

LncRNA HCP5 Promotes ADSC Adipogenic Differentiation via Its Sponging of miR-27a-3p and a Concomitant Increase in PPAR γ Expression

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Research Article

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Abstract

Current studies have shown that lncRNA plays an essential regulatory role in the physiological metabolism of cells. lncRNA HCP5 promotes proliferation and invasion in tumor cells and regulates differentiation in some stem cells. Our study was designed to determine the effects of HCP5 on the adipogenic differentiation of adipose-derived stem cell (ADSCs) and explore its underlying molecular mechanisms. ADSCs are known for their pluripotent differentiation potential and ready availability, making them promising intermediates for cell-based tissue damage repair. Our data revealed that HCP5 expression was elevated in differentiation-induced ADSCs, and that its overexpression promoted adipogenic differentiation in these cells. Then we predicted and then confirmed the effects of targeted interaction between HCP5 and miR-27a-3p. We also predicted and confirmed that miR-27a-3p demonstrated some binding affinity for PPAR γ and then used this information to design a gain-and-loss-of-function experiment to verify that HCP5 regulates ADSCs adipogenic differentiation via its regulation of miR-27a-3p and PPAR γ . These experiments showed that HCP5 overexpression promotes adipogenic differentiation in ADSCs, which was impaired by the upregulation of miR-27a-3p. Overexpression of miR-27a-3p inhibited PPAR γ expression, and overexpression of HCP5 restored this inhibitory effect. Finally, transfection with PPAR γ siRNAs reduced the adipogenic promotion due to HCP5 overexpression in these cells. Taken together our data suggest that the HCP5/miR-27a-3p/PPAR γ axis may be a major regulator of adipogenic differentiation in ADSCs, which might be the probable molecular mechanism underlying the effects of HCP5 in these cells.

Introduction

Stem cells have the potential for pluripotent differentiation and have promising research prospects in tissue damage repair. This study aimed mainly to find the mechanisms of stem cell differentiation at the molecular level. Mesenchymal stem cells (MSCs), pluripotent cells derived from the mesoderm, can differentiate into multiple tissues, including bone, cartilage, fat, muscle, and tendons, and have been increasingly mentioned in studies related to tissue repair. Experimental MSCs have been isolated from various sources, including umbilical cords, bone marrow, and adipose tissues [1]. Of these, adipose-derived stem cells (ADSCs), which are extracted from adipose tissues, are among the most commonly used because they are easily extracted with minimal invasion or tissue damage and can be obtained in reasonably large quantities. Animal models of ischemic disease have shown that ADSCs function to promote angiogenesis and reconstruction [2], and the transplantation of ADSCs has demonstrated excellent therapeutic outcomes when applied to studies treating both peripheral and central nerve injury models [3, 4]. ADSCs also play an essential role in repairing tissues such as skeletal and smooth muscle [5, 6]. There has been an explosion of interest in ADSC differentiation in the treatment of tissue damage, with several of these interventions even entering the clinical evaluation stage [7]. However, the mechanisms underlying ADSC differentiation are poorly defined. A study of the mechanism of ADSC adipogenic differentiation contributes to the better application of ADSCs in tissue repair .

Long non-coding RNAs (lncRNA) are a class of non-coding RNAs that are more than 200 nucleotides in length and are capable of participating in multiple cellular and physiological processes, often playing essential regulatory roles in many diseases [8]. Cao et al. found that lncRNA RMRP promotes bladder cancer cells proliferation and invasion [9], whereas Liao et al. suggested that lncRNA is involved in the proliferation and differentiation of various inflammatory cells and the secretion of several inflammatory factors [10]. lncRNA MEG3 regulates apoptosis of adipose-derived stem cells [11]. Several reports also suggest that these lncRNAs are involved in the regulation of stem cell differentiation. MALAT1 promotes BMSC differentiation into endothelial cells [12], whereas PRNCR1 affects the osteogenic differentiation of MSCs by modulating CXCR4 expression [13]. HCP5 (human leukocyte antigen (HLA) Complex P5) is also a type of lncRNA. Recent studies have focused on its regulation of cancer cells where it promotes proliferation and invasion [14–17], and HCP5 also regulates the differentiation of some cells. HCP5 has recently been reported to facilitate epithelial-mesenchymal transition in colorectal cancer [18] and was linked to osteoblastic differentiation of BMSCs, in a study evaluating osteoporosis [19, 20]. Chen et al.'s study on childhood obesity showed that HCP5 promotes adipogenic differentiation [21], but the molecular mechanisms underlying this regulation remain unknown.

miRNAs are a class of endogenous single-stranded non-coding RNA molecules that are 20-22 nucleotides in length and known to regulate the expression of their target genes via their 3' untranslated regions. miRNAs play a regulatory role in many cellular and physiological processes, such as in self-repair, signal transduction, and cell differentiation [22]. In experiments using both human and mouse-derived BMSCs, miRNA-130a promoted osteogenic differentiation and inhibited adipogenic differentiation [23]. *In vitro* experiments conducted by Chen et al. showed that mir-363 inhibits both mitosis and adipose differentiation in ADSCs [24], whereas Kim et al.'s *in vivo* studies identified miRNA-27a as a repressor of lipogenic differentiation [25]. Guo et al. found that miRNA-27a-3p binds specifically to PPAR γ (peroxisome proliferator-activated receptor) and suppresses its expression [26], which is significant since PPAR γ , a nuclear hormone receptor, acts as the major transcription factor for adipogenic differentiation [27]. Although the targeted regulatory relationship between miRNA-27a-3p and PPAR γ has been reported, the mechanism by which miRNA-27a-3p affects adipogenic differentiation in ADSCs and its regulation of PPAR γ requires further verification.

