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分担研究報告書

ヒトhepatocyte nuclear factor 4αのmicroRNAによる制御は、代謝酵素の発現や細胞周期を調節している

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研究要旨

Cytochrome P450 (CYP) などの薬物代謝酵素は薬物、発癌物質および胆汁酸など様々な化合物の代謝および排泄に重要な役割を担っている。薬物代謝酵素の発現は主に核内受容体ファミリーに属する転写因子によって調節される。MicroRNAは標的mRNAに結合し、翻訳抑制もしくはmRNAの分解を引きおこす小分子non-coding RNAである。本研究では核内受容体の発現制御および代謝酵素の発現へのmicroRNAの関与を解明することを目的した。ヒトhepatocyte nuclear receptor (HNF) 4α は内因性および外因性化合物の代謝酵素やトランスポーターの主たる転写調節因子である。本研究では、microRNAがヒトHNF4αの発現制御に関わっているかについて検討した。miR-24認識配列(MRE24)が翻訳領域と3'UTRに存在しており、miR-34a認識配列(MRE34a)がHNF4αの3'UTRに存在していた。HepG2細胞において、miR-24またはmiR-34aを過剰発現させた場合に、HNF4α の発現が有為に減少した。HNF4αのmRNAの発現は、miR-24の過剰発現によって減少したが、これはmiR-34aの過剰発現では認められなかった。HEK293細胞を用いたルシフェラーゼ分析で、HNF4αの3'UTRを含んだプラスミドのレポータジーン活性は、miR-34aの過剰発現で有為に減少した。HNF4αの翻訳領域については、miR-24の過剰発現で有為に減少した。これらの結果から、HNF4αの発現がmiR-24およびmiR-34aによりそれぞれmRNAの分解と翻訳抑制によって負に制御されることを明らかにした。これらの microRNAによるHNF4αの低下は、CYP7A1やCYP8B1の発現減少や、細胞周期にも影響をおよぼすことが明らかになった。さらに、miR-24およびmiR-34aはそれぞれprotein kinase C/mitogen-activated protein kinaseおよびreactive oxygen species経路により制御されることが示された。以上、本研究では、ヒトHNF4α はmiR-24およびmiR-34aによって負に制御され、細胞ストレスや代謝酵素の発現など広範な影響を及ぼしていることを明らかにした。

A. 研究目的

Human HNF4α (alternate name: NR2A1), which belongs to the nuclear receptor family, is highly expressed in liver and regulates the expression of various genes involved in the synthesis/metabolism of fatty acid, cholesterol, glucose and urea (Gonzalez, 2008). It is well recognized that endo/xenobiotic-metabolizing enzymes such as CYPs, UGTs, SULTs as well as ATP-binding cassette transporters, organic anion transporters and organic cation transporters 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. Therefore, it is believed that the change of HNF4α expression has a great impact upon the function of liver.

Bile acids are important regulatory molecules mediating cholesterol synthesis and glucose metabolism as well as their own synthesis (Hylemon et al., 2009). It is well known that HNF4α positively regulates the expression of bile acid-synthesizing enzymes such as CYP7A1 and CYP8B1. When bile acids are accumulated, the HNF4α-mediated transactivation is inhibited by short heterodimer partner (SHP), which is up-regulated by bile acid-activating farnesoid X receptor (Goodwin et al., 2000; Lee et al., 2000). Bile acids are known to activate the MAPK signaling pathway. It has been reported that the expression and function of HNF4α are up- or down-regulated through extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK pathways (Hatzis et al., 2006, Li et al., 2006, Guo et al., 2006). In addition, chenodeoxycholic acid (CDCA), a toxic bile acid, has been reported to decrease the HNF4α mRNA expression via unknown pathways (Popowski et al., 2005). Thus, the bile acid synthesis would be fine-tuned through the modulation of the expression and/or activity of HNF4α. However, the regulatory mechanism of the HNF4α expression has not still been fully understood. This study examined the

possibility that miRNAs might regulate the expression of human HNF4α resulting in the modulation of liver function.

B. 研究方法

Chemicals and reagents –

Phorbol 12-myristate 13-acetate (PMA), hydrogen peroxide (H_2O_2), U0126 and SB202190 were obtained from Wako Pure Chemicals. SP600125 was from Calbiochem (San Diego, CA). The pGL3-promoter (pGL3p) vector, pGL4.74-TK plasmid, pTARGET vector and Dual-Luciferase Reporter Assay System were purchased from Promega. Lipofectamine 2000, Lipofectamine RNAiMAX, Stealth Select RNAi for human HNF4α (HSS140902) (siHNF4α) and Negative Control Medium GC Duplex #3 (siControl) were from Invitrogen. Pre-miR miRNA Precursor Molecule for miR-24, miR-34a and Negative Control #1 (Control) were from Ambion. All primers were commercially synthesized at Hokkaido System Sciences. Goat anti-human HNF4α polyclonal antibodies (S-20), rabbit anti-human GAPDH polyclonal antibodies, and mouse anti-HA monoclonal antibodies were from Santa Cruz Biotechnology, IMGENEX and COVANCE (Berkeley, CA), respectively. Alexa Fluor 680 donkey anti-goat IgG was from Invitrogen. IRDye 680 goat anti-rabbit IgG and goat anti-mouse IgG were from LI-COR Biosciences. All other chemicals and solvents were of the highest grade commercially available.

Cell culture –

The human hepatocellular carcinoma cell line HepG2 was obtained from Riken Gene Bank. The human embryonic kidney cell line HEK293 was obtained from American Type Culture Collection. HepG2 cells were cultured in DMEM supplemented with 0.1 mM non-essential amino acid (Invitrogen) and 10% FBS (Invitrogen). 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.

Transfection of miRNAs or siRNA into HepG2 cells –

Pre-miR miRNA Precursor Molecule and siRNA were transfected into HepG2 cells using Lipofectamine RNAiMAX. Unless

otherwise specified, the Pre-miR miRNA Precursor Molecule and siRNA were transfected at a final concentration of 50 nM and 5 nM, respectively. After 48 h, total RNA was isolated using RNAiso according to the manufacturer's protocol. Whole cell lysates were prepared 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, 2 μg/mL pepstatin). The protein concentrations were determined using Bradford protein assay reagent (Bio-Rad) with γ-globulin as a standard.

