miR-330-5p inhibits H_2O_2-induced adipogenic differentiation of MSCs by regulating RXRγ

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Abstract. The elucidation of the underlying molecular mechanism of H_2O_2-induced adipocyte differentiation in mesenchymal stem cells (MSCs) is important for the development of treatments for metabolic diseases. The aim of the present study was to identify microRNA (miR)-330-5p, which targets retinoid X receptor γ (RXRγ) and to determine the function of H_2O_2-induced adipogenic differentiation of MSCs. During differentiation of MSCs into adipocytes induced by H_2O_2, miR-330-5p expression was decreased with a concomitant increase in RXRγ expression. A luciferase assay with RXRγ 3'-untranslated region (UTR) reporter plasmid, including the miR-330-5p-binding sequences, identified that the introduction of miR-330-5p decreases luciferase activity. However, it did not affect the activity of mutated RXRγ 3'-UTR reporter. Enforced expression of miR-330-5p significantly inhibited adipocyte differentiation by decreasing RXRγ mRNA and protein levels. In contrast, inhibition of the endogenous miR-330-5p promoted the formation of lipid droplets by rescuing RXRγ expression. Furthermore, the effects of inhibition of RXRγ were similar to those of overexpression of miR-330-5p on H_2O_2-induced adipogenic differentiation from MSCs. miR-330-5p inhibits H_2O_2-induced adipogenic differentiation of MSCs, and this is dependent on RXRγ. Taken together, the results of the present study revealed that miR-330-5p acts as a critical regulator of RXRγ, and is able to determinate the fate of MSCs to differentiate into adipocytes. This suggests that miR-330-5p and RXRγ may be target molecules for controlling metabolic diseases.

Introduction

Obesity is associated with an increased risk of chronic diseases, and is a major health concern worldwide (1). Clinical data have revealed that there is a positive correlation between oxidative stress and obesity (2,3). The effects of oxidative stress on adipocyte differentiation of mesenchymal stem cells (MSCs) is of research interest. Previous studies have revealed that oxidative stress is able to regulate stem cell differentiation into adipocyte lineage, which is involved in the pathogenesis of obesity, but the underlying molecular mechanisms remain unclear (4,5). The elucidation of the molecular mechanisms involved in the adipocyte differentiation of MSCs induced by oxidative stress is of vital importance for the development of therapeutics for obesity and diabetes. It is well-known that MSCs are regulated by a number of transcription factors and microRNAs (miRNAs) in the process of differentiation into adipocytes.

Retinoid X receptor γ (RXRγ), a member of the nuclear receptor superfamily, is expressed in brown adipocytes and an increased gene expression is observed during brown adipogenesis in stem cells (6,7). It has an important function in the control of adipogenic differentiation of MSCs (8), modulating MSC commitment and differentiation to determine whether cells partake in adipogenesis. RXR homodimers may promote adipogenesis by activating the target genes of peroxisome proliferator-activated receptor (PPAR) (9). RXRγ activates PPARγ expression, thus promoting the entry of MSCs to the adipocyte lineage while impeding progression for alternative lineage pathways (10). The RXR ligand LGD1069 increases the expression of adipogenesis-associated genes (11). The functions of RXR agonists in adipogenesis are cell type-specific and are based on the integration of signals from different RXR dimers (12). These results indicated that RXRγ is a positive regulator of adipogenesis. However, the epigenetic regulation of RXRγ in adipogenic differentiation in MSCs remains unclear.

miRNAs have been identified as essential elements of the epigenetic machinery which post-transcriptionally repress the expression of target genes, contributing to the regulation

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of a number of biological effects (13). Previous studies have revealed that miRNAs targeting osteoblast-associated genes are involved in adipogenic differentiation of MSCs. miR-204/211, miR-320 and miR-30 target Runt-related transcription factor 2 to promote adipocytic differentiation of human MSCs (14). miRNAs targeting adipocyte-associated genes negatively regulate adipogenic differentiation of MSCs (15,16). miRNAs targeting cell cycle and self-renewal-associated genes indirectly regulate adipogenic differentiation of MSCs, as identified with miR-21 (17). These results strongly suggested that the regulation of transcription factors by miRNAs is a notable component of the regulatory machinery. The results of the present study predicted that the target gene of miR-330-5p is RXRγ, using bioinformatics to further investigate the function of miR-330-5p in targeting RXRγ involved in H_2O_2-induced adipogenic differentiation of MSCs.

Materials and methods

Culture of MSCs. Sprague-Dawley rat MSCs were purchased from Cyagen Biosciences Inc. (Guangzhou, China). The cells have been tested for bacteria, fungi and *Mycoplasma*, and were assessed for cell-surface markers. CD29, CD44 and CD90 were markedly expressed, whereas the expression of CD34, CD45 and CD11 was <5%. Cells were grown in accordance with the supplier's specifications, seeded at 37°C under 5% CO_2 in low-glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (mother solution was $1x10^4$ U/ml; Thermo Fisher Scientific, Inc.). The cells were passaged at a density of $2.0x10^4$ cells/cm^2 or cryopreserved at a density of $(1.0-1.5)$ x10^6 cells/ml.

Induction of adipogenic differentiation. The model was established and optimized as described previously (18-21). To induce differentiation, 2-day post-confluent MSCs (designated day 0) were incubated in DMEM containing 10% fetal bovine serum; differentiation was induced with 10% fetal bovine serum and 100 μM H_2O_2 for 1 h. After 1 h of H_2O_2 induction, the induction medium was replaced with DMEM containing 10% fetal bovine serum. The medium was replaced every 2-3 days. Cell RNA and protein were determined on day 3, and Oil Red O staining was performed on day 7.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc.) and chloroform. Samples of 2 μl were tested for concentration and purity using an ultraviolet spectrophotometer (BioSpec-Nano, Shimadzu Corporation, Kyoto, Japan) at the wavelengths of 260/280 nm. A total of 1-3 μg RNA was used to synthesize first-strand cDNA (miRNA using specific RT primers and mRNA using random primers). Reverse transcription was performed using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, with specific retrovirus conditions as follows: 65°C for 5 min, followed by placing on ice, then 42°C for 60 min, heating to 70°C for 5 min and finally cooling to 4°C. The qPCR adopted the two-step method, with a 20 μl reaction volume and specific reaction conditions using

a Talent fluorescent quantitative detection kit (SYBR-Green) (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol as follows: 95°C pre-degeneration for 3 min; then 40 cycles of 95°C degeneration for 5 sec, 60°C annealing/extension for 15 sec and fluorescence signal collection. The differences in gene expression were analyzed using the $2^{-\Delta\Delta Cq}$ method (22). U6 was used as the internal reference for miRNA detection, and β-actin or GAPDH was used as internal references for the detection of mRNA. The primer sequences are presented in Table I.