The theory of ceRNA (competing endogenous RNAs)-mediated regulation, first proposed by Salmena et al. in 2011, suggests that various RNA transcripts interact and effect a complex regulatory network in cells. This has been confirmed in several studies and has gradually become a research hotspot [28]. lncRNAs can regulate the development and progression of multiple diseases when they act as ceRNAs [29], with many of these effectors acting as an miRNA sponge, allowing for complex dynamic regulation of the target gene [30]. We speculated that there might be a ceRNA relationship between HCP5 and miRNA-27a-3p. In this study, we experimentally verified the regulatory relationship between HCP5, miRNA-27a-3p and PPAR γ to further discuss the molecular mechanism of ADSCs adipogenic differentiation.

Methods And Materials

Cell culture and differentiation

Human ADSCs were purchased from the Cell Bank (Shanghai Institutes, China) for Biological Sciences and were cultured in DMEM/F12 1:1 medium (Gibco; Thermo Fisher Scientific, Inc. USA) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc. USA). Cells were incubated in a humidified chamber with a temperature of 37 °C and a CO₂ concentration of 5%. Adipogenic differentiation was induced in the cells by incubating them in DMEM/F12 medium supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 5 μg/mL insulin at 37 °C for 2 days. This medium was then replaced with DMEM/F12 supplemented with 10% FBS and 5 μg/mL insulin every two days.

Transfection

Human HCP5 sequence (GenBank, NR_040662.1) was synthesized and subcloned into a pcDNA3.1 vector (Genepharma, Shanghai, China) for transfection, with the empty vector acting as a vehicle control. HCP5 knockdown relied on a lentiviral vector, pGLVH1/GFP+Puro (Genepharma, Shanghai, China), expressing an shRNA against HCP5. The sequences of the shRNA and its negative control were as follows: 5'-GCAGTGTGCTTCCTTCCTT-3' and 5'-TTCTCCGAACGTGTCACGT-3'. The sense and anti-sense sequences for the miR-27a-3p mimics (Genepharma, Shanghai, China) were 5'-UUCACAGUGGCUAAGUCCGC-3' and 5'-GGAACUUAGCCACUGUGAAUU-3'. The sequences of the sense and anti-sense negative control were 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'. The sequence of the miR-27a-3p inhibitor (Genepharma, Shanghai, China) was 5'-GCGGAACUUAGCCACUGUGAA-3', and that of its negative control was 5'-CAGUACUUUUGUGUAGUACAA-3'. The sequences of the siRNA against PPAR γ and its negative control were 5'-AGUUUGCUGUGAAGUCAAUG-3' and 5'-UUGAACUUCACAGCAAACUCA-3'. HCP5 interactions with miR-27a-3p were evaluated using co-transfection of the HCP5 vector (or NC vector) and the miR-27a-3p mimics (or mimics NC) or the HCP5 shRNA (or shRNA NC) and miR-27a-3p inhibitor (or inhibitor NC). Interactions between HCP5 and PPAR γ were evaluated using co-transfections of the HCP5 vector (or NC vector) and PPAR γ siRNAs (or siRNA NC). All cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and all subsequent experiments were completed 48 h post-transfection.

Oil Red O staining

ADSCs were cultured in induction medium and then stained with oil-red O at 0, 2, 4, 6 and 8 days to evaluate adipogenic differentiation in response to changes in HCP5 expression. Next, we evaluated whether HCP5 regulates adipogenic differentiation in ADSCs via its interaction with miR-27a-3p. Therefore, we co-transfected ADSCs with pcDNA3.1-HCP5 and miR-27a-3p mimics. We also co-transfected ADSCs with HCP5 shRNA and an miR-27a-3p inhibitor and then subjected the cells to 4 days of culture in the adipose induction medium. We then evaluated their adipogenic differentiation using oil red O staining. After removing the induction medium, the ADSCs were washed with PBS twice. They were

fixed with 4% paraformaldehyde for 30 min at 20 °C, following which, the paraformaldehyde was removed by washing the cells twice in PBS. We then treated the cells with Oil Red O solution for 30 min and then washed the cells three times with PBS. After staining, plates were placed on a white background. Photos were taken to observe and compare the number of red-stained cells.

Immunofluorescence

Using immunofluorescence studies, we evaluated the expression of PPAR γ in cells. Cells were fixed using 4% paraformaldehyde for 30 min at 20 °C, following which, the paraformaldehyde was removed by washing the cells twice in PBS. They were then incubated with primary anti-PPAR γ antibody (ab209350, Abcam, Cambridge, UK) and primary anti- β tubulin antibody (ab78078, Abcam, Cambridge, UK) at 4 °C for 8 h, washed, and then incubated with goat anti-rabbit IgG (FITC) secondary antibody (ab6717, Abcam, Cambridge, UK) and goat anti-mouse IgG (DyLight549) secondary antibody (a23310, Abbkine, California, USA) for 1 h at 20 °C. Next, the cells were counterstained with DAPI (c0060, Solarbio) for 5 min and visualized using a fluorescence microscope (400 \times) fitted with a camera (Olympus, Japan).

