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Original Article

MicroRNA-142a-3p promotes the differentiation of 3T3-L1 preadipocytes by targeting high-mobility group AT-hook 1

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Abstract: Background: Obesity is characterized by the excess accumulation of adipose tissues, mainly composed of adipocytes. The differentiation of adipocytes is one of the major events in the process of adipogenesis. Among various adipogenic transcription factors, CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferators-activated receptor γ (PPARγ) have been identified as essential regulators of adipocyte differentiation. Methods: RT-qPCR assay was conducted to detect the expression of microRNA-142a-3p (miR-142a-3p), high-mobility group AT-hook 1 (HMGA1) mRNA, C/EBPα mRNA, and PPARγ mRNA. Western blot assay was performed to measure the protein levels of HMGA1, C/EBPα and PPARγ. Bioinformatics analysis and luciferase reporter assay were carried out to explore the interaction between miR-142a-3p and HMGA1. Results: miR-142a-3p expression was notably increased and HMGA1 expression was markedly reduced during 3T3-L1 preadipocyte differentiation. Functional analysis revealed that miR-142a-3p overexpression promoted 3T3-L1 preadipocyte differentiation. Further investigations on molecular mechanisms showed that HMGA1 was a target of miR-142a-3p in 3T3-L1 preadipocytes. Moreover, the knockdown of HMGA1 induced 3T3-L1 preadipocyte differentiation. Additionally, HMGA1 silencing abolished miR-142a-3p deficiency-mediated inhibitory effect on 3T3-L1 preadipocyte differentiation. Conclusion: MiR-142a-3p overexpression facilitated 3T3-L1 preadipocyte differentiation by targeting HMGA1, highlighting the importance of miR-142a-3p, HMGA1 and the miR-142a-3p/HMGA1 axis in adipogenesis.

Keywords: MicroRNA-142a-3p, high-mobility group AT-hook 1, adipogenesis, 3T3-L1 preadipocytes

Introduction

Obesity, characterized by the excess accumulation of adipose tissues, is one of the commonest globally public health problems with massive economic burdens for individuals, families and nations [1, 2]. Adipocytes, the main cellular component of adipose tissues, play critical roles in the regulation of metabolism in obesity [3, 4]. Adipogenesis is a complicated process during which fibroblast-like preadipocytes can differentiate into mature adipocytes [5]. Adipocyte differentiation is tightly regulated by various molecules such as transcription factors and histone-modifying enzymes [6-8].

MicroRNAs (miRNAs) are a group of endogenous non-coding RNAs with a length of about 22 nucleotides that can negatively regulate gene expression at posttranscriptional levels [9, 10]. Also, miRNAs have been demonstrated to be implicated in the pathogenesis of many diseases including obesity [11, 12]. Moreover, emerging studies show that miRNAs function as critical regulators during adipocyte differentiation by targeting adipogenic transcription factors and crucial signaling molecules [13, 14]. Recent studies showed that microRNA-142 (miR-142) plays multifaceted roles in different biologic processes such as organogenesis and homeostasis [15]. MicroRNA-142-3p (miR-142-3p), generated from the 3' terminus of miR-142 precursor, was highly expressed in the blood of patients with obesity [16-18]. Also, mmu-miR-142-3p level was markedly increased in white adipose tissues of mice fed with long-term high-fat diet compared with mice fed with a standard diet [19].

High-mobility group AT-hook 1 (HMGA1), a regulator of chromatin structure, has been reported to be involved in the etiology of multiple diseases such as cancers, diabetes and myocardial infarction [20-22]. Also, database analysis revealed that HMGA1 was associated with human obesity [23]. Moreover, previous findings showed that HMGA1 played key roles in the differentiation of adipocytes [24-26]. Additionally, HMGA1 has been identified as a target of miR-142-3p in human osteosarcoma [27].

Hence, the effects of miR-142a-3p and HMGA1 on the differentiation of adipocytes were further investigated. Our results showed that miR-142a-3p facilitated the differentiation of adipocytes by targeting HMGA1, hinting at the key roles of miR-142a-3p/HMGA1 axis in adipogenesis and providing some potential targets for the therapy of obesity.

