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RESEARCH PAPER

miR-146b-5p regulates bone marrow mesenchymal stem cell differentiation by SIAH2/PPARγ in aplastic anemia children and benzene-induced aplastic anemia mouse model

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ABSTRACT

This study aimed to reveal the mechanism of miR-146b-5p in the differentiation of bone marrow mesenchymal stem cells (BMSCs) derived from children with aplastic anemia (AA). Here, we found that miR-146b-5p was highly expressed in BMSCs from children with AA, and the BMSCs surface markers expressions in BMSCs derived from children with AA and the healthy controls exerted no significant differences. Besides, the overexpression of miR-146b-5p in normal human-derived BMSCs promoted the adipogenic differentiation of BMSCs. Furthermore, miR-146b-5p negatively regulated SIAH2 luciferase activity, and the interference with miR-146b-5p reduced the stability of PPARγ protein and inhibited SIAH2-mediated ubiquitination of PPARγ protein. Besides, the interference with miR-146b-5p was beneficial for ameliorating AA in a mouse model of AA. Overall, our results found that miR-146b-5p was highly expressed in BMSCs from children with AA, and our further studies indicated that miR-146b-5p improved AA via promoting SIAH2-mediated ubiquitination of PPARγ protein.

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KEYWORDS
Aplastic anemia; miR-146b-5p; SIAH2; PPARγ; BMSCs

Introduction

Aplastic anemia (AA) in children is a common bone marrow hematopoietic failure disease in children and mainly caused by the abnormal hematopoietic function of the bone marrow microenvironment [1,2]. Bone marrow mesenchymal stem cells (BMSCs) are important components of the bone marrow hematopoietic microenvironment and stimulate the hematopoietic function of the bone marrow microenvironment, and can differentiate into adipocytes under certain conditions [3]. Previous researches have clarified that the adipogenic differentiation of BMSCs can inhibit the hematopoietic function of the bone marrow microenvironment by increasing the formation of adipocytes [4,5]. Therefore, the inhibition of adipogenic differentiation of BMSCs has the potential to improve AA in children by reducing the formation of adipocytes.

MicroRNAs (miRNAs) are a class of non-coding RNAs of approximately 22 nucleotides in length [6]. As reported, miRNAs can lead to the degradation of mRNAs via binding to the 3 untranslated region (UTR) of these mRNAs [7,8]. Emerging evidence suggests that miRNAs are important regulators in the adipogenic differentiation of BMSCs [9], and the dysregulation of miRNAs is involved in the evolve of several diseases including AA [10]. Adhikari S. *et al* found that miR-1202 is highly expressed in tissues from AA patients compared to healthy control [11]. According to another study, increasing the expression of miR-214-3p can promote BMSC adipogenic differentiation, while the interference with miR-214-3p produces the opposite effect [12]. Furthermore, Zhao J. *et al* showed that miR-204 is a key regulator of the differentiation in BMSCs-derived from AA, and the knockdown of miR-204 can relieve AA through repressing the adipogenic differentiation of BMSCs [13]. Therefore, exploring more miRNAs that can alleviate AA by inhibiting the adipogenic differentiation of BMSCs is significant.

Peroxisome proliferator-activated receptor gamma (PPARγ) is ligand-activating receptors in the nuclear hormone receptor family [14]. PPARγ is a key transcription factor for adipocyte formation and plays

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Supplemental data for this article can be accessed here.

pivotal roles in BMSC adipogenic differentiation [15]. Xu X. *et al* found that the up-regulation of PPARγ expression can boost the adipogenic differentiation of BMSCs [16]. Also, other researches indicate that the expression of PPARγ is up-regulated in BMSCs, and this high expression of PPARγ boosts BMSC adipogenic differentiation [17,18]. Therefore, how to reduce the expression of PPARγ in BMSCs has become the focus of the next study. Emerging evidence suggests that the ubiquitin-proteasome system plays a critical role in regulating the expression of PPARγ in adipocytes [19]. As reported, E3 ubiquitinase tripartite motif protein 23 (TRIM23) can down-regulate PPARγ by mediating the ubiquitination of PPARγ protein [20]. Interestingly, seven-in-absentia homolog 2 (SIAH2) is one of the E3 ubiquitin enzymes and can down-regulate PPARγ protein by mediating PPARγ ubiquitination in adipocytes [21]. Thus, promoting SIAH2-mediated ubiquitination of PPARγ protein to down-regulate the level of PPARγ protein has the potential to improve AA.

Generally, our results found that miR-146b-5p and PPARγ were abnormally overexpressed in BMSCs from children with AA, while SIAH2 was lowly expressed, and our further studies indicated that miR-146b-5p could ameliorate AA via promoting SIAH2-mediated ubiquitination of PPARγ protein. Moreover, animal experiments had also shown that the interference with miR-146b-5p could attenuate AA.

Materials and methods

Isolation, culture, and identification of bone marrow mesenchymal stem cells

Bone marrow mesenchymal stem cells (BMSCs) were isolated from bone marrow samples from children (aged 4–17 years) with confirmed aplastic anemia (n = 9) and children (aged 4–17 years) with other diseases excluding blood (n = 9). The diagnostic criteria for patients with AA were pancytopenia and hypocellular BM and does not include other underlying diseases. Briefly, bone marrow samples isolated from the above two groups were resuspended in DMEM medium with the addition of 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL) and streptomycin (100 μg/mL), and then placed them in 37°C, 5% CO_2 environment for cultivation. The fresh medium was replaced every 3 d. In this paper, we used BMSCs cultured to the third generation for the subsequent experiments. Besides, flow cytometry was performed to detect BMSCs surface markers expressions. Our research was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2020-KY-150), and written informed consent was obtained from all the patients involved.

Flow cytometry

The surface markers of BMSCs isolated from children with confirmed AA was assessed by flow cytometry. BMSC surface markers CD45, HLA-DR, CD34, CD29, CD105 and CD44 were selected according to these previous literatures [22–24]. Briefly, BMSCs were incubated with antibodies for 0.5 h at the environment of room temperature, and then the cells were added in 0.3 mL PBS supplemented with 1% BSA. Finally, the Accuri C6 flow cytometer was used to collect data, and then the data were analyzed by FlowJo software.

Oil red O staining

BMSCs were stained by Oil Red O (Sigma). Briefly, BMSCs were fixed with 10% formaldehyde for 30 min. Subsequently, the BMSCs were stained with 0.3% oil red O (ORO), and lipid droplets of differentiated cells were obtained. Finally, we viewed these cells using an inverted optical microscope and saved these figures.

Cell transfection

Human BMSCs (1×10^6) were seeded in 6-well plates. After the miR-146b-5p mimic or miR-146b-5p inhibitor+si-SIAH2 was added into the DMEM medium, we added Lipofectamine 2000 into the medium, and the miRNAs and Lipofectamine 2000 were mixed fully at room temperature for 10 min and then added the mixtures into the above 6-well plate in equal amount. Fresh medium was added after 6 h of transfection. The

control cells were treated with the same volume of lipofectamine.

