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Abbreviations: atRA, all-*trans*-retinoic acid; CYP, cytochrome P450; DMSO, dimethyl

sulfoxide; miRNA, microRNA; MRE, microRNA recognition element; NR, nuclear receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RT, reverse transcription; UTR, untranslated region.

ABSTRACT

Retinoid X receptor α (RXRα) forms a heterodimer with numerous nuclear receptors to regulate drug- or lipid-metabolizing enzymes. In this study, we investigated whether human RXRα is regulated by microRNAs. Two potential recognition elements of miR-34a were identified in the RXRα mRNA: one in the coding region and the other in the 3'-untranslated region (3'-UTR). Luciferase assays revealed that miR-34a recognizes the element in the coding region. The overexpression of miR-34a in HepG2 cells significantly decreased the endogenous RXRα protein and mRNA levels. The stability of RXRα mRNA was decreased by the overexpression of miR-34a, indicating that miR-34a negatively regulates RXRα expression by facilitating mRNA degradation. We found that the miR-34a-dependent down-regulation of RXRα decreases the induction of CYP26 and the transactivity of CYP3A4. miR-34a has been reported to be up-regulated by p53, which has an ability to promote liver fibrosis. The p53 activation resulted in an increase of the miR-34a level and a decrease of the RXRα protein level. In addition, the miR-34a levels in eight fibrotic livers were higher than those in six normal livers, and the reverse trend was found for the RXRα protein levels. An inverse correlation was observed between the miR-34a and the RXRα protein levels in the 14 samples. Taken together, the data show that miR-34a negatively regulates RXRα expression in human liver, and affects the expression of its downstream genes. This miR-34a-dependent regulation might be the underlying mechanism responsible for the decreased expression of the RXRα protein in fibrotic livers.

Keyword: microRNA, RXRα, fibrosis, p53, post-transcriptional regulation

Chemical compounds studied in this article

All-*trans*-retinoic acid (PubChem CID: 444795); 9-*cis*-retinoic acid (PubChem CID: 449171); rifampicin (PubChem CID: 5381226); etoposide (PubChem CID: 36462)

1. Introduction

Human retinoid X receptor α (RXRα: NR2B1), a member of the nuclear receptor (NR) superfamily, is widely expressed in many tissues and is most abundant in the liver. RXRα plays a crucial role in the ligand-dependent transactivation of various genes involved in drug and lipid metabolism by forming a heterodimeric complex with numerous members of the NR superfamily, including retinoic acid receptor (RAR), vitamin D receptor (VDR), thyroid hormone receptor, pregnane X receptor (PXR), constitutive androstane receptor, peroxisome proliferator-activated receptor (PPAR), farnesoid X receptor, and liver X receptor. It has been demonstrated that more than 300 genes in humans can be transactivated by RXRα [1]. Although the gene regulation by RXRα is well known, the mechanisms responsible for the regulation of the expression of human RXRα remain to be clarified.

MicroRNAs (miRNAs), an evolutionarily conserved class of endogenous ~22-nucleotide noncoding RNAs, bind to target mRNAs to cause translational repression or mRNA degradation [2]. In animals, the miRNA target sites are located mainly in the 3'-untranslated region (3'-UTR) of target mRNAs [3] and sometimes in the coding region [4]. To date, more than 2,500 miRNAs have been identified in humans. One miRNA has the potential to target a large number of genes (an average of approximately 500 genes), and it has been estimated that more than 60% of human mRNAs can be targets of miRNAs [5, 6]. We recently reported that miRNAs are involved in the regulation of some human NRs, such as PXR [7], VDR [8], hepatocyte nuclear factor 4 α [9], and PPARα [10].

Employing an on-line search, we found that potential recognition elements of miR-34a were identified in human RXRα mRNA. miR-34a commonly functions as a tumor suppressor and is down-regulated in many human cancers [11]. Previous studies have revealed that miR-34a is regulated by p53, a tumor suppressor gene [12, 13]. It was recently reported that p53 induces the expression of connective tissue growth factor (CTGF), a hepatic fibrogenic master switch, and promotes liver fibrosis [14]. From these finding, we hypothesized that miR-34a might also lead to liver fibrosis. Interestingly, Wang et al. [15] reported that the

RXRα protein level is decreased in a CCl_4-induced fibrosis model rat and that the overexpression of RXRα results in the down-regulation of collagen type I expression and the inhibition of liver fibrosis.

In this study, we investigated whether human RXRα is regulated by miR-34a. We also evaluated the relationship between miR-34a and RXRα expression in fibrotic human livers to provide information for uncovering the pathogenic mechanisms and developing new therapeutic strategies for the treatment of liver fibrosis.

2. Materials and Methods

2.1. Chemicals and reagents.

All-*trans*-retinoic acid (atRA), 9-*cis*-retinoic acid (9-*cis*-RA), rifampicin, and etoposide were obtained from Wako Pure Chemicals (Osaka, Japan). α-amanitin was purchased from Calbiochem (San Diego, CA). RNasin Ribonuclease Inhibitor, the pGL3-promoter (pGL3p) vector, pGL4.74-TK, the pRL-SV40 plasmid, the TransFast Transfection Reagent, and the Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). The Pre-miR miRNA Precursor Molecules for miR-34a and Negative Control #1 (Control) were obtained from Ambion (Austin, TX). Lipofectamine RNAiMAX was purchased from Invitrogen (Carlsbad, CA). RNAiso, random hexamer, SYBR Premix Ex Taq, and ROX were from Takara (Shiga, Japan). ReverTra Ace was obtained from Toyobo (Osaka, Japan). The Ribonucleotide Solution Mix was purchased from New England Biolabs (Ipswich, MA). All of the primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). The rabbit anti-human RXRα polyclonal antibodies (D-20) and rabbit anti-human β-actin polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BioVision (Mountain View, CA), respectively. IRDye 680LT goat anti-rabbit IgG was purchased from LI-COR Biosciences (Lincoln, NE). All of the other chemicals and solvents were of the highest grade commercially available.

2.2. Cell culture

A human embryonic kidney-derived cell line HEK293 was obtained from American Type Culture Collection (Manassas, VA). The human hepatocellular carcinoma-derived cell lines HepG2 and HuH-7 were obtained from Riken Gene Bank (Tsukuba, Japan). The HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 4.5 g/l glucose, 10 mM HEPES, and 10% fetal bovine serum (FBS) (Invitrogen). The HepG2 cells were cultured in DMEM supplemented with 0.1 mM non-essential amino acids (Invitrogen) and 10% FBS. The HuH-7 cells were cultured in DMEM supplemented with 10% FBS. All of the cells were maintained at 37°C under an atmosphere of 5% CO_2-95% air.

2.3. Construction of plasmids

The fragments containing the MRE1 (+3449 to +3591) or MRE2 (+659 to +778) of the human RXRα (accession number NM_002957) cDNA were amplified by PCR and subcloned into the pGL3p vector at the *Xba* I site downstream of the *luciferase* gene. These plasmids were termed pGL3/MRE1 and pGL3/MRE2, respectively. A fragment containing the perfectly matching sequence with the mature miR-34a, 5'- CTA GA*A CAA CCA GCT AAG ACA CTG CCA* T -3' (the complementary sequence of miR-34a is italicized), was also cloned (pGL3/c-miR-34). DNA sequencing analyses using a Long-Read Tower DNA sequencer (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) confirmed the nucleotide sequences of these plasmids.