Real-time RT-PCR for HNF4α, its target genes and miRNAs –

The cDNAs were synthesized from total RNA using ReverTra Ace (Toyobo). The primers used are shown in Table 2. The real-time PCR was performed using the Mx3000P (Stratagene, La Jolla, CA) with the MxPro QPCR software as follows: after an initial denaturation at 95°C for 2 min, the

Table 2. **Primers for real-time RT-PCR**

Target gene	Accession No.	Forward (5' to 3')	Reverse (5' to 3')
HNF4α	NM_000457	TGTCCCGACAGATCACCTC	CACTCAACGAGAACCAGCAG
CYP7A1	NM_000780	CAGTGCCTCCCTCAACATCC	GACATATTGTAGCTCCCGATCC
CYP8B1	NM_004391	CTACACGAAGGACAAGGAGCAGGAC	GTGGCTCACGGAGAGCATCTTGTG
CYP27A1	NM_000784	GGCAACGGAGCTTAGAGGAGATTC	CATCCACATTGGACCGTACTTGGC
PEPCK	NM_002591	AGCTCGGTCGCTGGATGTCAGAG	GTAGGGTGAATCCGTCAGCTCGATG
p16	NM_000077	TGCCCAACGCACCGAATAGTTACG	TGCACGGGTCGGGTGAGAG
p21	NM_000389	CTGTCACTGTCTTGTACCCTTGTGC	GGAGAAGATCAGCCGGCGTTTG
p27	NM_004064	AGCAATGCGCAGGAATAAGGAAGCG	GTTTGACGTCTTCTGAGGCCAGG
GAPDH	NM_002046	CCAGGGCTGCTTTTAACTC	GCTCCCCCCTGCAAATGA
U6 snRNA	NR_004394	CGCTTCGGCAGCACATATACTAA	TATGGAACGCTTCACGAATTTGC
pre-miR-24-1	MI0000080	TCCGGTGCCTACTGAGCTGATATC	CTGTTCCTGCTGAACTGAGCCA
pre-miR-24-2	MI0000081	CGTGCCTACTGAGCTGAAACACAG	CTGTTCCTGCTGAACTGAGCCA
pre-miR-34a	MI0000268	CCAGCTGTGAGTGTTTCTTTGGCAG	CCCACAACGTGCAGCACTTCTAG
miR-24	MIMAT0000080	TGGCTCAGTTCAGCAGGAACAG	Universal qPCR primer
miR-34a	MIMAT0000255	TGGCAGTGTCTTAGCTGGTTGT	Universal qPCR primer

Nucleotide sequences of miRNAs and the others were adopted from miRBase sequences and the GenBank database, respectively.

amplification was performed by denaturation at 95°C for 15 s, annealing and extension at 65°C for 20 s for 40 cycles. The mRNA levels of HNF4α, phosphoenolpyruvate carboxykinase (PEPCK), p16, p21, p27, and CYPs were normalized with the GAPDH mRNA level, and the levels of precursor miR- (pre-miR-) 24-1, pre-miR-24-2, pre-miR-34a and mature miRNAs were normalized with the U6 snRNA level. For the quantification of mature miRNAs, reverse transcription was performed using the NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen) according to the manufacturer's protocol.

SDS-PAGE and Western blot analyses –

The whole cell lysates (20 μg) were separated with 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membrane (Millipore). The membranes were probed with goat anti-human HNF4α, rabbit anti-human GAPDH or mouse anti-hemagglutinin (HA) antibodies and the corresponding fluorescent dye-conjugated second antibodies. The band densities were quantified with Odyssey Infrared Imaging system (LI-COR Biosciences). The HNF4α protein level was evaluated as the sum of the densities of two bands.

Construction of plasmids –

Human HNF4α cDNA including coding region and 3'UTR was amplified by PCR using cDNA prepared from HepG2 with the forward primer, 5'-AGAATGCGACTCTCCAAAACCCTC-3', and the reverse primer, 5'-TGAATTCTCCTTAATATTTATCAGCAAAC-3'. From this fragment, the HNF4α coding region was digested with *PmaC* I. These cDNA fragments were cloned into the pTARGET vector (Promega), resulting in pTARGET/HNF4α+3'UTR and pTARGET/HNF4α plasmids. HA-tagged GFP expression plasmid was constructed by PCR using pGSU6-GFP plasmid (Genlantis, San Diego, CA) as a template with the forward primer, 5'-TTTACGCGTATGTACCCCTACGACGTGCCCGACTACGCCATGGCTAGCAAAGGAGAAGAAC-3' (HA-tag is underlined), and the reverse primer, 5'-TTTGCGGCCGCTCAGTTGTACAGTT

CATCCATGC-3'. To construct luciferase reporter plasmids, various fragments of the HNF4α coding region or 3'UTR were inserted into the *Xba* I site downstream of the *luciferase* gene in the pGL3p vector. The nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analyses.

Transient expression of HNF4α in HEK293 cells and transfection of miRNAs –

The pTARGET/HNF4α and pTARGET/HNF4α+3'UTR plasmids were transiently transfected with HA-tagged GFP expression plasmid and Pre-miR miRNA Precursor Molecules into HEK293 cells. Briefly, the day before transfection, the cells were seeded into 24-well plates. After 24 h, 450 ng of HNF4α expression plasmid, 50 ng of pTARGET/HA-tagged GFP plasmid and 50 nM of Pre-miR miRNA Precursor Molecules were transfected using Lipofectamine 2000. After incubation for 48 h, the cells were harvested and whole cell lysates were prepared as described above.

Luciferase assay –

Various luciferase reporter plasmids (pGL3p) were transiently transfected with pGL4.74-TK plasmid into HEK293 cells. Briefly, the day before transfection, the cells were seeded into 24-well plates. After 24 h, 90 ng of pGL3p plasmid, 10 ng of pGL4.74-TK plasmid and 10 nM of Pre-miR miRNA Precursor Molecules were transfected into HEK293 cells using Lipofectamine 2000. After incubation for 48 h, the cells were resuspended in the passive lysis buffer, and then the luciferase activity was measured with a luminometer (Wallac) using the Dual-Luciferase Reporter Assay System.

Treatment of HepG2 cells with chemicals –

HepG2 cells were seeded into 12-well plates and after 24 h, the cells were treated with 100 nM PMA or 500 μM H_2O_2 for the indicated times. The specific inhibitors for MAPK, U0126, SB202190, and SP600125 were co-treated with PMA for 1 h or with H_2O_2 for 6 h at a final concentration of 10 μM. Total RNA was isolated as described above.