Adipogenic differentiation of BMSCs

To induce BMSC adipogenic differentiation, we seeded the BMSCs (1×10^4) in a 24 well plate supplemented with the adipogenic induction medium containing 10% FBS and 10% adipogenic differentiation medium additive and then placed in 37°C, 5% CO_2 environment to continue culturing for 12 h, and we changed the fresh adipogenic induction medium every 4 d. Next, oil red O staining was performed to observe the lipid droplets in the BMSCs.

Total RNA extraction and quantitative real-time PCR

Based on the instructions of the manufacturer, TRIzol reagent (Takara) was used to extract the total RNA. Subsequently, the above-extracted RNA was synthesized into cDNA by the Prime-Script RT kit (Takara). Next, the real-time PCR was performed using the miScript SYBR® Green PCR Kit (QIAGEN) and ABI 7300 Real-Time PCR System (Applied Biosystems). U6 was used as an endogenous control. The relative expressions were calculated by the $2^{-\Delta\Delta CT}$ method. The primer sequences used in this research were listed: miR-146b-5p: forward 5′-CGCGTGAGAACTGAATTCCAT-3′, reverse 5′-AGTGCAGGGTCCGAGGTATT-3′. U6: forward 5′-GCTTCGGCAGCACATATACTAA-3′, reverse 5′-AAAATATGGAACGCTTCACGA-3′.

Western blot analysis

BMSCs were lysed by RIPA lysis buffer and then the concentrations of the total proteins were quantified by a BCA kit (Thermo Fisher Scientific). Next, the above proteins were separated using SDS-PAGE gel and then transferred them into a polyvinylidene difluoride (PVDF) membrane (Millipore), and then blocked them with 5% milk. Subsequently, we incubated this membrane with primary antibodies overnight at 4°C and the membrane and the HRP-conjugated secondary antibody were then incubated together for 1 h. Finally, Fuji (Japan) SUPER RX-N-C films were performed to visualize the intensity of proteins. The details of the antibodies used in this study were listed: SIAH2 (Abcam, ab31234), PPARγ (CST, #2443); C/EBP-α (Abcam, ab40764), FABP4 (CST, #2120), GAPDH (Abcam, ab181602).

Dual-luciferase reporter experiment

The dual-luciferase reporter assay was conducted concerning the previous methods with several minor revisions [25]. Briefly, we constructed a luciferase reporter vector containing the SIAH2 3′UTR and cloned the SIAH2 3′UTR with the binding site mutation (mut) of miR-146b-5p into the same reporter vector as control. The above recombinant vector was then transfected into human BMSCs. After 2 d, the luciferase activity was tested by a dual-luciferase reporter assay system (Promega).

Cycloheximide-chase experiment

To assess the influence of miR-146b-5p on PPARγ stability, cycloheximide (CHX)-chase assay was performed by using the routine protein synthesis inhibitor CHX [26]. Briefly, after the miR-146b-5p inhibitor was transfected into human BMSCs, the cells were treated with 20 μg/mL CHX for 0, 2, and 4 h, respectively. Finally, Western blot assays were conducted to quantify the protein levels.

Immunoprecipitation and Ubiquitination assays

Immunoprecipitation and Ubiquitination Assays were performed to explore the influence of miR-146b-5p on PPARγ Ubiquitination. Specifically, we transfected inhibitor negative control (NC) +si-NC (inhibitor NC was the negative control of miR-146b-5p inhibitor and si-NC was the negative control of si-SIAH2), miR-146b-5p inhibitor+si-NC, miR-146b-5p inhibitor+si-SIAH2 into human BMSCs, and then these cells were handled with 10 μM MG132 for 5 h. Subsequently, these cells were lysed with a lysis

buffer with the addition of MG132, which is a protease inhibitor [27]. The above cell lysate was incubated with the antibody for 2–3 h and then incubated with protein A/G agarose resin overnight. Next, this protein A/G agarose resin was repeatedly washed with a lysis buffer, and Western blot was performed to quantify the eluted proteins.

Establishment a mouse model of aplastic anemia

Twenty-four C57BL/6 (aged 6–8 weeks, 18–20 g) male mice were stochastically grouped into these four groups: control (n = 6), AA group (n = 6), BMSC-inhibitor NC group (n = 6, the negative control of BMSC-miR-146b-5p inhibitor) and BMSC-miR-146b-5p inhibitor group (n = 6). Briefly, except for the control group, the other three groups of mice were injected subcutaneously in the dorsal region with 1500 mg/kg benzene in corn oil daily for 3 weeks [28]. Besides, we transfected inhibitor NC, miR-146b-5p inhibitor into human BMSCs, and then injected BMSCs (2×10^9 cells) into the mice via tail vein [29], which were used as BMSCs-inhibitor NC and BMSCs-miR-146b-5p inhibitor group, respectively. We provided a timeline for each group in the model establishment (Supplementary Figure 1). All animal procedures were performed following the protocols approved by the Experimental Animal Ethics Committee of Zhengzhou University.

Hemoglobin counting experiment

Peripheral blood of the mice was taken on the 14th day after the transplantation and then collected in a test tube containing an anticoagulant ethylene diamine tetraacetic acid (EDTA). Subsequently, the hemoglobin number was quantified using a BC5300 blood cell analyzer.

Hematoxylin-eosin staining

The bilateral femurs of the mice in the AA model were isolated and the pathological changes of the bone marrow tissues of each group were observed by HE staining. Specifically, the bilateral femurs were fastened with formalin and then embedded them in paraffin. Subsequently, the embedded wax block was fixed on a microtome and cut into 5 μm. The above sections were then immersed in xylene for dewaxing and then hydrated in gradient ethanol. Finally, the samples were stained with hematoxylin for 10 min and then stained with eosin for another 3 min.

Statistical analysis

All statistical analyzes in this study were conducted by SPSS 23.0 software. All experimental data in this study were expressed as mean ± Standard Deviation (SD) unless otherwise stated. For the relevant data of animal experiments, we used a one-way analysis of variance post hock (Sidak) for statistical analysis. For the relevant data of human experiments, we used the Student's *t*-test. The P-value <0.05 was considered to have a remarkable difference.