2.4. Luciferase assay

Various pGL3 luciferase reporter plasmids were transiently transfected with the pGL4.74-TK plasmid into HEK293 cells. Briefly, the day before transfection, the cells were seeded into 24-well plates. After 24 h, 190 ng of the pGL3p plasmid, 10 ng of the pGL4.74-TK plasmid, and the precursor for miR-34a or control (50 nM) were transfected into HEK293 cells using LipofectAMINE 2000. After incubation for 48 h, the cells were

resuspended in passive lysis buffer, and the luciferase activity was then measured with a luminometer (Wallac, Turku, Finland) using the Dual-Luciferase Reporter Assay System.

2.5. Transfection of pre-miRNAs and preparation of cell homogenates and total RNA

The HepG2 cells were seeded into six-well plates and transfected with the precursor for miR-34a or control (50 nM) using Lipofectamine RNAiMAX. After 72 h, the cells were harvested, suspended in a small amount of TGE buffer [10 mM Tris-HCl, 20% glycerol, and 1 mM EDTA (pH 7.4)], disrupted through three freeze-thaw cycles, and homogenized. The protein concentration was determined using the Bradford protein assay reagent (Bio-Rad, Hercules, CA) with γ-globulin as the standard. The total RNA was prepared using RNAiso according to the manufacturer's protocols.

2.6. Western blot analyses

The cell homogenates from the HepG2 and HuH-7 cells (30 μg) or human liver samples (20 μg) were separated on a 10% SDS-polyacrylamide gel and transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA) at 5 V for 1 hr. The protein levels were decided based on the linear range of band intensity. The membranes were probed with primary antibody rabbit anti-human RXRα (diluted 1:200) or rabbit anti-human β-actin (diluted 1:100) and secondary antibody IRDye 680LT goat anti-rabbit IgG (diluted 1:10000). The band densities were quantified with the Odyssey Infrared Imaging system (LI-COR Biosciences). The RXRα protein level was normalized to the β-actin protein level.

2.7. Real-time RT-PCR

The cDNAs were synthesized from the total RNA using ReverTra Ace. The sequences of the primers used are shown in Table 1. A 1-μl aliquot of the reverse-transcribed mixture was added to a PCR mixture containing 10 pmol of each primer, 10 μl of the SYBR Premix Ex Taq solution, and 0.25 μl of ROX in a final volume of 20 μl. The PCR conditions for RXRα were the following: after an initial denaturation at 95°C for 1 min, the amplification was

performed through 40 cycles of denaturation at 94°C for 15 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. For the other genes, after an initial denaturation at 95°C for 30 s, the amplification was performed through 40 cycles of denaturation at 94°C for 20 s and annealing/extension at 65°C (p21) or 64°C (β-actin) for 20 s. The real-time RT-PCR was performed using Mx3000P (Stratagene, La Jolla, CA) with the MxPro QPCR software. The RXRα and p21 mRNA levels were normalized to the β-actin mRNA level.

2.8. Stability of RXRα mRNA

The HepG2 cells transfected with the precursor for miR-34a or control (50 nM) were simultaneously treated with 10 μg/ml α-amanitin. The total RNA was prepared 3, 6, 9, and 12 h after treatment. The RXRα mRNA levels were determined by real-time RT-PCR as described above.

2.9. Nuclear run-on assay

The nuclei from the HepG2 cells (30 mg) transfected with the precursor for miR-34a or control (50 nM) and cultured for 48 h were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). The nuclear pellet was suspended in 600 μl of transcription buffer [50 mM Tris-HCl, 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dithiothreitol, 10% glycerol, and 0.1 mM EDTA (pH 8.0)] supplemented with 2 μl of 50 μg/ml RNase A Solution (Qiagen, Tokyo, Japan) and incubated at 37°C for 20 min. After the addition of 5 μl of RNasin Ribonuclease Inhibitor and incubation at 37°C for 5 min, half of the nuclei suspension was added to 1 ml of RNAiso, and the remaining half was used for the nuclear run-on assay. The *in vitro* transcription reaction was conducted through the addition of 1 μl of Ribonucleotide Solution Mix at 25°C for 30 min. The reaction was terminated by the addition of 1 ml of RNAiso. The total RNA was isolated, and RXRα mRNA level was quantified by real-time PCR as described above.

2.10. Evaluation of CYP26 mRNA induction and CYP3A4 promoter transactivation

The HepG2 cells were seeded into 12-well plates and transfected with the precursor for miR-34a or control (50 nM) using Lipofectamine RNAiMAX. After 72 h, the cells were treated with 1 nM atRA or 2 nM 9-*cis*-RA (or 0.1% DMSO) for 24 h, and the total RNA was prepared. The sequences of the primers used for CYP26 are shown in Table 1. The real-time RT-PCR for CYP26 was performed as follows: after an initial denaturation at 95°C for 30 s, the amplification was performed through 40 cycles of denaturation at 94°C for 10 s and annealing/extension at 68°C for 30 s.

The CYP3A4 promoter transactivation was evaluated using the reporter plasmid pCYP3A4-362-7.7K [7]. HepG2 cells were seeded into 24-well plates. After 24 h, 290 ng of the pCYP3A4-362-7.7K plasmid, 10 ng of the pRL-SV40 plasmid, and the precursor for miR-34a or control (50 nM) were transfected using the TransFast Transfection Reagent. After 48 h, the cells were treated with 10 μM rifampicin for 24 h, and the luciferase activity was measured as described above.

2.11. Etoposide treatment

The HepG2 and HuH-7 cells were seeded into six-well plates. After 48 h, the cells were treated with 1 μM etoposide for 48 h. The total cell homogenates and total RNA were prepared as described above.

2.12. Real-time RT-PCR for mature miR-34a

The expression levels of mature miR-34a were determined using the TaqMan microRNA assay (Applied Biosystems, Foster City, CA). The cDNA templates were prepared using the TaqMan microRNA Reverse Transcription kit, which utilizes the stem-loop reverse primers, according to the manufacturer's protocols. After the reverse transcription reaction, the product was mixed with the TaqMan Universal PCR Master Mix, the TaqMan MicroRNA assay mix containing the forward and reverse primers, and the TaqMan probe for miR-34a. The PCR conditions were the following: after an initial denaturation at 95°C for 10 min, the amplification was performed through 40 cycles of denaturation at 95°C for 15 s and

annealing/extension at 60°C for 60 s. The expression levels of U6 small nuclear RNA (U6 snRNA) were also determined using the TaqMan microRNA assay and were used to normalize the miR-34a levels.

2.13. Human livers and preparation of cell homogenates and total RNA

Fibrotic (n = 8) and normal human liver samples (n = 6) were obtained from autopsy materials that were discarded after pathological investigation at Iwate Medical University (Morioka, Japan) and University of Toyama (Toyama, Japan) (Table 2). The use of the human livers was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan), Iwate Medical University (Morioka, Japan), and University of Toyama (Toyama, Japan). The total cell homogenates were prepared by homogenization with three volumes of 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.1 M KCl. The total RNA was prepared as described above.