Western blotting. Total protein was extracted using radioimmunoprecipitation assay lysis and extraction buffer (Thermo Fisher Scientific, Inc.) on ice, and bicinchoninic acid protein quantification (Beyotime Institute of Biotechnology, Haimen, China) was used to adjust the sample concentration. Samples were boiled in loading buffer for denaturation. A total of 30-50 μg/lane protein was separated by SDS-PAGE (10% gel; conditions, 80 V for 30 min and 120 V for 60 min) prior to wet transfer (conditions, 300 mA for 80 min) onto polyvinylidene difluoride membranes. Following blocking each membranes with 5% skimmed milk in Tris-buffered saline containing 0.01% Tween-20 (TBST) for 2 h at room temperature, membranes were incubated with primary antibodies at 4°C overnight. Following elution with TBST, membranes were incubated with secondary antibodies for 1 h at room temperature, prior to elution with TBST. The enhanced chemiluminescence working fluid (Beyotime Institute of Biotechnology) was used with the Tanon 5200 Multi Chemiluminescent Imaging system (Tanon Science & Technology Co., Ltd., Shanghai, China) exposure strip. ImageJ software (version 1.4; National Institutes of Health, Bethesda, MD, USA) was used to analyze the gray values of each group. Anti-PPARγ (cat. no. sc-7237; dilution, 1:200) and anti-CCAAT/enhancer-binding protein α (C/EBPα; cat. no. sc-365318; dilution, 1:200) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); anti-GAPDH (cat. no. AC002; dilution, 1:20,000), anti-β-actin (cat. no. AC004; dilution, 1:20,000), anti-glucose transporter 4 (Glut4; cat. no. A0071; dilution, 1:500), anti-30 kDa adipocyte complement-related protein (Acrp30; cat. no. A2543; dilution, 1:1,000) and horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG; cat. no. AS003; dilution, 1:25,000) antibodies were all purchased from ABclonal Biotech Co., Ltd. (Woburn, MA, USA); and anti-RXRγ (cat. no. ab15518, dilution, 1:150), anti-adipocyte protein 2 (aP2; cat. no. ab186424; dilution, 1:3,000) and HRP-conjugated goat anti-rabbit IgG (cat. no. ab97051; dilution, 1:20,000) antibodies were all purchased from Abcam (Cambridge, MA, USA). Antibodies were diluted with 5% bovine serum albumin, according to the manufacturers' protocols.

Oil Red O assay. According to a protocol described previously (23), the conditioned medium was discarded and rat MSCs were washed with PBS twice. Subsequently, cells were fixed with 4% paraformaldehyde for 20 min and rinsed once with ultrapure water. Next, 60% isopropanol was added for a 5 min wash, and Oil Red O working liquid was added at room temperature for 30 min. Subsequently, 60% isopropanol was used again two or three times to rapidly wash away the excess dye, with ultrapure water; hematoxylin (final concentration,

Table I. Primers for RT-qPCR.

Gene	Sequence	Supplier
β-actin	Forward, 5'-cccatctatgagggttacgc-3' Reverse, 5'-tttaatgtcacgcacgatttc-3'	Sangon Biotech, Co., Ltd., Shanghai, China
PPARγ	Forward, 5'-gtcagtactgtcggtttcagaag-3' Reverse, 5'-gcatggtgtagatgatctcatgg-3'	
aP2	Forward, 5'-tcgtcatccggtcagagagt-3' Reverse, 5'-ccagcttgtcaccatctcgt-3'	
RXRγ	Forward, 5'-cgagcagagagtgaggcaga-3' Reverse, 5'-acactcatgtcgccgtagga-3'	
U6	RT-qPCR forward and reverse primers, sequences unavailable from the supplier; cat. no. MQP-0201	Guangzhou RiboBio Co., Ltd., Guangzhou, China
rno-miR-330-5p	RT-PCR forward and reverse primers, sequences unavailable from the supplier; cat. no. MQP-0101	

RT-qPCR, reverse transcription-quantitative PCR; PPARγ, peroxisome proliferator-activated receptor γ; aP2, adipocyte protein 2; RXRγ, retinoid X receptor γ; rno, *Rattus norvegicus*; miR, microRNA.

99.25 mM) staining occurred for 20 sec and tap water was used to wash cells three times. Finally, cells were sealed with glycerol gelatin. Using the image analysis system, five random images were captured. Oil Red O working liquid and hematoxylin were provided by Professor Saixia Zhang (School of Basic Medical Science, Guangzhou University of Chinese Medicine, Guangzhou, China).

Bioinformatic prediction of the target gene of miR-330-5p. According to a protocol described previously (24), the target gene of *Rattus norvegicus* (rno)-miR-330-5p was predicted and screened using bioinformatics websites, including miRbase (www.mirbase.org), miRWalk2.0 (zmf.umm.uni-heidelberg. de/apps/zmf/mirwalk2/index.html), TargetScan (www. targetscan.org/mmu_71), microRNA.org (www.microrna.org), miRTarBase (mirtarbase.mbc.nctu.edu.tw/php/index.php), FINDTAR3 (bio.sz.tsinghua.edu.cn). The predicted gene was selected using a Venn diagram, when the number of genes in the intersection number >3 were imported into the DAVID Bioinformatics Resources 6.8 website (david.ncifcrf.gov) for gene identity conversion. Following protein enrichment and Kyoto Encyclopedia of Genes and Genomes (www.kegg. jp/kegg/pathway.html) signal pathway analysis, a series of genes associated with adipose differentiation were selected as candidate target genes, including RXRγ. Subsequently, biological products of miR-330-5p and RXRγ were constructed for verification by western blot assay and dual-luciferase reporter gene analysis. The sequences of miRNA and small interfering (si)RNA are presented in Table II.

Transient transfection of MSCs with miR-330-5p mimic, inhibitor and the respective negative controls (m-NC and i-NC) or siRNA (siR)-RXRγ. On the day before transfection, MSCs were inoculated into different plates according to certain density (24-well plates, $3.0x10^4$ cells/well; 6-well plates, $1.5x10^5$ cells/well), fusion for cell proliferation to ~55%

Table II. miRNAs and siRNA sequences.

Name	Sequence
miR-330-5p mimic	5'-ucucugggccugugucuuaggc-3' 5'-agagacccggacacagaauccg-3'
miR-330-5p inhibitor	5'-gccuaagacacaggcccagaga-3'
siR-RXRγ fragments	001, actggctccacatccatga 002, gacagtcatcccagctaca 003, ctcctccgggaatcaactt
miRNA mimic negative control	uuuguacuacacaaaaguacug
miRNA inhibitor negative control	uuuguacuacacaaaaguacug
siRNA negative control	Sequence unavailable from the supplier; cat. no. siN05815122147-1-5

miRNAs and siRNAs were from Guangzhou RiboBio Co., Ltd., Guangzhou, China. miRNA/miR, microRNA; siRNA/siR, short interfering RNA; RXRγ, retinoid X receptor γ.

for transfection. Transfection of miR-330-5p or siR-RXRγ used a RiboFect™ CP transfection kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China). Preparation of specific transfection complexes was according to the manufacturer's protocol. The final concentration of mimic, m-NC and siRNAs was 50 nM, and the final concentration of inhibitor and i-NC was 100 nM. Western blotting and RT-qPCR analysis were performed after 48 h.