Results

Different expressions of miR-146b-5p, PPARγ and SIAH2 in bone marrow mesenchymal stem cells from children with aplastic anemia

To elucidate the expressions of miR-146b-5p, PPARγ, and SIAH2 in BMSCs from children with AA, we used routine protocols to separate BMSCs from bone marrow samples from the children with AA [30]. Based on flow cytometry analysis, it was found that the expressions of BMSC surface markers CD45, HLA-DR, CD34, CD29, CD105, CD44 in the Normal group (n = 9), and AA group (n = 9) exerted no significant differences (Figure 1(a)). Moreover, oil red O staining results displayed that the accumulation of lipid droplets was increased in the AA group, prompting that BMSCs derived from children with AA had a better adipogenic differentiation ability (Figure 1(b)). qRT-PCR results displayed that the expression of miR-146b-5p was dramatically up-regulated in the AA group (Figure 1(c)). Western blot analysis indicated that the protein level of SIAH2 was

down-regulated in the AA group, while PPARγ was up-regulated (Figure 1(d-f)).

miR-146b-5p promotes BMSC adipogenic differentiation

To clarify the influence of miR-146b-5p on BMSC adipogenic differentiation, we transfected miR-146b-5p mimic into normal human BMSCs and then induced BMSC adipogenic differentiation. From the analysis of qRT-PCR, it was found that compared with the Normal group, miR-146b-5p was up-regulated in the Induced group, and the transfection of miR-146b-5p mimic significantly up-regulated the expression of miR-146b-5p (Figure 2(a)). From the analysis of Western blot, it was found

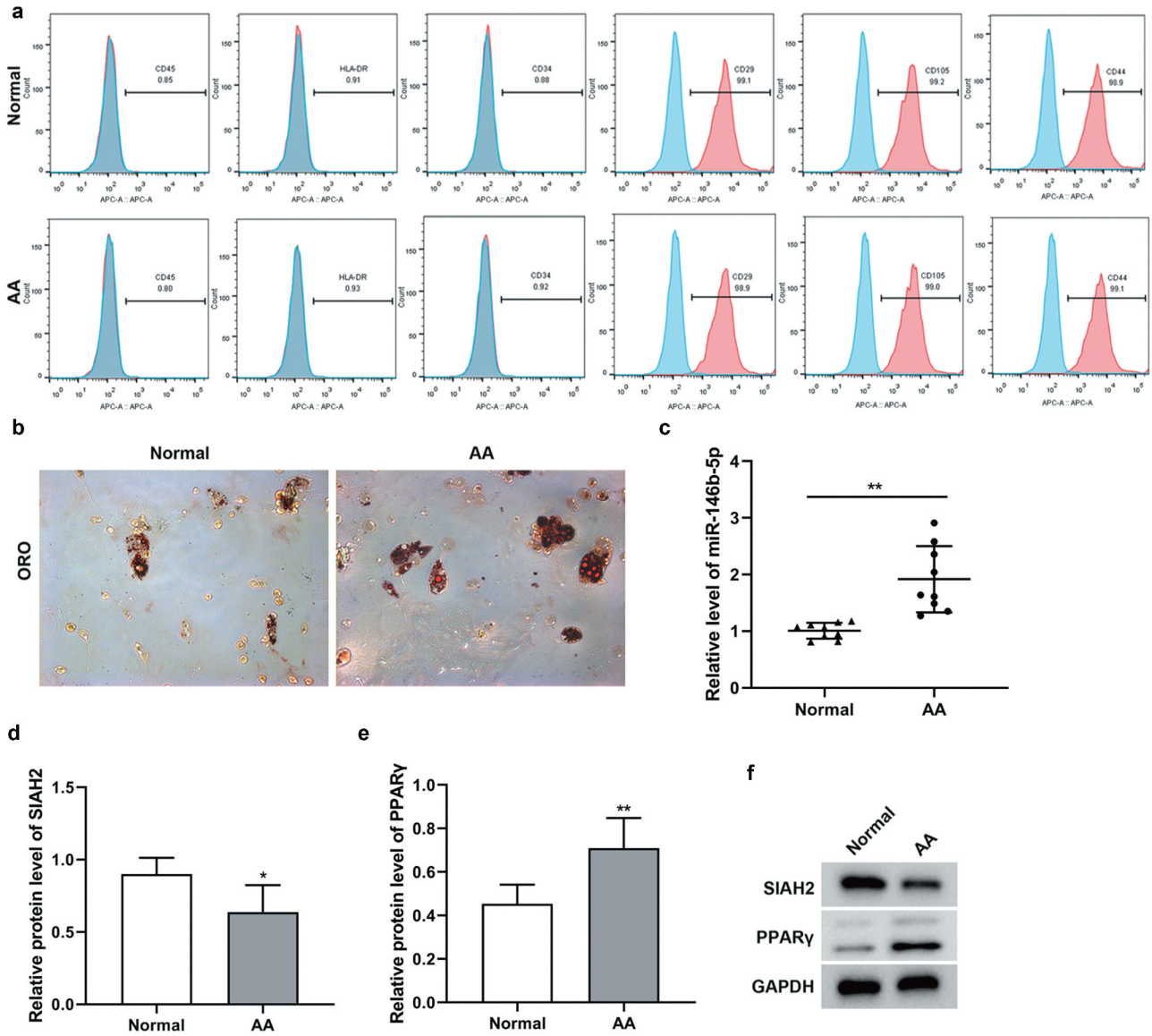

Figure 1. The expressions of miR-146b-5p, PPARγ, and SIAH2 in bone marrow mesenchymal stem cells from children with aplastic anemia. Bone marrow mesenchymal stem cells (BMSCs) were isolated from bone marrow samples of children (aged 4–17 years) with aplastic anemia (AA, n = 9) and children (aged 4–17 years) with other diseases excluding blood (Normal, n = 9), respectively. (a) The expressions of BMSC surface markers CD45, HLA-DR, CD34, CD29, CD105, CD44 in the Normal group and AA group were detected by flow cytometry. (b) The adipogenic differentiation ability of BMSCs was analyzed by oil red O staining (Under (×200) magnification). (c) The expression of miR-146b-5p was detected by qRT-PCR. $^{**}P < 0.01$ *vs* Normal group. (d-f) The protein levels of SIAH2 and PPARγ were analyzed by Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* Normal group. ORO: Oil red O.