Materials and methods

Preadipocyte culture and differentiation

Mouse 3T3-L1 preadipocytes were purchased from American Tissue Culture Collection (ATCC, Manassas, VA, USA) and were maintained in DMEM medium (Thermo Scientific, Rockford, IL, USA) containing 10% newborn calf serum (NCS, Thermo Scientific) (Basal medium I) in a 5% CO_2 incubator at 37°C prior to differentiation (normal culture conditions). The differentiation of 3T3-L1 preadipocytes was induced following the protocols as previously described [28]. Briefly, to induce differentiation, untransfected or transfected 3T3-L1 preadipocytes were cultured in differentiation medium I for 2 days and then maintained in differentiation medium II for another 2 days. Following this, 3T3-L1 preadipocytes were grown in Basal medium II for an additional 4 days.

Reagents and cell transfection

miR-142a-3p mimic (miR-142a-3p), miR-142a-3p inhibitor (anti-miR-142a-3p), small interference RNA targeting HMGA1 (siHMGA1) and negative control (NC) were obtained from GenePharma Co. ltd (Suzhou, China). All oligonucleotides or plasmids were transfected into 3T3-L1 preadipocytes using ribo Fect CP Transfection Kit (Ribobio, Guangzhou, China) referring to the instructions of manufacturer.

Luciferase reporter assay

Wild type or mutant type HMGA1 3'UTR luciferase reporter plasmids (HMGA1 WT 3'UTR or HMGA1 MUT 3'UTR) containing wild or mutant miR-142a-3p binding sites were purchased from TsingKe Biotech (Wuhan, China). Then, HMGA1 WT 3'UTR or HMGA1 MUT 3'UTR reporter was transfected into 3T3-L1 preadipocytes together with miR-142-3p mimic, anti-miR-142-3p, or their negative control. Luciferase activities were measured through a dual luciferase reporter assay kit (Promega, Madison, WI, USA) at 48 h following transfection.

RT-qPCR assay

Total RNA was extracted from 3T3-L1 preadipocytes using Trizol reagent (Thermo Scientific) and quantified by NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). For expression detections of HMGA1, CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferators-activated receptor γ (PPARγ), 1 μg RNA was converted to cDNA by reverse transcription using M-MLV Reverse Transcriptase (Thermo Scientific) and random primers, followed by the quantitative PCR analysis using SYBR™ Select Master Mix (Thermo Scientific) and specific quantitative primers. Among this process, β-actin acted as the housekeeping gene to normalize expressions of HMGA1, C/EBPα and PPARγ. The expression level of miR-142a-3p was detected by TaqMan® MicroRNA real-time PCR Assay (Thermo Scientific) and primers for mmu-miR-142a-3p and U6 snRNA (Thermo Scientific) with U6 snRNA as the endogenous control. The quantitative primers were shown as follows: mouse HMGA1, 5'-GCAGGAAAAGGATGGGACTG-3' (sense) and 5'-AGCAGGGCTTCCAGTCCCAG-3' (antisense); mouse PPARγ, 5'-CTGCTCAAGTATGGTGTCCATGA-3' (sense) and 5'-TGAGATGAGGACTCCATCTTTATTCA-3' (antisense); mouse C/EBPα, 5'-GAGCCGAGATAAAGCCAAACA-3' (sense) and 5'-GCGCAGGCGGTCATTG-3' (antisense); mouse β-actin, 5'-CAGCCTTCCTTCTTGGGTAT-3' (sense) and 5'-TGGCATAGAGGTCTTTACGG-3' (antisense).

Western blot assay

The whole proteins were extracted from 3T3-L1 preadipocytes by ice-cold RIPA Lysis and

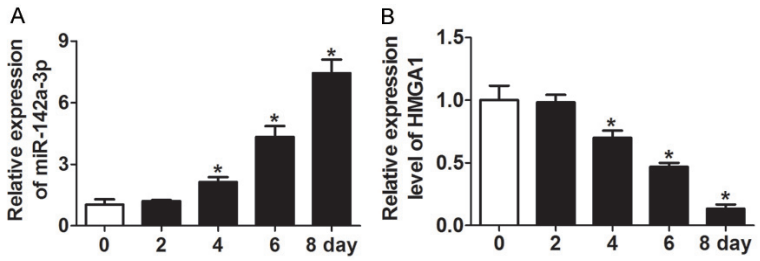

Figure 1. miR-142a-3p expression was notably upregulated and HMGA1 expression was markedly downregulated during 3T3-L1 preadipocyte differentiation. A and B. Expression levels of miR-142a-3p and HMGA1 were determined by RT-qPCR assay at the indicated time points (0, 2, 4, 6, 8 days) during 3T3-L1 preadipocyte differentiation. *$P < 0.05$.