qRT-PCR

Trizol reagent (Invitrogen; Thermo Fisher Scientific) was used to extract each group's total RNA, and the quantity and quality of this RNA were then evaluated using a Nanodrop ND-2000 spectrophotometer. We used 2 μ g of total RNA for both the mRNA and lncRNA expression analysis, which was reverse transcribed to cDNA. A RevertAid First Strand cDNA Synthesis kit (Thermo Scientific™, USA) was used. The template used for the miRNA analysis was reverse transcribed into cDNA using a miDETECT A Track™ qRT-PCR kit (RiboBio, Guangzhou, China). SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA) was used to perform the qRT-PCR according to the manufacturer, using an ABI 7500 (Applied Biosystems, Foster, CA, USA). Primer sequences for HCP5 were as follows: forward 5'-TATCCCTGTGAAGATGAACC-3', reverse 5'-TGCCACCTCTAAATGTCCTA-3'. Primer sequences for PPAR γ were as follows: forward 5'-GACGAGCTCCAGAAAAGTCCCAGTCGCTGACAAAG-3', reverse: 5'-CATCTCGAGTA TTAAAAGTAAATTGTAAATGTATC-3'. Primer sequences for miR-27a-3p: forward 5'-GCCGCTTCACAGTGGCTAA-3', reverse: 5'-GTGCAGGGTCCGAGGTATTC-3'. Primer sequences for GAPDH were as follows: forward 5'-CGGAGTCAACGGATTTGGTCG-3', reverse 5'-TCTCGCTCCTGGAAGATGGTGAT-3'. Primer sequences for U6 were as follows: forward 5'-CTCGCTTCG GCAGCAGCACATATA-3 , reverse: 5'-AAATATGGAACGCTTCACGA-3 . Either GAPDH or U6 was used as the control depending on the assay, and relative expression was calculated using the 2- $\Delta\Delta$ Ct method. Each evaluation was repeated three times.

Luciferase reporter assay

We then validated these predictions using a dual-luciferase reporter assay using luciferase reporters with both the WT and mutant binding sequence from HCP5 and the WT and mutant binding sequences from PPAR γ . A luciferase-based reporter assay was performed to evaluate the specific interactions within our ceRNA network. We constructed two PGL3 (Promega, Madison, USA) reporter constructs. The wild-type

(WT) HCP5 or the mutant (Mut) HCP5 sequence was cloned. We constructed a second similar set of reporters to evaluate miR-27a-3p binding of PPAR γ , with the WT PPAR γ or Mut PPAR γ sequence inserted. ADSCs were then cultured and transfected in a 96-well plate. Forty-eight hours after transfection, the cells were harvested, and the luciferase activity was measured using a Dual-Luciferase Reporter Assay kit (Promega, Madison, USA). These assays were performed in triplicate.

Western blotting

Cells were lysed by RIPA (Beyotime Institute of Biotechnology, Haimen, China) . The BCA protein assay kit (Wanleibio, Co., Ltd., Shanghai, China) was used to quantify the harvested protein. A total of 20 μ g/lane protein were separated using 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% skimmed milk powder in Tris-buffered saline-0.1% Tween-20 (TBST) at 20 °C for 2 h. Then the membranes were incubated with following primary antibodies overnight at 4 °C: anti-PPAR γ (ab209350, Abcam, Cambridge, UK, 1:1000) and anti-GAPDH (ab9485, Abcam, Cambridge, UK, 1:2000). The membranes were then washed three times in TBST and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ab288151, Abcam, Cambridge, UK, 1:10000) for 2 h at 20 °C. Finally, the protein bands were visualized using a chemiluminescence detection system (SuperSignal™ West Pico PLUS, Thermo Fisher Scientific, Inc.). Image-pro Plus software (version 6.0; Media Cybernetics, Inc.) was used to quantified protein expressions.

Statistical analysis

All continuous variables are described as the mean \pm SEM, and all statistical analyses were performed using SPSS version 13.0 software (SPSS, Inc.). Differences between groups were evaluated using an unpaired Student's t-test, and statistical significance was set at a P-value of < 0.05. All experiments were performed in triplicate.

Results

HCP5 promotes adipogenic differentiation in ADSCs

On performing oil red O staining, we noted that increases in induction time led to increased oil red O staining in the treated adipocytes (Fig. 1A). Therefore, we examined the relationship between adipogenesis and HCP5 expression by quantifying HCP5 expression at each time point using qRT-PCR. The results of these assays showed that the expression levels of HCP5 gradually increased with increasing induction time (Fig. 1B). These results suggest that HCP5 expression may be linked to adipogenesis in these cells and support our hypothesis that this lncRNA plays an important role in the adipogenic differentiation of ADSCs. We then confirmed this by transfecting ADSCs with pcDNA3.1-HCP5 and an empty vector control (NC) and comparing the oil red O staining from these two groups following 4 days of induction (Fig. 1C, D). We found more red-stained cells in the pcDNA3.1-HCP5 group than in the NC group, suggesting that HCP5 plays a distinct role in promoting the adipogenic differentiation of ADSCs.

<Figure 1>

Prediction and validation of the targeting effects of HCP5 and miR-27a-3p

We then explored the underlying mechanism of action for HCP5 in these cells. We predicted the targets of HCP5, which identified miR-27a-3p as an important potential target (Fig. 2A). In further predictions, we found that PPAR γ is a potential target for miR-27a-3p (Fig. 2B), allowing us to begin to develop a potential regulatory axis. Dual-luciferase reporter assays using luciferase reporters showed that the fluorescence activity of miR-27a-3p was significantly reduced in HCP5-Wt-transfected cells compared with that in HCP5-Mut cells (Fig. 2C, $p < 0.05$) and that the fluorescence activity of miR-27a-3p was significantly reduced in PPAR γ -Wt compared to PPAR γ -Mut (Fig. 2D, $p < 0.05$).