2.14. Statistical analysis

The statistical significance was determined by analysis of variance followed by Tukey's test. The comparisons of two groups were performed using unpaired, two-tailed Student's *t* test or Mann-Whitney's U-test. The correlation analyses were performed by Spearman's rank method. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of functional MRE in RXRα mRNA

Based on a computational prediction using TargetScan (http://www.targetscan.org/), a number of miRNAs were predicted to bind to the 3'-UTR of human RXRα mRNA. Among these, we focused on miR-34a because it is substantially expressed in the human liver [16] and shares the highest complementarity with the sequence of RXRα (the energy: -37.8 kcal/mol, whereas those of the other miRNAs are over -23 kcal/mol, by RNAhybrid,

http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html). We also searched the potential MREs for miR-34a in the coding region using RNA22 (http://cbcsrv.watson.ibm.com/rna22.html). The results revealed two potential sites, namely MRE1 and MRE2, in the 3'-UTR (+3506 to +3539) and coding region (+714 to +735, the energy is -27.9 kcal/mol), respectively (Fig. 1A).

To investigate whether these MREs are functional, a luciferase assay using reporter plasmids containing a fragment with one of the MREs was performed with HEK293 cells (Fig. 1B). The co-transfection of the pGL3/c-miR-34a plasmid containing the miR-34a complementary sequence downstream of the *luciferase* gene with the precursor for miR-34a significantly decreased the luciferase activity ($P < 0.001$), suggesting that the overexpressed miR-34a is functional. Interestingly, the overexpression of miR-34a significantly ($P < 0.01$) decreased the luciferase activity of the pGL3/MRE2 plasmid but not that of the pGL3/MRE1 plasmid, indicating that MRE2 in the coding region is functional.

3.2. RXRα expression is decreased by overexpression of miR-34a

We investigated whether miR-34a modulates RXRα expression. The transfection of the precursor for miR-34a into HepG2 cells resulted in the overexpression of miR-34a and significantly decreased the endogenous RXRα protein level ($P < 0.001$, 49% of control) (Fig. 2A). To investigate whether the decrease in the RXRα protein levels can be attributed to a decrease in the mRNA levels, the RXRα mRNA levels were determined (Fig. 2B). In the control cells, the RXRα mRNA level was increased during the first 12-48 h of incubation and then slightly decreased at 96 h. In the miR-34-overexpressing cells, the increase in RXRα mRNA was significantly ($P < 0.001$) suppressed at 24-72 h, indicating that miR-34a negatively regulates the expression of human RXRα through mRNA degradation. To determine whether the suppression of RXRα mRNA induced by miR-34a is due to accelerated mRNA degradation, we examined the effects of miR-34a on the stability of the RXRα mRNA. In the presence of α-amanitin, the RXRα mRNA was rapidly degraded in cells overexpressing miR-34a (Fig. 2C). We then performed a nuclear run-on assay to

examine whether miR-34a may affect the transcription efficiency of RXRα. The results demonstrated that the transcription efficiency was not affected by the overexpression of miR-34a (Fig. 2D). These results suggest that miR-34a down-regulates RXRα expression through the acceleration of mRNA degradation.

3.3. miR-34a-dependent down-regulation of RXRα suppresses the induction of CYP26 and the transactivation of CYP3A4

We investigated whether the miR-34a-dependent down-regulation of RXRα may affect the induction of downstream genes of RXRα. The treatment of HepG2 cells with atRA, a ligand of RAR, significantly increased the CYP26 mRNA level (3.2-fold) (Fig. 3A). A similar induction was obtained by the treatment of HepG2 cells with 9-*cis*-RA, a ligand of RXRα. Interestingly, the induction of CYP26 mRNA was completely abrogated by the overexpression of miR-34a. These results suggest that the miR-34a-dependent down-regulation of RXRα affects the induction of downstream genes of RXRα by ligands of whether RAR or RXRα.

To further investigate whether the miR-34a affects the induction of other targets of RXRα, we focused on CYP3A4, a downstream gene of RXRα with a heterodimer partner (PXR). Because the CYP3A4 mRNA levels in HepG2 cells were too low to determine the induction, we evaluated the transactivity of CYP3A4 through a luciferase assay using a reporter plasmid containing the PXR response elements. The treatment of HepG2 cells with rifampicin, a ligand of PXR, significantly increased the transactivity of CYP3A4 (4.1-fold) (Fig. 3B). The transactivation was markedly decreased by the overexpression of miR-34a. These results suggest that miR-34a also affects the induction of downstream genes of RXRα with a heterodimer partner whether RAR or PXR.

3.4. miR-34a-dependent down-regulation of RXRα is associated with liver fibrosis

As described in the introduction, miR-34a is up-regulated by p53, which induces the expression of CTGF and promotes liver fibrosis. On the other hand, the overexpression of

RXRα results in the suppression of collagen synthesis and the inhibition of liver fibrosis. These findings led to our hypothesis that the miR-34a-dependent regulation of RXRα may be an underlying mechanism of the p53-induced liver fibrosis (Fig. 4A). We investigated the association of the miR-34a-dependent regulation of RXRα with liver fibrosis.

We examined whether the activation of p53 alters the expression of miR-34a and RXRα protein. The treatment of HepG2 cells (with wild-type endogenous p53) with etoposide, which is known to activate p53, significantly increased the mRNA level of p21, a well-known target of p53 (9.9-fold) (Fig. 4B). Under these conditions, the mature miR-34a level was significantly increased (1.5-fold), and the RXRα protein level was significantly decreased (83% of control) (Figs. 4C and 4D). To investigate whether the increase in miR-34a and the decrease in RXRα protein were due to the activation of p53, we performed a knocked down assay using siRNA for p53 (data not shown), but unfortunately the p53 protein level could not be suppressed (data not shown). Alternatively, we examined the effects of p53 activation on the expression of miR-34a and RXRα protein in HuH-7 cells (with mutated endogenous p53). Unlike in the case of HepG2 cells, no changes in the p21 mRNA, mature miR-34a, and RXRα protein levels were observed in HuH-7 cells (Figs. 4E-4G).These results support that the induction of miR-34a through p53 activation affects the expression of RXRα.

In addition, we examined the relationship between the expression levels of mature miR-34a and RXRα protein in fibrotic and normal human livers. The mature miR-34a levels in eight fibrotic livers were higher than those found in six normal livers (Fig. 5A). In contrast, the RXRα protein levels in the fibrotic livers were significantly ($P < 0.05$) lower than those observed in the normal livers (Fig. 5B). Interestingly, in the 14 samples, the miR-34a levels were inversely correlated with the RXRα protein levels (Rs = -0.79, $P < 0.001$, Fig. 5C). These results suggest that the high expression of miR-34a in fibrotic livers may be one of the causes of the low expression of the RXRα protein.

4. Discussion

RXRα plays a crucial role in many intracellular signaling pathways and the ligand-dependent transactivation of various genes by forming a heterodimeric complex with numerous members of the NR superfamily. Previous studies [17, 18] have reported that systemic RXRα-null mice are embryonic lethal because of cardiac failure, indicating the essential role of RXRα in embryonic development. Despite the indispensability of RXRα in mammals, the mechanisms through which RXRα expression is regulated are largely unknown. In this study, we investigated the possibility that human RXRα may be regulated by miRNA.