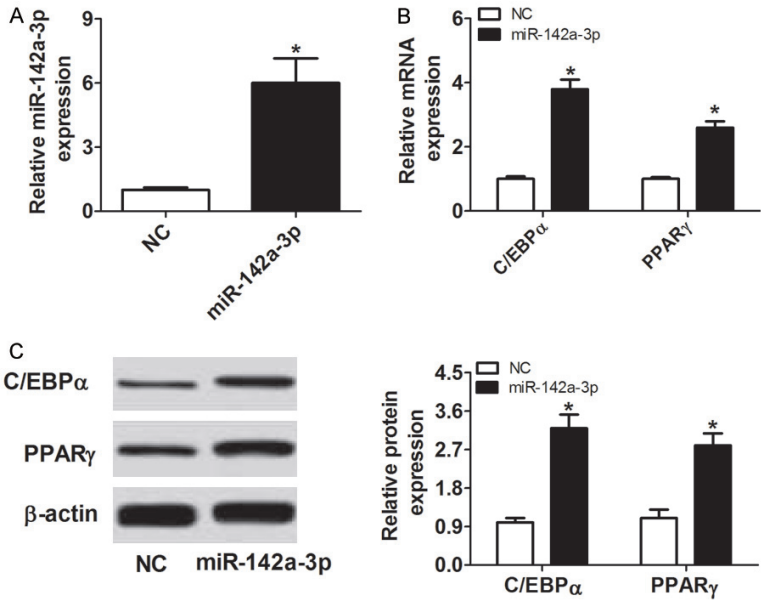

Figure 2. Ectopic expression of miR-142a-3p promoted 3T3-L1 preadipocyte differentiation. A. 3T3-L1 preadipocytes were transfected with miR-142a-3p mimic or a negative control (NC) in normal culture conditions. Then, miR-142a-3p level was determined by RT-qPCR assay at 24 h upon transfection. B and C. 3T3-L1 preadipocytes were transfected with miR-142a-3p mimic or a negative control in normal culture conditions. At 24 h after transfection, 3T3-L1 preadipocytes were cultured in differentiation mediums for another 4 days. Next, mRNA and protein levels of C/EBPα and PPARγ were determined on day 4 after the differentiation induction by RT-qPCR and western blot assays, respectively. *$P < 0.05$.

Extraction Buffer (Thermo Scientific) supplemented with protease inhibitors (Thermo Scientific). Then, protein concentrations were determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific). Next, equal amounts of proteins (40 μg/sample) were separated through SDS-PAGE and electro-transferred to polyvinylidine difluoride (PVDF) membranes (Millipore, Burlington, MA, USA). After the blockade of non-specific proteins in 5% non-fat milk, the membranes were incubated overnight at 4°C with anti-HMGA1 antibody (ab129153, 1:10000 dilution, Abcam, Cambridge, UK), anti-C/EBPα antibody (ab40764, 1:1000 dilution, Abcam), anti-PPARγ antibody (ab233218, 1:2000 dilution, Abcam) or anti-β-actin antibody (ab8227, 1:5000 dilution, Abcam). Following this, the PVDF membranes were hybridized for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ab97051, 1:10000 dilution, Abcam). Finally, protein bands were detected by Pierce™ ECL Western Blotting Substrate (Thermo Scientific). Relative expression levels of proteins were analyzed by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

The data were analyzed by GraphPad Prism software (La Jolla, CA, USA) with the results expressing as mean ± standard deviation (SD). Difference significance analyses were conducted using Student's *t*-test (two group data) or one-way analysis of variance (ANOVA) (more than two group data) with $P < 0.05$ as significant.

