microRNA-34a and microRNA-34c promote the activation of human hepatic stellate cells by targeting peroxisome proliferator-activated receptor γ

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Abstract. Liver fibrosis is the common outcome of almost all cases of chronic liver disease. The hallmark of liver fibrosis is the activation of hepatic stellate cells (HSCs). microRNA-34a (miR-34a), which regulates a plethora of target proteins involved in the cell cycle, apoptosis, differentiation and cellular development, is found to be upregulated in both activated HSCs and liver fibrosis, while it is consistently downregulated in numerous cancer types. In the present study, the possible mechanisms underlying the role of miR-34a and miR-34c in the activation of the HSCs was investigated. Through bioinformatics analysis and a luciferase reporter assay, five genes were identified to be the target genes of miR-34a and miR-34c. Of these, peroxisome proliferator-activated receptor γ (PPARγ) was selected for further investigation. Mutation luciferase reporter assay confirmed the direct interaction of PPARγ and miR-34a and miR-34c. Western blot analysis and quantitative polymerase chain reaction demonstrated that the expression of PPARγ was negatively correlated with the expression of miR-34a and miR-34c during the activation of HSCs. In activated human HSCs, inhibitors of miR-34a and miR-34c upregulated the expression of PPARγ and downregulated the expression of α-smooth muscle actin. These data suggested that the miR-34 family may be involved the process of liver fibrosis by targeting PPARγ.

Introduction

Liver fibrosis is the essential pathophysiological consequence of chronic liver injury and is characterized by the excessive accumulation of extracellular matrix (ECM) proteins, particularly collagens (1). Liver fibrosis has traditionally been regarded as an irreversible process. However, mounting clinical evidence has indicated that even advanced fibrosis is reversible (2). The activation of hepatic stellate cells (HSCs), the primary source of the ECM, that is characterized by the expression of α-smooth muscle actin (α-SMA), is the pivotal process in liver fibrosis (1). Therefore, the inhibition of the accumulation of activated HSCs by modulating either their activation and/or proliferation or promoting their apoptosis is one strategy to regress liver fibrosis (1).

microRNAs (miRNAs) are a group of small, evolutionarily conserved, non-coding, naturally occurring RNA molecules, which post-transcriptionally modulate gene expression and determine cell fate by regulating multiple gene products and cellular pathways (3,4). Deregulation of miRNAs has been consistently associated with a number of different human malignancies, including diseases of the liver (5,6). Several miRNAs have been identified that may be involved in the process of liver fibrosis and hepatocellular carcinoma (7,8).

The miR-34 family was first identified as direct transcriptional targets of p53, and is composed of miR-34a, miR34b and miR34c (9). The miR-34 family members target numerous genes, including cyclin-dependent kinases 1 and 4, B-cell lymphoma 2, cAMP-response element binding protein (CREB), forkhead box protein P1 (Foxp1), to modulate the cell cycle, differentiation, proliferation and apoptosis (10). Lower miR-34 expression in cancer has been reported by several groups, suggesting its possible involvement in oncogenesis as a tumor suppressor (4,11-13). However, elevated miR-34 expression has been reported in activated HSCs, in rats with induced hepatic fibrosis or liver tumors and in patients with liver diseases (14-18). It has been reported that miR-34 family members may be involved in the process of liver fibrosis by targeting acyl-CoA synthetase long-chain family member 1 (ACSL1) (15). However, whether other cellular factors or proteins are involved remains elusive.

Peroxisome proliferator-activated receptor γ (PPARγ) belongs to a superfamily of nuclear receptors controlling the transcriptions of numerous different genes. PPARγ is an important anti-fibrotic factor and is involved in the maintenance of HSCs in a quiescent phenotype (19). Several studies have reported that PPARγ antagonists or activators impeded the HSCs activation during live fibrosis (20-22). PPARγ is now considered to be a promising therapeutic target for antifibrotic chemotherapy (23).

The present study, utilizing bioinformatics and a reporter assay, investigated whether the PPARγ gene was a target

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protein of miR-34a/c. Expression of PPARγ in activated or miR-34a/c-silenced HSCs was assessed in human and rat cell models *in vitro*, in order to elucidate whether the miR-34 family promoted liver fibrosis by targeting PPARγ.

