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PPAR Gamma-Regulated MicroRNA 199a-5p Underlies Bone Marrow Adiposity in Aplastic Anemia

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Increased propensity of bone marrow-derived mesenchymal stem cells (BM-MSCs) toward adipogenic differentiation has been implicated in the fatty bone marrow and defective hematopoiesis of aplastic anemia (AA). However, the underlying molecular mechanism remains to be investigated. In this study, we found that microRNA 199a-5p (miR-199a-5p) exhibits significantly higher expression in AA BM-MSCs compared with the normal control and is demonstrated to facilitate adipogenic differentiation of BM-MSCs through lentivirus-mediated miR-199a overexpression. Mechanistic investigation reveals that miR-199a-5p could be regulated by PPAR gamma (PPARγ) in a transcription-independent manner and regulates adipogenic differentiation by targeting the expression of transforming growth factor beta induced (TGFBI), which is subsequently validated as a negative regulator of adipogenesis. Besides, the positive correlation between PPARγ and miR-199a-5p expression as well as the inverse relationship between miR-199a-5p and TGFBI expression in normal and AA BM-MSCs was observed. Altogether, our work demonstrates that PPARγ-regulated miR-199a-5p promotes adipogenesis of BM-MSCs by inhibiting TGFBI expression, which might be a novel mechanism underlying the bone marrow adiposity in AA, and provides promising therapeutic targets for AA treatment.

INTRODUCTION

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are multipotent stem cells with the capacity to self-renew and differentiate into multiple lineages, including adipocytes, osteoblasts, and so forth.[1] Accumulating evidence indicates that the dysregulation of the adipo-osteogenic balance has been linked to several pathophysiologic processes, such as obesity, osteoporosis, advancing age, and aplastic anemia (AA).[2–4] AA is a kind of hematopoietic disorder characterized by pancytopenia and fatty bone marrow.[5] BM-MSCs and their derived cells constitute the major cellular components of the bone marrow microenvironment, and bone marrow adipocytes play an important role in the homeostasis of the hematopoietic microenvironment and bone remodeling.[6,7] We and other groups have previously demonstrated the increased propensity of AA-derived BM-MSCs toward adipogenic differentiation.[8,9] Rescuing cell lineage commitment of BM-MSCs is a potential remedy for AA treatment. Despite considerable progress, the scientific issue concerning increased bone marrow fat in AA remains to be investigated. Dissecting the molecular mechanism of bone marrow adipogenesis is of great significance in understanding bone marrow adipocyte-mediated pathophysiologic processes and providing potential therapeutic targets for the related disorders, including AA.

Approximately two-thirds of protein abundance variation of mammalian cells can be accounted for by posttranscriptional mechanisms.[10] As important posttranscriptional regulators, microRNAs (miRNAs) are a class of small (21∼23 nt) endogenous non-coding RNAs that can regulate gene expression through base pairing of miRNA seed sequences with the target RNAs.[11] In the past two decades, miRNAs have been intensively studied, and increasing evidence indicates that they can participate in many physiological and pathological processes.[12] A wide variety of miRNAs have been demonstrated to mediate the lineage commitment of MSCs, including adipogenic and/or adipo-osteogenic differentiation.[13–15] However, there are still many miRNAs to be identified in bone marrow adipocyte development. miR-199a-5p is one of our previously investigated miRNAs that negatively regulated monocyte-macrophage differentiation through the ACVR1B-mediated Smad pathway.[16,17] The role and mechanism of miR-199a-5p in adipogenesis of human BM-MSCs and its relationship with AA have never been reported.

Here we demonstrate that miR-199a-5p can be regulated by PPARγ and promote adipogenic differentiation of BM-MSCs by targeting the expression of transforming growth factor beta induced (TGFBI),

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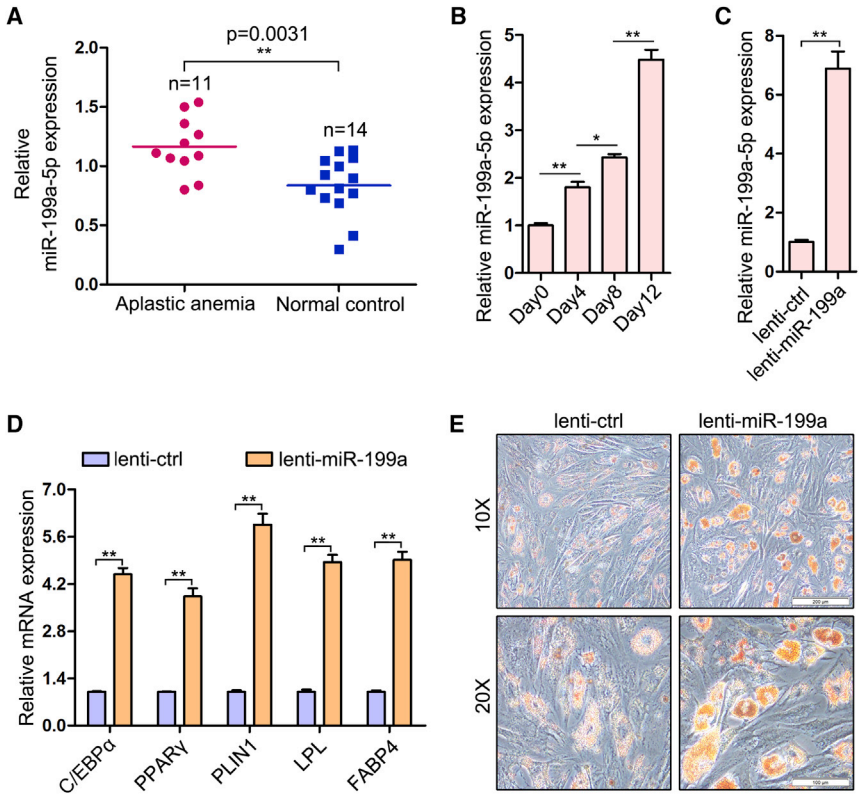