Results

miR-142a-3p expression was notably upregulated and HMGA1 expression was markedly downregulated during 3T3-L1 preadipocyte differentiation

First, RT-qPCR assay was performed to determine expression patterns of miR-142a-3p and HMGA1 during 3T3-L1 preadipocyte differentiation. Results showed that miR-142a-3p was minimally expressed in 3T3-L1 preadipocytes on day 0 and 2 upon the differentiation induction (**Figure 1A**). miR-142a-3p level was gradually upregulated and reached the maximum value on day 4 in 3T3-L1 preadipocytes after

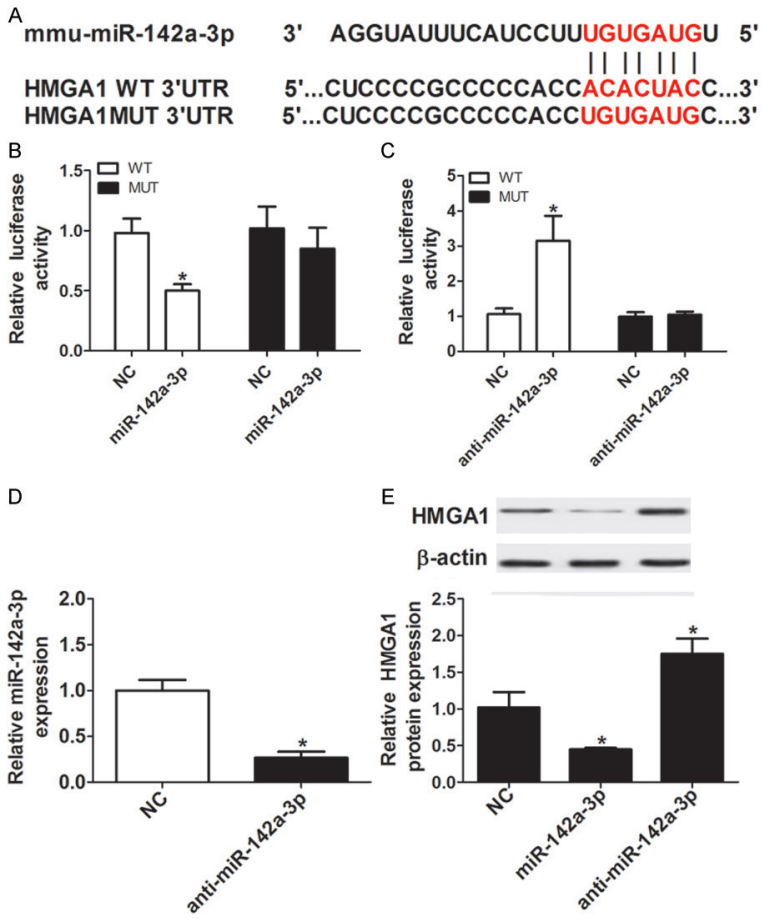

Figure 3. HMGA1 is a target of miR-142a-3p. A. Predicted binding sites between miR-142a-3p and HMGA1 3'UTR, and mutant sites in mutant type HMGA1 reporter. B and C. 3T3-L1 preadipocytes were co-transfected with WT or MUT reporter and NC, miR-142a-3p mimic, or anti-miR-142a-3p. At 48 h after transfection, luciferase activities were determined by luciferase reporter assay. D. 3T3-L1 preadipocytes were transfected with NC or anti-miR-142a-3p, followed by the measurement of miR-142a-3p level through RT-qPCR assay at 48 h post transfection. E. 3T3-L1 preadipocytes were transfected with NC, miR-142a-3p or anti-miR-142a-3p. Then, protein level of HMGA1 was determined via western blot assay at 48 h following transfection. *$P < 0.05$.

differentiation induction (**Figure 1A**). Similarly, no obvious change in HMGA1 level was observed in 3T3-L1 preadipocytes on day 2 following the differentiation induction compared with control group (day 0) (**Figure 1B**). But, HMGA1 level was significantly downregulated in a time-dependent manner since the fourth day and reached the minimum level on the eighth day in 3T3-L1 preadipocytes after differentiation induction (**Figure 1B**). These data indicated that miR-142a-3p and HMGA1 play critical roles in the process of 3T3-L1 preadipocyte differentiation.