Materials and methods

Cells and reagents. The immortalized human HSC line LX-2 (Institute of Cell and Molecule Biology, Central South University, Changsha, China), a generous gift from Dr. Tan (24), was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 100 μg/ml streptomycin (Amresco, Solon, OH, USA) and 100 U/ml penicillin (Amresco) and incubated at 37˚C with 5% CO_2. Prior to the start of the experiments, cells were tested by PCR for mycoplasma, using commercially available primers, and were shown to be mycoplasma-negative. The cells were activated by administration of transforming growth factor β1 (TGF-β1; Sigma-Aldrich, St. Louis, MO, USA). Human Embryonic Kidney (HEK; Cell Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) 293 cells were maintained in DMEM supplemented with 10% FBS and antibiotics at 37˚C in 5% CO_2. The mimics and inhibitors of miR34a and miR-34c, and the respective RNA controls, were obtained from Shanghai JIMA Pharmacy Technology Co., Ltd. (Shanghai, China).

Bioinformatics approaches. To search for miRNA-34 family target genes, the online miRNA database miRanda (http://www.microrna.org), Targetscan (http://www. targetscan.org) and Pictar (http://pictar.bio.nyu.edu) were used.

Isolation and identification of rat HSCs. Primary HSCs were isolated from normal male Sprague Dawley rats aged between five and six months (weighing 400-500 g; Shanghai Laboratory Animal Center of Chinese Academy of Sciences, Shanghai, China) by *in situ* perfusion and density-gradient centrifugation, as previously described (25). The rats were maintained at 25˚C on a 12/12 h light/dark cycle, with *ad libitum* access to rodent chow and water. The rats received humane care according to the Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Sciences. The study was approved by the ethics committee of Yiwu Central Hospital. All institutional and national guidelines for the care and use of laboratory animals were followed. Isolated HSCs were suspended in DMEM supplemented with 10% FBS, and penicillin and streptomycin at a cell density of $5x10^5$ cells/ml, seeded in culture flasks and cultured at 37˚C in 5% CO_2.

Indirect immunofluorescence assay (IFA). The cells seeded on glass coverslips or 96-well plates were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min at room temperature (RT). After being washed three times with PBS, the cells were incubated in blocking buffer [PBS containing 3% bovine serum albumin (BSA), 0.3% Triton™ X-100 and 10% FBS] for at least 30 min and then in binding buffer (PBS containing 3% BSA and 0.3% Triton X-100) with monoclonal antibodies (mAbs) against α-SMA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:100 for 1 h at RT. Following three washes with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Ig)G (Thermo Fisher Scientific, San Jose, CA, USA) at a 1:100 dilution with binding buffer for 1 h at RT. The cell nuclei were stained with DAPI (Sigma-Aldrich). The stained samples were then examined with a Leica TCS SPII confocal microscope (Leica Microsystems, Wetzlar, Germany).

Total RNA extraction and quantitative polymerase chain reaction (qPCR) analysis. For general PCR, total RNA was extracted with TRIzol (Invitrogen Life Technologies) from HEK 293 cells and cDNA was synthesized using moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI, USA). For miRNA detection, total RNA was prepared by using the mirVana™ miRNA Isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using the Taqman miRNA RT kit (Applied Biosystems, Foster City, CA, USA). For the detection of the miRNA levels by qPCR, TaqMan® microRNA assay (Applied Biosystems) was used to quantify the relative expression levels of miR-34a (assay ID: 000426), miR-34c (assay ID: 000428), and U6 (assay ID: 001973) as an internal control, in an Applied Biosystems 7500 Detection system (Applied Biosystems). The relative amount of miRNAs was normalized against U6 small nuclear RNA, and the fold change for each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (26). The relative miRNA expression was calculated from three different experiments.

Vector construction. The 3'-untranslated region (UTR) fragments of the indicated target mRNAs containing putative miR-34a and miR-34c binding sites were amplified by PCR from the cDNA of HEK293 cells. The amplified fragments were cloned into the *Xba*I sites of pGL3-promoter vector (Promega Corp.) downstream of the luciferase coding region to generate reporter vector pGL-UTRs. The vector pGL-PPARγ-mut with mutated binding sites was mutated with the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The oligonucleotides used for cloning are demonstrated in Table I. All of the primers were synthesized by Sangong Biotech Co., Ltd. (Shanghai, China). The constructed clones were confirmed by sequencing (Sangong Biotech Co., Ltd.).

Luciferase reporter assays. The HEK293 cells were plated in 24-well plates the day prior to transfection. The cells were co-transfected with internal control pRL-TK (Promega Corp.), reporter vectors and mimics of miR-34a/c or negative control probes (Shanghai JIMA Pharmacy Technology Co., Ltd.) using Lipofectamine® 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. A total of 48 h later, the cells were harvested and applied to the luciferase measurement with a Dual-Luciferase Reporter Assay System (Promega Corp.) using a GloMax® 20/20 detection system (Promega Corp.). Values are represented as the number of relative light units (RLU).