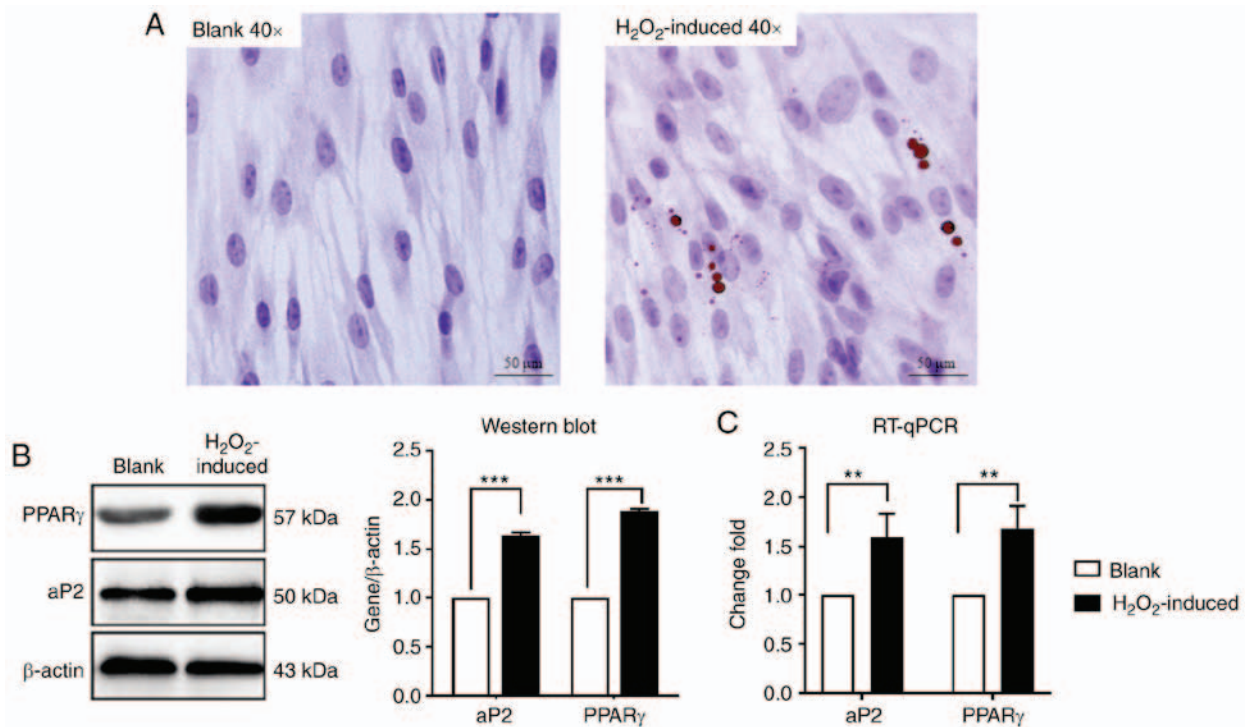*Co-transfection of fluorescent reporter plasmid containing RXRγ-3'-untranslated region (UTR) fragments.* MSCs

Figure 1. H_2O_2 induces adipogenic differentiation of MSCs. (A) An Oil Red O assay identified that the lipid droplets were accumulated in the H_2O_2-induced group compared with the blank group. (B) The expression of the fat-associated genes aP2 and PPARγ was determined by western blotting. β-actin was used as a reference. (C) The expression of aP2 and PPARγ was determined by reverse transcription-quantitative polymerase chain reaction. β-actin served as the internal reference. $^{**}P<0.01$, $^{***}P<0.001$ vs. the H_2O_2-induced group. MSC, mesenchymal stem cell; ap2, adipocyte protein 2; PPARγ, peroxisome proliferator-activated receptor γ.

were inoculated at $3.0x10^4$ cells/well density in 24-well plates, then co-transfection of mimic, m-NC, inhibitor and i-NC with pLUC-RXRγ-wild-type (WT) 3'-UTR and pLUC-RXRγ-mutant (MUT) 3'-UTR plasmid (Shenzhen Huaan Ping Kang Bio Technology, Inc., Shenzhen, China) using Lipofectamine® 3000 reagent (Thermo Fisher Scientific, Inc.) was performed according to the manufacturer's protocol. The final concentration of miR-330-5p mimic and its control was 50 nM, the final concentration of miR-330-5p inhibitor and its control was 100 nM, and plasmid content was 500 ng. After 48 h, using the Dual-Luciferase® Reporter assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol, the degree of activation of the target reporter gene in different samples was compared on the basis of the ratio obtained. Using a GloMax®-20/20 Single Tube Luminometer (Promega Corporation) to obtain the fluorescence ratio at 465 nm (Renilla luciferase)/560 nm (firefly luciferase). Comparing the fluorescence ratio of the experimental group and the control group to determine the accuracy of the target gene.

Co-transfection of the miR-330-5p mimic and its control with siR-RXRγ and its control. Using a protocol described previously (25), MSCs were inoculated at a density of $3.0x10^4$ cells/well in a 24-well plate or $1.5x10^5$ cells/well in a 6-well plate. Following transfection, 1.25 or 5 µl mimic, m-NC, siR-RXRγ-002 and siRNA negative control (siR-NC) were diluted with 50 or 200 µl 1X riboFECT™ CP buffer (Guangzhou RiboBio Co., Ltd.), respectively, and mixed with 5 or 20 µl riboFECT™ CP reagent (Guangzhou RiboBio

Co., Ltd.). Cells were mixed evenly and incubated at room temperature for 15 min. The transfected complex was added to the medium without antibiotics to a volume of 500 µl or 2 ml. Following transfection for 12 h, the original cell culture medium was re-added and incubation at 37°C was performed for 48 h. Subsequently, western blotting and RT-qPCR assays were performed.