Figure 1. miR-199a-5p Regulates Adipogenic Differentiation of BM-MSCs

(A) miR-199a-5p expression was detected using qRT-PCR in BM-MSCs derived from aplastic anemia patients and normal counterparts. U6 small nuclear RNA (snRNA) was used as an internal control. (B) qRT-PCR analysis of miR-199a-5p expression during the adipogenic differentiation of BM-MSCs. (C and D) miR-199a-5p (C) and adipogenic markers C/EBPα, PPARγ, PLIN1, LPL, and FABP4 mRNA (D) were detected using qRT-PCR in BM-MSCs infected with lenti-miR-199a or lenti-ctrl, followed by adipogenic induction for 10 days. GAPDH was used as an internal control for adipogenic markers. (E) The lentiviruses infected BM-MSCs followed by adipogenic induction for 10 days were stained with oil red O and captured using an Olympus microscope under 10× and 20× objective lenses and a representative experiment was presented. *$p < 0.05$ and **$p < 0.01$.

which is subsequently validated as a negative regulator of adipogenesis. We also found that miR-199a-5p exhibits increased expression in BM-MSCs derived from AA patients compared with the normal counterpart, whose expression presents significant positive and negative correlation with PPARγ and TGFBI expression, respectively, in normal and AA BM-MSCs. The results confer that PPARγ-regulated miR-199a-5p might underlie the bone marrow adiposity in AA, which will provide promising therapeutic targets for AA treatment.

RESULTS

miR-199a-5p Expression Underlies Increased Adipogenic Differentiation of AA BM-MSCs

AA patients are often characterized by an increase in fat cells in the bone marrow. Our previous work demonstrated the increased propensity of AA BM-MSCs toward adipogenic differentiation.[8] To verify whether miR-199a-5p is associated with increasing adipocytes in AA bone marrow, we first detected miR-199a-5p expression in AA BM-MSCs and their normal counterparts. As shown in Figure 1A, the expression of miR-199a-5p is significantly higher in AA BM-MSCs than in normal control. Besides, miR-199a-5p was upregulated significantly during the adipogenic induction time course of BM-MSCs (Figure 1B), implying that miR-199a-5p may act as a positive regulator in adipocyte development. To further investigate the role of miR-199a-5p in adipogenesis of BM-MSCs, we used the recombined lentiviruses that express miR-199a (lenti-miR-199a) to infect BM-MSCs, followed by adipogenic induction for 10 days. qRT-PCR analysis revealed that lenti-miR-199a infection remarkably increased miR-199a-5p expression (Figure 1C). To evaluate adipogenesis, C/EBPα, PPARγ, PLIN1, LPL, and FABP4 were selected as adipogenic markers because they were previously reported as key adipogenesis-related genes,[18] whose expression profiles during adipogenic differentiation were performed and are presented in Figure S1. As shown in Figure 1D, forced miR-199a-5p expression resulted in significant upregulation of the mRNA level of the adipogenic differentiation markers (C/EBPα, PPARγ, PLIN1, LPL, and FABP4) compared with the lenti-control (lenti-ctrl) infection. Besides, oil red O staining demonstrated that overexpression of miR-199a-5p also exhibited more lipid droplets compared with the control (Figure 1E). The results demonstrated that miR-199a-5p facilitates adipogenic differentiation of BM-MSCs, which, together with the high expression of miR-199a-5p in AA BM-MSCs, underlies the increased adipocytes in AA bone marrow.

TGFBI Is Identified as a Target of miR-199a-5p

As is well known, miRNAs exert their function mainly by binding to the target RNA through complementary hybridization, at least between nucleotides 2–8, numbered from the 5′ end (seed sequences) of the small RNAs and negatively regulating their expression.[19] To seek the downstream targets of miR-199a-5p in adipogenesis, we focused on the genes which bear the binding site of miR-199a-5p seed sequences and are downregulated in adipogenic differentiation. TGFBI, which presents decreased expression upon adipogenic induction and also contains a binding site of miR-199a-5p in the coding region, was identified as a potential target. The stable binding between TGFBI and miR-199a-5p was also predicted by use of the RNAhybrid web tool (Figure 2A). To demonstrate whether TGFBI is a target of miR-199a-5p, we first detected TGFBI expression in lenti-miR-199a-infected and adipogenic differentiation-induced BM-MSCs.