Transient transfection. rHSCs or LX-2 cells were seeded into six- or 96-well plates the day prior to transfection. The cells

Table I. Primers used in this study.

Primer	Sequence (5'-3')
LEF1-F	GCAGGTCTAGAGAAACATGGTGGAA
LEF1-R	GCAGGTCTAGACTGGGGTGCTGATG
Wnt2B-F	GCAGGTCTAGATGGGAAGGAGTTGTC
Wnt2B-R	GCAGGTCTAGAGGAGTGTTCTAGGG
PPARγ-F	GCAGGTCTAGAGGACTTGTACTAGCAG
PPARγ-R	GCAGGTCTAGAGGTGTCAGATTTTCCC
PPARγ-mut1	GAGTCCTGAGCCTGTCGCAACATTTCC
PPARγ-mut2	GGAAATGTTGCGACAGGCTCAGGACTC
AREG-F	GCAGGTCTAGAAACAGAAAGAAGAA
AREG-R	GCAGGTCTAGAAATAGCATAAAAGTG
LDHA-F	GCAGGTCTAGACCTTGCATTTTGGGA
LDHA-R	GCAGGTCTAGAGGAAGAATTATGCAC
ACSL1-F	GCAGGTCTAGATTTCAGGTCGCAGATAG
ACSL1-R	GCAGGTCTAGACTGGTCCGCTTGTTG
FABP3-F	GCAGGTCTAGACACCACATTGCCTCATT
FABP3-R	GCAGGTCTAGACAAGCCTGGGTTCTGT
XBP1-F	GCAGGTCTAGAGGGCGCCTGCGTCGG
XBP1-R	GCAGGTCTAGACGGGGTGTTCTGGCC

LEF, lymphoid enhancer factor; AREG, amphiregulin; LDHA, lactate dehydrogenase; FABP, fatty acid binding protein; XBP, X box binding protein; PPARγ, proliferator-activated receptor γ.

were transfected with inhibitors of miR-34a/c or negative control miRNAs (Shanghai JIMA Pharmacy Technology Co., Ltd.) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen Life Technologies). Following 6 h of culture with the transfection mix, the cells were cultured in normal culture medium (rHSCs) or medium containing 5 ng/ml TGF-β1 (LX-2 cells). A total of 48 h later, the cells were harvested and subjected to western blot analysis.

Western blot analysis. The cells were harvested in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)] were first quantified by the bicinchoninic acid method (Pierce Biotechnology, Inc., Rockford, IL, USA) and then denatured by boiling for 5 min. A total of 30 μg of protein per sample were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with tris-buffered saline (TBS; 20 mM Tris-HCl (pH 7.4), 150 mM NaCl) containing 5% milk for 1 h at RT. Next, the membranes were incubated with monoclonal mouse antibodies against α-SMA (1:100 dilution; sc-53142), against PPARγ (1:100 dilution; sc-7273) or against GAPDH (1:100 dilution; sc-365062) (all Santa Cruz Biotechnology, Inc.) for 1 h at 37˚C, followed by three time washes with TBS-T (TBS containing 0.1% (v/v) Tween-20) buffer. Next, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG at a dilution of 1:10,000 in TBS for 1 h at RT, followed by three washes with TBS-T. The proteins were then detected using the Supersignal® West Pico chemiluminescent substrate (Pierce Biotechnology, Inc.) on an AlphaEase® FC Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis. Values are presented as the mean ± standard deviation. The software GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analysis and graphical illustrations. The statistical significance was analyzed using Student t-test. $P<0.01$ was considered to indicate a statistically significant difference.

Results

PPARγ is a target protein of miR-34a and miR-34c. To identify the potential target mRNAs of miR-34a and miR-34c, which contain the same 'seed region', three different prediction tools (miRanda, Targetscan and Pictar) were used. Of all the predicted genes, eight genes were selected for confirmation. The criteria for target selection was not only concerning the binding possibility, but also concerning the potential functions in liver fibrosis. Therefore, the selected genes encoded proteins either correlated with certain important signaling pathways [lymphoid enhancer factor 1 (LEF1), Wnt-2B, PPARγ, amphiregulin (AREG)] (23,27-29) or associated with lipid metabolism [lactate dehydrogenase (LDHA), ACSL-1, fatty acid binding protein 3 (FABP3), X box binding protein 1 (XBP1)] (30,31).