Statistical analysis. Data are presented as the mean ± standard deviation. Data were analyzed using Excel 2016 (Microsoft Corporation, Redmond, WA, USA), SPSS 20 (version 20; IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 7.0; GraphPad Software, Inc., La Jolla, CA, USA). The comparison between two sample groups was analyzed using a t-test. The comparison between multiple sample groups was analyzed using analysis of variance followed by Dunnett's multiple comparisons test for post hoc analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

H_2O_2 induces adipogenic differentiation of MSCs. To investigate whether H_2O_2 affects adipocyte differentiation in MSCs, H_2O_2 (100 µM) was added to the culture medium. As presented in Fig. 1A, H_2O_2 treatment induced differentiation, as detected using an Oil Red O assay, and the number of lipid droplets increased markedly in the H_2O_2-induced group compared with the blank group. RT-qPCR and western blot assay also indicated that the expression of PPARγ and aP2 was also increased significantly compared with the blank control group (Fig. 1B
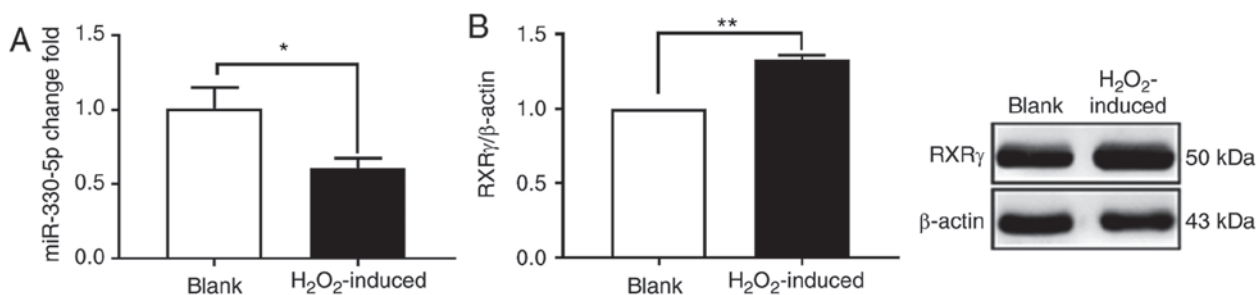
Figure 2. miR-330-5p expression is decreased and RXRγ expression is increased in H_2O_2-induced adipogenic differentiation of MSCs. (A) Expression levels of miR-330-5p in the H_2O_2-induced group and the blank group were determined using the reverse transcription-quantitative polymerase chain reaction. U6 was used as an internal control. (B) Expression levels of RXRγ in the H_2O_2-induced group and blank group were determined by western blotting. β-actin was used as an internal control. *P<0.05, **P<0.01 vs. the H_2O_2-induced group. miR, microRNA; RXRγ, retinoid X receptor γ; MSC, mesenchymal stem cell.
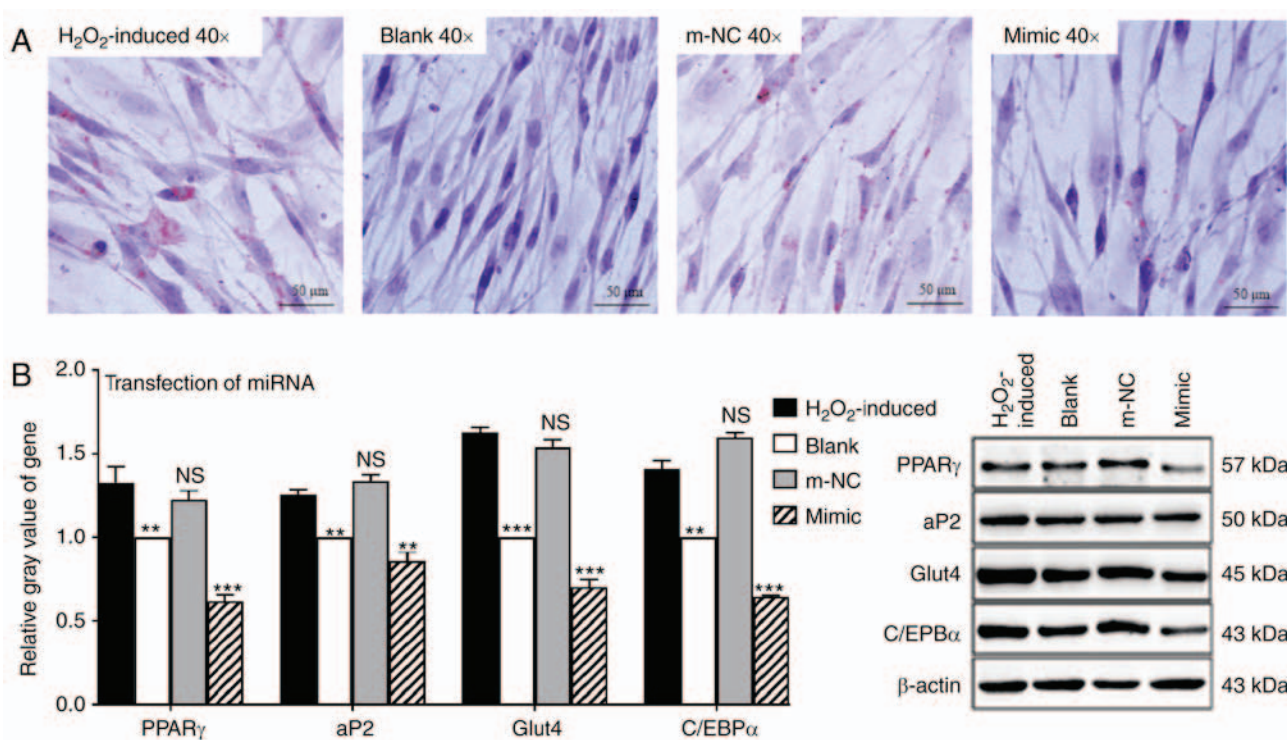

Figure 3. miR-330-5p inhibits the adipogenic differentiation process of MSCs. (A) An Oil Red O assay identified that the H_2O_2-induced group and the m-NC group contained large numbers of lipid droplets, and overexpression of miR-330-5p decreased the number of lipid droplets. (B) Western blotting indicated that the gene expression levels of PPARγ, aP2, Glut4 and C/EBPα in the H_2O_2-induced group and the m-NC group were increased compared with the blank group. Overexpression of miR-330-5p inhibited expression of the aforementioned genes. **P<0.01, ***P<0.001 vs. the H_2O_2-induced group; NS, not significant. miR, microRNA; MSC, mesenchymal stem cell; m-NC, mimic negative control; PPARγ, peroxisome proliferator-activated receptor γ; aP2, adipocyte protein 2; Glut4, glucose transporter 4; C/EBPα, CCAAT/enhancer-binding protein α.

and C). These data suggested that H_2O_2 induces adipogenic differentiation of MSCs.

miR-330-5p expression is decreased and RXRγ expression is increased in the H_2O_2-induced adipogenic differentiation of MSCs. To determine whether miR-330-5p expression changes during the process of H_2O_2-induced adipogenic differentiation, an RT-qPCR assay was performed. When adipogenic differentiation of MSCs was induced by H_2O_2, the expression of rno-miR-330-5p was decreased significantly compared with the blank group (Fig. 2A). A western blot assay was used to detect the expression of RXRγ, which identified that the expression of RXRγ was significantly increased in the H_2O_2-induced group (Fig. 2B). The results indicated that the

miR-330-5p expression was decreased and RXRγ expression was increased in the H_2O_2-induced adipogenic differentiation of MSCs.