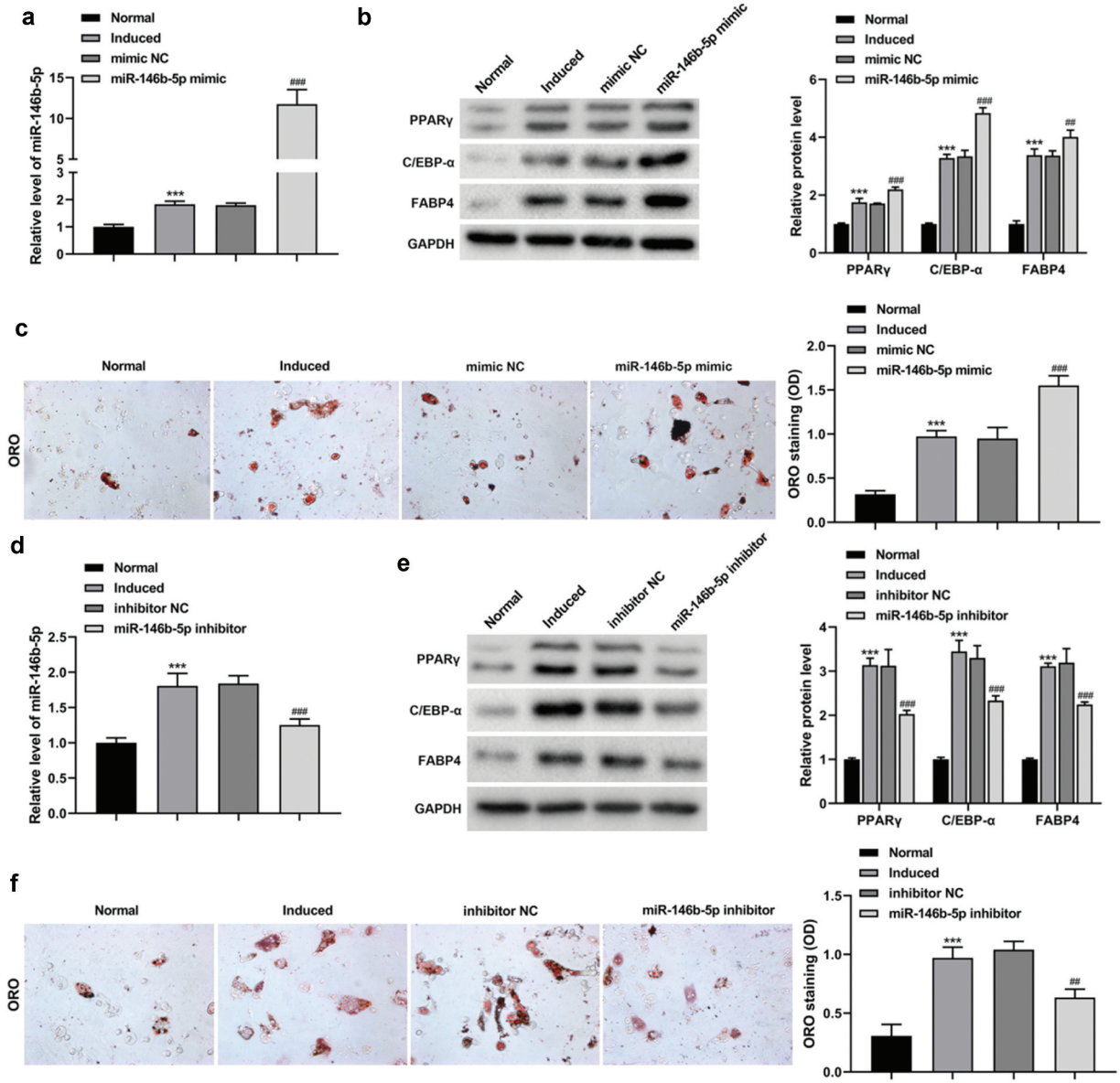

Figure 2. miR-146b-5p is involved in the adipogenic differentiation of BMSCs. miR-146b-5p mimic was transfected into normal human BMSCs and induced the adipogenic differentiation of BMSCs. (a) The expression of miR-146b-5p was detected by qRT-PCR. $^{***}P < 0.001$ *vs* Normal group, $^{###}P < 0.001$ *vs* mimic Negative Control (NC) group. (b) The expressions of the adipogenic differentiation-related proteins PPARγ, C/EBP-α, and FABP4 were measured by Western blot. n = 3. $^{***}P < 0.001$ *vs* Normal group, $^{##}P < 0.01$, $^{###}P < 0.001$ *vs* mimic NC group. (c) The ability of BMSCs adipogenic differentiation was analyzed by oil red O staining (Under (×200) magnification). $^{***}P < 0.001$ *vs* Normal group, $^{###}P < 0.001$ *vs* mimic NC group. The miR-146b-5p inhibitor was transfected into normal human BMSCs and induced the adipogenic differentiation of BMSCs. (d) Detection of miR-146b-5p expression. $^{***}P < 0.001$ *vs* Normal group, $^{###}P < 0.001$ *vs* inhibitor NC group. (e) Detection of PPARγ, C/EBP-α, and FABP4 protein levels. n = 3. $^{***}P < 0.001$ *vs* Normal group, $^{###}P < 0.001$ *vs* inhibitor NC group. (f) The ability of BMSCs adipogenic differentiation was analyzed by oil red O staining (Under (×200) magnification). $^{***}P < 0.001$ *vs* Normal group, $^{##}P < 0.01$ *vs* inhibitor NC group. ORO: Oil red O.

that compared with the Normal group, the expressions of adipogenic differentiation-related proteins PPARγ, C/EBP-α, and FABP4 were markedly up-regulated in the Induced group, and the transfection of miR-146b-5p mimic enhanced these trends (Figure 2(b)). Besides, oil red O staining results