HEK293 cells were co-transfected with reporter vectors pGL-UTRs bearing the predicted binding sites and mimics of miR-34a or miR-34c together with the internal control pRL-TK. The increased expression of miR-34a or miR-34c by mimics transfection was confirmed by qPCR at 48 h following transfection (Fig. 1A), and a concentration of 20 nM mimics was selected for the following experiments. The results demonstrated that the overexpression of miR-34a/34c significantly decreased the relative luciferase activity in HEK293 cells transfected with UTRs of LEF1, PPARγ, AREG, LDHA, ACSL-1 and XBP1. LEF1, LDHA and ACSL-1 have been reported to be targeted by miR-34a/34c previously (15,32). Since PPARγ was reported to be involved in the maintenance of a quiescent HSCs phenotype and the activation of HSCs resulted in the loss of PPARγ (19,33), PPARγ was selected for further confirmation. The reporter vector bearing the mutated UTR of PPARγ was constructed (Fig. 1C). It was identified that the overexpression of miR-34a/34c significantly decreased the relative luciferase activity in HEK293 cells transfected with pGL-PPARγ (Fig. 1D), while it caused no apparent relative luciferase activity changes in HEK293 cells transfected with pGL-PPARγmut (Fig. 1E). These results indicated that PPARγ was a direct target gene of miR-34a and miR-34c.

Expression of miR-34a/c and PPARγ during the activation of HSCs. The upregulation of miR-34 family members and downregulation of PPARγ have been previously reported during liver fibrosis (15,23). Since the activation of HSCs is the pivotal process in liver fibrosis, the expression of miR-34a/c and PPARγ was detected during the activation of HSCs. Rat HSCs and human HSCs (LX-2) were used in the studies.

Firstly, primary rat HSCs were isolated, and ~$2x10^8$ HSCs were harvested from each rat. The fraction of freshly isolated