miR-330-5p inhibits H_2O_2-induced adipogenic differentiation of MSCs. In order to further clarify the function of miR-330-5p in the H_2O_2-induced adipogenic differentiation of MSCs, miR-330-5p mimic or m-NC were used to transfect MSCs, which were subjected to adipogenic differentiation when induced with H_2O_2. An Oil Red O assay revealed that the number of lipid droplets in the miR-330-5p mimic group was decreased compared with the H_2O_2-induced group and m-NC group, and cell morphology tended to be in the original form of MSCs (Fig. 3A). Western blotting also indicated that
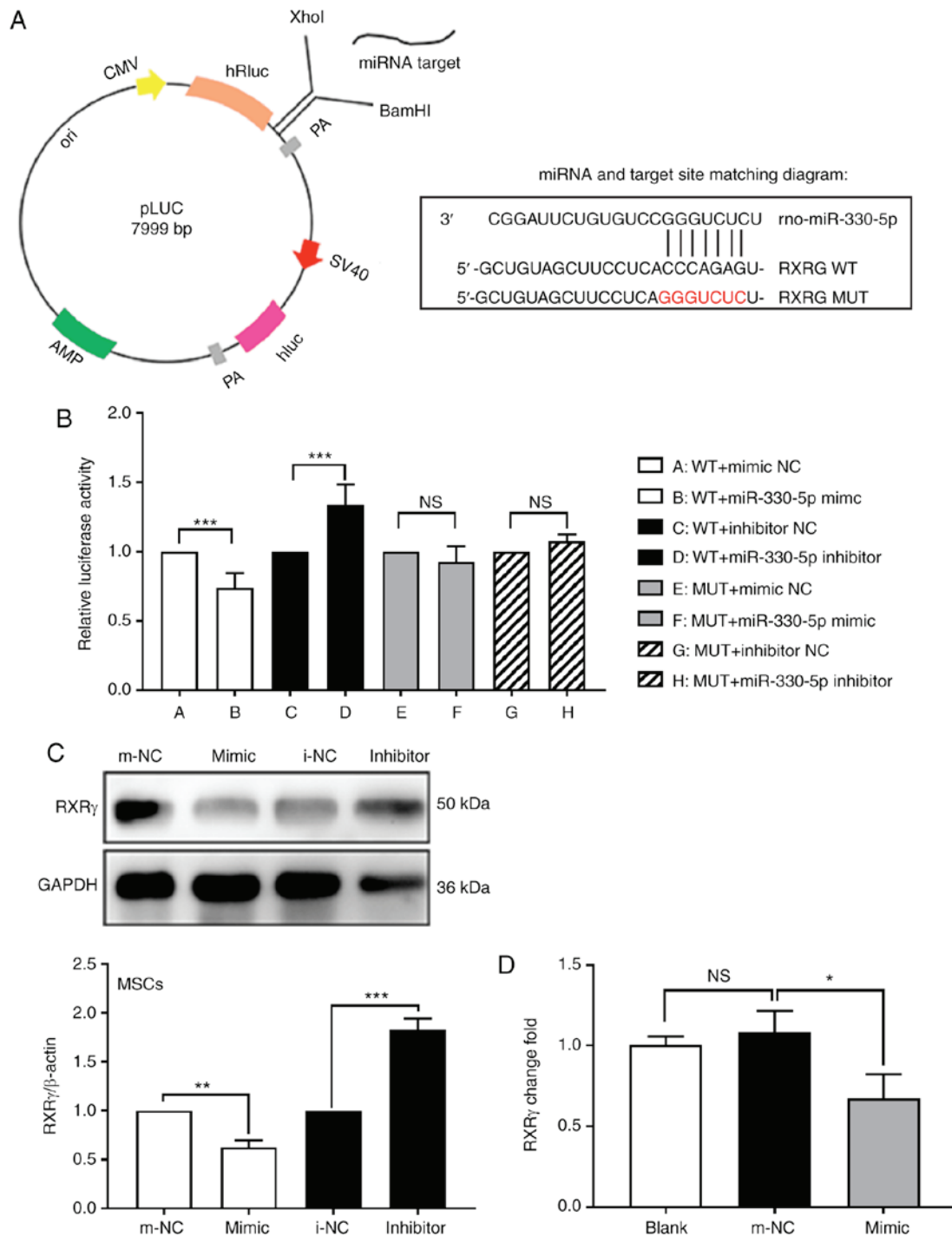
Figure 4. RXRγ is a potential target of miR-330-5p. (A) RXRγ was predicted by miRbase, miRWalk2.0, microRNA.org and FINDTAR3 target prediction websites, and the construction of pLUC-RXRγ-WT and pLUC-RXRγ-MUT was conducted by Shenzhen Huaan Ping Kang Bio Technology, Inc., Shenzhen, China. (B) Dual-luciferase reporter gene results indicated that there is an association between miR-330-5p and RXRγ. (C) Western blotting results identified that in MSCs, transfection of miR-330-5p mimic decreased RXRγ expression compared with the m-NC group, and transfection of the miR-330-5p inhibitor was reversed. (D) Reverse transcription-quantitative polymerase chain reaction results identified that RXRγ mRNA decreased in the miR-330-5p group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. the respective NC groups; NS, not significant. RXRγ, retinoid X receptor γ; miR, microRNA; MSC, mesenchymal stem cell; m-NC, mimic negative control; WT, wild-type; MUT, mutant; i-NC, inhibitor negative control.

that the expression of PPARγ, aP2, Glut4 and C/EBPα in the H_2O_2-induced group and the m-NC group was significantly increased compared with that in the blank group (Fig. 3B). In contrast, the expression of PPARγ, aP2, Glut4 and C/EBPα in the miR-330-5p mimic group was decreased (Fig. 3B). These data suggested that miR-330-5p inhibited H_2O_2-induced adipogenic differentiation of MSCs.

RXRγ is a potential target of miR-330-5p. The aforementioned results suggested that miR-330-5p negatively regulated adipogenesis and increased RXRγ. Bioinformatics predicted the miR-330-5p sequence. GGGUCUC was associated with the RXRγ gene sequence CCCAGAG (Fig. 4A). To determine whether RXRγ is targeted to miR-330-5p through this sequence, luciferase reporters were constructed that had either