Ectopic expression of miR-142a-3p promoted 3T3-L1 preadipocyte differentiation

Next, 3T3-L1 preadipocytes were transfected with miR-142a-3p mimic or a negative control (NC), followed by the detection of transfection efficiency at 24 h after transfection. As presented in **Figure 2A**, miR-142a-3p level was markedly increased in 3T3-L1 preadipocytes transfected with miR-142a-3p mimic relative to control group, hinting that miR-142a-3p mimic could be used for the subsequent gain-of-function experiments. To further test the function of miR-142a-3p in the process of 3T3-L1 preadipocyte differentiation, 3T3-L1 preadipocytes were transfected with miR-142a-3p mimic or a negative control (NC) for 24 h under normal culture conditions and then cultured in differentiation mediums for another 4 days. Expression levels of differentiation markers (PPARγ and C/EBPα) were measured on day 4 after the differentiation induction. Results showed that the enforced expression of miR-142a-3p induced an obvious upregulation of C/EBPα and PPARγ expressions at mRNA (**Figure 2B**) and protein (**Figure 2C**) levels during the differentiation of 3T3-L1 preadipocytes, implying that miR-142a-3p contributed to the differentiation of 3T3-L1 preadipocytes.

HMGA1 is a target of miR-142a-3p

To further investigate the molecular basis of miR-142a-3p, bioinformatics analysis by TargetScan online website was conducted to predict potential targets of miR-142a-3p. Among candidate targets of miR-142a-3p, HMGA1 was selected considering its critical roles in adipocyte differentiation [24-26] (**Figure 3A**). To further validate this prediction, the overexpres-

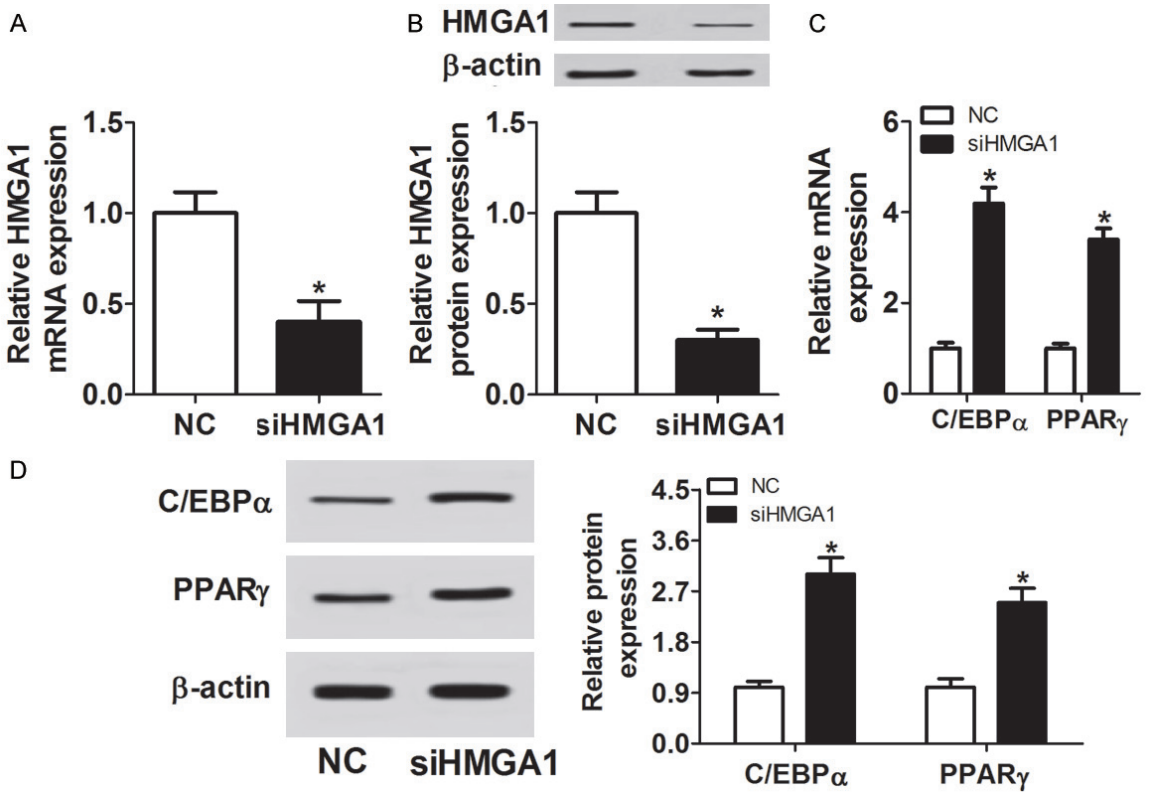

Figure 4. The knockdown of HMGA1 facilitated the differentiation of 3T3-L1 preadipocytes. (A and B) 3T3-L1 preadipocytes were transfected with siHMGA1 or a negative control in normal culture conditions. Then, HMGA1 mRNA (A) and protein (B) levels were measured at 24 h following transfection. (C and D) 3T3-L1 preadipocytes were transfected with siHMGA1 or a negative control for 24 h in normal culture conditions and then maintained in differentiation mediums for an additional 4 days. Next, mRNA and protein levels of C/EBPα and PPARγ were detected on day 4 after the differentiation induction. *$P < 0.05$.