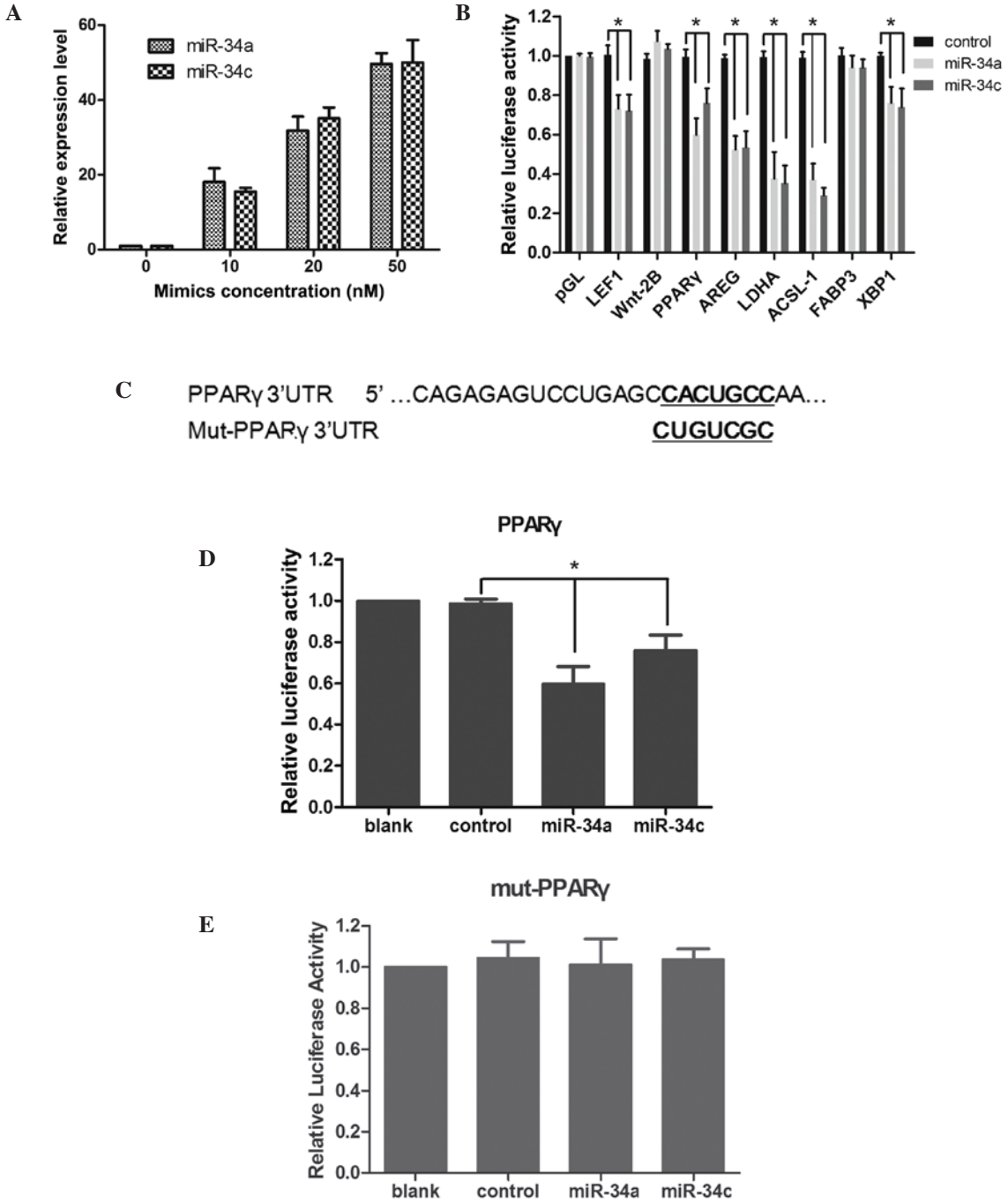

Figure 1. PPARγ is a direct target protein of miR-34a and miR-34c. Mimics of miR-34a and miR-34c were introduced into HEK-293 cells and upregulation of miR-34a and miR-34c was confirmed by quantitative polymerase chain reaction. (A) A concentration of 20 nM mimics was selected for the following assays. (B) Luciferase reporter assay was used to confirm the direct interaction between miR-34a/miR-34c and target mRNAs. HEK293 cells were co-transfected with mimics, pRL-TK together with reporter vector pGL-UTRs, and the relative luciferase activity was detected. (C) The predicted miR-34a/miR-34c binding site on the PPARγ mRNA 3'-UTR and mutated binding site are demonstrated as bold and underlined. (D and E) Mutation luciferase reporter assay was used to further confirm the direct interaction between miR-34a/miR-34c and PPARγ. HEK293 cells were co-transfected with mimics, pRL-TK together with (D) wild reporter vector pGL-PPARγ (E) and mutant reporter vector pGL-PPARγ-mut. Relative luciferase activity was detected. Data are represented as the mean ± standard deviation from triplicate independent experiments. *P<0.01, for comparison between the miR-34a/c transfected cells and the control miRNA transfected cells. UTR, untranslated region; Mut, mutation; LEF, lymphoid enhancer factor; AREG, amphiregulin; LDHA, lactate dehydrogenase; FABP, fatty acid binding protein; XBP, X box binding protein PPARγ, proliferator-activated receptor γ; HEK293, human embryonic kidney 293; miRNA, microRNA.

living HSCs was ≤90%, as defined by trypan blue staining. The morphology and growth characteristics of the freshly isolated cells were observed with an inverted phase contrast microscope. The cells were small and round with the quiescent phenotype when freshly isolated; however, they demonstrated a weak adhesive growth pattern following culture for two days and presented a wall-adhesive growth pattern following culture for ten days (Fig. 2Aa-c). IFA and western blot analysis of α-SMA were applied for demonstrating the activated phenotype of HSCs. The IFA results demonstrated that the cells expressed no or little α-SMA on day 2 (Fig. 2Ad) and expressed abundant α-SMA on day 10 (Fig. 2Ad). Western blot analysis also demonstrated that