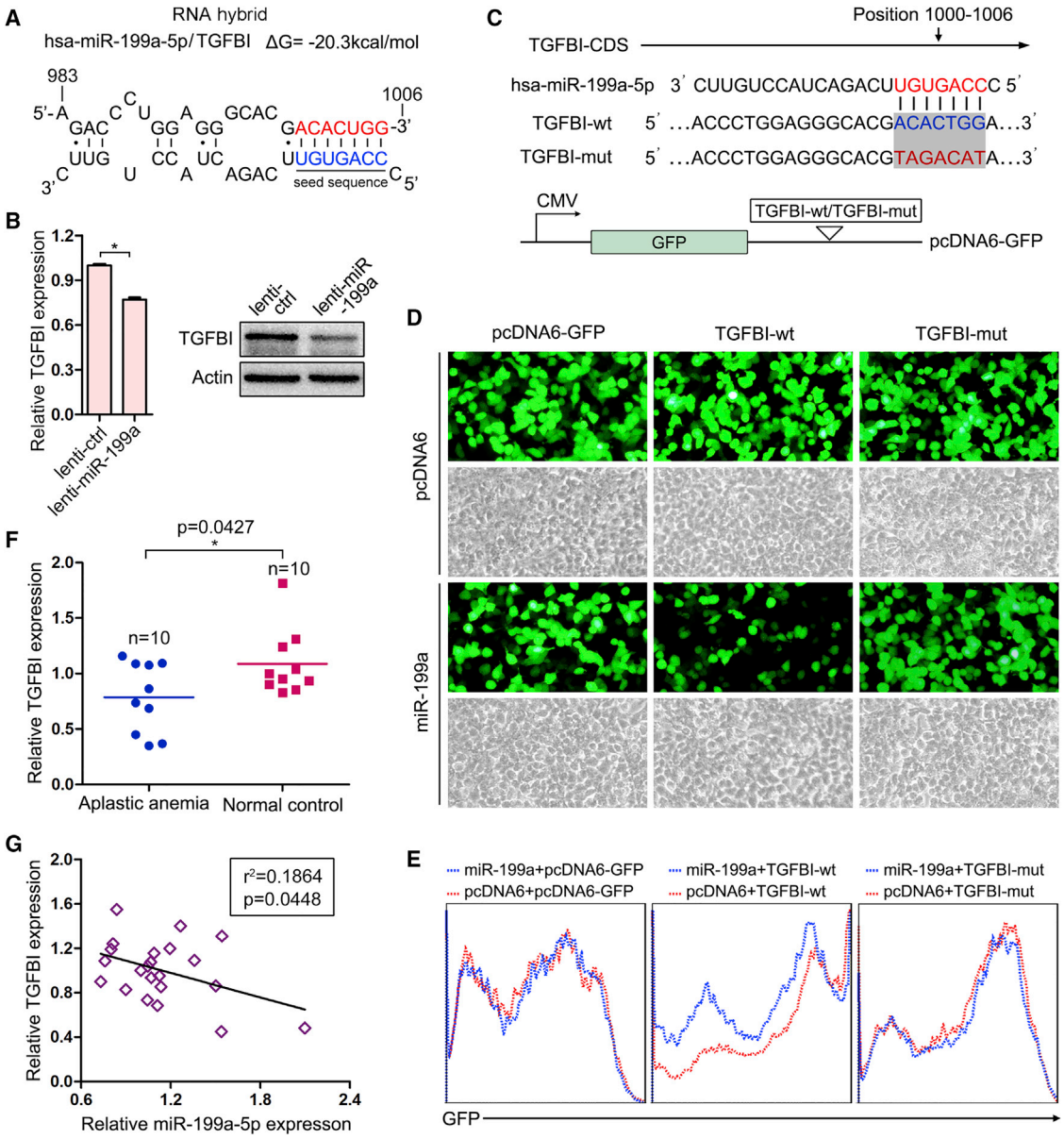

Figure 2. TGFBI Is Demonstrated as a Target of miR-199a-5p

(A) Schematic illustration of the interaction between TGFBI and miR-199a-5p predicted by the RNAhybrid web tool. (B) qRT-PCR and western blot analysis of TGFBI expression in BM-MSCs infected with lenti-miR-199a or lenti-ctrl, followed by adipogenic induction for 10 days. GAPDH and actin were used as internal controls, respectively. (C) The sequences of miR-199a-5p, together with the predicted binding site of miR-199a-5p in TGFBI (WT and mut) were shown, and constructs for TGFBI-WT and TGFBI-mut were also illustrated. (D and E) GFP reporter assay. 293TN cells were co-transfected with each pcDNA6-GFP-based construct (pcDNA6-GFP, TGFBI-WT, and TGFBI-mut) and pcDNA6-miR-199a (or pcDNA6). The relative GFP expression was presented as fluorescence pictures (D) and analyzed by flow cytometry (E). (F) TGFBI expression was detected using qRT-PCR in BM-MSCs derived from aplastic anemia patients and normal counterparts. GAPDH was used as an internal control. (G) The correlation between TGFBI and miR-199a-5p expression in 12 aplastic anemia and 10 normal samples was performed using linear regression analysis. *$p < 0.05$ and **$p < 0.01$.

As shown in Figure 2B, lenti-miR-199a infection significantly decreased TGFBI expression at both the mRNA and protein level compared with control infection. To further confirm whether TGFBI is a direct target of miR-199a-5p, the fragment of TGFBI containing wild-type (WT) or mutant (mut) miR-199a-5p binding site was cloned into pcDNA6-GFP for the GFP reporter assay, as depicted in Figure 2C. The GFP expression was presented as fluorescence pictures and flow cytometry analysis (Figures 2D and 2E), which demonstrated that miR-199a-5p negatively regulates GFP expression in a miR-199a-5p binding site-dependent manner. Further, the