Cell cycle analysis –

HepG2 cells were fixed with 70% ethanol at 48 h after the transfection of Pre-miR miRNA Precursor Molecules or siRNAs. The cells were washed with FACS buffer (phosphate-buffered saline containing 0.1% bovine serum albumin) and incubated with FACS buffer containing 50 μg/mL RNase A for 30 min at 37°C. The cells were stained with 25 μg/mL propidium iodide and analyzed using FACSCalibur and Cell Quest Pro software (BD Biosciences, San Jose, CA). Synchronization of HepG2 cells were carried out by serum deprivation. After 24 h, the cells were restimulated with serum for the indicated time.

Statistical analysis –

Statistical significance was determined by analysis of variance followed by Dunnett multiple comparisons test or Tukey method test. A value of $P < 0.05$ was considered statistically significant.

C. 研究結果

miR-24 and miR-34a down-regulate HNF4α -

The length of the 3'UTR of human HNF4α is approximately 1.7 kb (Fig. 7*A*). Computational prediction using miRanda (http://cbio.mskcc.org/mirnaviewer/), PicTar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/) identified the potential recognition elements for various miRNAs including miR-1, miR-24, miR-34a, miR-326 and miR-485 in the 3'UTR of HNF4α. Among them, miR-24 and miR-34a were selected to investigate their effects on the HNF4α expression, because they were predicted by two algorisms. Western blot analysis using whole cell lysates from HepG2 cells showed two bands of HNF4α, probably corresponding to variant 1 and variant 2 (Fig. 7*B*). Variant 2 is a natural splicing variant with 30 nucleotides inserted in the coding region. When miR-24 and miR-34a were overexpressed in HepG2 cells, the HNF4α protein levels were dramatically decreased. To investigate whether the decrease of the HNF4α protein levels was accompanied by a decrease of the mRNA levels, the HNF4α mRNA levels was determined by real-time RT-PCR analysis. As shown in Fig. 7*C*, the HNF4α mRNA level was

significantly decreased with the overexpression of miR-24, but not with miR-34a. These results suggested that miR-24 and miR-34a down-regulate the HNF4α expression by different mechanisms, i.e. mRNA degradation and translational repression, respectively.

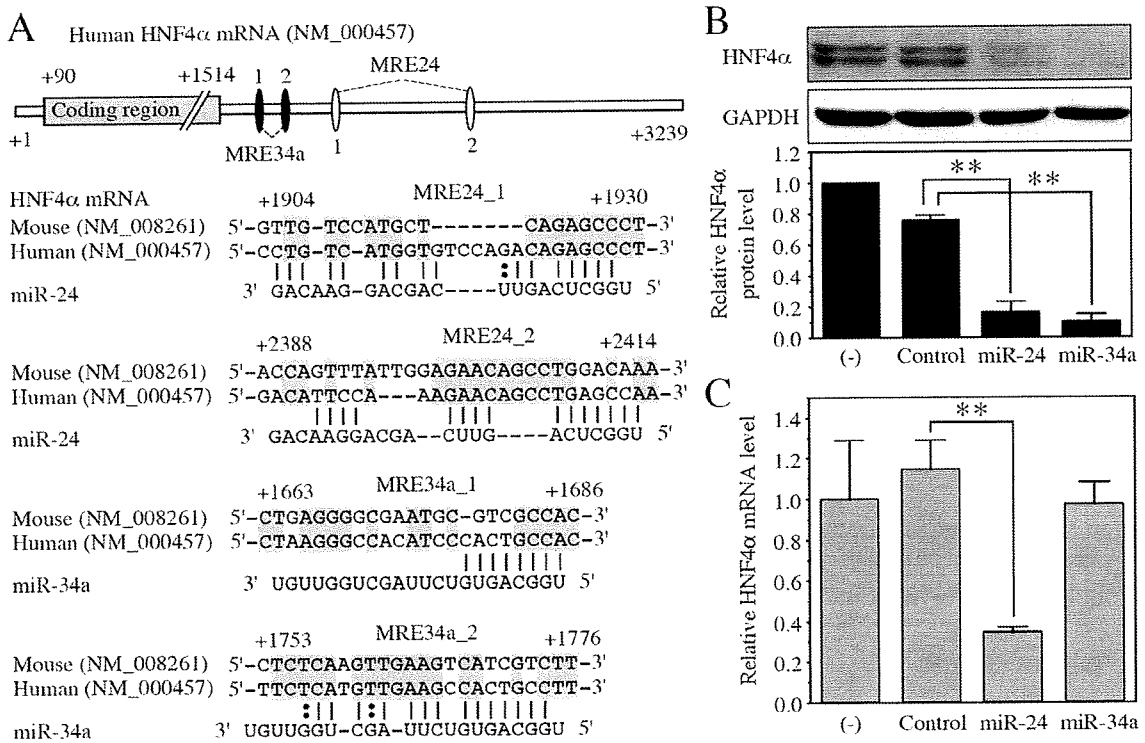

Fig. 7. **The potential MREs in the 3'UTR of HNF4α mRNA and the effects of miR-24 and miR-34a on the HNF4α level.** ***A***, Schematic diagrams of human HNF4α mRNA and the predicted target sites of miR-24 and miR-34a in the 3'UTR are shown (*upper*). The numbering refers to the 5' end of mRNA as 1. Complementarity of miR-24 and miR-34a to the predicted target sequence of human HNF4α is shown (*lower*). The conserved nucleotides are highlighted in gray boxes. ***B, C***, Pre-miR miRNA Precursor Molecules were transfected into HepG2 cells at a concentration of 50 nM. After 48 h, total RNA and whole cell lysates were prepared. The HNF4α and GAPDH protein levels were determined by Western blot analyses (***B***). The HNF4α mRNA levels were determined by real-time RT-PCR and normalized with the GAPDH mRNA level (***C***). The data are relative to no transfection (-). Each column represents the mean ± SD of three independent experiments. $^{**}P < 0.01$.

Identification of functional MRE in coding region and 3'UTR of HNF4α mRNA –

In the 3'UTR of the HNF4α mRNA, two potential miRNA recognition elements for miR-24 (MRE24_1 and MRE24_2) and miR-34a (MRE34a_1 and MRE34a_2) were

predicted (Fig. 7*A*). To investigate whether these MRE are functional in the down-regulation of the HNF4α, luciferase assays were performed using the pGL3p/3'UTR plasmid containing 3'UTR of HNF4α with HEK293 cells (Fig. 8*A*). The luciferase activity was significantly decreased by the overexpression of miR-34a, but not by miR-24. To examine the possibility that down-regulation of HNF4α by miR-24 might be mediated by elements in the coding region, luciferase assays were performed using the pGL3p/coding sequence (CDS) plasmid containing the coding region of HNF4α. The luciferase activity was significantly decreased by the overexpression of miR-24, although miR-34a did not affect the activity (Fig. 8*A*). These results suggested the presence of the potential recognition element of miR-24 in the coding region of HNF4α mRNA. Computational search using RNA22 (http://cbcsrv.watson.ibm.com/rna22.html) identified five potential MRE24s (termed MRE24_3 to MRE24_7) in the coding region of HNF4α mRNA (Fig. 8*B*).