The present study found that the human RXRα protein and mRNA levels are decreased by the overexpression of miR-34a (Fig.2A, B). Because miR-34a decreases the stability but not the transcription of RXRα mRNA (Fig. 2C, D), the results suggest that miR-34a negatively regulates RXRα expression by facilitating mRNA degradation. In general, in vertebrates, miRNAs are believed to recognize elements in the 3'-UTR to repress the translation or to degrade mRNA. We found that the functional MRE for miR-34a is located in the coding region of the RXRα mRNA (Fig. 1). The cases in which miRNAs regulate the expression of their target gene expression through an MRE in the coding region that have been previously reported are the following: miR-148 regulating *DNA methyltransferase 3b* [19], miR-24 regulating *HNF4α* [9], and miR-29 regulating *Elastin* [20]. In all of these cases, the regulation mechanism is the acceleration of mRNA degradation. Thus, if the miRNAs recognize an MRE in the coding region, the acceleration of mRNA degradation rather than translational repression may be the dominant mechanism responsible for the down-regulation. It should be noted that the miR-34a-dependent down-regulation of RXRα affects the induction of the downstream genes (Fig. 3). Since CYP3A4 catalyzes the metabolism of over 50% of current prescription drugs, miR-34a may modulate drug metabolism. In addition, miR-34a may also modulate lipid metabolism because RXRα plays key roles in fatty acid and cholesterol metabolism [21].

We found that the p53 activation increased the miR-34a level decreased the RXRα protein level in HepG2 cells (wild-type p53) (Fig. 4B-D). These phenomena were not observed in HuH-7 cells (mutated p53) (Fig. 4E-G), supporting that the induction of miR-34a through p53

activation affects the expression of RXRα. Because p53 is also activated by DNA damage, reactive oxygen species, and hypoxia, RXRα expression may be under the control of these genotoxic stresses. To the best of our knowledge, this study provides the first demonstration that the RXRα protein level is decreased in the human fibrotic liver (Fig. 5). Thus, the low expression of RXRα protein in the human fibrotic liver likely results from the increased expression of miR-34a. The pathological examination revealed that three samples had mild fibrosis and five samples had severe fibrosis (Table 2). No association was observed between the severity and the miR-34a or RXRα protein level (data not shown). miR-34a may play a role in the incidence or progression of liver fibrosis. Although the inhibitory mechanism of RXRα toward liver fibrosis remains to be identified, Konta et al. [22] reported that atRA (RXRα agonist) suppresses the activator protein 1 (AP1) pathway involved in collagen synthesis and that an RXRα antagonist abrogates the suppression of the AP1 pathway. Thus, it would be worth examining whether the miR-34a-dependent down-regulation of RXRα can actually suppress collagen synthesis in the liver.

miR-34a is considered a therapeutic target of cancer because it promotes cell cycle arrest and apoptosis and inhibits proliferation by targeting many genes, such as Bcl2 (B-cell lymphoma 2), CDK (cyclin-dependent kinases), and cyclines [23]. In fact, a phase I clinical trial using a miR-34a mimic for the treatment of liver cancer is ongoing. Because we found that miR-34a down-regulates RXRα to exert protective effects on collagen synthesis, the overexpression of miR-34a may lead to liver fibrosis. In addition, our previous study [9] found that miR-34a down-regulates HNF4α and thereby affects the expression of its downstream genes involved in bile acid synthesis. The disruption of bile acid homeostasis causes various disorders, such as arteriosclerosis and gallstone disease [24]. Therefore, the careful monitoring of liver function would be required during miR-34a-modulation therapy.

miR-27b and miR-148, which are liver-enriched miRNAs, were also predicted as other candidate regulators for human RXRα by the computational search. However, the transfection of the precursors for these miRNAs into the HepG2 cells did not affect RXRα expression (data not shown). Although it has been reported that rat RXRα in hepatic stellate cells is

regulated by miR-27a/b [25], the regulation unlikely applies to human RXRα in hepatocytes, even though the sequence of the MRE is conserved between rat and human. It is possible that miR-27b was not found to be functional for the regulation of human RXRα because the other genes regulated by miR-27b may be more abundantly expressed in human hepatocytes than in rat hepatic stellate cells.

In conclusion, we found that miR-34a negatively regulates the expression of human RXRα, affects the expression of its downstream genes, and plays a role in the incidence of liver fibrosis. This study could provide useful information for uncovering the pathogenic mechanisms and developing new therapeutic strategies for the treatment of liver fibrosis.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

[1] Williams GM, Iatropoulos MJ. Alteration of liver cell function and proliferation: differentiation between adaptation and toxicity. Toxicol Pathol 2002; 30: 41-53.

[2] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-97.

[3] Chekulaeva M, Filipowicz W. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. Curr Opin Cell Biol 2009; 21: 452-60.

[4] Qin W, Shi Y, Zhao B, Yao C, Jin L, Ma J et al. miR-24 regulates apoptosis by targeting the open reading frame (ORF) region of FAF1 in cancer cells. PLoS One 2010; 5: e9429.

[5] Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. Nat Rev Drug Discov 2010; 9:775−89.

[6] Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009; 19: 92-105.

[7] Takagi S, Nakajima M, Mohri T, Yokoi T. Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. J Biol Chem 2008; 283: 9674-80.

[8] Mohri T, Nakajima M, Takagi S, Komagata S, Yokoi T. MicroRNA regulates human vitamin D receptor. Int J Cancer 2009; 125: 1328-33.

[9] Takagi S, Nakajima M, Kida K, Yamaura Y, Fukami T, Yokoi T. MicroRNAs regulate human hepatocyte nuclear factor 4α modulating the expression of metabolic enzymes and cell cycle. J Biol Chem 2010; 285: 4415-22.

[10] Kida K, Nakajima M, Mohri T, Oda Y, Takagi S, Fukami T et al. PPARα is regulated by miR-21 and miR-27b in human liver. Pharm Res 2011; 28: 2467-76.

[11] Lodygin D, Tarasov V, Epanchintsev A, Berking C, Knyazeva T, Körner H et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. Cell Cycle 2008; 7: 2591-600.

[12] Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N et al.

Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol Cell 2007; 26: 731-43.

[13] Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell 2007; 26: 745-52.

[14] Kodama T, Takehara T, Hikita H, Shimizu S, Shigekawa M, Tsunematsu H et al. Increases in p53 expression induce CTGF synthesis by mouse and human hepatocytes and result in liver fibrosis in mice. J Clin Invest 2011; 121: 3343-56.

[15] Wang Z, Xu J, Zheng Y, Chen W, Sun Y, Wu Z et al. Effect of the regulation of retinoid X receptor-α gene expression on rat hepatic fibrosis. Hepatol Res 2011; 41: 475-83.

[16] Castro RE, Ferreira DM, Afonso MB, Borralho PM, Machado MV, Cortez-Pinto H et al. miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in the rat liver and activated by disease severity in human non-alcoholic fatty liver disease. J Hepatol 2013; 58:119-25.

[17] Sucov HM, Dyson E, Gumeringer CL, Price J, Chien KR, Evans RM. RXRα mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. Genes Dev 1994; 8:1007-18.

[18] Kastner P, Grondona JM, Mark M, Gansmuller A, LeMeur M, Decimo D et al. Genetic analysis of RXRα developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. Cell 1994; 78: 987-1003.

[19] Duursma AM, Kedde M, Schrier M, le Sage C, Agami R. miR-148 targets human DNMT3b protein coding region. RNA 2008; 14: 872-77.

[20] Ott CE, Grünhagen J, Jäger M, Horbelt D, Schwill S, Kallenbach K et al. MicroRNAs differentially expressed in postnatal aortic development downregulate elastin via 3' UTR and coding-sequence binding sites. PLoS One 2011; 6: e16250.

[21] Wan YJ, An D, Cai Y, Repa JJ, Hung-Po Chen T, Flores M et al. Hepatocyte-specific mutation establishes retinoid X receptor α as a heterodimeric integrator of multiple physiological processes in the liver. Mol Cell Biol. 2000; 20: 4436-44.