sion or deficiency of miR-142a-3p on luciferase activities of wild type or mutant type HMGA1 reporter was measured by luciferase reporter assay, respectively. As displayed in **Figure 3B**, the introduction of miR-142a-3p mimic resulted in a notable downregulation in luciferase activity of wild type HMGA1 reporter (WT) in 3T3-L1 preadipocytes, but had no influence on luciferase activity of mutant type HMGA1 reporter (MUT). Conversely, a conspicuous elevation in luciferase activity of WT reporter was observed in 3T3-L1 preadipocytes after the depletion of miR-142a-3p (**Figure 3C**). Also, as expected, no obvious difference in luciferase activity of MUT reporter was noticed in 3T3-L1 preadipocytes with or without the depletion of miR-142a-3p (**Figure 3C**). These data suggested that miR-142a-3p could interact with HMGA1 3'UTR by putative binding sites. RT-qPCR assay also confirmed that miR-142a-3p level was markedly reduced in 3T3-L1 preadipocytes transfected with anti-miR-142a-3p (**Figure 3D**). Additionally, miR-142a-3p overexpression resulted in the marked reduction of HMGA1 protein level, whereas miR-142a-3p depletion induced the obvious increase of HMGA1 protein level in 3T3-L1 preadipocytes (**Figure 3E**). Taken together, these results revealed that miR-142a-3p inhibited HMGA1 expression by direct interaction.

The knockdown of HMGA1 facilitated the differentiation of 3T3-L1 preadipocytes

Next, RT-qPCR and western blot assays further unveiled that the transfection of siHMGA1 resulted in the dramatic reduction of HMGA1 mRNA and protein levels (**Figure 4A** and **4B**), indicating that siHMGA1 could be applied to following loss-of-function experiments. Functional investigations revealed that the silence of HMGA1 promoted the expressions of C/EBPα and PPARγ at mRNA (**Figure 4C**) and protein (**Figure 4D**) levels in the process of 3T3-L1 pre-

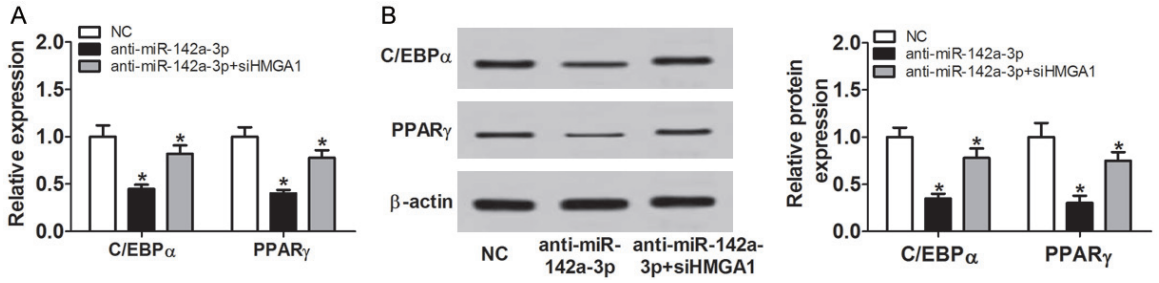

Figure 5. HMGA1 knockdown abrogated the inhibitory effect of miR-142a-3p deficiency on 3T3-L1 preadipocyte differentiation. (A and B) 3T3-L1 preadipocytes were transfected with NC, anti-miR-142a-3p, or anti-miR-142a-3p+siHMGA1 for 24 h in normal culture conditions and then cultured in differentiation mediums for another 4 days. Next, mRNA (A) and protein (B) levels of C/EBPα and PPARγ were detected on day 4 after differentiation induction by RT-qPCR and western blot assays, respectively. $*P < 0.05$.

adipocyte differentiation, hinting that HMGA1 knockdown accelerated the differentiation of 3T3-L1 preadipocytes.