To identify the functional MREs, luciferase assay performed using a series of deleted reporter constructs. Overexpression of miR-24 significantly decreased the luciferase activities of reporter constructs pGL3/CDS-b and pGL3/CDS-c containing MRE24_3 to _5, but did not affect the activity of the pGL3/CDS-f (Fig. 8*C*) indicating three MREs would be functional. The deletion of MRE24_4 and 5 (pGL3p/CDS-d and -e) resulted in the loss of repression suggesting MRE24_3, 4, and 5 function cooperatively. Overexpression of miR-34a significantly decreased the activity of reporter constructs pGL3/3'UTR-b containing MRE34a_1 and 2, but did not affect the activity of the pGL3/3'UTR-d (Fig. 8*D*). The deletion of MRE34a_1 did not affect the repressive effects (pGL3p/3'UTR-c) indicating that MRE34a_2 plays a key role in the miR-34a-mediated repression. Collectively, miR-24 and miR-34a would recognize the MREs in the coding region and the 3'UTR of HNF4α mRNA, respectively.

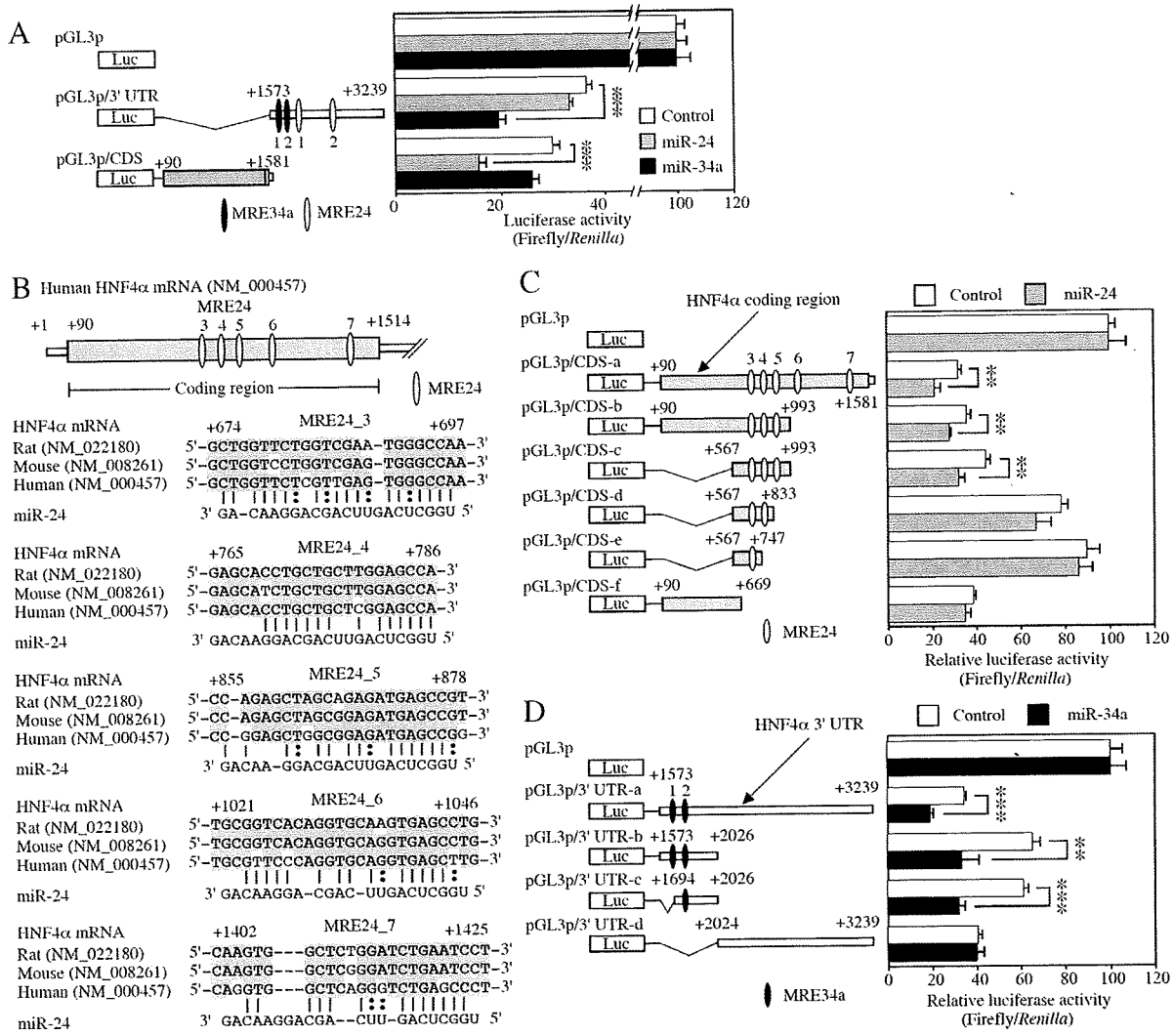

Fig. 8. **Reporter analyses of MREs in the coding region and 3'UTR of HNF4α mRNA.** ***A,*** Reporter plasmids and Pre-miR miRNA Precursor Molecules were cotransfected into HEK293 cells, and luciferase assays were performed after 48 h. The data were the firefly luciferase activities normalized with the *Renilla* luciferase activities relative to that of pGL3p cotransfected with each miRNA. Each column represents the mean ± SD of three independent experiments. ***$P < 0.001$. ***B,*** Schematic diagrams of the coding region of human HNF4α mRNA and mapping of predicted miR-24 target sites are described. Complementarity of miR-24 to the predicted target sequence of human HNF4α is also indicated. The conserved nucleotides are highlighted in gray boxes. ***C, D,*** Luciferase assays were performed using plasmids containing MRE24 in the coding region (***C***) or MRE34a in the 3'UTR (***D***) of HNF4α mRNA. The data were relative to that of pGL3p cotransfected with each miRNA. Each column represents the mean ± SD of three independent experiments. **$P < 0.01$, ***$P < 0.001$.

miR-24 and miR-34a act on the coding region and the 3'UTR of HNF4α, respectively –

To verify that miR-24 and miR-34a act on the coding region and the 3'UTR of HNF4α, respectively, the expression systems of

HNF4α that excluded or included the 3'UTR were constructed. In the HEK293 cells transfected with pTARGET/HNF4α plasmid, the HNF4α protein level was significantly decreased by the overexpression of miR-24, but not by miR-34a (Fig. 9). In the HEK293 cells transfected with pTARGET/HNF4α+3'UTR plasmid, the HNF4α protein level was significantly decreased by both miR-24 and miR-34a. These results support that miR-24 and miR-34a down-regulate the HNF4α expression through recognizing the elements in the coding region and the 3'UTR of HNF4α mRNA, respectively.