[22] Konta T, Xu Q, Furusu A, Nakayama K, Kitamura M. Selective roles of retinoic acid

receptor and retinoid x receptor in the suppression of apoptosis by all-trans-retinoic acid. J Biol Chem 2001; 276:12697-701.

[23] Liu C, Tang DG. MicroRNA regulation of cancer stem cells. Cancer Res 2011; 71: 5950-54.

[24] Lorbek G, Lewinska M, Rozman D. Cytochrome P450s in the synthesis of cholesterol and bile acids – from mouse models to human diseases. FEBS J 2012; 279: 1516-33.

[25] Ji J, Zhang J, Huang G, Qian J, Wang X, Mei S. Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation. FEBS Lett 2009; 583: 759-66.

[26] Oda Y, Nakajima M, Mohri T, Takamiya M, Aoki Y, Fukami T et al. Aryl hydrocarbon receptor nuclear translocator in human liver is regulated by miR-24. Toxicol appl pharmacol 2012; 260: 222-31.

Figure legends

Figure 1. Luciferase assay using the plasmids containing MRE in the 3'-UTR and MRE in the coding region of human RXRα mRNA. (A) Schematic representation of the human RXRα mRNA and the predicted target sequences of miR-34a. The numbering refers to the 5' end of mRNA as 1, and the coding region is 163 to 1551. The MRE sequences are located from +714 to +735 in the coding region and from +3506 to +3539 in the 3'-UTR. The *bold letters* indicate the seed sequence. (B) Luciferase assays using the reporter plasmids containing various fragments downstream of the firefly luciferase gene. The reporter plasmids (190 ng) were transiently transfected with the pGL4.74-TK plasmid (10 ng) and 50 nM precursors for miR-34a or control into HEK293 cells. The firefly luciferase activity of each construct was normalized to the *Renilla* luciferase activities. The values are expressed as percentages of the relative luciferase activity of the pGL3p plasmid. Each column represents the mean ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$.

Figure 2. Effects of overexpression of miR-34a on RXRα protein and mRNA levels. HepG2 cells were transfected with 50 nM precursors for miR-34a or control. (A) After 72 h, the RXRα protein levels were determined by Western blot analysis and normalized to the β-actin protein levels. The values are expressed as percentages relative to no-transfection (NT). (B) After the indicated times, the RXRα mRNA levels were determined by real-time RT-PCR and normalized to the β-actin mRNA levels. The values are expressed as percentages relative to the NT at 6 h. (C) The cells were simultaneously treated with 10 μg/mL α-amanitin. After the indicated times, the RXRα mRNA levels were determined by real-time RT-PCR and normalized to the β-actin mRNA levels. The values are expressed as percentages relative to the value at 3 h. (D) After 48 h, the cells were harvested, and the nuclei were used for the Nuclear run-on assay. The *de novo* expression levels of RXRα mRNA were determined by real-time RT-PCR. The values are expressed as percentages relative to the NT before the run on assay. Data are the means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$,

and ***$P < 0.001$ compared with the precursor for control.

Figure 3. Effects of overexpression of miR-34a on induction of CYP26 and transactivity of CYP3A4 in HepG2 cells. (A) The precursor for miR-34a or control (50 nM) was transfected into HepG2 cells. After 72 h, the cells were exposed to 1 nM atRA, 2 nM 9-*cis*-RA, or 0.1% DMSO for 24 h, and the total RNA was then prepared. The CYP26 mRNA levels were determined by real-time RT-PCR and normalized to the β-actin mRNA levels. (B) The reporter plasmid pCYP3A4-362-7.7K (290 ng) was transiently transfected with the pRL-SV40 plasmid (10 ng) and the precursors for miR-34a or control into HepG2 cells. After 48 h, the cells were treated with 10 μM rifampicin for 24 h, and the luciferase activity was measured. The values are expressed as percentages relative to that of the precursor for control (-). Data are the means ± SD of three independent experiments. **$P < 0.01$ and ***$P < 0.001$, compared with vehicle treatment (-); $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ compared with the precursor for control.

Figure 4. Effects of induction of miR-34a through p53 activation on expression of endogenous RXRα protein in HepG2 and HuH-7 cells. (A) Schema showing previous findings and new insights on the progression of liver fibrosis through p53 activation. (B-E) HepG2 and HuH-7 cells were treated with 1 μM etoposide or 0.1% DMSO for 48 h, and the total RNA and total cell homogenates were isolated. (B and E) The p21 mRNA levels were determined by real-time RT-PCR and normalized to the β-actin mRNA levels. (C and F) The mature miR-34a levels were determined by real-time RT-PCR and normalized to the U6 snRNA levels. (D and G) The RXRα protein levels were determined by Western blot analysis and normalized to the β-actin protein levels. The values are expressed as percentages relative to that of HepG2 cells treated with DMSO. Data are the means ± SD of three independent experiments. *$P < 0.05$ and ***$P < 0.001$ compared with the DMSO treatment.

Figure 5. Expression levels of mature miR-34a and RXRα protein in fibrotic and normal

human livers and their relationship. (A) The mature miR-34a levels were determined by real-time RT-PCR and normalized to the U6 snRNA levels. The bars represent the means. (B) The RXRα protein levels were determined by Western blot analysis and normalized to the β-actin protein levels. The bars indicate the means of each group. (C) Relationship between the mature miR-34a and RXRα protein levels. The values are expressed as percentages relative to the lowest value. Each point represents the mean of two independent experiments.

Table 1. Sequence of primers used for real-time RT-PCR analysis.

Primer	Sequence
RXRα forward	5'-TGC GCA AGG ACC TGA CCT ACA C-3'
RXRα reverse	5'-GAC TCC ACC TCA TTC TCG TTC CG-3'
β-actin forward[a]	5'-TCA CCC TGA AGT ACC CCA TC-3'
β-actin reverse[a]	5'-GAT AGC ACA GCC TGG ATA GC-3'
CYP26 forward	5'-CCG CTG CTG CTC TTC CTG GCT GCG A-3'
CYP26 reverse	5'-GAC CGA CAC CAG CCG GTC GTC TCC GA-3'
p21 forward[b]	5'-CTG TCA CTG TCT TGT ACC CTT GTG C-3'
p21 reverse[b]	5'-GGA GAA GAT CAG CCG GCG TTT G-3'

[a]Oda et al. [26]; [b]Takagi et al. [9].

Table 2. Characteristics of 14 donors used in the present study.