<Figure 2>

We then confirmed that miR-27a-3p and PPAR γ were involved in the adipogenic differentiation of ADSCs by examining their expression in the cells at days 0, 2, 4, 6 and 8 of induction. qRT-PCR showed that miR-27a-3p expression gradually decreased with increasing induction time (Fig. 2E) whereas western blotting showed that PPAR γ expression gradually increased with increasing induction time (Fig. 2F).

We further confirmed whether the expression of HCP5 and PPAR γ was associated with the expression of miR-27a-3p by transfecting a series of ADSCs with miR-27a-3p or NC mimics. qRT-PCR revealed that miR-27a-3p expression was significantly increased in ADSCs treated with the miR-27a-3p mimics compared to that in the NC mimic group (Fig. 2G, $p < 0.05$). qRT-PCR also revealed that HCP5 expression was significantly lower in the miR-27a-3p mimic group compared to that in the NC group (Fig. 2H, $p < 0.05$), confirming the interaction between these two transcripts. Western blotting revealed that PPAR γ expression was significantly decreased in cells treated with the miR-27a-3p mimic compared to that in the NC group (Fig. 2I, $p < 0.05$), confirming the interactions between miR-27a-3p and PPAR γ . Taken together, these results suggest a direct regulatory interaction between HCP5, miR-27a-3p, and PPAR γ in ADSCs.

Hcp5 Modulates The Expression Of Mir-27a-3p And Ppar γ

To further investigate the regulatory effect of HCP5 on miR-27a-3p and PPAR γ in ADSCs, we transfected pcDNA3.1-HCP5 and HCP5 shRNA into ADSCs, respectively. RT-qPCR tests showed that HCP5 knockdown increased miR-27a-3p expression levels significantly (Fig. 3A, $p < 0.05$). However, the upregulation of HCP5 significantly reduced the expression levels of miR-27a-3p (Fig. 3C, $p < 0.05$). The detection of PPAR γ protein by Western blotting showed that PPAR γ was significantly reduced at HCP5 knockdown (Fig. 3B, $p < 0.05$) and that PPAR γ was significantly increased when HCP5 was upregulated (Fig. 3D, $p < 0.05$). These results suggest that HCP5 inhibits the miR-27a-3p expression and promotes PPAR γ expression.

<Figure 3>

HCP5 regulates adipogenic differentiation in ADSCs where it acts as a ceRNA

Following the downregulation of HCP5, adipogenic differentiation in the HCP5 shRNA + inhibitor NC group was significantly decreased compared to that in the shRNA NC + inhibitor NC group. However, this decrease in adipogenic differentiation was weakened by the concomitant downregulation of miR-27a-3p (Fig. 4A). In contrast, upregulation of HCP5, as demonstrated by the pcDNA3.1-HCP5 + mimics NC group, promoted the adipogenic differentiation of cells compared to that in the pcDNA3.1 vector + mimic NC control. In contrast, upregulation of miR-27a-3p expression in the pcDNA3.1-HCP5+miR-27a-3p mimics group significantly impaired this effect compared with the pcDNA3.1-HCP5 + mimics NC group (Fig. 4B).

<Figure 4>

Then, we evaluated the competitive regulation of PPAR γ effected by the interactions between HCP5 and miR-27a-3p by evaluating the changes in PPAR γ expression in each group of cells using both western-blotting and immunofluorescence staining. The results revealed that PPAR γ expression was significantly increased in the shRNA NC + miR-27a-3p inhibitor group compared to that in the shRNA NC + inhibitor NC group and in the HCP5 shRNA + inhibitor NC group compared to that in the shRNA NC + miR-27a-3p inhibitor group (Fig. 4C, D). In contrast, PPAR γ expression was significantly reduced in the pcDNA3.1 vector + miR-27a-3p mimics group compared to that in the pcDNA3.1 vector + mimics NC group. However, the inhibitory effect of miR-27a-3p was significantly reduced following the upregulation of HCP5 as demonstrated by the comparison between the pcDNA3.1-HCP5 + miR-27a-3p mimics group and the pcDNA3.1 vector + miR-27a-3p mimics group (Fig. 4E, F). These results suggest that HCP5 acts as a ceRNA for miR-27a-3p and uses this mechanism to modulate PPAR γ expression.

We then validated this regulatory network by co-transfecting ADSCs with pcDNA3.1-HCP5 and PPAR γ siRNA and then staining each group of cells with oil-red O to observe the extent of adipogenic differentiation. The number of adipogenic cells in the pcDNA3.1-HCP5 + PPAR γ siRNA group was significantly lower than that in the pcDNA3.1-HCP5 + siRNA NC group (Fig. 4G, $p < 0.05$), suggesting that PPAR γ knockdown markedly reduced the HCP5 overexpression-induced promotion of adipogenic differentiation. These results support our hypothesis that HCP5 promotes ADSC adipogenic differentiation by promoting the expression of PPAR γ .