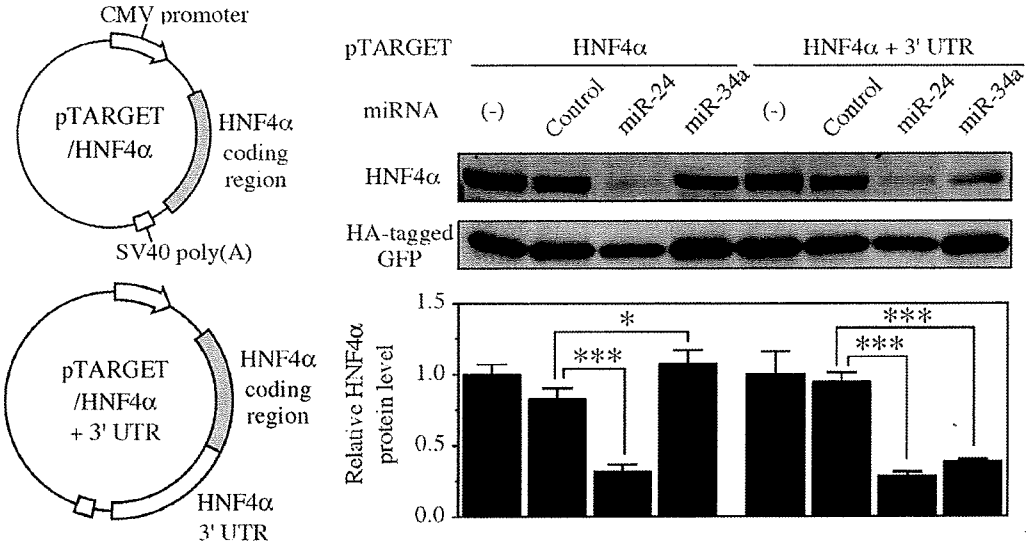

Fig. 9. **Effects of miRNAs on the exogenous HNF4α expression in HEK293 cells.** The HNF4α expression plasmids including and excluding 3'UTR used in this study are shown. These plasmids were transfected with HA-tagged GFP expression plasmid and Pre-miR miRNA Precursor Molecules into HEK293 cells. After 48 h, whole cell lysates were prepared. The exogenously expressed HNF4α and HA-tagged GFP protein levels were determined by Western blot analyses. The data represent HNF4α protein level normalized with HA-tagged GFP level relative to that of no transfection (-). Each column represents the mean ± SD of three independent experiments. $*P < 0.05$, $***P < 0.001$.

Regulation of miR-24 and miR-34a expression –

The mature miR-24 is produced from two precursors, pre-miR-24-1 and pre-miR-24-2, the genes of which are located on chromosome 9q22.32 and 19p13.12, respectively. The mature miR-34a is produced from a precursor pre-miR-34a, the gene of which is located on chromosome 1p36.22. Since the HNF4α expression and/or activity are changed in response to signals derived from bile acids, it was examined whether bile acids affect the expression of miR-24 and miR-34a. First, the effect of CDCA on the expression of the precursors of miR-24 and miR-34a was examined. The treatment with CDCA for 24 h increased the pre-miR-24-2 level in HepG2 cells (3.4-fold) at a concentration of 200 μM (data not shown). Bile acids are known to activate PKC and ROS generation. Next, the effects of PKC activator PMA and ROS generator H_2O_2 on the expression of the precursors of miR-24 and miR-34a were investigated. The treatment with PMA for 0.5 - 3 h markedly increased the expression of the pre-miR-24-2 level (Fig. 10*A*). The treatment with H_2O_2 for a relatively longer time increased greatly the expression levels of pre-miR-24-2 and pre-miR-34a (Fig. 10*B*). This was accompanied by increases of the mature miR-24 and miR-34a levels (Fig. 10*C*). Interestingly, the HNF4α protein levels were significantly decreased (Fig. 10*D*). It was likely that PMA or H_2O_2 repressed the HNF4α expression though increasing the miR-24 and miR-34a levels.

PKC activates MAPK pathway including ERK, JNK and p38. The PMA-dependent induction of pre-miR-24-2 was decreased by co-treatment with MAPK/ERK kinase (MEK) inhibitor U0126 or p38 inhibitor SB202190, but not with JNK inhibitor SP600125 (Fig. 10*E*). In contrast, these MAPK inhibitors did not affect the H_2O_2-dependent induction of pre-miR-34a (Fig. 10*F*). These results suggest that the ERK and p38 MAPK pathways modulate the pre-miR-24-2 level. The decrease of the HNF4α expression via activation of MAPK or generation of ROS might partly be explained by the induction of miRNAs repressing HNF4α.