HMGA1 knockdown abrogated the inhibitory effect of miR-142a-3p deficiency on 3T3-L1 preadipocyte differentiation

We further demonstrated that the introduction of miR-142a-3p inhibitor resulted in the reduction of C/EBPα and PPARγ expressions at mRNA (**Figure 5A**) and protein (**Figure 5B**) levels in the process of 3T3-L1 preadipocyte differentiation, while these effects were weakened by silenced HMGA1 (**Figure 5A** and **5B**). In sum, these results signified that the depletion of miR-142a-3p curbed 3T3-L1 preadipocyte differentiation by targeting HMGA1.

Discussion

Obesity is a great threat to human health and life quality, even for non-symptomatic healthy individuals [29]. It is estimated that overweight or obese people account for about 30% of the global population [2, 29]. Moreover, obesity is related with the increased risk of numerous diseases such as cancer, cardiovascular diseases (CVDs), chronic kidney disease, and diabetes [30, 31]. Current management approaches such as lifestyle intervention, pharmacotherapy, and bariatric surgery are not extremely effective to maintain weight loss and reduce the morbidity and potentially mortality of obesity, especially for patients with severe obesity [32, 33].

The 3T3-L1 cell line, derived from murine Swiss 3T3 cells from embryos, is a common cell model in exploring the molecular basis associated with adipocyte differentiation [34]. miRNAs have emerged as key regulators in many obesity-related biological processes including adipocyte differentiation [35, 36]. For instance, microRNA-204-5p overexpression inhibited proliferation and facilitated differentiation by targeting kruppel-like factor 3 in 3T3-L1 preadipocytes [37]. MicroRNA-185 suppressed 3T3-L1 preadipocyte differentiation by downregulating sterol regulatory element binding protein 1 (SREBP-1) expression [38].

In the present study, we demonstrated that miR-142a-3p level was gradually upregulated during the differentiation of 3T3-L1 preadipocytes, implying that miR-142a-3p might contribute to adipogenesis, which was in line with prior studies [16-19]. Also, our data showed that HMGA1 level was reduced in a time-dependent manner during the differentiation of 3T3-L1 preadipocytes, indicating an inhibitory effect of HMGA1 on adipogenesis.

PPARγ and C/EBPα have been identified as vital positive transcriptional factors in the process of adipogenesis [39, 40]. Also, the depletion of C/EBPα and PPARγ blocked the differentiation of 3T3-L1 preadipocytes [41, 42]. Consequently, the differentiation pattern of 3T3-L1 preadipocytes was assessed by C/EBPα and PPARγ. Our results showed that miR-142a-3p overexpression induced the increase of C/EBPα and PPARγ levels, indicating the promotive effect of miR-142a-3p on 3T3-L1 preadipocyte differentiation. Further analyses disclosed that HMGA1 was a target of miR-142a-3p in 3T3-L1 preadipocytes. Also, the knockdown of HMGA1 resulted in the increase of C/EBPα

and PPARγ expressions in 3T3-L1 preadipocytes, hinting that HMGA1 curbed the differentiation of 3T3-L1 preadipocytes. In agreement with our results, Arce-Cerezo *et al.* showed that ectopic expression of HMGA1 in adipose tissues impaired adipogenesis and reduced fat mass by increasing the expression of preadipocyte markers and decreasing the expression of adipogenesis-related molecules such as PPARγ and C/EBPα in mice [26]. However, some studies pointed out that HMGA1 expression was positively associated with 3T3-L1 adipocyte differentiation, and the reduction of HMGA1 level dramatically inhibited differentiation of 3T3-L1 preadipocytes [24, 25]. In this finding, we further disclosed that the knockdown of HMGA1 abrogated the inhibitory effect of miR-142a-3p deficiency on PPARγ and C/EBPα expressions during differentiation of 3T3-L1 preadipocytes.

Collectively, our data showed that miR-142a-3p facilitated the differentiation of 3T3-L1 preadipocytes by targeting HMGA1, deepening our understanding of molecular mechanisms associated with adipogenesis and suggesting miR-142a-3p and HMGA1 have diagnostic or treatment value for obesity and other metabolic disorders. However, our study only pointed out the effect of miR-142a-3p/HMGA1 axis on expressions of PPARγ and C/EBPα during the differentiation of 3T3-L1 preadipocytes. More experiments were indispensable to further validate the influence of miR-142a-3p/HMGA1 axis on adipocyte differentiation *in vitro* and *in vivo*.

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Disclosure of conflict of interest

None.

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