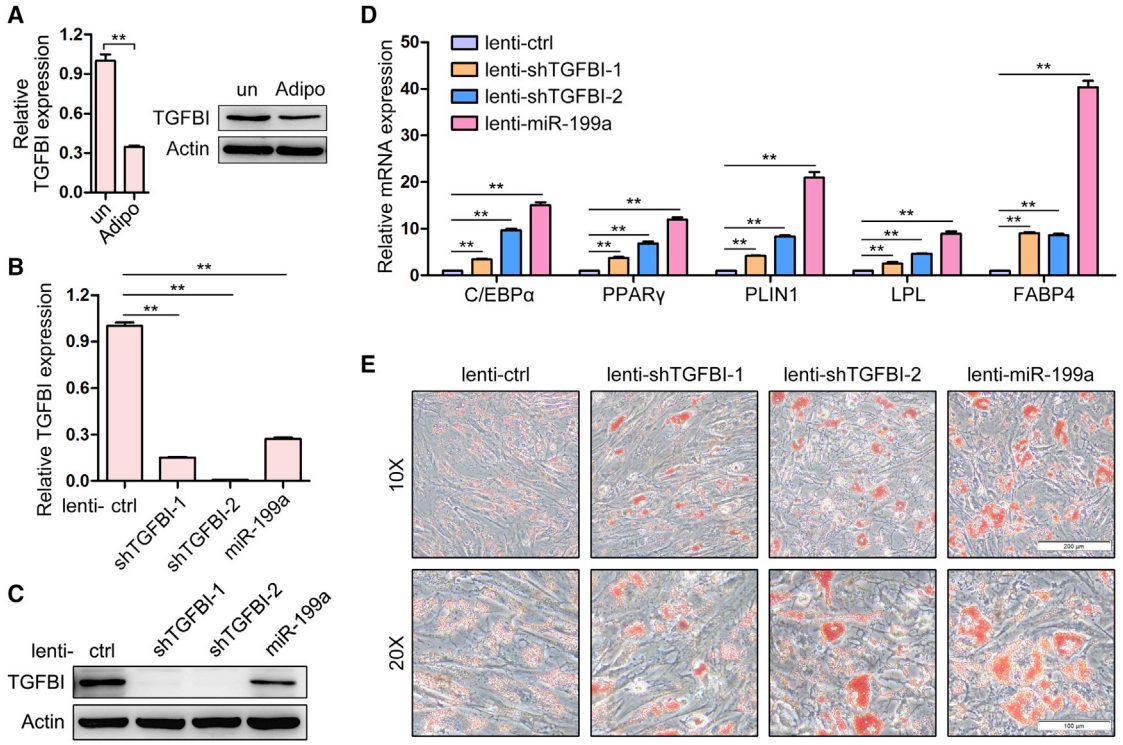

Figure 3. TGFBI Is Involved in the Adipogenesis of BM-MSC

(A) The expression of TGFBI mRNA and protein were detected in BM-MSCs cultured without (un) and with adipogenic medium (Adipo) for 8 days. GAPDH and actin were used as internal controls, respectively. (B and C) qRT-PCR (B) and western blot (C) analysis of TGFBI expression in BM-MSCs infected with lenti-shTGFBI-1, lenti-shTGFBI-2, lenti-miR-199a, or lenti-ctrl, followed by adipogenic induction for 10 days. (D and E) Expression analysis of adipogenic markers (C/EBPα, PPARγ, PLIN1, LPL, and FABP4 mRNA) (D) and oil red O staining (E) were performed in lenti-ctrl/lenti-shTGFBI-1/lenti-shTGFBI-2/lenti-miR-199a-infected and adipogenic medium-induced BM-MSCs. The pictures of oil red O staining were captured using an Olympus microscope under 10× and 20× objective lens and a representative experiment was presented. Three independent experiments were performed and representative data are shown. *$p < 0.05$ and **$p < 0.01$.

expression of TGFBI in AA and normal BM-MSCs was also analyzed by qRT-PCR. As expected, TGFBI showed decreased expression in AA BM-MSCs compared with the normal control (Figure 2F), whose expression had a significantly negative correlation with miR-199a-5p expression in AA and normal BM-MSC samples, shown by linear regression analysis (Figure 2G). Together, the results suggest that TGFBI is a target of miR-199a-5p.

TGFBI Negatively Regulates Adipogenesis of BM-MSCs

To characterize the role of TGFBI in adipogenesis of BM-MSCs, we first detected TGFBI expression in BM-MSCs induced toward adipogenic differentiation for 8 days. TGFBI exhibited decreased expression at both the mRNA and protein levels in BM-MSCs upon adipogenic induction (Figure 3A). Next, we make use of the recombined lentiviruses that express two specific short hairpin RNAs (shRNAs) for TGFBI (lenti-shTGFBI-1 and lenti-shTGFBI-2) to infect BM-MSCs, followed by adipogenic induction for 10 days, with lenti-miR-199a performed side by side as a control. lenti-shTGFBI-1, lenti-shTGFBI-2, and miR-199a infection all significantly reduced TGFBI expression at both the mRNA and protein levels (Figures 3B and 3C), which resulted in remarkable upregulation of the mRNA level of the adipogenic differentiation markers (C/EBPα, PPARγ, PLIN1, LPL, and FABP4) (Figure 3D) and an obvious increase of lipid droplets (Figure 3E) as compared with the lenti-ctrl infection. The enhanced adipogenic differentiation caused by miR-199a-5p overexpression is much stronger than that by TGFBI knockdown (Figures 3D and 3E), which may be due to the fact that miR-199a-5p can also have other targets when regulating adipogenesis. The results indicate that TGFBI acts as a negative regulator of adipogenic differentiation, whose knockdown, to some extent, mimicked the phenotypic effect of miR-199a-5p overexpression on adipogenesis of BM-MSCs. To further demonstrate whether miR-199a-5p regulates adipogenic differentiation by targeting TGFBI expression, we performed rescue assay. As expected, re-infection of lenti-TGFBI reversed the downregulation of TGFBI expression resulting from lenti-miR-199a treatment (Figure S2A). Re-infection of lenti-TGFBI also alleviated the facilitation of adipogenic differentiation caused by lenti-miR-199a infection, which is presented as adipogenic marker expression (Figure S2B). These results imply that TGFBI functions directly downstream of miR-199a-5p and partly mediates the regulatory role of miR-199a-5p on adipogenesis.