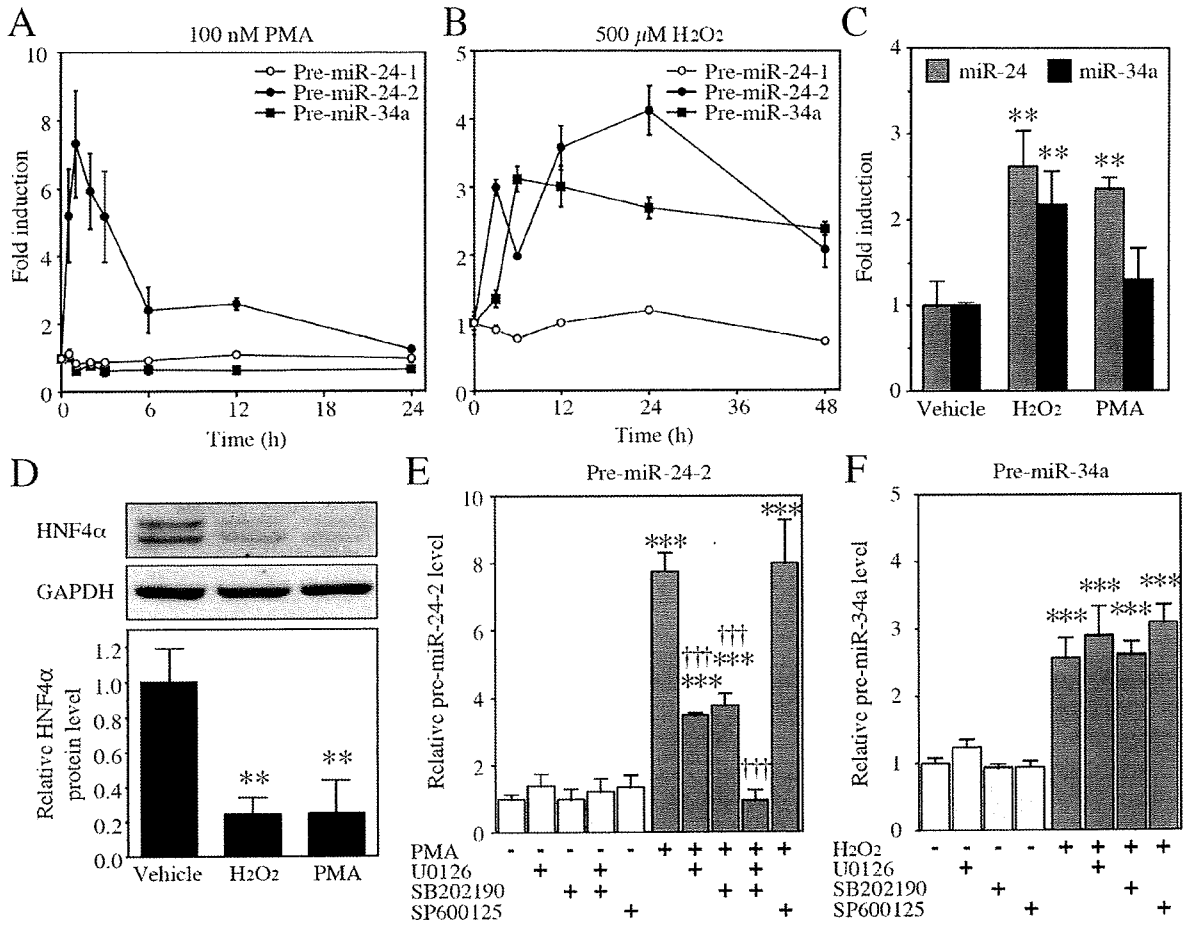

Fig. 10. **Regulation of miR-24 and miR-34a through MAPK and ROS pathway, respectively.** ***A, B,*** HepG2 cells were treated with 100 nM PMA (***A***) or 500 μM H_2O_2 (***B***) for the indicated time. The pre-miRNA levels were determined by real-time RT-PCR and normalized with the U6 snRNA level. The data are shown as fold changes compared with vehicle. Each point represents the mean ± SD of three independent experiments. ***C, D,*** HepG2 cells were treated with 100 nM PMA or 500 μM H_2O_2 for 48 h. The mature miR-24 and miR-34a levels were determined by real-time RT-PCR and normalized with the U6 snRNA level (***C***). The HNF4α and GAPDH protein levels were determined by Western blot analyses (***D***). The data are relative to vehicle. Each column represents the mean ± SD of three independent experiments. $^{**}P < 0.01$, compared with vehicle. ***E, F,*** Cells were co-treated with 100 nM PMA and 10 μM MAPK inhibitors for 1 h (***E***) or co-treated with 500 μM H_2O_2 and 10 μM MAPK inhibitors for 6 h (***F***). Each column represents the mean ± SD of three independent experiments. $^{***}P < 0.001$, compared with non-treatment. †††$P < 0.001$, compared with PMA-treated.

miR-24- and miR-34a-dependent down-regulation of HNF4α decreases the expression of target genes –

The effects of the miRNA-dependent down-regulation of HNF4α on the expression of target genes were investigated

(Fig. 11*A*). The overexpression of miR-24 and miR-34a drastically decreased the CYP7A1 mRNA level in HepG2 cells. In addition, the overexpression of miR-24 and miR-34a significantly decreased the CYP8B1, CYP27A1, and PEPCK mRNA levels. To investigate whether the decrease of these mRNAs resulted from the decrease of the HNF4α protein level but not the direct effects of miRNAs, siHNF4α was introduced into the HepG2 cells. It was clearly demonstrated that the HNF4α protein level was remarkably decreased by the transfection of siHNF4α (Fig. 11*B*). Under this condition, the mRNA levels of CYP7A1, CYP8B1, CYP27A1, and PEPCK were significantly decreased. These results suggest that the decrease of HNF4α by miR-24 and miR-34a caused the decrease of the expression of the target genes. As they are key enzymes for the bile acid biosynthetic pathway and rate-limiting for gluconeogenesis, miR-24 and miR-34a may affect the hepatic functions through the regulation of HNF4α.

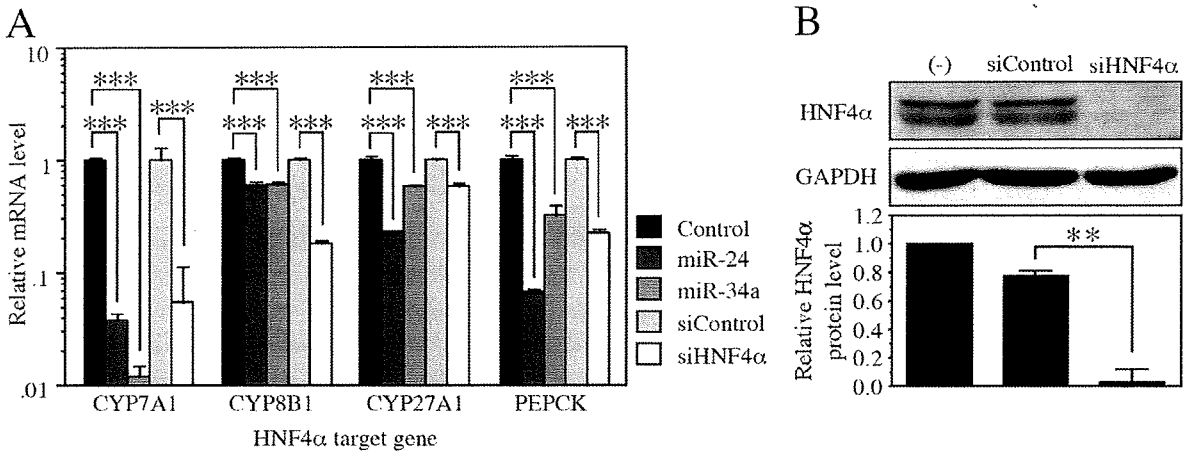

Fig. 11. **Down-regulation of various HNF4α target genes by miR-24 and miR-34a as well as siHNF4α.** ***A,*** The mRNA levels of various targets of HNF4α in HepG2 cells were examined by real-time RT-PCR and normalized with the GAPDH mRNA level. The data are relative to that transfected with control or siControl. Each column represents the mean ± SD of three independent experiments. ***$P < 0.001$. ***B,*** The HNF4α and GAPDH protein levels in HepG2 cells were determined by Western blot analyses. The data are relative to no transfection (-). Each column represents the mean ± SD of three independent experiments. **$P < 0.01$.