Discussion

Several recent studies have shown that lncRNAs play various essential regulatory roles in many diseases and biological functions with many of the physiological activities of stem cells, including differentiation, regulated by these transcripts [31]. Li et al. reported that MEG3 inhibits the adipogenic differentiation of ADSCs and promotes their osteogenic differentiation [32], while Liu et al. reported that TINCR regulation was critical to their adipogenic differentiation [33]. HCP5 expression was upregulated in response to adipogenesis in ADSCs. Consequently we proposed that HCP5 is involved in the regulation of their adipogenic differentiation. We validated this hypothesis in an overexpression experiment, which showed that upregulation of HCP5 significantly increased adipogenic differentiation in the ADSCs.

We predicted that HCP5 could target miR-27a-3p, which is known to play a regulatory role in multiple physiological processes [34, 35]. miR-27a-3p promotes the osteogenic differentiation of MC3T3-E1 cells [36], and Shi et al. reported that miR-27a-3p is involved in adipogenic differentiation and obesity in humans [37]. Our experiments revealed that adipogenesis decreased the expression of miR-27a-3p in ADSCs. The result suggested the involvement of miR-27a-3p in regulating the adipogenic differentiation of ADSCs and its downstream targets remained to be further explored. Our prediction experiments showed that miR-27a-3p targeted PPAR γ , which is a significant regulator of adipogenesis [38, 39] and whose levels were significantly increased in our adipogenic induction assay. Transfection with an miR-27a-3p mimic revealed that the expression of both HCP5 and PPAR γ was inhibited by increased expression of this miRNA, confirming the likely interaction between these three effectors.

We then used overexpression and silencing assays to explore the regulatory effects of HCP5 on miR-27a-3p and PPAR γ . Our data revealed that miR-27a-3p expression was significantly inhibited, and PPAR γ expression was induced in response to increased HCP5 expression. However, HCP5 silencing induced a significant increase in miR-27a-3p expression and a significant decrease in PPAR γ . This suggests that miR-27a-3p expression is negatively correlated with HCP5, while PPAR γ expression is positively correlated with HCP5. We speculate that miR-27a-3p may be an intermediate mediator of HCP5 regulation of PPAR γ .

Various recent studies have linked HCP5 to the regulation of various physiological activities such as proliferation, migration, invasion, and EMT. Moreover, reports suggest that this regulation is mediated by the fact that HCP5 acts as a gene sponge for effector miRNAs, thus regulating the expression of their downstream targets [40]. We also found that silencing of HCP5 inhibited ADSCs adipogenic differentiation, while knockdown of miR-27a-3p restored this adipogenic effect. Moreover, we found that miR-27a-3p had an inhibitory effect on PPAR γ expression, and that the overexpression of HCP5 restored its expression, while miR-27a-3p knockdown induced a marked increase in PPAR γ . However, this upward trend was significantly reduced when HCP5 expression was downregulated. This result confirmed that the competitive binding of HCP5 to miR-27a-3p regulates ADSCs adipogenic differentiation likely via changes in its regulation of PPAR γ .

To confirm this, we then co-transfected ADSCs with pcDNA3.1-HCP5 (or pcDNA3.1 vector) and PPAR γ siRNA (or siRNA NC), which revealed a clear promoting effect for HCP5 during adipogenesis that was significantly reduced in cells with reduced PPAR γ . This result confirmed that HCP5 promotes adipogenesis by promoting the expression of PPAR γ .

Taken together, our data confirm that HCP5 promotes adipogenic differentiation of ADSCs. Further investigations of its mechanism revealed that HCP5 acts as a ceRNA for miR-27a-3p, competitively inhibiting its expression and thereby regulating the expression of its downstream target gene, PPAR γ . Stem cell differentiation relies on a series of complex regulatory networks, but our study is the first to identify the HCP5/miR-27a-3p/PPAR γ axis, which may be a critical mechanism in the regulation of adipogenic differentiation in ADSCs. Whether similar mechanisms exist in other stem cells remains to be

evaluated. Alternatively, this study was only performed only at the cellular level, and the action process of this mechanism in vivo still requires further experiments and validation.