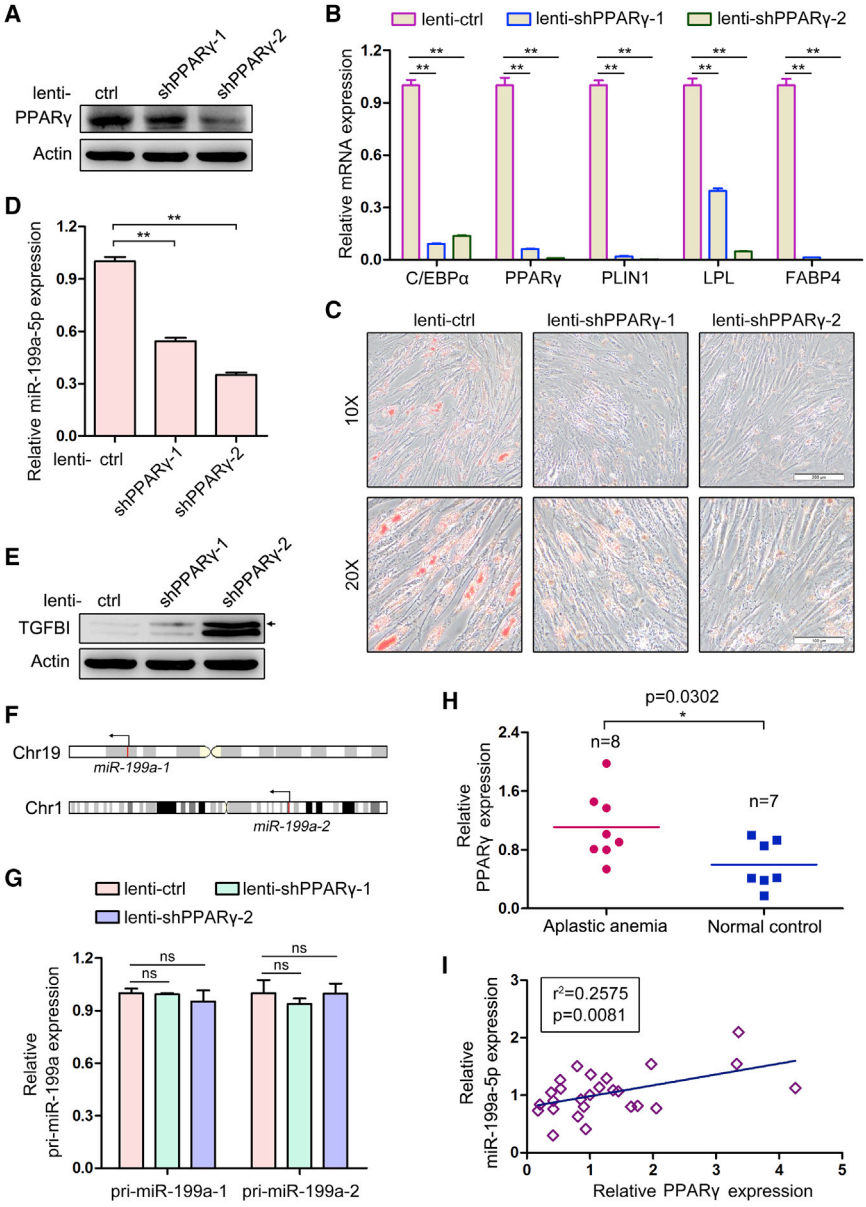

Figure 4. PPARγ Modulates miR-199a-5p Expression during Adipogenesis of BM-MSCs and Correlates with Its Expression

(A–E) BM-MSCs were infected with lenti-shPPARγ-1, lenti-shPPARγ-2, or lenti-ctrl, followed by adipogenic induction for 10 days. (A) Western blot analysis of PPARγ expression. Actin was used as an internal control. (B) qRT-PCR detection of adipogenic marker (C/EBPα, PPARγ, PLIN1, LPL, and FABP4) expression. GAPDH was used as an internal control. (C) Oil red O staining. The pictures were captured using an Olympus microscope under 10× and 20× objective lenses and a representative experiment was presented. (D) qRT-PCR analysis of miR-199a-5p expression. (E) Western blot analysis of TGFBI expression. (F) Schematic outline of the chromosome location of genes (*miR-199a-1* and *miR-199a-2*) encoding miR-199a-5p. (G) qRT-PCR analysis of pri-miR-199a-1/pri-miR-199a-2 in BM-MSCs infected with lenti-shPPARγ-1, lenti-shPPARγ-2, or lenti-ctrl. (H) PPARγ expression was detected using qRT-PCR in BM-MSCs derived from aplastic anemia patients and normal counterparts. GAPDH was used as an internal control. (I) The correlation between miR-199a-5p and PPARγ expression in 11 aplastic anemia and 15 normal samples was performed using linear regression analysis. *$p < 0.05$ and **$p < 0.01$.

PPARγ Regulates and Correlates with miR-199a-5p Expression in BM-MSCs

Lineage commitment and subsequent cell maturation of MSCs are mainly directed by lineage-specific transcription factors.[20] PPARγ is considered a master regulator of adipogenic fate decision.[21] Our previous work demonstrated that PPARγ expression positively correlates with the adipogenic capacity of MSCs with different tissue origins.[22] In this study, we further validated the regulatory role of PPARγ with its two specific shRNAs in the adipogenesis of BM-MSCs. As expected, lentiviruse-mediated knockdown of PPARγ (Figure 4A) significantly repressed the mRNA expression of adipogenic markers (C/EBPα, PPARγ, PLIN1, LPL, and FABP4) (Figure 4B) and resulted in less formation of lipid droplets (Figure 4C).

Besides, the expression of miR-199a-5p was also remarkably attenuated by lenti-shPPARγ-1 and lenti-shPPARγ-2 infection (Figure 4D), accompanied by the upregulation of TGFBI protein level (Figure 4E). Genomic analysis revealed that there are two human genes (*has-miR-199a-1* located on chromosome 19 and *has-miR-199a-2* located on chromosome 1) encoding miR-199a (Figure 4F). With the use of ChIPbase, we found that PPARγ has binding sites on promoters of the two genes (*has-miR-199a-1* and *has-miR-199a-2*). To investigate whether PPARγ regulates the transcription of miR-199a-5p, we first detected the expression of two miR-199a primary transcripts (pri-miR-199a-1 and pri-miR-199a-2) in adipogenesis of BM-MSCs after PPARγ knockdown. Unfortunately, PPARγ had no influence on the expression of pri-miR-199a-1 and pri-miR-199a-2 (Figure 4G), which indicates that PPARγ may regulate miR-199a-5p expression in a transcription-independent manner. To further clarify how PPARγ regulates miR-199a-5p expression, we also detected the expression of pre-miR-199a-1/-2 and miR-199a-3p after PPARγ knockdown. As shown in Figure S3, miR-199a-3p expression was not as obviously affected as miR-199a-5p by PPARγ knockdown, whereas PPARγ knockdown results in a mild increase of pre-miR-199a-1 and a slight decrease of pre-miR-199a-2, indicating that PPARγ may participate in the processing of pre-miR-199a-1 and maintain the stability of pre-miR-199a-2,