miR-24- and miR-34a-dependent down-regulation of HNF4α is associated with changes of morphology and cell cycle –

When miR-24 and miR-34a were overexpressed in the HepG2 cells, morphological changes including scattering and enlargement of the cells were observed (Fig. 12*A*). Such morphological changes were also observed when the siHNF4α was transfected. Since it has been reported that HNF4α is involved in the control of differentiation, cell adhesion and cell proliferation (Parviz et al., 2003; Battle et al., 2006; Erdmann et al., 2007), the effects of overexpression of miR-24 and miR-34a on the cell cycle were investigated. The percentage of S-phase cells was significantly decreased by the transfection with miR-24 or miR-34a into the HepG2 cells (Fig. 12*B*). The decrease was also observed by the transfection of siHNF4α. In addition, it was found that the transfection with miR-24 or miR-34a significantly induced the mRNA level of cyclin-dependent kinase inhibitor p21 (Fig. 12*C*), although there was no clear induction of p16 and p27. The induction of p21 and p16 was also observed when the siHNF4α was transfected. These results suggest that the changes of morphology and cell population by miR-24 and miR-34a might partly be due to the down-regulation of HNF4α. However, the transfection of siHNF4α did not cause cell cycle arrest at the G_1/S-transition, although the overexpression of miR-24 and miR-34a caused complete arrest at the G_1/S-transition, when the cells were synchronized by serum starvation (Fig. 12*D*). Thus, miR-24 and miR-34a may arrest the cell cycle through other mechanisms that are independent of HNF4α.

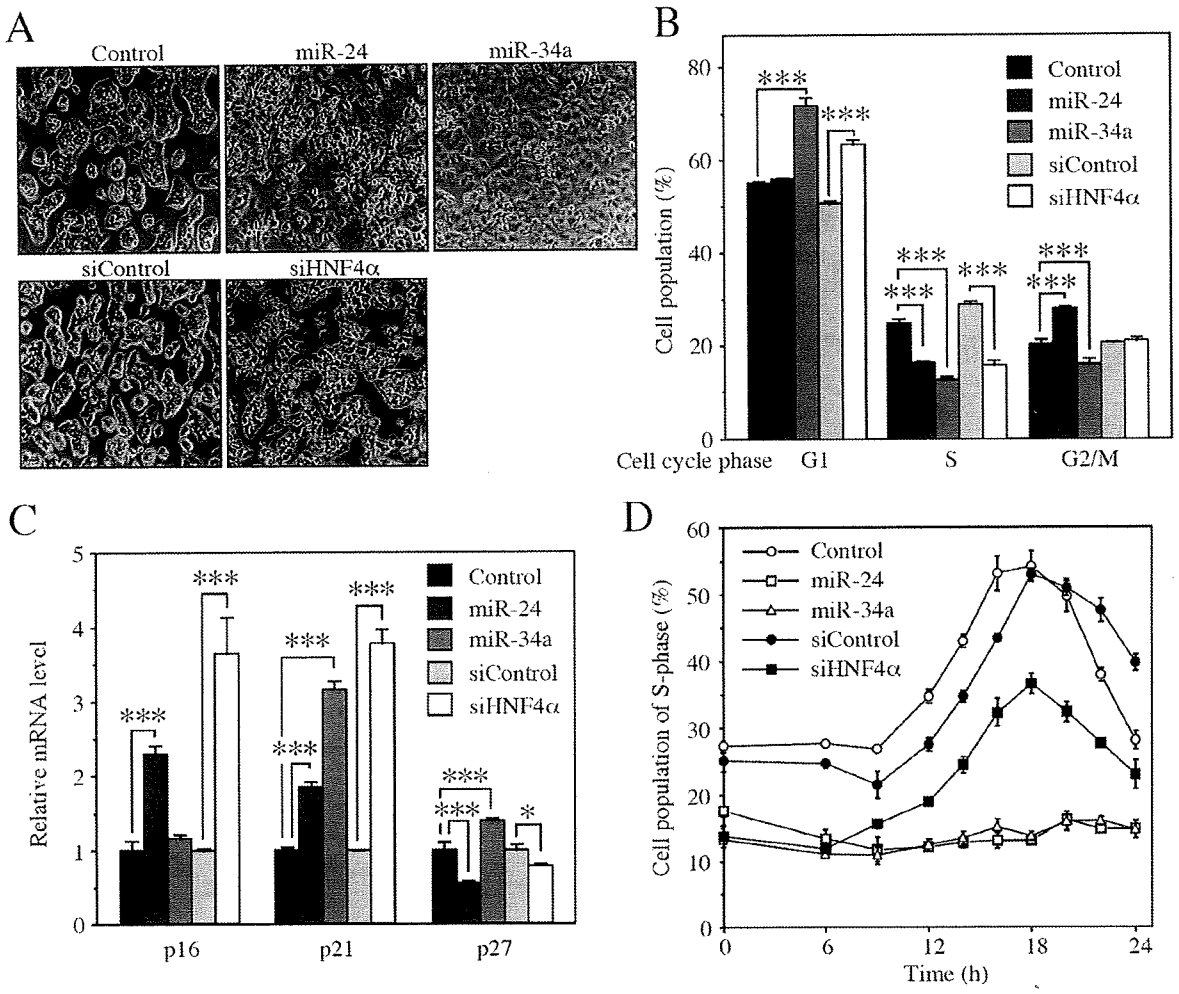

Fig. 12. **Inhibition of G1/S transition by miR-24 and miR-34a in HepG2 cells.** ***A,*** Morphological change of HepG2 cells at 72 h after the transfection with Pre-miRNA Precursor Molecules or siRNAs was visualized and photographed under a light microscope. ***B,*** Cells were collected at 48 h after the transfection and the cell population in each phase of cell cycle was analyzed by FACS. Each column represents the mean ± SD of three independent experiments. ***$P < 0.001$. ***C,*** The p16, p21 and p27 mRNA levels were determined by real-time RT-PCR and were normalized with the GAPDH mRNA level. The data are relative to that transfected with the control or siControl. Each column represents the mean ± SD of three independent experiments. *$P < 0.05$, ***$P < 0.001$. ***D,*** The time-dependent change of the percentages of cell population under S-phase are shown. Twenty-four hours after the transfection with Pre-miRNA Precursor Molecules or siRNAs, the cells were synchronized by serum deprivation for 24 h. Then the cells were restimulated with serum for the indicated time. Each point represents the mean ± SD of three independent experiments.

