 cells were determined by real-time RT-PCR analysis using NCode miRNA First-Strand cDNA Synthesis Kit. The values were the mature miR-148a levels normalized with the U6 snRNA levels relative to that in HLE cells. ***C***, ***D***, Luciferase assays were performed to investigate whether PXRMRE148 is functional in the regulation by miR-148a. The reporter constructs were transiently transfected with 4 pmol of the precursors for miR-148a or control into HEK293 cells (***C***) or 10 pmol of the AsO for miR-148a or control into HepG2 cells (***D***). The data were the firefly luciferase activities normalized with the *Renilla* luciferase activities relative to that of pGL3p plasmid. Each column represents the mean ± SD of three independent experiments. $^{**}P < 0.01$, compared with pGL3p; $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$, compared with AsO for control.

FIGURE 3. **Effects of overexpression or inhibition of miR-148a on the PXR protein level in HepG2 cells.** The precursors for miR-148a or control (50 nM, ***A***) or AsOs for miR-148a or control (50 nM, ***B***) were transfected into HepG2 cells. After 72 h, the cells were harvested and total RNA and nuclear extracts were isolated. ***A***, ***B***, The mature miR-148a levels were determined by real-time RT-PCR analyses. The values were the mature miR-148a levels normalized with the U6 snRNA