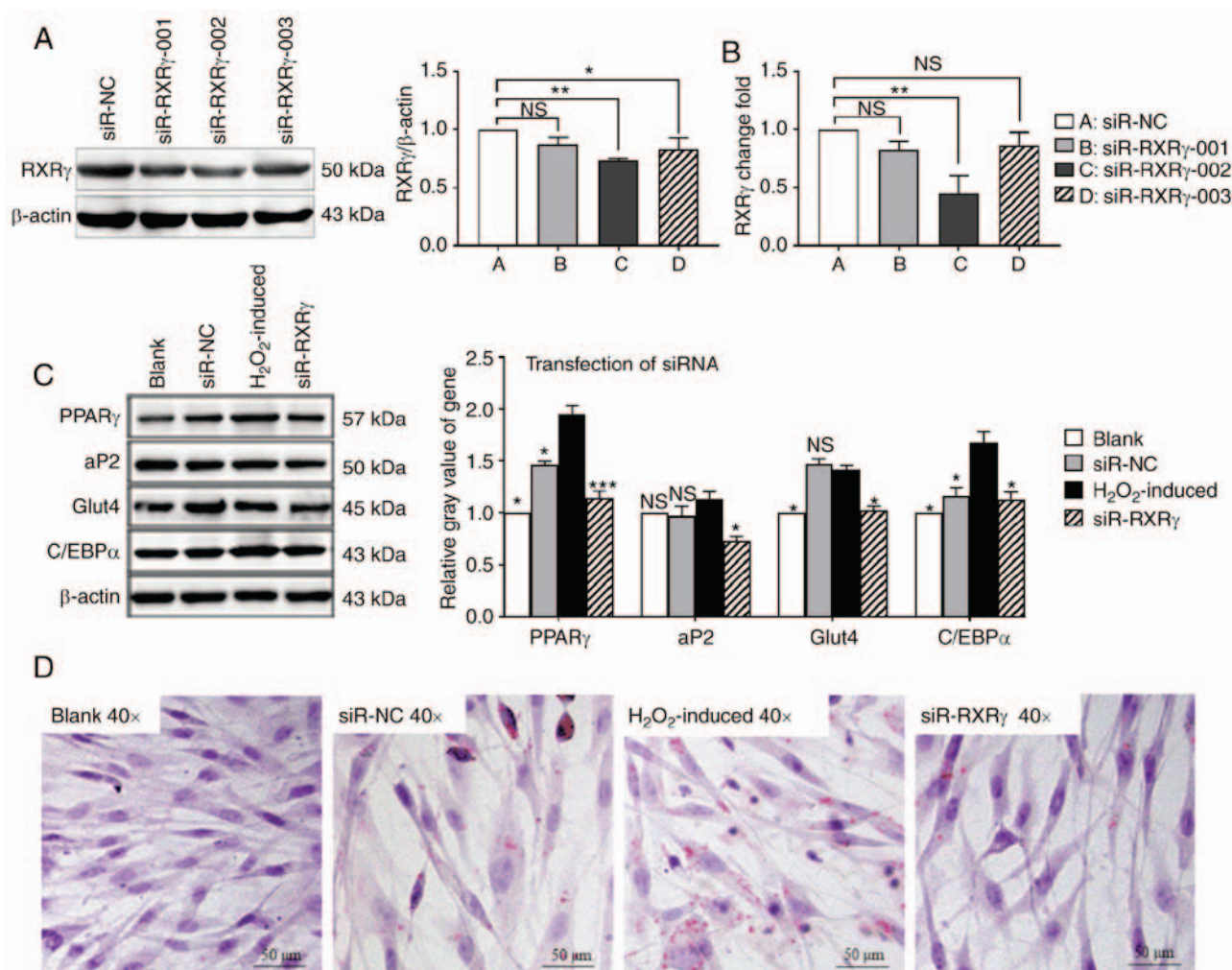Figure 5. Silencing of RXRγ inhibits adipogenic differentiation of MSCs. Screening of the optimal silent fragments by transfection into rat MSCs. The second RXRγ siRNA fragment silencing effect was selected by (A) western blotting and (B) reverse transcription-quantitative polymerase chain reaction assay, and β-actin was used as the internal reference for the two experiments. *P<0.05, **P<0.01 vs. the siR-NC group. (C) Western blotting indicated that the expression levels of PPARγ, aP2, Glut4 and C/EBPα were decreased following silencing of RXRγ. β-actin was used as an internal reference. *P<0.05, ***P<0.001 vs. the H₂O₂-induced group; NS, not significant. (D) An Oil Red O assay identified that the silencing of RXRγ decreased the number of lipid droplets. RXRγ, retinoid X receptor γ; MSC, mesenchymal stem cell; siRNA/siR, short interfering RNA; NC, negative control; PPARγ, peroxisome proliferator-activated receptor γ; aP2, adipocyte protein 2; Glut4, glucose transporter 4; C/EBPα, CCAAT/enhancer-binding protein α.

a WT 3'-UTR or a 3'-UTR containing mutant sequences of the miR-330-5p-binding site. It was revealed that overexpression of miR-330-5p significantly inhibited the luciferase reporter activity of the WT RXRγ 3'-UTR, but not that of the mutated 3'-UTR or the 3'-UTR of another gene (Fig. 4B). These results indicated that miR-330-5p may directly regulate RXRγ expression. Further experiments confirmed that miR-330-5p overexpression significantly suppressed the expression of RXRγ, at the mRNA and protein levels compared with the m-NC group (Fig. 4C and D). These results indicated that RXRγ is a target gene of miR-330-5p.

Silencing RXRγ inhibits adipogenic differentiation of MSCs. To further understand the effect of RXRγ on MSCs adipogenesis, siR-RXRγ-001, siR-RXRγ-002 or siR-RXRγ-003 were transfected into MSCs. Western blotting and RT-qPCR analysis verified that siR-RXRγ-002 effectively suppressed the expression of RXRγ at the protein and mRNA levels compared with the siR-NC group (Fig. 5A and B). The decrease in RXRγ

significantly inhibited the expression levels of the adipogenic marker genes PPARγ, aP2, Glut4 and C/EBPα compared with the H₂O₂-induced group, as indicated by western blotting (Fig. 5C). The Oil Red O assay clearly indicated that the downregulation of RXRγ also resulted in decreases in the accumulation of lipid droplets (Fig. 5D). The data demonstrated that the downregulation of RXRγ effectively inhibited H₂O₂-induced adipogenic differentiation of MSCs.