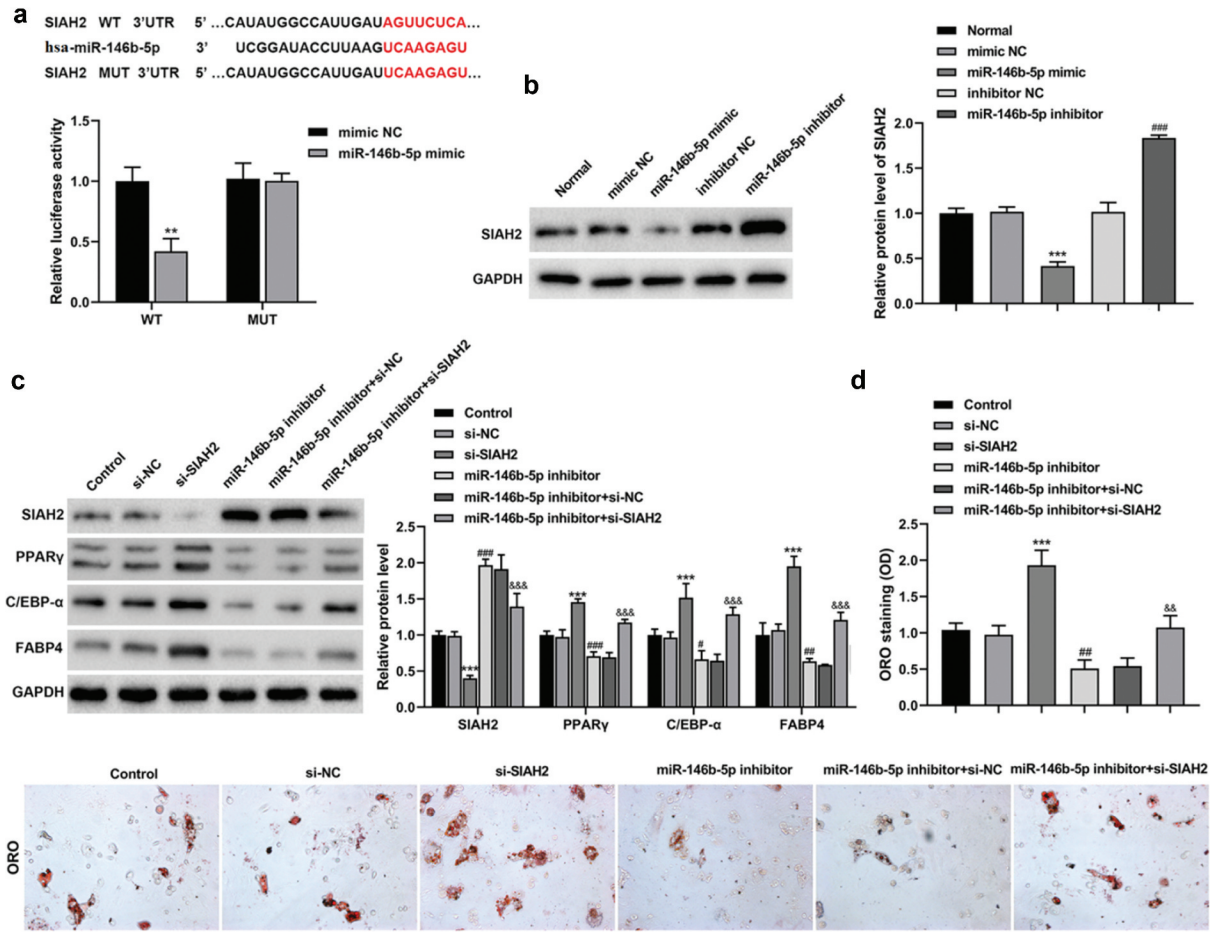

Figure 3. miR-146b-5p participates in the adipogenic differentiation of BMSCs via SIAH2. (a) Online prediction software and dual-luciferase reporter assay were used to analyze miR-146b-5p could target regulated SIAH2 protein. n = 3. $^{**}P < 0.01$ *vs* mimic Negative Control (NC) group. (b) After transfecting miR-146b-5p mimic or miR-146b-5p inhibitor into normal human BMSCs, the expression of SIAH2 was measured by Western blot. $^{***}P < 0.001$ *vs* mimic NC group, $^{###}P < 0.001$ *vs* inhibitor NC group. si-NC, si-SIAH2, miR-146b-5p inhibitor, miR-146b-5p inhibitor+si-NC, and miR-146b-5p inhibitor+si-SIAH2 was transfected into normal human BMSCs and induced the adipogenic differentiation of the cells. (c) The expressions of SIAH2, PPARγ, C/EBP-α, and FABP4 were detected by Western blot. $^{***}P < 0.001$ *vs* si-NC group. $^{#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$ *vs* Control group. $^{&&&}P < 0.001$ *vs* miR-146b-5p inhibitor+si-NC group. (d) Oil red O staining was used to analyze the ability of BMSC adipogenic differentiation (Under (×200) magnification). $^{***}P < 0.001$ *vs* si-NC group. $^{##}P < 0.01$ *vs* Control group. $^{&&}P < 0.01$ *vs* miR-146b-5p inhibitor+si-NC group.

displayed that compared with the Normal group, the accumulation of lipid droplets was raised in the Induced group, indicating that the ability of BMSC adipogenic differentiation was enhanced, and the transfection of miR-146b-5p mimic enhanced this trend (Figure 2(c)). Also, we transfected miR-146b-5p inhibitor into normal human BMSCs and then induced the adipogenic differentiation of BMSCs. From the analysis of qRT-PCR, it was found that compared with the Normal group, miR-146b-5p was raised in the Induced group, while the transfection of miR-146b-5p inhibitor reversed this raise (Figure 2 (d)). Western blot assay showed that compared with the Normal group, PPARγ, C/EBP-α, and FABP4 were up-regulated in the Induced group, while the transfection of miR-146b-5p inhibitor reversed these effects (Figure 2(e)). Also, oil red O staining results displayed that compared with the Normal group, the accumulation of lipid droplets was raised in the Induced group, while the transfection of miR-146b-5p inhibitor reversed this trend (Figure 2(f)). These above experimental results revealed that the

overexpression of miR-146b-5p boosted BMSC adipogenic differentiation, while the interference with miR-146b-5p could produce the opposite influence.

miR-146b-5p influences BMSC adipogenic differentiation via targeting SIAH2

Subsequently, we explored how miR-146b-5p affected BMSC adipogenic differentiation. According to the results predicted by the oline software, it was found that the miR-146b-5p contained the binding sites of the SIAH2 3′UTR, and the dual-luciferase reporter gene experiments displayed that miR-146b-5p negatively regulated the luciferase activity of SIAH2 (Figure 3(a)). After transfecting miR-146b-5p mimic or miR-146b-5p inhibitor into normal human BMSCs, Western blot assay demonstrated that the overexpression of miR-146b-5p could down-regulate SIAH2 protein level, while the knockdown of miR-146b-5p produced the opposite effect (Figure 3(b)). si-NC, si-SIAH2, miR-146b-5p inhibitor, miR-146b-5p inhibitor+si-NC, and miR-146b-5p inhibitor+si-SIAH2 was transfected into normal human BMSCs and induced the adipogenic differentiation of the cells. From the analysis of Western blot, we found that SIAH2 was down-regulated and PPARγ, C/EBP-α and FABP4 were up-regulated after the transfection of si-SIAH2, and compared with the Control group, SIAH2 was up-regulated and PPARγ, C/EBP-α, and FABP4 were down-regulated in the miR-146b-5p inhibitor group, and compared with the miR-146b-5p inhibitor+si-NC group, SIAH2 was down-regulated and PPARγ, C/EBP-α, and FABP4 were up-regulated after the transfection of si-SIAH2 (Figure 3(c)). Besides, oil red O staining results demonstrated that the accumulation of lipid droplets was raised in the si-SIAH2 group, indicating that the ability of BMSCadipogenic differentiation was enhanced after the transfection of si-SIAH2, and compared with the Control group, the accumulation of lipid droplets was reduced in the miR-146b-5p inhibitor group, and compared with the miR-146b-5p inhibitor+si-NC group, the accumulation of lipid droplets was increased in the miR-146b-5p inhibitor+si-SIAH2 group (Figure 3(d)). In summary, the above experimental results indicated that miR-146b-5p regulated the adipogenic differentiation of BMSCs by targeting SIAH2.

miR-146b-5p regulates the ubiquitination degradation of PPARγ through SIAH2

To examine the function of miR-146b-5p in PPARγ ubiquitination, we transfected miR-146b-5p inhibitor into normal human BMSCs and then processed these cells with 20 μg/mL cycloheximide (CHX) for 0, 2, 4 h. Western blot analysis displayed that after the interference with miR-146b-5p, the level of PPARγ protein was significantly down-regulated (Figure 4(a)). Besides, after MG132 treatment of human BMSCs transfected with inhibitor NC+si-NC, miR-146b-5p inhibitor+si-NC, miR-146b-5p