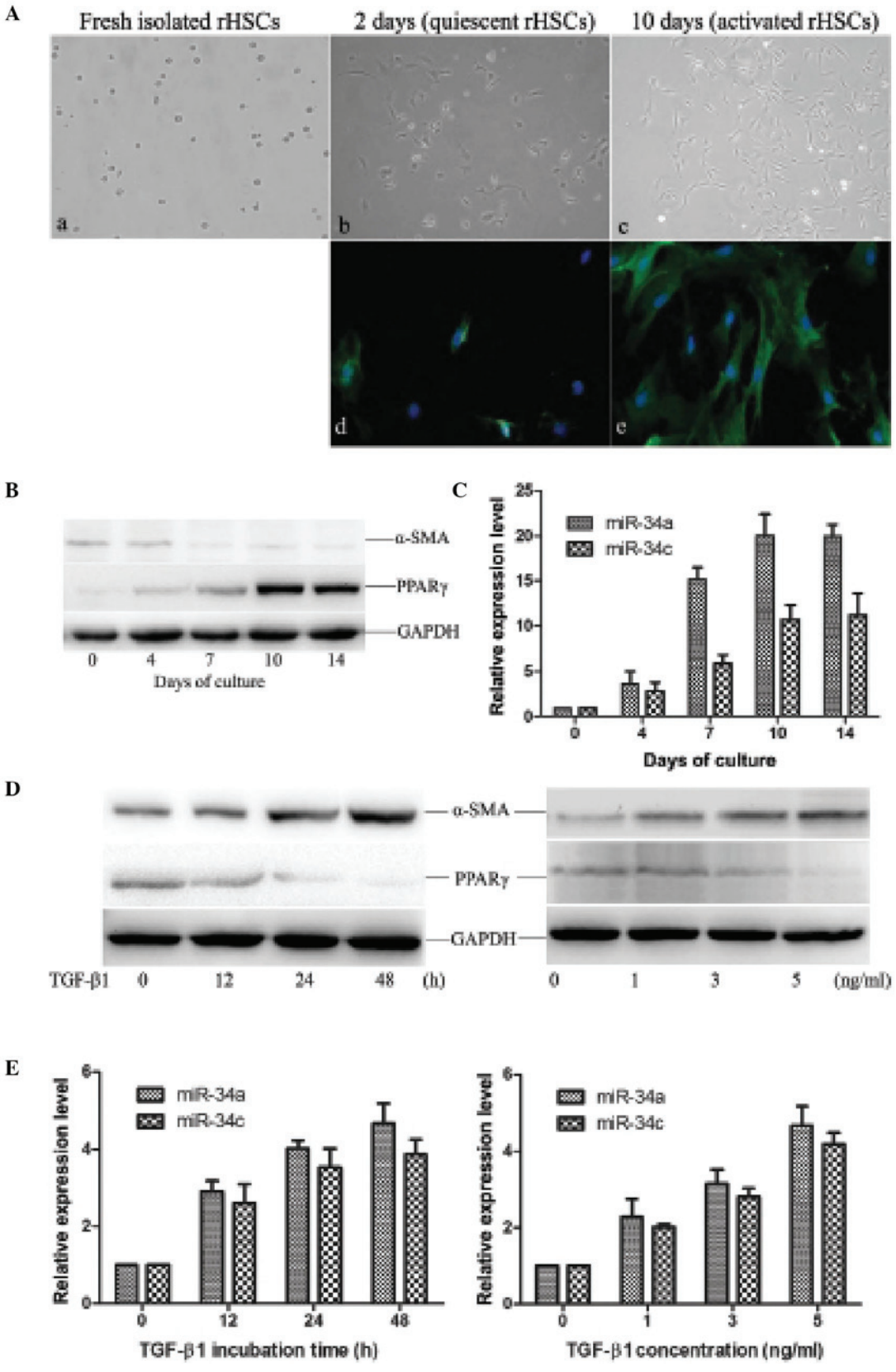

Figure 2. miR-34a and miR-34c are upregulated and PPARγ is downregulated during the activation of HSCs. (A) Characterization of HSCs isolated from rat liver; (a) cell morphology of isolated cultured HSCs; (b and c) following isolation, the cells were cultured for (b) two days (quiescent HSCs) or for (c) 10 days (activated HSCs); (d and e) images from indirect immunofluoresence assay against α-SMA (green) in (d) quiescent HSCs and (e) activated HSCs are demonstrated. Magnification, x100 for a-c, x400 for d-e. (B and D) Expression of α-SMA and PPARγ in (B) rat HSCs and in (D) TGF-β1-activated LX-2 cells. Cells at indicated time-points were collected and subjected to western blot analysis. GAPDH indicates the amount of protein loaded in each well. (C and E) Expression of miR-34a and miR-34c in (C) rat HSCs and (E) TGF-β1-activated LX-2 cells. Cells at indicated time-points were collected and subjected to quantitative polymerase chain reaction. Triplicate assays were performed for each RNA sample and the relative amount of each miRNA was normalized to U6 small nuclear RNA. Data are represented as the mean ± standard deviation. PPARγ, proliferator-activated receptor γ; HSCs, hepatic stellate cells; TGF-β1, transforming growth factor β1; α-SMA; α-smooth muscle actin; HEK293, human embryonic kidney 293; miRNA, microRNA.