miR-330-5p inhibition of H₂O₂-induced adipogenic differentiation of MSCs is dependent on RXRγ. From the aforementioned experiments, it may be concluded that miR-330-5p and RXRγ are different in the process of adipogenic differentiation of MSCs. RXRγ positively regulated the adipogenic differentiation of MSCs, whereas miR-330-5p negatively regulated the process. Experiments have confirmed that miR-330-5p specifically regulated RXRγ expression; it was therefore hypothesized that miR-330-5p inhibited MSCs differentiation by directly targeting RXRγ, from the miR-330-5p/RXRγ
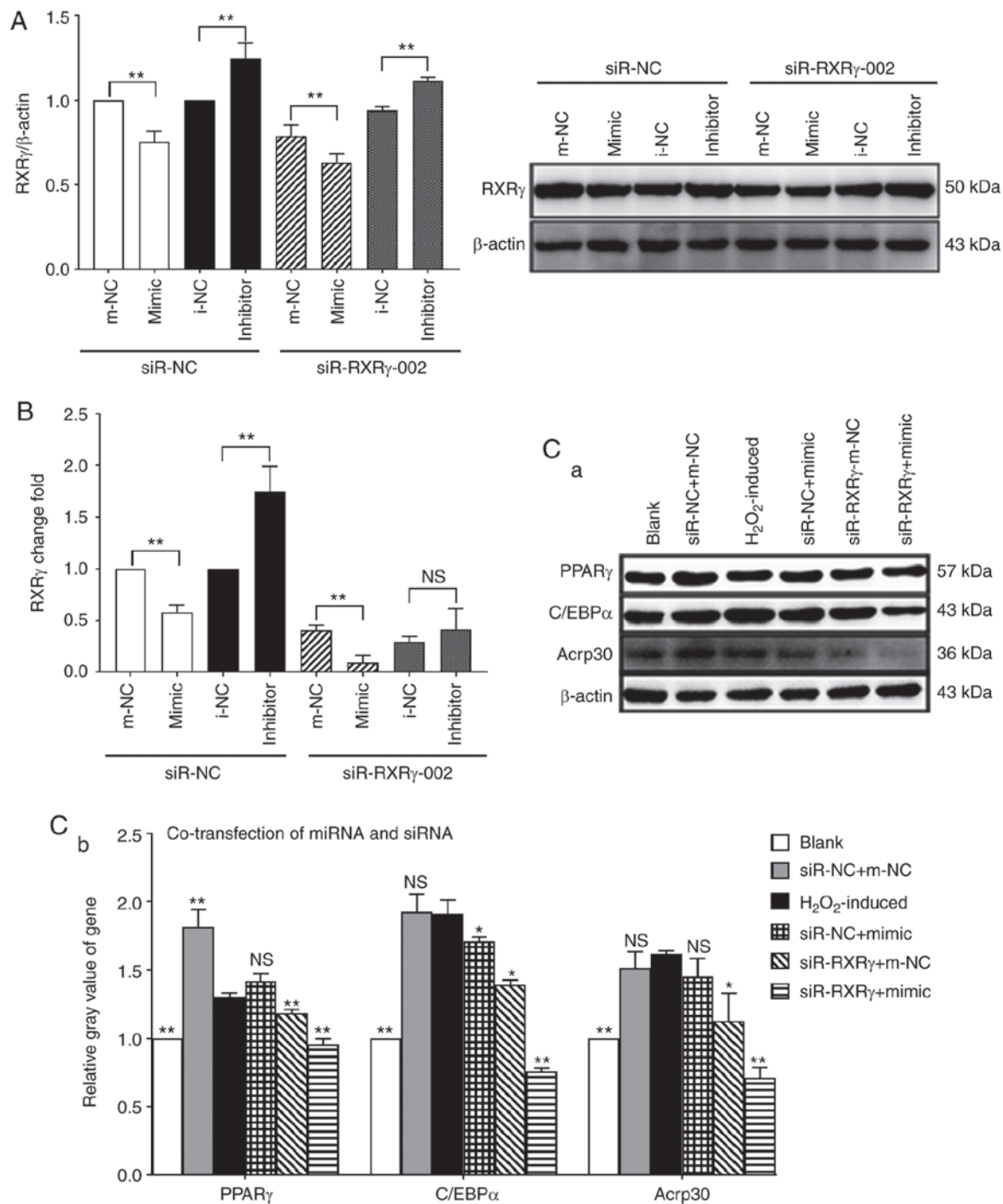
Figure 6. Inhibitory effects of miR-330-5p on H_2O_2-induced adipogenic differentiation of MSCs depend on RXRγ. (A) Western blotting and (B) RT-qPCR detected the protein and mRNA level of RXRγ, respectively. **$P<0.01$ vs. the respective NC groups; NS, not significant. (C) Western blotting indicated that the expression levels of PPARγ, C/EBPα and Acrp30 in the siR-RXRγ + mimic group were significantly decreased compared with the H_2O_2-induced group, the siR-NC + m-NC group, the siR-NC + mimic group or the siR-RXRγ + m-NC group. *$P<0.05$, **$P<0.01$ vs. the H_2O_2-induced group; NS, not significant. miR, microRNA; MSC, mesenchymal stem cell; RXRγ, retinoid X receptor γ; NC, negative control; PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT/enhancer-binding protein α; Acrp30, 30 kDa adipocyte complement-related protein; siR, short interfering RNA.

signaling pathway. To demonstrate that miR-330-5p inhibited RXRγ-dependent adipogenic differentiation, miR-330-5p and RXRγ-interfering fragments were co-transfected into MSCs. The western blot assay results revealed that when siR-NC was co-transfected with miR-330-5p mimic, inhibitor and negative control, the expression of RXRγ in the mimic + siR-NC group was decreased compared with that of the m-NC + siR-NC group and the effects of the inhibitor + siR-NC group were

the opposite. However, on co-transfection with miR-330-5p and siR-RXRγ-002, the expression of RXRγ was significantly less in the mimic + siR-RXRγ-002 group (Fig. 6A). RT-qPCR results also identified that RXRγ mRNA levels were significantly decreased in the mimic + siR-RXRγ-002 group (Fig. 6B). The western blot assay revealed that transfection of mimic, without silencing of RXRγ (i.e. siR-NC + mimic group), was able to decrease the expression of PPARγ, C/EBPα

and Acrp30, but following transfection of mimic and silent RXRγ (i.e. siR-RXRγ + mimic group), the decrease in PPARγ, C/EBPα and Acrp30 was greater. Silencing RXRγ without transfection of mimic (i.e. siR-RXRγ + m-NC group) was able to decrease the expression of PPARγ, C/EBPα and Acrp30; however, the decrease in PPARγ, C/EBPα and Acrp30 was greater following transfection of mimic and silent RXRγ (i.e. siR-RXRγ + mimic group). Briefly, co-transfection of miR-330-5p mimic with siR-RXRγ-002 significantly decreased the expression of PPARγ, C/EBPα and Acrp30 compared with the H_2O_2-induced group, the siR-NC + m-NC group, the siR-NC + mimic group or the siR- RXRγ + m-NC group (Fig. 6C). The gain of miR-330-5p function promoted the inhibitory effect on adipogenesis of RXRγ siRNA. These results indicated that miR-330-5p inhibited H_2O_2-induced adipogenic differentiation of MSCs, dependent on RXRγ.

Discussion

Oxidative stress has been associated with increased dysfunctional adipogenesis (5,26-28), but its molecular mechanism is unclear. Adipogenic differentiation of MSCs contributes greatly to metabolic diseases. Therefore, it is of the utmost importance to explore the positive and negative regulators of adipogenic differentiation of MSCs. The results may provide promising therapeutic targets for metabolic diseases. The present study used an H_2O_2-treated MSC model to simulate a number of significant characteristics in the process of metabolic disorders of fat, to identify further the function of miRNAs in regulating adipogenic differentiation of MSCs induced by oxidative stress. The principal results of the present study were: i) Low concentration of H_2O_2-induced adipogenic differentiation of MSCs; ii) miR-330-5p was downregulated, accompanied by upregulated RXRγ during H_2O_2- induced adipogenic differentiation of MSCs; iii) miR-330-5p was demonstrated to be a negative regulator of H_2O_2-induced adipogenic differentiation of MSCs; iv) RXRγ was demonstrated to be a positive regulator of H_2O_2-induced adipogenic differentiation of MSCs; and v) RXRγ was identified as a direct target of miR-330-5p. These results suggested that the miR-330-5p/RXRγ signaling pathway is an important part of the regulatory mechanisms involved in early adipogenesis and that the miR-330-5p/RXRγ signaling pathway may be a key target for drug development in metabolic diseases.