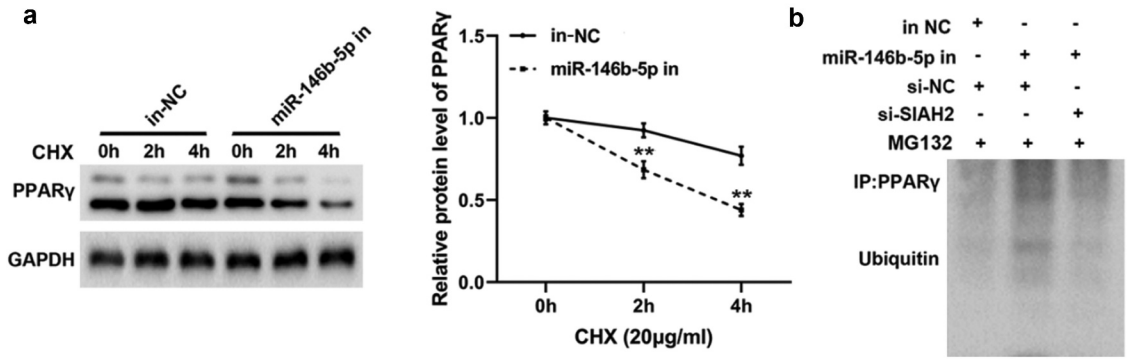

Figure 4. miR-146b-5p is involved in the regulation of the ubiquitination of PPARγ protein through SIAH2. (a) The miR-146b-5p inhibitor was transfected into human BMSCs and then the cells were treated with 20 μg/mL cycloheximide (CHX). The protein level of PPARγ was analyzed by Western blot. $^{**}P < 0.01$ *vs* inhibitor NC group. (b) miR-146b-5p inhibitor and miR-146b-5p inhibitor+si-SIAH2 were transfected into human BMSCs and then the cells were treated with 10 μM MG132. The ubiquitination of PPARγ was assessed by immunoprecipitation (IP) and ubiquitination assays. in-NC: inhibitor Negative Control, miR-146b-5p in: miR-146b-5p inhibitor.

inhibitor+si-SIAH2, the interference with miR-146b-5p promoted the ubiquitination degradation of PPARγ, while this promotion was reversed after the interference with SIAH2 (Figure 4(b)). The above data indicated that miR-146b-5p could regulate the ubiquitination of PPARγ protein by SIAH2.

Interference with miR-146b-5p alleviates AA

To further research the function of miR-146b-5p in AA mice, we established a mouse model of AA. Peripheral blood was taken on the 14th day after the transplantation, and hemoglobin counts were performed. The results displayed that the count of hemoglobin in the AA group was significantly reduced compared with the control group, while the count of hemoglobin in the BMSC-miR-146b-5p inhibitor group was significantly increased compared with the BMSC-inhibitor NC group (Figure 5(a)). HE staining of bone marrow tissues announced that the bone marrow hyperplasia was reduced, and the hematopoietic cells were rare, and the mouse bone marrow was filled with fat cells with large cavities in the AA group. Also, compared with the BMSC-inhibitor NC group, bone marrow hematopoietic failure was improved in the BMSC-miR-146b-5p inhibitor group (Figure 5(b)). Based on qRT-PCR, it was found that miR-146b-5p was dramatically increased in the AA group, and compared with the BMSC-inhibitor NC group, miR-146b-5p was decreased in the BMSC-miR-146b-5p inhibitor group (Figure 5(c)). Western blot analysis indicated that SIAH2 was down-regulated and adipogenic differentiation-related proteins PPARγ, C/EBP-α, and FABP4 were increased in the AA group and compared with the BMSC-inhibitor NC group, SIAH2 was up-regulated and PPARγ, C/EBP-α, and FABP4 were down-regulated in the BMSC-miR-146b-5p inhibitor group (Figure 5(d)). In summary, the interference with miR-146b-5p could significantly ameliorate AA.

Discussion

More and more studies have shown that microRNAs (miRNAs) are closely related to the occurrence of various human diseases. However, the role of miRNAs in children with AA has not been fully elucidated. Here, we found that miR-146b-5p was extraordinarily highly expressed in BMSCs derived from children with AA and the overexpression of miR-146b-5p boosted BMSC adipogenic differentiation, and our intensive researches had shown that the interference with miR-146b-5p could attenuate AA by promoting SIAH2-mediated ubiquitination of PPARγ protein to down-regulate the PPARγ expression.

MiRNAs are endogenous non-coding RNAs with approximately 22 nucleotides in length and have important regulatory roles in multiple diseases [31,32]. Recent studies have shown that several miRNAs play a crucial role in the regulation of BMSC adipogenic differentiation [9,33]. As reported, miR-149-3p is significantly decreased during BMSC adipogenic differentiation and the overexpression of miR-149-3p can repress BMSC adipogenic differentiation [34]. Similarly, Zhu E *et al* found that the overexpression of miR-20a-5p enhances the formation of fat and boosts BMSC adipogenic differentiation [35]. Notably, BMSC adipogenic differentiation is one of the major pathological features of AA [36]. Therefore, inhibiting BMSC adipogenic differentiation via regulating miRNAs has the potential to alleviate AA. As expected, our results illustrated that miR-146b-5p was dramatically increased in BMSCs from children with AA and the high expression of miR-146b-5p promoted the adipogenic differentiation of BMSCs. Besides, our further studies indicated that the interference with miR-146b-5p could improve AA by restraining BMSC adipogenic differentiation.

PPARγ is a major transcription factor that influences adipocyte differentiation, and PPARγ promotes the adipogenic differentiation of BMSCs [37,38]. PPARγ has been reported to be extraordinarily highly expressed in BMSCs from patients with AA and the inhibition of PPARγ expression can alleviate AA by inhibiting the adipogenic differentiation of BMSCs [39]. Therefore, how to inhibit BMSC adipogenic differentiation by reducing PPARγ expression has become the