	No	Age (yr)	Sex	Fibrosis	Diagnosis
Normal liver	1	82	F	None	Mild fatty degeneration around central vein Nuclear glycogen deposition Lung cancer without liver metastasis
	2	68	F	None	Death from cold
	3	72	F	None	Asphyxia
	4	72	M	None	Bile duct cancer
	5	83	M	None	Acute myocardial infarction
	6	64	F	None	Colorectal cancer with liver metastasis
Fibrotic liver	7	78	F	Mild	Mild hepatic congestion Multiple myeloma without liver metastasis
	8	49	F	Severe	Primary biliary cirrhosis
	9	70	F	Mild	Moderate hepatic congestion Mild fatty liver Acute myocardial infarction
	10	78	M	Severe	Hepatocellular carcinoma with cirrhosis (hepatitis C virus-positive)
	11	75	M	Severe	Hepatocellular carcinoma with cirrhosis
	12	55	M	Mild	Mild alcoholic liver fibrosis
	13	62	M	Severe	Alcoholic cirrhosis
	14	61	F	Severe	Primary biliary cirrhosis

Figure 1

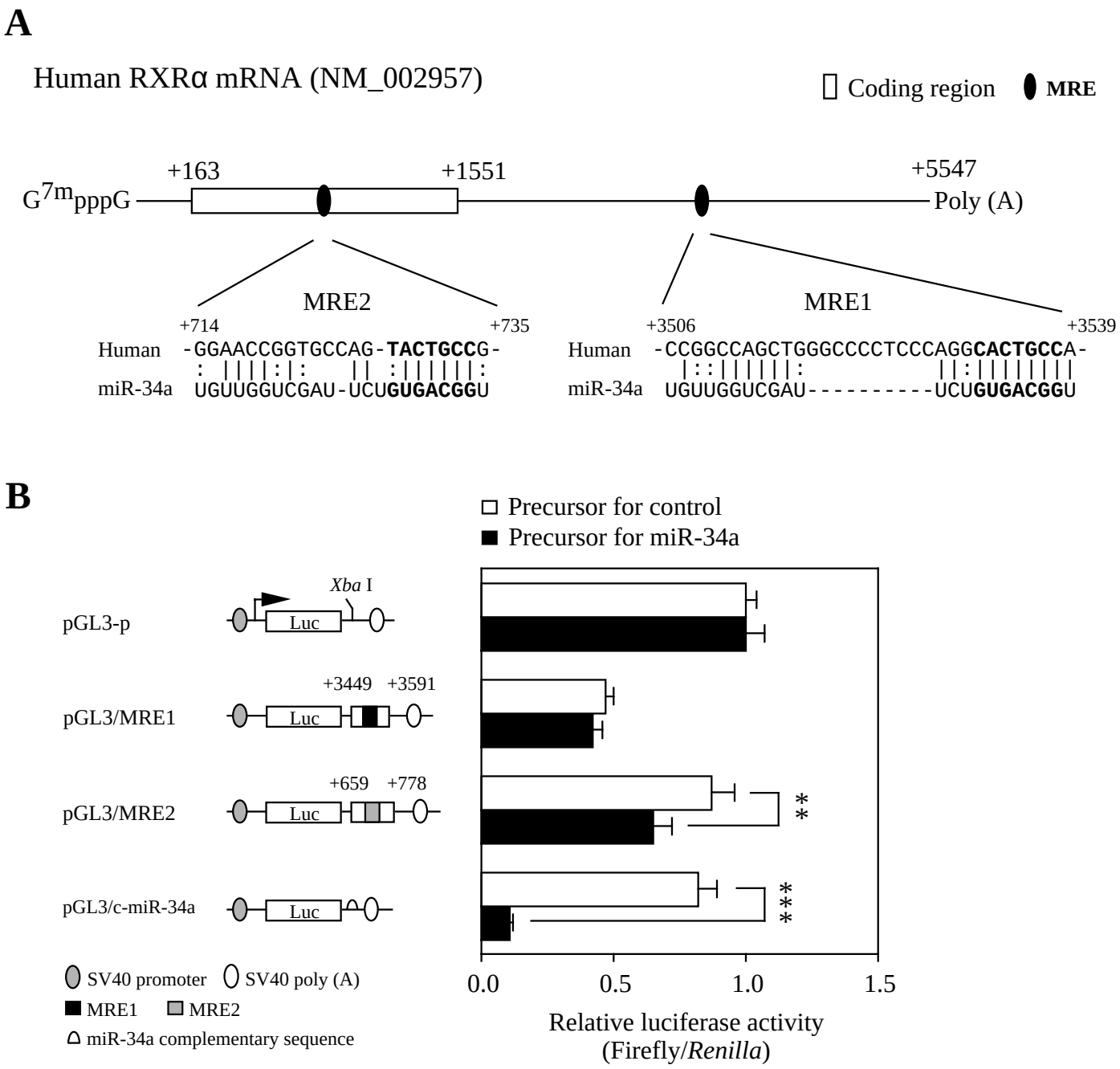

A
Human RXRα mRNA (NM_002957)
Coding region
MRE
+163
+1551
+5547
G7mpppG
Poly (A)
MRE2
MRE1
+714
+735
+3506
+3539
Human -GGAACCGGTGCCAG-TACTGCCG-
miR-34a UGUUGGUCGAU-UCUGUGACGGU
Human -CCGGCCAGCTGGGCCCCTCCCAGGCACTGCCA-
miR-34a UGUUGGUCGAU----------UCUGUGACGGU
B
Precursor for control
Precursor for miR-34a
Xba I
pGL3-p
Luc
+3449
+3591
pGL3/MRE1
+659
+778
pGL3/MRE2
pGL3/c-miR-34a
SV40 promoter
SV40 poly (A)
MRE1
MRE2
miR-34a complementary sequence
0.0
0.5
1.0
1.5
Relative luciferase activity
(Firefly/Renilla)