The key result of the present study is that miR-330-5p is a negative regulator of H_2O_2-induced adipogenic differentiation of MSCs. A recent study identified that miR-330-5p may be associated with cancer progression (29) and may regulate leukemia (30). However, the regulatory function of miR-330-5p on adipogenic differentiation of MSCs has not been reported. Compared with the younger MSCs, miR-330-5p is altered in the aging MSCs (31). Aging is associated with oxidative stress, and it was hypothesized that miR-330-5p serves a function in adipogenic differentiation of MSCs induced by oxidative stress. To confirm the hypothesis, H_2O_2-induced adipogenic differentiation of MSCs was used, which is an established cell model (20). MSCs have been widely used for elucidating the molecular mechanisms involved in adipogenesis (32). In the present study, miR-330-5p was downregulated in H_2O_2-induced adipogenic differentiation of MSCs. Therefore,

it was further inferred that miR-330-5p is a negative regulator of H_2O_2-induced adipogenic differentiation of MSCs. Furthermore, the data suggested that overexpression of miR-330-5p inhibited the process of adipogenic differentiation induced by H_2O_2, which indicated that miR-330-5p is indeed a negative regulator of H_2O_2-induced adipogenic differentiation of MSCs. These results may provide a new regulatory function of miR-330-5p in the process of H_2O_2- induced adipogenic differentiation.

The key molecular mechanism identified in the present study is that RXRγ is a direct target of miR-330-5p. RXRγ is initially associated with the development of the animal (including human) fetus and is used to detect genetic variation associated with growth, reproduction, selection of metabolic characteristics and breeding (33). RXRγ, expressed in white and brown fat cells, increases markedly following the differentiation of lipids (34). Previous studies have identified that RXRγ is crucial for fat differentiation since, in the late stage of adipocyte differentiation, it is able to form a heterodimer with PPAR (8,10). The RXRγ-PPARγ association is enriched near the 5'-region of the transcriptional start site to promote the upregulation of gene transcription associated with fatty acid and lipid metabolism. RXRγ is activated to modify the whole genome histone 3 Lys^{27} trimethylation, promoting adipogenic differentiation. It has been identified that the expression of the adipose differentiation-associated protein is regulated by RXRγ, which accelerates the accumulation of neutral lipid (35). It has also been identified that RXRγ contributes to the genetic background of familial combined hyperlipidemia (36). Therefore, RXRγ is a novel target for the treatment of adipose disease (37). In view of this, investigation has focused on RXRγ. In the present study, expression of RXRγ was positively associated with H_2O_2-induced adipogenic differentiation of MSCs. Using target prediction tools, including miRWalk2.0 and microRNA.org, it was observed that RXRγ is one target of miR-330-5p. The luciferase activity result revealed that overexpression of miR-330-5p mimic suppressed RXRγ expression. However, this effect was abolished when a luciferase reporter containing a mutant 3'-UTR of RXRγ was co-transfected with mimic miR-330-5p, thus confirming the specificity of action. Enforced expression of miR-330-5p significantly inhibited adipocyte differentiation by decreasing RXRγ mRNA and protein levels. In contrast, inhibition of the endogenous miR-330-5p promoted the formation of lipid droplets by rescuing RXRγ expression. Furthermore, the effects of the inhibition of silencing RXRγ were similar to those of overexpression of miR-330-5p on H_2O_2-induced adipogenic differentiation from MSCs. miR-330-5p inhibited H_2O_2-induced adipogenic differentiation of MSCs, and this effect was dependent on RXRγ. Taken together, the results of the present study identified that miR-330-5p negatively regulates H_2O_2-induced adipogenic differentiation of MSCs by targeting RXRγ.

The potential molecular pathway speculated in the present study is that RXRγ regulates the adipogenesis of MSCs through the PPARγ signaling pathway. It was identified that PPARγ, C/EBPα, aP2 and Glut4 were all altered following silencing of RXRγ. PPARγ, C/EBPα and aP2 are all key genes for fat differentiation, and they all exist in the PPAR signaling pathway. Glut4 is involved in the AMP-activated protein

kinase (AMPK) signaling pathway. It has been identified that the AMPK and PPARγ signaling pathways are interrelated in the process of 3T3-IL adipocyte differentiation and co-regulate the formation of adipocytes (38). Considering that RXR is more likely to form a heteropolymer with PPAR, we hypothesize that RXRγ regulates H_2O_2-induced adipogenic differentiation of MSCs that may be associated with the PPARγ and AMPK signaling pathways, particularly the PPARγ pathway. However, additional data are required to confirm this.

The results of the present study have a number important clinical implications. First, obesity is associated with increased risk of heart disease, stroke and diabetes. By 2016, there were >1.9 billion adults >18 years that were overweight globally, of whom >650 million were obese (39). Obesity and associated disorders lead to heavy economic burdens (40). Understanding of adipogenic differentiation of MSCs to develop effective drugs for the prevention and treatment of obesity and associated disorders is vital; alterations in adipocyte differentiation of MSCs may lead to obesity and associated disorders. The earliest symptoms of obesity and associated disorders present as a relative deficit. Therefore, sensitive and specific biomarkers for early detection are urgently required. In the present study, it was demonstrated that miR-330-5p was decreased significantly in early adipogenic differentiation. Thus, miR-330-5p may be a potential biomarker for the early diagnosis of obesity and its associated disorders. Secondly, currently, there are no therapies to prevent the progression of obesity or its associated disorders. It was revealed that miR-330-5p functions as a negative regulator of adipogenesis by repressing RXRγ expression, which in turn, may result in suppression of the RXRγ signaling pathway. Therefore, a pharmacological regulator of miR-330-5p may represent a therapeutic strategy for obesity. Thirdly, the present study employed an H_2O_2-induced adipogenic differentiation of MSCs and provided an example of highly efficient miRNA identification and functional dissection of the miRNA/RXRγ signaling pathway regulating stem cell fate. Further studies on the identified miRNA/RXRγ network will aid in understanding the critical molecular switches in adipogenic differentiation of MSCs, and facilitate the characterization of the miRNA basis of obesity and associated disorders as well as the development of novel therapies to treat them.

In conclusion, the present study aimed to detect the effect of miR-330-5p on the adipogenic differentiation of MSCs under oxidative stress and searching for its target genes. The results of the present study revealed that miR-330-5p functions as a negative regulator of H_2O_2-induced adipogenic differentiation of MSCs by repressing RXRγ expression. miR-330-5p should be considered an important candidate molecular target of adipogenic differentiation of MSCs for the development of preventive or therapeutic approaches against obesity and its associated disorders.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

WH performed the experiments and wrote the manuscript; KL performed the experiments; AL performed experimental technical guidance; ZY provided the reagents/materials; CH analyzed the data; DC and HW conceived and designed the experiments. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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