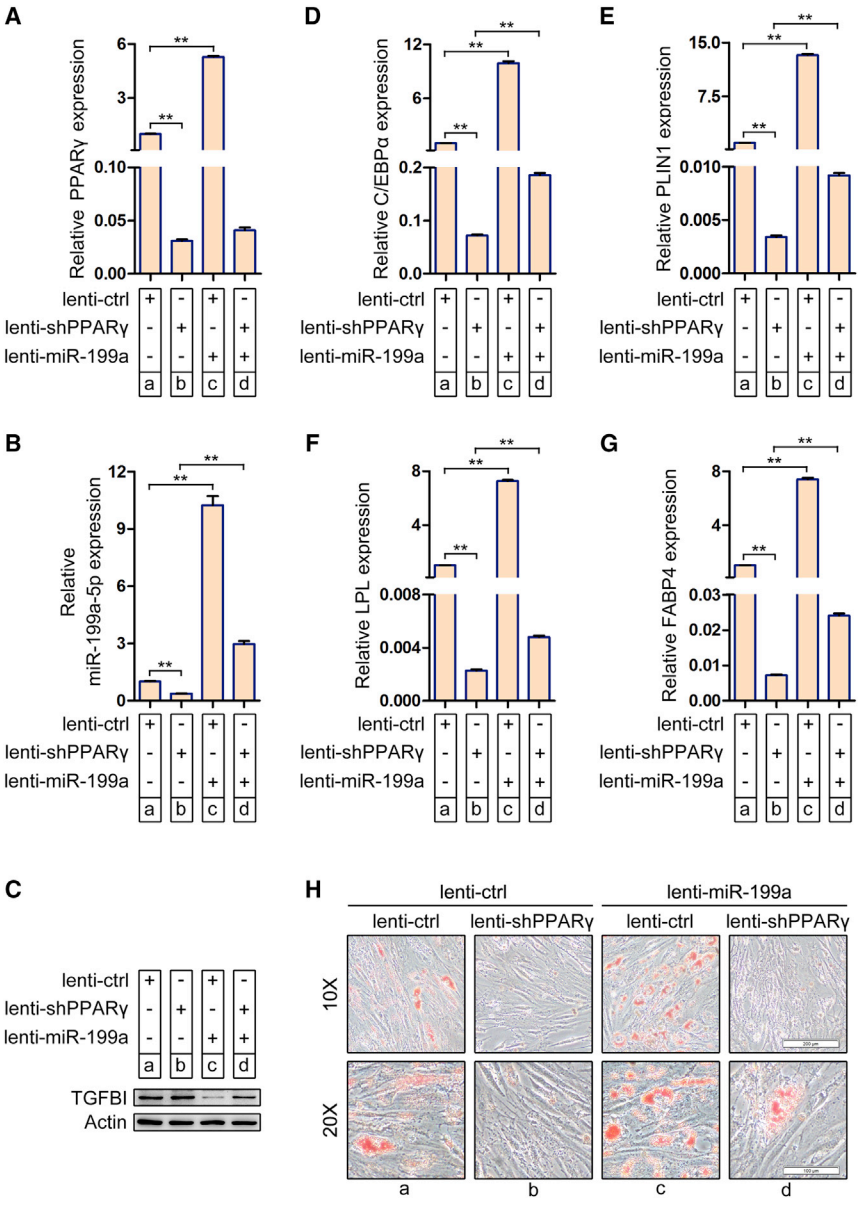

Figure 5. miR-199a-5p Mediates the PPARγ-Directed Adipogenic Differentiation of BM-MSCs

(A and B) PPARγ mRNA (A) and miR-199a-5p (B) were evaluated in BM-MSCs infected with lenti-shPPARγ (lenti-ctrl) and lenti-miR-199a (lenti-ctrl), followed by adipogenic induction for 10 days. (C) Western blot analysis of TGFBI expression in the above BM-MSCs. (D–G) Adipogenic marker C/EBPα (D), PLIN1 (E), LPL (F), and FABP4 (G) expression was detected in lentivirues infected BM-MSCs followed by adipogenic induction for 10 days. (H) Oil red O staining of the above BM-MSCs. The pictures were captured using an Olympus microscope under 10× and 20× objective lenses and a representative experiment was presented. *$p < 0.05$ and **$p < 0.01$.

both of which can lead to the downregulation of miR-199a-5p after PPARγ knockdown. In general, our results imply that PPARγ may regulate miR-199a-5p expression by influencing the processing or stability of pre-miR-199a at the posttranscriptional level. Next, we evaluated the expression of PPARγ in AA and normal BM-MSCs and analyzed its correlation with miR-199a-5p expression in the samples. As manifested in Figures 4H and 4I, PPARγ presented increased expression in AA BM-MSCs compared with the normal control (Figure 4H), which has a significant positive correlation with miR-199a-5p expression (Figure 4I). Our results imply that PPARγ-regulated miR-199a-5p expression may account for the increased adipocytes in the AA bone marrow.

miR-199a-5p Mediates the PPARγ-Directed Adipogenic Differentiation of BM-MSCs

To further demonstrate whether PPARγ directs adipogenic differentiation of BM-MSCs partly by regulating miR-199a-5p expression, we performed rescue assay. BM-MSCs were infected with lenti-shPPARγ or lenti-ctrl. Twenty-four hours later, the cells were re-infected with lenti-miR-199a or lenti-ctrl and induced toward adipogenic differentiation for 10 days. As expected, infection with lenti-shPPARγ significantly decreased PPARγ expression (Figure 5A, b versus a), inhibited miR-199a-5p expression (Figure 5B, b versus a) with concomitant upregualtion of TGFBI expression (Figure 5C, b versus a), and impaired adipogenic differentiation, as revealed by the mRNA expression of adipogenic markers (C/EBPα, PLIN1, LPL, and FABP4) (Figures 5D–5G, b versus a) and formation of droplets evaluated through oil red O staining (Figure 5H, b versus a). However, re-infection with lenti-miR-199a not only recovered the expression of miR-199a-5p (Figure 5B, d versus b), reversing the upregulation of TGFBI expression caused by PPARγ knockdown (Figure 5C, d versus b), but it also ameliorated the blockade of adipogenic differentiation resulting from lenti-shPPARγ infection, as revealed by the mRNA expression of adipogenic markers (C/EBPα, PLIN1, LPL, and FABP4) (Figures 5D–5G, d versus b) and formation of droplets evaluated through oil red O staining (Figure 5H, d versus b). A lower miR-199a-5p level together with higher TGFBI expression was also observed in cells co-infected with lenti-shPPARγ and lenti-miR-199a than in cells co-infected with lenti-miR-199a and lenti-ctrl (Figures 5B and 5C, d versus c), again demonstrating the positive regulatory role of PPARγ on miR-199-5p expression. Collectively, these results demonstrate that miR-199a-5p partly mediates the PPARγ-directed adipogenic processes of BM-MSCs.