Figure 2

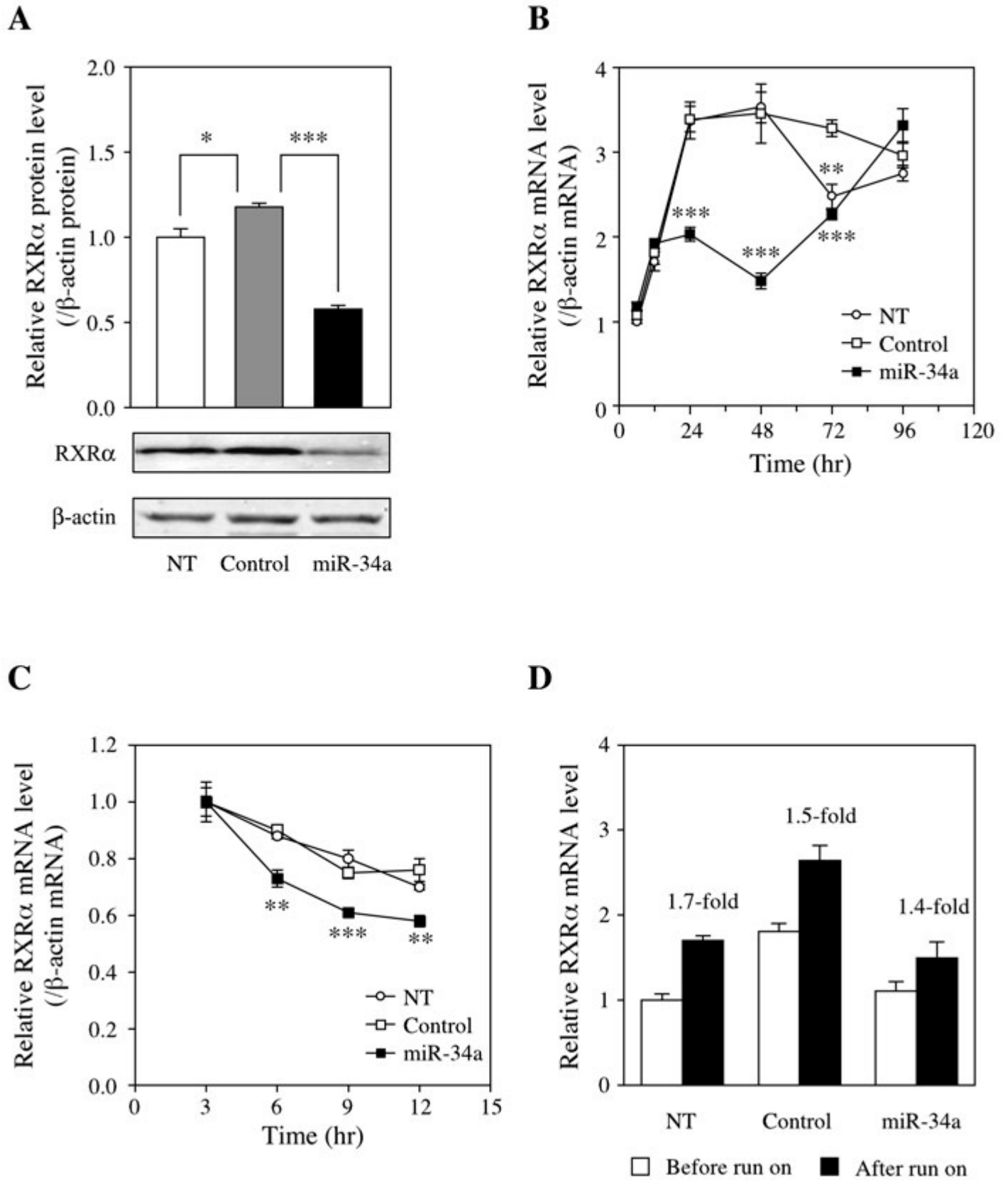

A
Relative RXRα protein level (/β-actin protein)
2.0
1.5
1.0
0.5
0.0
*
***
RXRα
β-actin
NT
Control
miR-34a
B
Relative RXRα mRNA level (/β-actin mRNA)
4
3
2
1
0
**
***
***
***
NT
Control
miR-34a
0
24
48
72
96
120
Time (hr)
C
Relative RXRα mRNA level (/β-actin mRNA)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
**
NT
Control
miR-34a
0
3
6
9
12
15
Time (hr)
D
Relative RXRα mRNA level
4
3
2
1
0
1.7-fold
1.5-fold
1.4-fold
NT
Control
miR-34a
Before run on
After run on

Figure 3

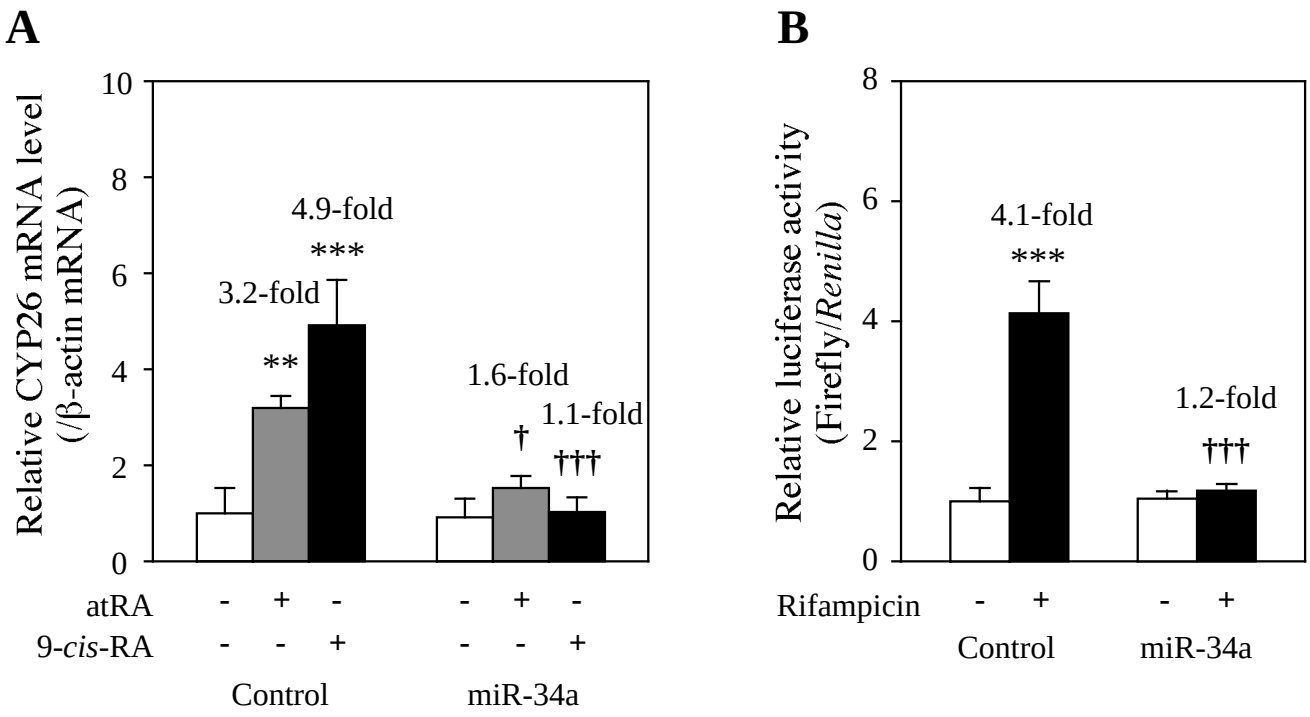

A
Relative CYP26 mRNA level
(/β-actin mRNA)
10
8
6
4
2
0
4.9-fold
***
3.2-fold
**
1.6-fold
†
1.1-fold
†††
atRA - + - - + -
9-cis-RA - - + - - +
Control
miR-34a
B
Relative luciferase activity
(Firefly/Renilla)
8
6
4
2
0
4.1-fold
***
1.2-fold
†††
Rifampicin - + - +
Control
miR-34a