Declarations

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Figures

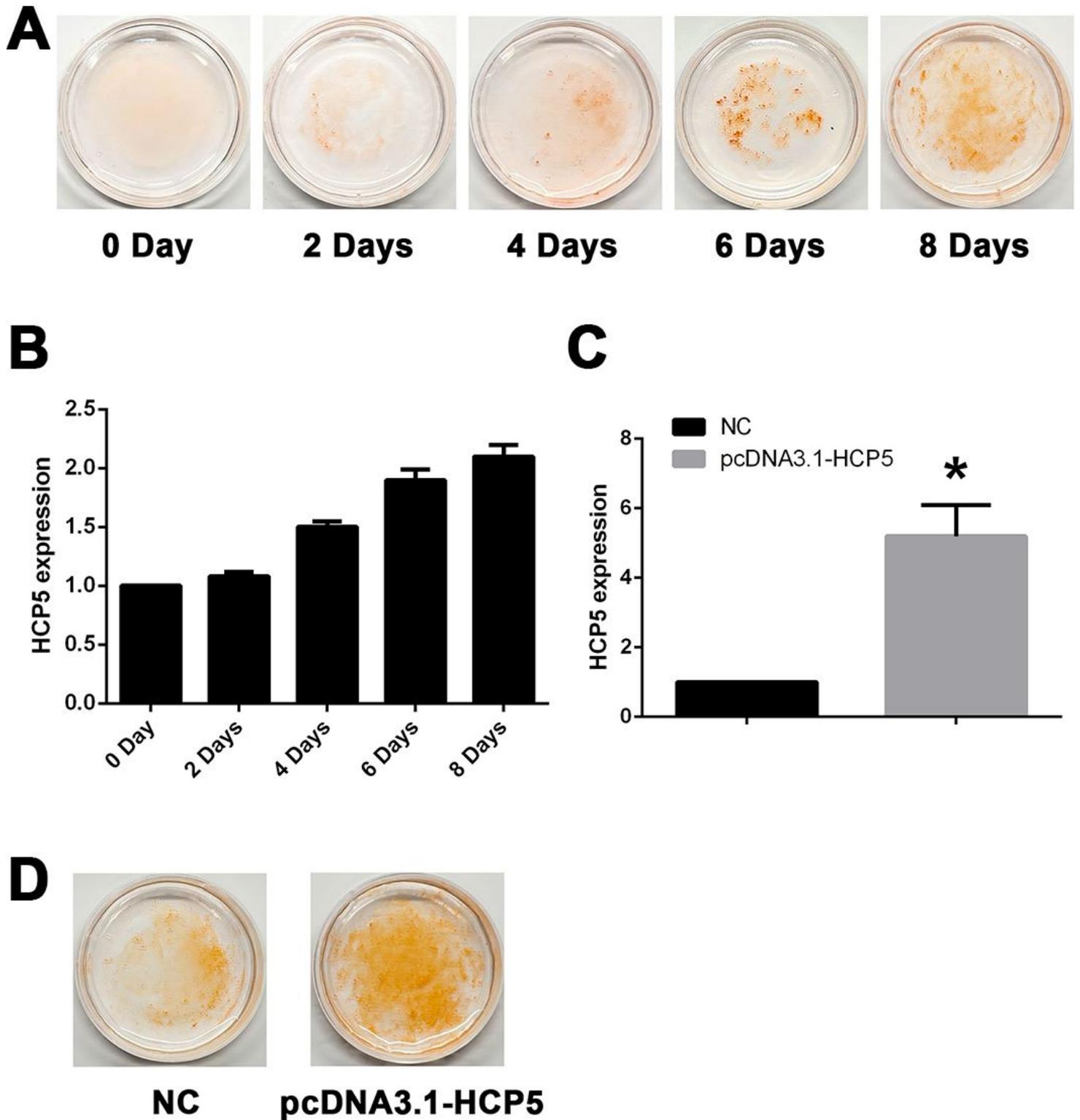


Figure 1

HCP5 promotes adipogenic differentiation in ADSCs

(A) Adipogenic-induced ADSCs were oil-red O stained and photographed at each induction time point (0, 2, 4, 6 and 8 days). (B) HCP5 expressions at each induction time point (0, 2, 4, 6 and 8 days) were quantified using qRT-PCR. (C) HCP5 expressions of transfected ADSCs ($*p < 0.05$). (D) Adipogenic differentiation of pcDNA3.1-HCP5 and NC group ADSCs was determined by oil-red O staining.