D. 考察

HNF4α is highly expressed in the liver, although it is also expressed in extrahepatic tissues such as kidney, intestine, and pancreas (Gonzalez et al., 2008). It is constitutively active and generally acts as a positive transcriptional regulator of the

expression of various transcriptional factors and enzymes. It has been reported that knockout of HNF4α disrupts the hepatic architecture and function (Parviz et al., 2003). Mutations in HNF4α are a cause of type 1 maturity-onset diabetes of the young (MODY1), which is the monogenic form of diabetes that results from functional defects in islet β cells (Stanger, 2008). Thus, HNF4α is critical for tissue development and for the maintenance of a number of metabolic pathways. In this study, the role of miRNAs in the regulation of HNF4α expression was investigated.

It was found that both miR-24 and miR-34a negatively regulate the HNF4α expression. Generally, in vertebrates, miRNAs are believed to recognize elements in the 3'UTR to repress the translation or to degrade mRNA. In this study, however, it was found that the functional MREs for miR-24-dependent regulation are located in the coding region of HNF4α mRNA. This was not surprising because, for other targets, it has been demonstrated that the miRNA regulates through the coding region or 5'UTR (Lytle et al., 2007; Forman et al., 2008; Tay et al., 2008). miR-24 decreased the HNF4α mRNA level in addition to the protein level, suggesting that miR-24 is likely to repress the HNF4α expression through mRNA degradation rather than through translational repression. On the other hand, the functional MRE for miR-34a-dependent regulation is located in the 3'UTR. miR-34a is likely to repress the HNF4α expression through translational repression, because it did not decrease the mRNA levels although it did decrease the HNF4α protein level. Thus, it was considered that miR-24 and miR-34a regulate the human HNF4α expression through different mechanisms.

The pre-miR-24-2 level was strongly increased by the treatment with PKC/MAPK activator PMA and ROS generator H_2O_2 in HepG2 cells. The activation of PKC pathway induces cholestasis (Kubitz et al., 2004). The ROS pathway plays an important role in the pathogenesis of nonalcoholic steatohepatitis (Day, 2002). Since the increase of the pre-miR-24-2 resulted in the increase of the mature miR-24 level, the mature miR-24 expression seems to be induced in these pathological conditions. Additionally, it has been reported that

transforming growth factor (TGF)-β, which is associated with fibrosis, increased the miR-24 level (Huang et al., 2008). Regarding HNF4α, it has been reported that cholestasis, hepatic steatosis and fibrosis down-regulate the expression (Geier et al.,2005; Xie et al., 2009; Yue et al., 2008). Thus, the down-regulation of HNF4α in these diseases might be due to the induction of miR-24.

Previous studies revealed that miR-34a is regulated by p53, a tumor suppressor gene (Raver-Shapira et al., 2007; Chang et al.2007). The present study demonstrated that the pre-miR-34a level was increased by the treatment with H_2O_2. The treatment with MAPK inhibitors failed to inhibit the induction of pre-miR-34a. ROS is known to activate p53 pathway, suggesting that the increase of pre-miR-34a might result from the p53 activation, but not MAPK pathways. It is known that CDCA activates the PKC pathway and generates ROS, but it failed to increase the pre-miR-34a level in this study (data not shown). Although the reason for the discrepancy is unknown, it is surmised that miR-34a may be up-regulated directly by bile acids.

The changes of morphology and cell cycle by miR-24 and miR-34a might partly be due to the down-regulation of HNF4α. However, detailed examination of the cell population revealed that cell cycle arrest might be caused by additional roles of miR-24 and miR-34a with other targets besides HNF4α. In fact, miR-34a is known to suppress cell cycle-regulatory genes such as cyclin E2 and cyclin-dependent kinase 4, resulting in cell cycle arrest in the G1-phase (He et al., 2007). Meanwhile, miR-24 has been reported to promote the proliferation of TGF-β-treated HuH7 hepatocellular carcinoma cells (Huang et al., 2008) as well as A549 lung carcinoma cells (Cheng et al., 2005). These findings might be consistent with a report showing that miR-24 suppressed the translation of p16, which arrests cells in the G1 phase (Lal et al., 2008). In contrast, Cheng et al (2005) have reported that miR-24 attenuated the proliferation in HeLa cells. Thus, miR-24 might function differently in different cells. In contrast to a previous study (Hwang-Verslues et al., 2008), the decrease of HNF4α by the transfection with siHNF4α resulted in the up-regulation of

p21 gene expression. Since c-myc interacts with HNF4α and blocks the activation of p21 promoter, the conflicting result might be due to the difference in the balance between c-Myc and HNF4α expression. Taken together, miR-24 and miR-34a would cause cell arrest through the regulation of multiple targets in global network for cell cycle.

Of particular interest in the findings was that the miR-24- and miR-34a-dependent down-regulation of HNF4α resulted in decreases of the downstream genes. CYP7A1 catalyzes the first and rate-limiting step in the classical bile acid synthetic pathway (Pikuleva et al., 2006). It is considered that the induction of miR-24 and miR-34a would result in decreased bile acid synthesis. In addition, gluconeogenic enzyme PEPCK was also down-regulated by the decrease of the HNF4α expression by miRNAs. Thus, miR-24 and miR-34a might affect the various hepatic functions through the negative regulation of HNF4α expression. Interestingly, these miRNAs were induced by PKC/MAPK activator or ROS generator. Therefore, a novel feedback regulation of bile acids synthesis could be proposed (Fig. 13). Namely, bile acids activate PKC/MAPK and ROS pathways. The PKC/MAPK and ROS pathways increase the miR-24 and miR-34a expression, respectively. These miRNAs down-regulate the HNF4α expression. Accordingly, the expression of bile acid-synthesizing enzymes is decreased. Thus, a new insight into the negative feedback regulation of bile acids synthesis could be provided.

In summary, this study found that miR-24 and miR-34a regulate human HNF4α expression resulting in the decrease of various downstream genes and aberrant cell cycle. Since these miRNAs are under the control of cellular stress, the miRNAs-dependent regulation of human HNF4α might contribute to the pathology in the liver.