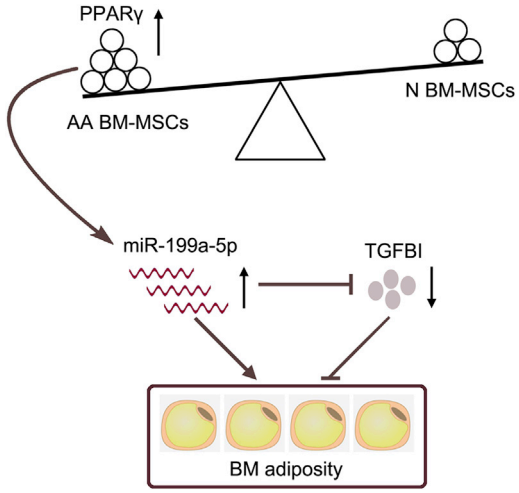

Figure 6. Schematic Representation of miR-199a-5p's Involvement in the Regulation of Bone Marrow Adiposity

miR-199a-5p could be regulated by PPARγ and facilitates adipogenic differentiation of BM-MSCs by targeting the expression of TGFBI, which is subsequently validated as a negative regulator of adipogenesis.

DISCUSSION

AA is generally considered an immune-mediated bone marrow failure syndrome, characterized by defective hematopoiesis and an increase in fat cells in the bone marrow.[5] BM-MSC-mediated bone marrow microenvironment changes and the adipo-osteogenic differentiation imbalance might play an essential role in the pathogenesis of AA.[23,24] Previous work has demonstrated that AA BM-MSCs display decreased proliferation,[25] an altered transcriptome profile,[26] and impaired differentiation, with more susceptibility to differentiate into adipocytes at the expense of osteoblasts,[4,8] which is, to some extent, similar to the characteristics of BM-MSCs derived from aging and osteoporotic populations.[27,28] Adipocytes in the bone marrow are predominantly negative regulators of hematopoiesis,[7] whereas osteoblasts often provide a supporting microenvironment for hematopoietic development in addition to their role in the bone formation.[29,30] Revealing the molecular mechanism underlying the adipocyte-osteoblast differentiation imbalance of BM-MSCs is of great value in understanding AA pathogenesis and providing therapeutic insight into MSC-mediated regenerative medicine. miRNA is an important posttranscriptional regulator, and a number of miRNAs have been identified to regulate adipogenesis or adipo-osteogenic differentiation of BM-MSCs.[31–33] In this study, we investigated the potential role of miR-199a-5p in the fatty bone marrow of AA. First, miR-199a-5p was chosen for its significant high expression in AA BM-MSCs compared with the normal control. Subsequently, miR-199a-5p was demonstrated to positively regulate the adipogenic differentiation of BM-MSCs through lentivirus-mediated overexpression.

To our knowledge, miRNAs function in various biological processes by regulating the expression of their target genes. Since TGFBI expression is negatively correlated with the miR-199a-5p level in normal and AA BM-MSCs and a complementary site with miR-199a-5p seed sequence exists in the coding region of TGFBI, we speculated that TGFBI may be a target of miR-199a-5p. Indeed, we found that overexpression of miR-199a-5p resulted in decreased expression of TGFBI at both the mRNA and protein level. GFP reporter assay further confirmed TGFBI as a direct target of miR-199a-5p. Accumulating evidence indicates that the differentiation of MSCs into adipocytes and osteoblasts involves multiple pathways, including transforming growth factor beta (TGF-β), Wnt, and Hedgehog signals.[34] In the past few decades, the TGF-β/Smad pathway has been intensively studied and is considered to play an important role in regulating the balance between osteogenic and adipogenic differentiation of MSCs, which reciprocally facilitates osteogenesis and inhibits adipogenesis.[35] TGFBI is a TGF-β induced protein, whose role is still unclear in the multiple lineage differentiation of MSCs. This study demonstrates that TGFBI is downregulated in adipogenic differentiation of BM-MSCs and is subsequently verified as a negative regulator of adipogenesis, which is in accordance with the reported function of the TGF-β/Smad pathway in adipogenesis.[36] miR-199a-5p has also been reported to regulate ACVR1B-mediated p-Smad2/3 expression.[16] As to the role of the miR-199a-5p-TGFBI axis in osteogenesis of BM-MSCs, it will be further elucidated in our future work.

Adipogenesis is a highly orchestrated process that involves a coordinated cascade of transcription factors that, together, lead to the establishment of adipogenic commitment from the multipotent stem cells and subsequent adipocyte maturation.[37] The nuclear receptor PPARγ is a ligand-activated transcription factor that is characterized as a master regulator and is indispensible for adipocyte development.[38] Aberrant expression of PPARγ signaling has been implicated in obesity, diabetes, and cardiovascular disease.[39,40] Here we found that PPARγ expression is significantly higher in AA BM-MSCs compared with normal control and positively correlates with the miR-199a-5p level. Since PPARγ has binding sites on the promoters of two miR-199a coding genes inferred by the data from ChIPBase, we hypothesized that PPARγ might regulate the transcription of miR-199a. As expected, we found that knockdown of PPARγ led to significant downregulation of miR-199a-5p and also concomitant upregulation of TGFBI expression. However, lenti-shPPARγ infection has little influence on the expression of two primary miR-199a transcripts, which indicates that PPARγ probably regulates miR-199a-5p expression in a transcription-independent manner. Though the detailed mechanism as to how PPARγ regulates miR-199a-5p expression in the adipogenic differentiation of BM-MSCs still remains to be investigated, overexpression of miR-199a-5p can partly rescue the adipogenic differentiation of BM-MSCs inhibited by PPARγ knockdown.