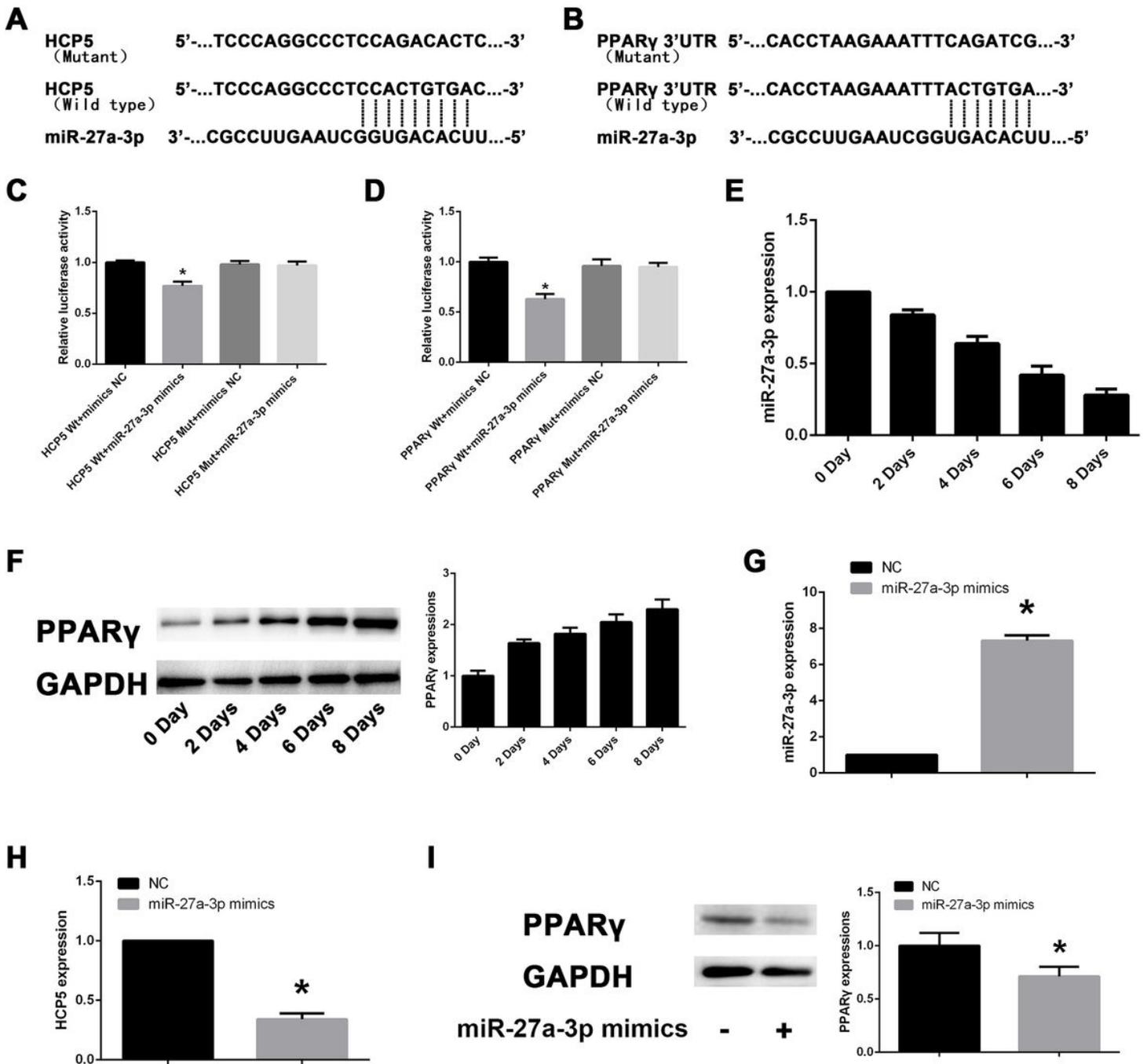


Figure 2

Prediction and validation of the targeting effects of HCP5 and miR-27a-3p

(A, B) The predicted binding sites of between HCP5 and miR-27-3p, PPAR γ and miR-27-3p. The wild type or mutant of HCP5 or PPAR γ -3'UTR were inserted into luciferase reporter vector, respectively, and then co-transfected with miR-27a-3p mimic or NC. (C,D) The luciferase activities were detected after 48h transfection ($*p<0.05$). (E) miR-27a-3p expressions at each induction time point (0, 2, 4, 6 and 8 days) were quantified using qRT-PCR. (F) PPAR γ expressions at each induction time point (0, 2, 4, 6 and 8 days) were detected by western blotting. (G, H) Expressions of miR-27a-3p and HCP5 were quantified by qRT-PCR, respectively, after miR-27a-3p mimics were transfected ($*p<0.05$). (I) Expressions of PPAR γ were detected by western blotting after miR-27a-3p mimics were transfected ($*p<0.05$).

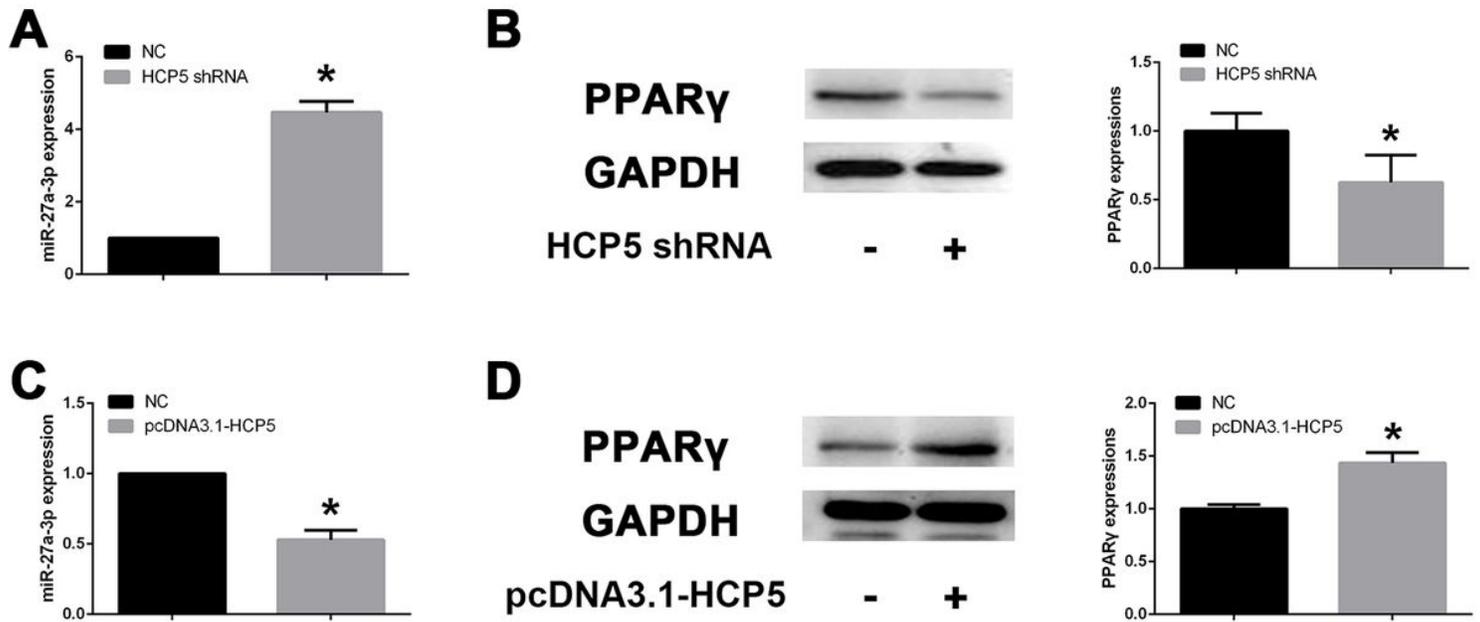


Figure 3

HCP5 modulates the expression of miR-27a-3p and PPAR γ

(A) Expressions of miR-27a-3p were quantified by qRT-PCR after HCP5 shRNA were transfected ($*p<0.05$). (B) Expressions of PPAR γ were detected by western blotting after HCP5 shRNA were transfected ($*p<0.05$). (C) Expressions of miR-27a-3p were quantified by qRT-PCR after pcDNA3.1-HCP5 were transfected ($*p<0.05$). (D) Expressions of PPAR γ were detected by western blotting after pcDNA3.1-HCP5 were transfected ($*p<0.05$).