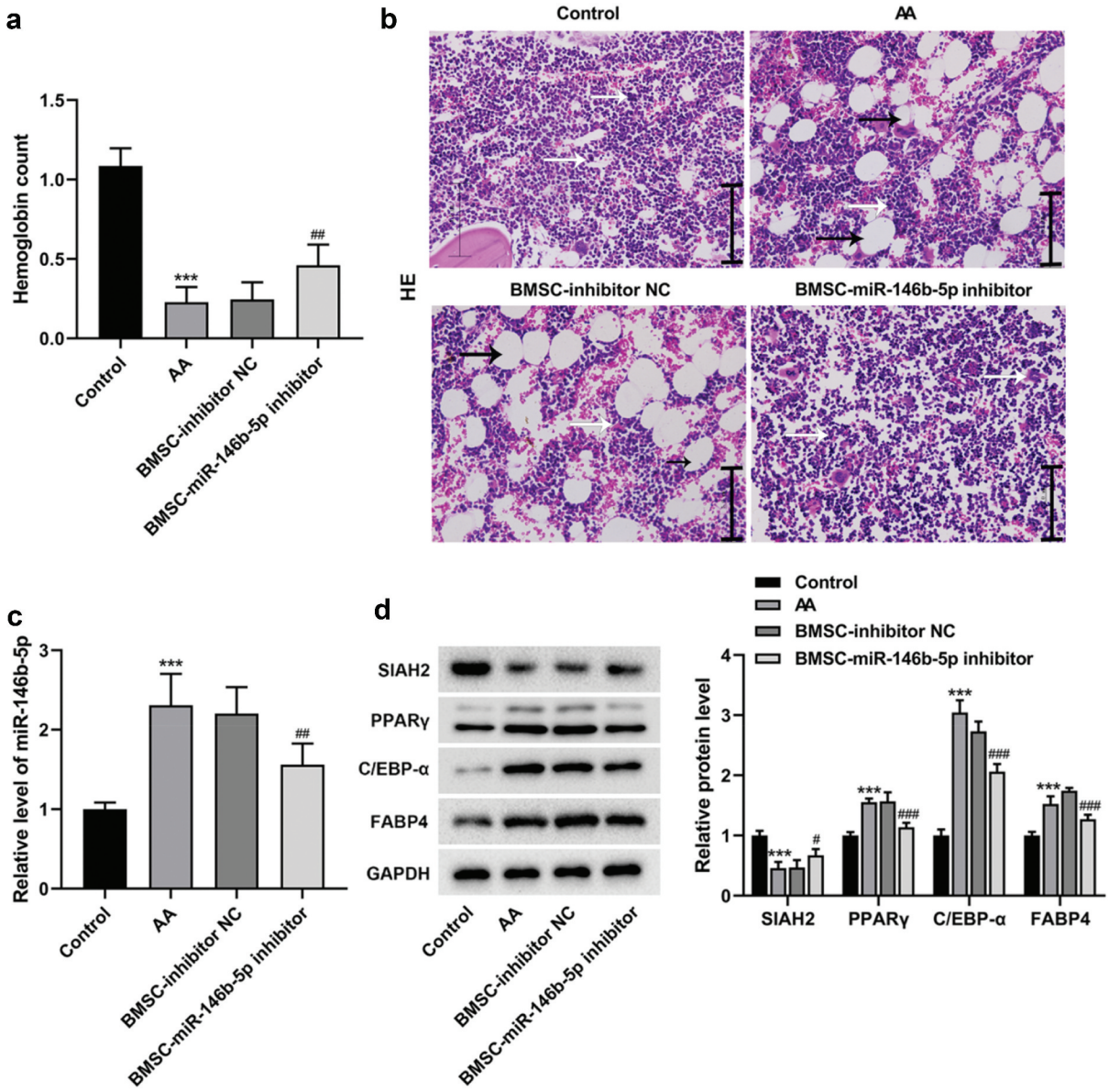

Figure 5. Effect of the interference with miR-146b-5p on the mouse with AA. Twenty-four male C57BL/6 mice were randomly divided into the following four groups for the experiments: control group (n = 6), AA group (n = 6), BMSC-inhibitor NC group (n = 6), BMSC-miR-146b-5p inhibitor group (n = 6). (a) Hemoglobin counts were performed on each of the above groups. $^{***}P < 0.001$ *vs* control group, $^{\#\#}P < 0.01$ *vs* BMSC-inhibitor NC group. (b) Hematoxylin-eosin (HE) staining was used to evaluate the changes in bone marrow histopathology in each group (Scale bar, 5.0 μm). (c) qRT-PCR was used to detect the expression of miR-146b-5p. $^{***}P < 0.001$ *vs* control group. $^{\#\#}P < 0.01$ *vs* BMSC-inhibitor NC group. (d) Western blot was performed to detect the expressions of SIAH2 and adipogenic differentiation-related proteins PPARγ, C/EBP-α, and FABP4. $^{***}P < 0.001$ *vs* control group. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs* BMSC-inhibitor NC group.

focus of the following study. Emerging evidence suggests that E3 ubiquitin ligase SIAH2 is involved in the regulation of many diseases progression via mediating proteins ubiquitination [40,41]. As reported, SIAH2-mediated HDAC3 protein ubiquitination is regulated by miR-335 [42]. Besides, another study has shown that SIAH2 can mediate the ubiquitination of Smad7 protein and this process is regulated by miR-146b [43]. Furthermore, it is worth noting that SIAH2 can down-regulate PPARγ expression by promoting PPARγ ubiquitination [44]. Therefore, discovering the miRNAs

that can regulate SIAH2-mediated PPARγ ubiquitination is of great significance to improve AA. In this study, we found that SIAH2 bound to miR-146b-5p and SIAH2-mediated PPARγ ubiquitination was also regulated by miR-146b-5p. Besides, our further studies had shown that miR-146b-5p could attenuate AA by promoting SIAH2-mediated PPARγ ubiquitination, which was consistent with the above findings.

In general, our experimental data clarified that miR-146b-5p was overexpressed in BMSCs from children with AA, and our further studies indicated that miR-146b-5p improved AA via promoting SIAH2-mediated PPARγ protein ubiquitination. Besides, the animal experiments also indicated that the down-regulation of miR-146b-5p could improve AA. This research might provide new insights for improving AA, which was of great significance.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

[1] Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. Blood. 2006;108(8):2509–2519.

[2] Korthof ET, Bekassy AN, Hussein AA. Management of acquired aplastic anemia in children. Bone Marrow Transplant. 2013;48(2):191–195.

[3] Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature. 2014;505 (7483):327–334.

[4] Gimble JM, Nuttall ME. The relationship between adipose tissue and bone metabolism. Clin Biochem. 2012;45(12):874–879.

[5] Naveiras O, Nardi V, Wenzel PL, et al. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. Nature. 2009;460(7252):259–263. .

[6] Lu TX, Rothenberg ME. MicroRNA. J Allergy Clin Immunol. 2018;141(4):1202–1207.

[7] Zhu JY, Heidersbach A, Kathiriya IS, et al. The E3 ubiquitin ligase Nedd4/Nedd4L is directly regulated by microRNA 1. Development. 2017;144(5):866–875. .

[8] Li Q, Li Z, Wei S, et al. Overexpression of miR-584-5p inhibits proliferation and induces apoptosis by targeting WW domain-containing E3 ubiquitin protein ligase 1 in gastric cancer. J Exp Clin Cancer Res. 2017;36(1):59. .

[9] Li B. MicroRNA regulation in osteogenic and adipogenic differentiation of bone mesenchymal stem cells and its application in bone regeneration. Curr Stem Cell Res Ther. 2018;13(1):26–30.

[10] Hosokawa K, Kajigaya S, Feng X, et al. A plasma microRNA signature as a biomarker for acquired aplastic anemia. Haematologica. 2017;102(1):69–78. .

[11] Adhikari S, Mandal P. Integrated analysis of global gene and microRNA expression profiling associated with aplastic anaemia. Life Sci. 2019;228:47–52.

[12] Xi FX, Wei CS, Xu YT, et al. MicroRNA-214-3p targeting ctnnb1 promotes 3T3-L1 preadipocyte differentiation by interfering with the Wnt/beta-catenin signaling pathway. Int J Mol Sci. 2019;20(8):1816.

[13] Zhao J, Wang C, Song Y, et al. Arsenic trioxide and microRNA-204 display contrary effects on regulating adipogenic and osteogenic differentiation of mesenchymal stem cells in aplastic anemia. Acta Biochim Biophys Sin (Shanghai). 2014;46(10):885–893. .