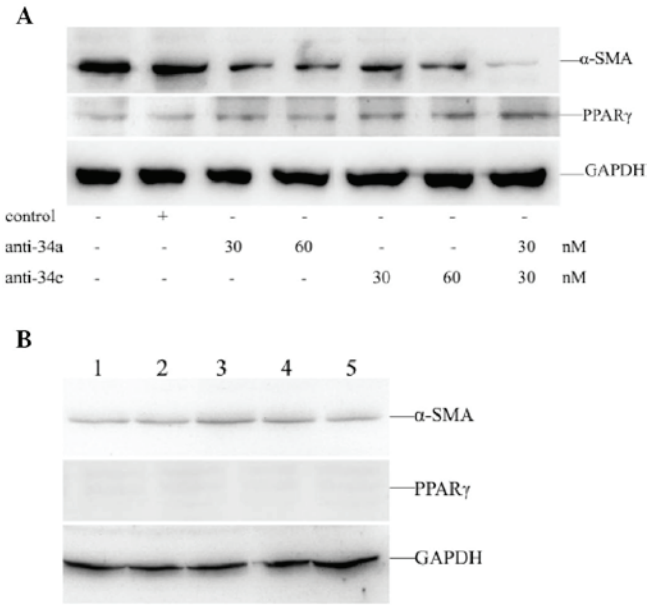

Figure 3. miR-34a and miR-34c inhibitors upregulate PPARγ expression and downregulate α-SMA expression. Activated HSCs were transfected with inhibitors of miR-34a and miR-34c and the expression of α-SMA and PPARγ was detected by western blot analysis. (A) TGF-β1-activated LX-2 cells and (B) rat HSCs. Lane 1, blank; lane 2, negative control; lane 3, 100 nM siR-34a inhibitor; lane 4, 100 nM siR-34c inhibitor; lane 5, 50 nM siR-34a inhibitor combined with 50 nM siR-34c inhibitor. GAPDH indicates the amount of protein loaded in each well. PPARγ, proliferator-activated receptor γ; HSCs, hepatic stellate cells; TGF-β1, transforming growth factor β1; α-SMA; α-smooth muscle actin; miRNA, microRNA.

the expression of α-SMA was increased progressively with the time in culture, and reached the highest expression at day 10 (Fig. 2B). These results indicated that following culture for ten days, HSCs had been highly activated.

Next, the expression of miR-34a and miR-34c in the rat HSCs was detected by qPCR. As demonstrated in Fig. 2C, the expression of miR-34a and miR-34c in rat HSCs revealed a significant ~15-fold and 5-fold increase, respectively, following culture for seven days, and peaked with the highest expression at day 10, with an increase of ~20-fold and 10-fold, respectively. The expression of miR-34a was always marginally higher than that of miR-34c.

Next, the expression of PPARγ was detected by western blot analysis. As demonstrated in Fig. 2B, PPARγ decreased progressively with the time in culture and demonstrated a negative correlation with the expression of miR-34a/c and α-SMA.

The expression of miR-34a/c and PPARγ was also analyzed during the activation of human HSC LX-2 cells. TGF-β1 is one of the critical factors for the activation of HSC during chronic inflammation (34). LX-2 cells were treated with TGF-β1 for the indicated time (from 0 to 48 h) in 5 ng/ml or in the indicated concentration (from 0 to 5 ng/ml) for 48 h. The upregulated expression of α-SMA in both conditions indicated the activation of LX-2 by TGF-β1 stimulation (Fig. 2D). In addition, the expression of PPARγ, miR-34a and miR-34c demonstrated changes that were consistent with the results in the rat HSCs during the activation of LX-2 (Fig. 2D and E). In conclusion, during activation of HSCs, miR-34a and miR-34c were upregulated and PPARγ was downregulated, and the expression of PPARγ was negatively correlated with the expression of miR-34a and miR-34c and the degree of HSC activation.

Inhibitors of miR-34a and miR-34c upregulate the expression of PPARγ and downregulate the expression of α-SMA in human HSCs. To further investigate the association between the miR-34 family and PPARγ and their effect on the activation of HSCs, activated HSCs were transfected with miR-34a and miR-34c inhibitors alone or together, and the expression of PPARγ and α-SMA was detected. As demonstrated in Fig. 3A, PPARγ was upregulated following transfection with miR34a and miR34c alone or together, compared with the negative control miRNA in TGF-β1-stimulated LX-2 cells, while α-SMA was downregulated in HSCs transfected with miR-34a/c compared with negative control microRNA, which suggested that activation of HSCs was reversed. Furthermore, application of the miR34a inhibitor resulted in higher expressional regulation than the miR34c inhibitor did, and the two inhibitors in combination induced the highest expressional regulation. However, in the activated rat HSCs, no expression change was observed (Fig. 3B). These results indicated that the miR-34a family accelerated the activation of human HSCs by modulating PPARγ, and the decreased expression of PPARγ may have resulted from differential regulatory mechanisms in rat and human HSCs.