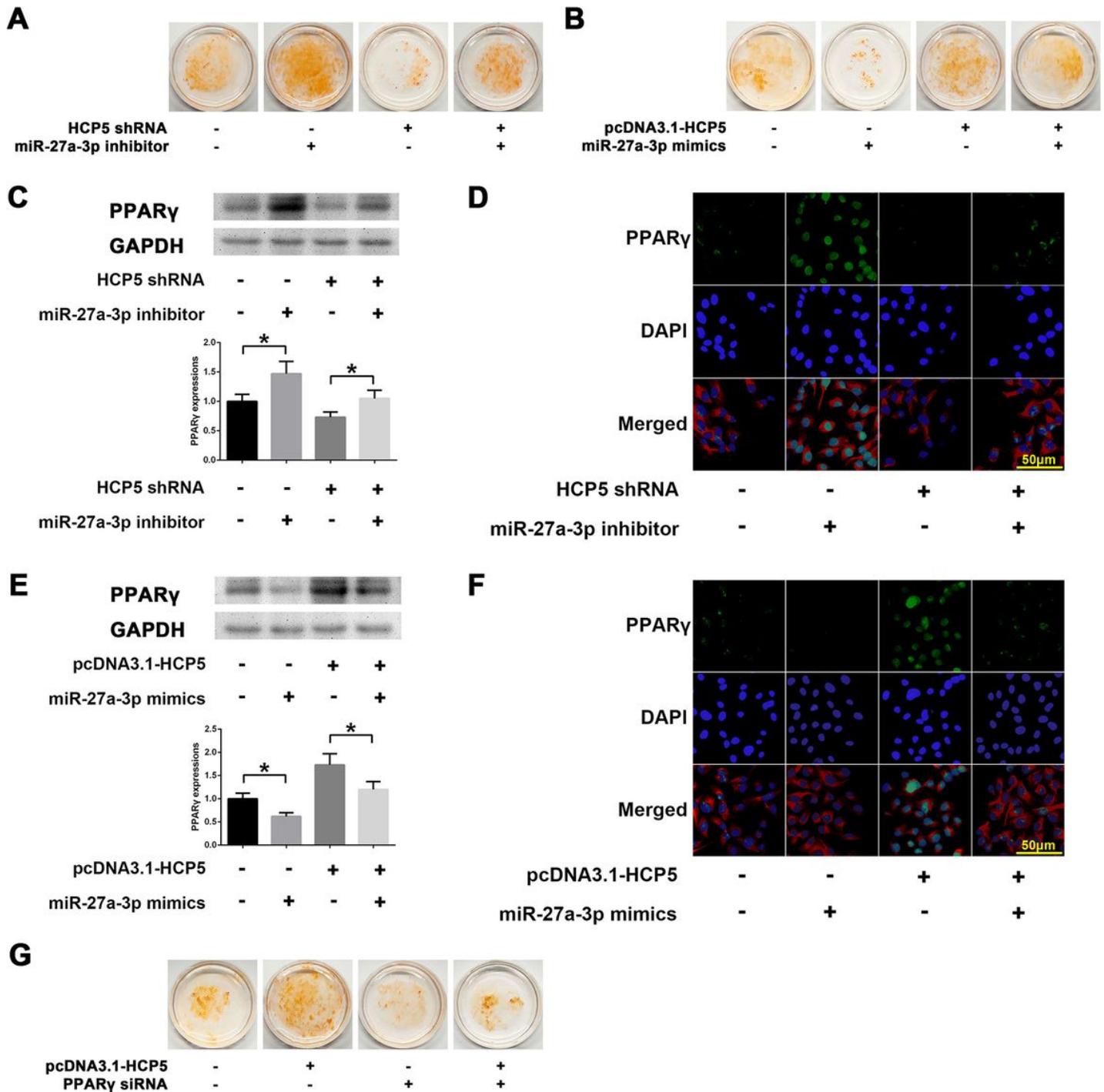


Figure 4

HCP5 regulates adipogenic differentiation in ADSCs where it acts as a ceRNA

ADSCs were co-transfected by the HCP5 shRNA (or shRNA NC) and miR-27a-3p inhibitor (or inhibitor NC) or the HCP5 vector (or NC vector) and the miR-27a-3p mimics (or mimics NC). (A, B) Adipogenic differentiation of ADSCs in each group was determined by oil-red O staining. Cells labeling PPAR γ were incubated with primary anti-PPAR γ antibody followed by goat anti-rabbit IgG (FITC) secondary antibody

(green). The nuclear counter stain was DAPI (blue). Primary anti- β tubulin antibody was followed by goat anti-mouse IgG (DyLight549) secondary antibody (red). (C, E) Expression of PPAR γ in each group was detected by western blotting ($*p<0.05$). (D, F) Expression of PPAR γ in each group was detected by Immunofluorescent analysis. (G) ADSCs were co-transfected by the HCP5 vector (or NC vector) and PPAR γ siRNAs (or siRNA NC) and oil-red O stained.