[14] Harris SG, Phipps RP. The nuclear receptor PPAR gamma is expressed by mouse T lymphocytes and PPAR gamma agonists induce apoptosis. Eur J Immunol. 2001;31(4):1098–1105.

[15] Lehrke M, Lazar MA. The many faces of PPARgamma. Cell. 2005;123(6):993–999.

[16] Xu X, Li R, Zhou Y, et al. Dysregulated systemic lymphocytes affect the balance of osteogenic/adipogenic differentiation of bone mesenchymal stem cells after local irradiation. Stem Cell Res Ther. 2017;8 (1):71. .

[17] Gu C, Xu Y, Zhang S, et al. miR-27a attenuates adipogenesis and promotes osteogenesis in steroid-induced rat BMSCs by targeting PPARgamma and GREM1. Sci Rep. 2016;6:38491.

[18] Xu Y, Takahashi Y, Wang Y, et al. Downregulation of GATA-2 and overexpression of adipogenic gene-PPARgamma in mesenchymal stem cells from patients with aplastic anemia. Exp Hematol. 2009;37 (12):1393–1399. .

[19] Floyd ZE, Stephens JM. Interferon-gamma-mediated activation and ubiquitin-proteasome-dependent degradation of PPARgamma in adipocytes. J Biol Chem. 2002;277(6):4062–4068.

[20] Watanabe M, Takahashi H, Saeki Y, et al. The E3 ubiquitin ligase TRIM23 regulates adipocyte differentiation via stabilization of the adipogenic activator PPARgamma. Elife. 2015;4:e05615.

[21] Kilroy G, Kirk-Ballard H, Carter LE, et al. The ubiquitin ligase Siah2 regulates PPARgamma activity in adipocytes. Endocrinology. 2012;153(3):1206–1218. .

[22] Stiehler M, Rauh J, Bünger C, et al. In vitro characterization of bone marrow stromal cells from osteoarthritic donors. Stem Cell Res. 2016;16(3):782–789. .

[23] Saeed H, Taipaleenmäki H, Aldahmash AM, et al. Mouse embryonic fibroblasts (MEF) exhibit a similar but not

identical phenotype to bone marrow stromal stem cells (BMSC). Stem Cell Rev Rep. 2012;8(2):318–328. .

[24] Zhu Y, Liu T, Song K, et al. Adipose-derived stem cell: a better stem cell than BMSC. Cell Biochem Funct. 2008;26(6):664–675. .

[25] Zhang GP, Zhang J, Zhu C-H, et al. MicroRNA-98 regulates osteogenic differentiation of human bone mesenchymal stromal cells by targeting BMP2. J Cell Mol Med. 2017;21(2):254–264. .

[26] Vajrala N, Bottomley PJ, Stahl DA, et al. Cycloheximide prevents the de novo polypeptide synthesis required to recover from acetylene inhibition in Nitrosopumilus maritimus. FEMS Microbiol Ecol. 2014;88(3):495–502. .

[27] Yan H, Ma Y-L, Gui Y-Z, et al. MG132, a proteasome inhibitor, enhances LDL uptake in HepG2 cells in vitro by regulating LDLR and PCSK9 expression. Acta Pharmacol Sin. 2014;35(8):994–1004. .

[28] Wang Y, Niu Z-Y, Guo Y-J, et al. IL-11 promotes the treatment efficacy of hematopoietic stem cell transplant therapy in aplastic anemia model mice through a NF-κB/microRNA-204/thrombopoietin regulatory axis. Exp Mol Med. 2017;49(12):e410. .

[29] Wang J, Zhu L, Chen X, et al. Human bone marrow mesenchymal stem cells functionalized by hybrid baculovirus-adeno-associated viral vectors for targeting hypopharyngeal Carcinoma. Stem Cells Dev. 2019;28 (8):543–553. .

[30] Wang J, Li S-F, Wang T, et al. Isopsoralen-mediated suppression of bone marrow adiposity and attenuation of the adipogenic commitment of bone marrow-derived mesenchymal stem cells. Int J Mol Med. 2017;39 (3):527–538. .

[31] Patrick C, Alireza A, Gyorgy H. RNA binding proteins in the miRNA pathway. Int J Mol Sci. 2015;17(1):31.

[32] Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. Cell. 2012;148(6):1172–1187.

[33] Wang J, Liu S, Li J, et al. Roles for miRNAs in osteogenic differentiation of bone marrow mesenchymal stem cells. Stem Cell Res Ther. 2019;10(1):197. .

[34] Li Y, Yang F, Gao M, et al. miR-149-3p regulates the switch between adipogenic and osteogenic differentiation of BMSCs by targeting FTO. Mol Ther Nucleic Acids. 2019;17:590–600.

[35] Zhu E, Zhang J, Zhou J, et al. miR-20a-5p promotes adipogenic differentiation of murine bone marrow stromal cells via targeting Kruppel-like factor 3. J Mol Endocrinol. 2018;60(3):225–237.

[36] Deng S, Zeng YQ, Wu LQ, et al. The regulatory roles of VEGF-Notch signaling pathway on aplastic anemia with kidney deficiency and blood stasis. J Cell Biochem. 2018;120(2):2078–2089.

[37] Siersbaek R, Nielsen R, Mandrup S. PPARgamma in adipocyte differentiation and metabolism–novel insights from genome-wide studies. FEBS Lett. 2010;584(15):3242–3249.

[38] Moerman EJ, Teng K, Lipschitz DA, et al. Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stroma/stem cells: the role of PPAR-gamma2 transcription factor and TGF-beta/BMP signaling pathways. Aging Cell. 2004;3(6):379–389. .

[39] Wang X, Ma F-X, Lu S-H, et al. [Effects of rapamycin on biological characteristics of bone marrow mesenchymal stem cells from patients with aplastic anemia]. Zhongguo Shi Yan Xue Ye Xue Za Zhi. 2014;22(3):762–766. .

[40] Jing Y, Nguyen MM, Wang D, et al. DHX15 promotes prostate cancer progression by stimulating Siah2-mediated ubiquitination of androgen receptor. Oncogene. 2018;37(5):638–650. .

[41] Kim H, Claps G, Möller A, et al. Siah2 regulates tight junction integrity and cell polarity through control of ASPP2 stability. Oncogene. 2014;33(15):2004–2010. .

[42] Kim Y, Kim H, Park D, et al. miR-335 targets SIAH2 and confers sensitivity to anti-cancer drugs by increasing the expression of HDAC3. Mol Cells. 2015;38 (6):562–572. .

[43] Liao Y, Zhang M, Lonnerdal B. Growth factor TGF-beta induces intestinal epithelial cell (IEC-6) differentiation: miR-146b as a regulatory component in the negative feedback loop. Genes Nutr. 2013;8 (1):69–78.

[44] Kilroy G, Carter LE, Newman S, et al. The ubiquitin ligase Siah2 regulates obesity-induced adipose tissue inflammation. Obesity (Silver Spring). 2015;23(11):2223–2232. .