According to the results, we summarized a model for miR-199a-5p's involvement in the regulation of bone marrow adiposity (Figure 6). Our findings reveal that miR-199a-5p presents higher expression in AA BM-MSCs, could be regulated by PPARγ, and positively facilitates bone marrow adipogenesis by inhibiting the

expression of TGFBI, which is validated as a negative regulator of adipogenic differentiation. The positive correlation between PPARγ and miR-199a-5p expression as well as the inverse relationship between miR-199a-5p and TGFBI expression suggests that the PPARγ-miR-199a-5p-TGFBI axis may underlie the fatty bone marrow of AA, which will help us gain new insight into the pathogenesis of AA and provide promising therapeutic targets for AA treatment.

MATERIALS AND METHODS

Human Samples and Cell Culture

Human bone marrow samples were obtained from the hematology department of the affiliated hospital of Jining Medical University. Informed consent to perform the biological studies was obtained from the individuals examined, and the related study was approved by the ethics committees of the hospitals and the Institutional Review Board of Jining Medical University. BM-MSCs were isolated from the bone marrow of AA patients and iron deficiency anemia (IDA) controls, and the cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (GIBCO). Fourteen AA patients (10 male and 4 female aged 4 to 68) and 17 controls (7 male and 10 female aged 27 to 75) were enrolled in this study. The AA patients were diagnosed according to the guidelines previously reported.[41] 293TN cells were cultured in DMEM with 10% FBS.

Adipogenic Differentiation

Adipogenic differentiation was performed according to the method described elsewhere with minor modifications.[42] The BM-MSCs were seeded into 12 or 6-well plates. Upon reaching confluence, the BM-MSCs were changed into adipogenic medium composed of DMEM with 10% FBS, 1 μM dexamethasone, 0.01 mg/mL insulin, 100 μg/mL indomethacin, and 0.5 mM 3-isobutyl-1-methyl-xanthine for 9–12 days (all purchased from Sigma, St. Louis, MO, USA).

Oil Red O Staining

The cells were rinsed with PBS twice in the plates after discarding the supernatant and fixed with 4% paraformaldehyde at room temperature for 20 min. Then the cells were washed with PBS and stained with oil red O (Solarbio) for 20 min followed by washing with PBS. Lipid droplets were observed and photographed under 10× and 20× objective lenses using an Olympus microscope (Olympus, Tokyo, Japan).

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from cell samples using TRIzol Reagent (Invitrogen) and quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Bremen, Germany). The first strand of cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Oligo (dT) was used as the primer for reverse transcription of mRNA. Stem-loop RT primers were used for the reverse transcription of miRNAs. GAPDH and U6 were used as their respective controls. qRT-PCR was performed in a Bio-Rad CFX-96 System (Bio-Rad, Foster City, CA, USA) using the SYBR Premix (CWBio). The primers used for reverse transcription and qRT-PCR are listed in Table S1.

Plasmid Construction

The miR-199a cDNA was amplified and cloned into pmiRNA1 (System Biosciences) and pcDNA6 (Invitrogen) to get the expression plasmids. The TGFBI open reading frame (ORF) was amplified and cloned into pTOMO (Addgene). The fragment of TGFBI coding sequence (CDS) containing miR-199a-5p binding sites was inserted into modified pcDNA6-GFP plasmid (Invitrogen). The shRNA sequences for TGFBI and PPARγ were synthesized, annealed, and inserted into pSIH1-H1-copGFP (System Biosciences). The primers and oligonucleotides used for plasmid construction are listed in Table S2.

Lentivirus Production and Cell Infection

The recombination lentiviruses for overexpression and knockdown were produced using the pmiRNA1- and pSIH1-H1-copGFP-based constructs. Lentivirus packaging was performed using the pPACKH1 Lentiviral Vector Packaging Kit (LV500A-1, System Biosciences, CA, USA) according to the manufacturer's instructions. The culture supernatant containing the virus particles was directly used to infect the BM-MSCs in 12 or 6-well plates with 5 μg/mL polybrene (Sigma Aldrich). After 24 h infection, the cells were replaced with fresh complete medium and induced toward adipogenic differentiation.

GFP Reporter Assay

293TN cells were co-transfected with each pcDNA6-GFP-based construct (pcDNA6-GFP, TGFBI-WT and TGFBI-mut) and pcDNA6-miR-199a (or pcDNA6) using UltraFection 2.0 (Beijing 4A Biotech) in 12-well plates. The transfection medium was replaced with complete medium after 5–6 h. The cells were cultured at 37°C in 5% CO_2 for an additional 24-48 h. Then the GFP expression pictures were observed and captured under an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan). The transfected cells were also collected, rinsed twice with PBS, re-suspended in 200 μL PBS, and analyzed immediately using a FACSCalibur flow cytometer (BD Biosciences, USA).

Western Blot

Cell lysates were subjected to SDS-PAGE (10% separation gel) and transferred to a polyvinylidene difluoride (PVDF) membrane. Primary antibodies against the following proteins were used: PPARγ (16643-1-AP; Proteintech), TGFBI (10188-1-AP; Proteintech), and actin (60008-1-Ig; Proteintech). Horseradish peroxidase-conjugated secondary antibodies were used (ZSGB-BIO). Signals were detected using an ECL (enhanced chemiluminescence) kit (Millipore).

Statistical Analysis

Student's t test (two-tailed) was performed to analyze the data. Statistical significance was set at $p < 0.05$, as indicated by an asterisk (*$p < 0.05$; **$p < 0.01$). The expression of miR-199a-5p, TGFBI, and PPARγ in normal and AA BM-MSCs was detected using qRT-PCR, and the obvious outliers were removed during statistical analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.07.005.

AUTHOR CONTRIBUTIONS

X.Z., Lulu Liu, and M.C. performed experiments and interpreted data. C.D., P.C., Lei Liu, H.L., S.R., C.W., S.J., and L.C. helped collect the samples and performed partial experiments. M.C. and H.Z. conceived the study and directed the experiments. M.C. wrote the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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