Discussion

The miR-34 family is transcriptionally controlled by p53 tumor suppressor protein and regulates a plethora of target proteins, which are involved in cell cycle, apoptosis, differentiation and cellular development (10). The downregulation of miR-34a has been previously identified in numerous cancer types (12,13,35), and therefore, miR-34a is considered to be a tumor suppressor. However, upregulation of miR-34 has been found in numerous liver diseases from fatty liver disease to hepatocellular carcinoma (36-38). It was reported that miR-34 family members may target ACSL1, which has a central role in lipid metabolism and fatty acid metabolism in the liver, and impairs the lipid metabolism in the liver, resulting in the development of hepatic fibrosis (15). Whether other factors are involved in this process has yet to be elucidated.

In the present study, using a bioinformatics approach, eight proteins were selected for experimental confirmation. Among them, LEF1, Wnt-2B, PPARγ and AREG have been reported to be correlated with several signaling pathways, including Wnt and PPAR (23,27-29), which may have an important role during liver fibrosis, while LDHA, ACSL-1, FABP3 and XBP1 were reported to be associated with lipid metabolism (30,31). ACSL-1 is also used as a positive control since it has been reported to be the target of miR-34a and miR-34c (15). The reporter assay indicated that LEF1, PPARγ, AREG, LDHA, ACSL-1 and XBP1 are possible target genes of miR-34a and miR-34c. Among these, PPARγ has been reported to be potently involved in liver fibrosis (23). Therefore, PPARγ was selected for further analysis in the present study. The mutation reporter assay confirmed that PPARγ is the direct target of miR-34a and miR-34c.

PPARγ is a ligand-activated nuclear transcription factor that belongs to the nuclear hormone receptor superfamily. PPARγ has a key role in HSC biology and is involved in the maintenance of a quiescent HSC phenotype (19). PPARγ receptors were found to have anti-proliferative and anti-fibrotic effects on activated HSCs, as well as to induce HSC apoptosis through a mechanism involving an extrinsic apoptosis pathway (20,23).

It has been reported previously that miR-34 family members are upregulated and PPARγ is downregulated during liver fibrosis (15,23). Considering the hallmark of liver fibrosis is the activation of HSCs, the present study next detected the expression of miR-34 family members and PPARγ during the activation of HSCs. The results demonstrated that miR-34 family member expression was increased, while PPARγ expression was reduced during the activation of HSCs both in rats and humans, which is consistent with previous studies (15,23). Furthermore, the expression of PPARγ was negatively correlated with the expression of miR-34 family members and the degree of activation.

The present study further examined the association of miR-34 and PPARγ in activated HSCs by using miR-34 inhibitors. The results from the human HSCs demonstrated that PPARγ was upregulated and α-SMA was downregulated when the cells were transfected with miR-34 and miR-34c inhibitors alone or in combination, indicating that miR-34a and miR-34c inhibitors may decrease the activation of HSCs by upregulating the expression of PPARγ. The miR-34 family may exhibit profibrotic effects by targeting PPARγ. However, no expression changes of PPARγ and α-SMA were observed in rat HSCs transfected with miR-34 and miR-34c inhibitors alone or in combination. Further bioinformatics analysis demonstrated that the binding site of human PPARγ mRNA is located in a poorly conserved region in mammals, and there were no predicted binding sites for miR-34a and miR-34c on the 3'UTR of rat PPARγ mRNA. This is not in accordance with the general observation that binding sites are always conserved in species, while the results from the mutation reporter assay confirmed this interaction. These data suggested that although the expression of miR-34 family members, PPARγ and α-SMA demonstrated a similar expression pattern in rat HSCs and in human HSCs, the regulation mechanism in the two cell lines may be contrasting. It has been previously reported that TGF-β1 inhibits the expression of PPARγ in activated rat HSCs through the β-catenin pathway (39). TGF-β1 is one important cytokine expressed following liver injury and is the most important cytokine stimulating fibrogenesis in HSCs (1). It may be possible that in rat HSCs, the inhibition of PPARγ is mainly the result of the upregulation of TGF-β1 during the activation of HSCs, while in human HSCs, the inhibition of PPARγ results from the combination effect of upregulation of TGF-β1 and miR-34a/c.

For the first time, to the best of our knowledge, the present study identified and confirmed PPARγ to be a target gene of the miR-34 family. The regulation of the miR-34 family is negatively associated with PPARγ in activated HSCs. These data suggested that miR-34 family members may be involved in liver fibrosis by targeting PPARγ.

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