Figure 4

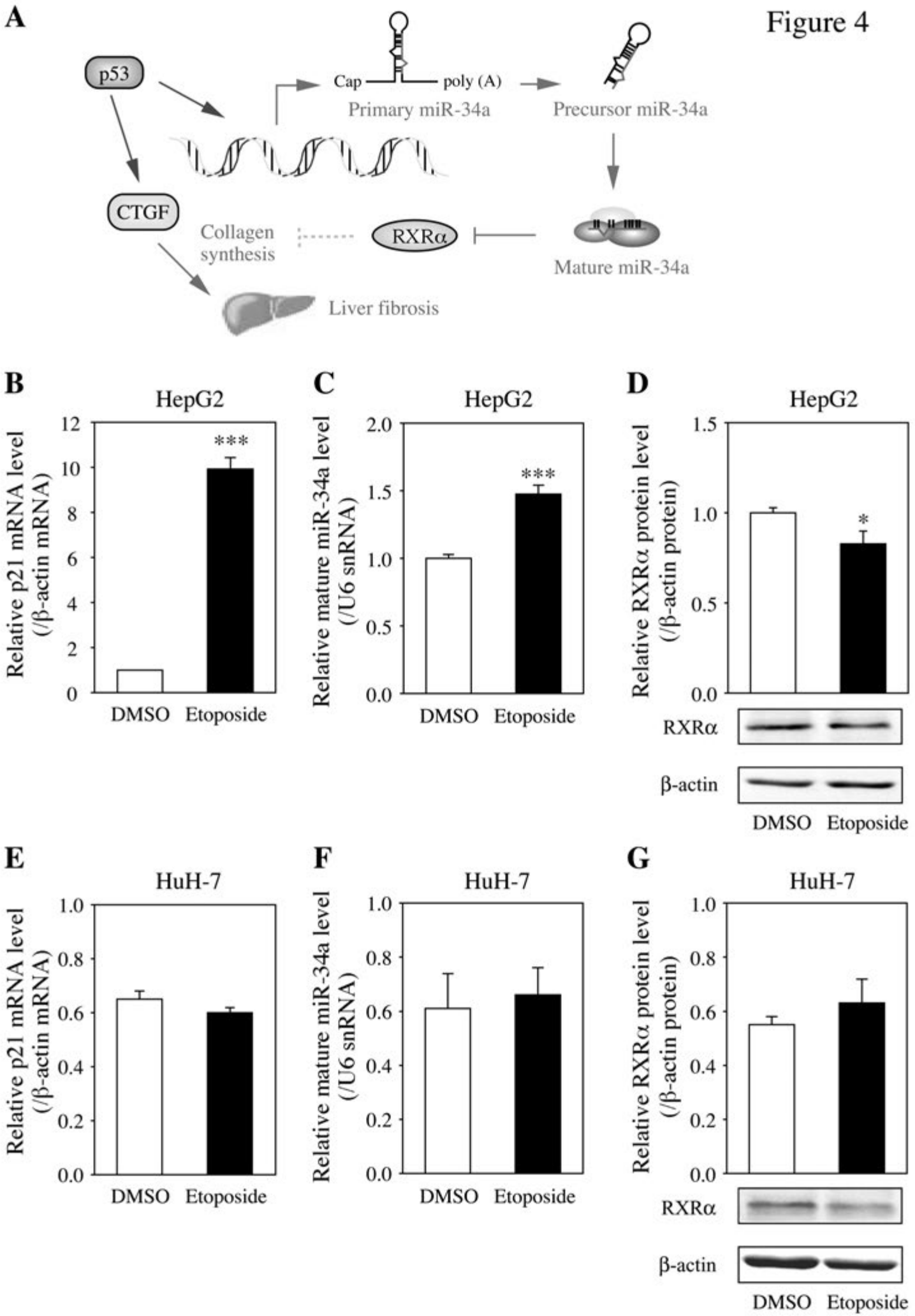
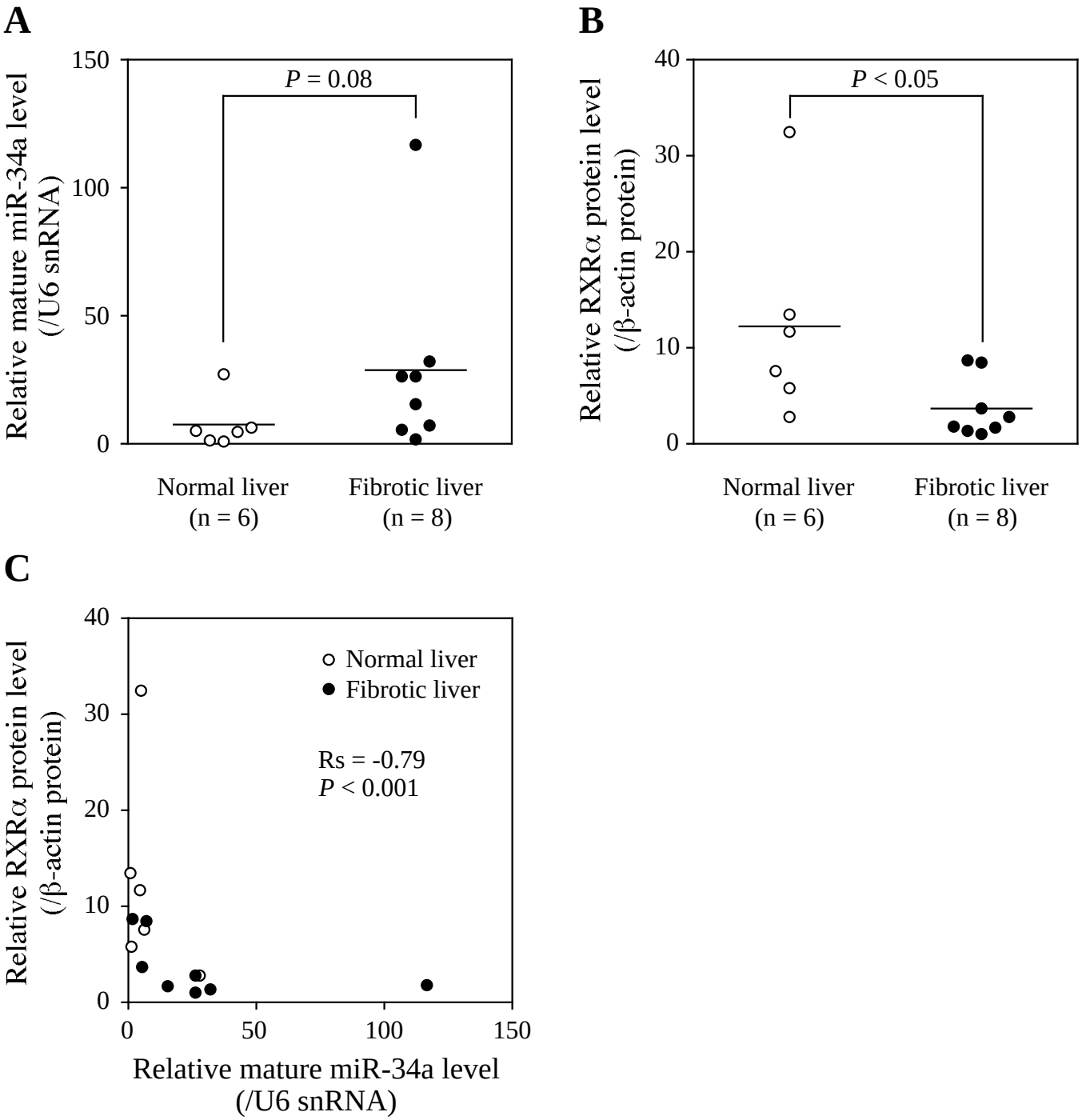

A
Figure 4
p53
Cap
poly (A)
Primary miR-34a
Precursor miR-34a
CTGF
Collagen synthesis
RXRα
Mature miR-34a
Liver fibrosis
B
HepG2
Relative p21 mRNA level (/β-actin mRNA)
***
DMSO
Etoposide
C
HepG2
Relative mature miR-34a level (/U6 snRNA)
***
DMSO
Etoposide
D
HepG2
Relative RXRα protein level (/β-actin protein)
*
RXRα
β-actin
DMSO
Etoposide
E
HuH-7
Relative p21 mRNA level (/β-actin mRNA)
DMSO
Etoposide
F
HuH-7
Relative mature miR-34a level (/U6 snRNA)
DMSO
Etoposide
G
HuH-7
Relative RXRα protein level (/β-actin protein)
RXRα
β-actin
DMSO
Etoposide

Figure 5


A
Relative mature miR-34a level
(/U6 snRNA)
150
100
50
0
P = 0.08
Normal liver
(n = 6)
Fibrotic liver
(n = 8)
B
Relative RXRα protein level
(/β-actin protein)
40
30
20
10
0
P < 0.05
Normal liver
(n = 6)
Fibrotic liver
(n = 8)
C
Relative RXRα protein level
(/β-actin protein)
40
30
20
10
0
Normal liver
Fibrotic liver
Rs = -0.79
P < 0.001
0
50
100
150
Relative mature miR-34a level
(/U6 snRNA)