

miR-144-3p Suppresses Osteogenic Differentiation of BMSCs from Patients with Aplastic Anemia through Repression of TET2

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Reduced osteogenic capacity of bone marrow mesenchymal stem cells (BMSCs) has been causally linked to the development of aplastic anemia. In this work, we aimed to identify novel microRNAs (miRNAs) that participate in the regulation of differentiation of BMSCs from patients with aplastic anemia. We show that miR-144-3p is significantly upregulated in BMSCs from patients with aplastic anemia relative to control equivalents. Depletion of miR-144-3p significantly enhances osteogenic differentiation of BMSCs from patients with aplastic anemia after culturing in osteogenesis-inducing medium. Conversely, overexpression of miR-144-3p blocks osteogenic differentiation of BMSCs. Mechanistically, miR-144-3p negatively regulates the expression of ten-eleven translocation 2 (TET2) in BMSCs. Reduced TET2 expression is associated with a significant decrease in global 5-hydroxymethyl-cytosine (5hmC) levels and osteogenic gene expression. Knockdown of miR-144-3p elevates the expression of TET2 and total 5hmC levels in BMSCs. Silencing of TET2 inhibits the osteogenic differentiation of BMSCs. Overexpression of TET2 reverses miR-144-3p-mediated inhibition of osteogenesis. In addition, there is a significant negative correlation between the expression of miR-144-3p and TET2 in BMSCs from patients with aplastic anemia. Overall, miR-144-3p impairs the osteogenic capacity of BMSCs from patients with aplastic anemia through repression of TET2. Therefore, the targeting of miR-144-3p may be a therapeutic strategy against aplastic anemia.

INTRODUCTION

Aplastic anemia is a rare hematologic disease characterized by bone marrow hypocellularity.^{1,2} The profound reduction in hematopoietic stem/progenitor cells results in peripheral blood pancytopenia. The pathogenesis of aplastic anemia is still elusive.³ There is evidence that bone marrow mesenchymal stem cells (BMSCs) isolated from patients with aplastic anemia have a reduced osteogenic potential compared to BMSCs from healthy individuals.^{4,5} In contrast, the adipogenic potential of BMSCs is increased in patients with aplastic anemia.^{6,7} The reduction in the osteogenic capacity of BMSCs has been causally linked to the development of aplastic anemia.

The ten-eleven translocation (TET) family comprising three members (TET1, TET2, and TET3) is an important epigenetic modifier.⁸ TET proteins are capable of converting 5-methyl-cytosine (5-mC) to 5-hydroxymethyl-cytosine (5hmC), consequently leading to DNA demethylation.⁹ Previous studies reported that TETs play an essential role in stem cell differentiation.^{10,11} For instance, Dawlaty et al.¹⁰ demonstrated that Tet1/2/3 triple knockout impairs the differentiation of mouse embryonic stem cells. Similarly, Yang et al.¹¹ reported that loss of Tet1 and Tet2 blocks differentiation of BMSCs and leads to an osteoporotic phenotype in a mouse model. Cakouros et al.¹² showed that TET2 functions as a promoter of both osteogenesis and adipogenesis. Most interestingly, *TET2* somatic mutations (7.3%) were frequently detected in patients with aplastic anemia.¹³ These studies encourage us to hypothesize that TETs may be implicated in the pathogenesis of aplastic anemia through regulation of the differentiation of BMSCs.

MicroRNAs (miRNAs) are endogenous, small, noncoding RNAs that regulate target gene expression at the post-transcriptional level through mRNA degradation or translational inhibition.¹⁴ The miRNA target sites are primarily detected in the 3' untranslated region (UTR) of mRNAs.¹⁵ Previous studies have demonstrated that several miRNAs contribute to the lineage commitment of stem cells.^{16–18} For instance, miR-384-5p affects osteogenic differentiation of rat BMSCs by targeting Gli2.¹⁶ Our previous work has shown that miR-204 has the ability to regulate MSC differentiation in aplastic anemia.¹⁹ miR-144-3p is capable of inhibiting differentiation of C3H10T1/2 pluripotent stem cells to the osteoblast lineage.²⁰ A recent

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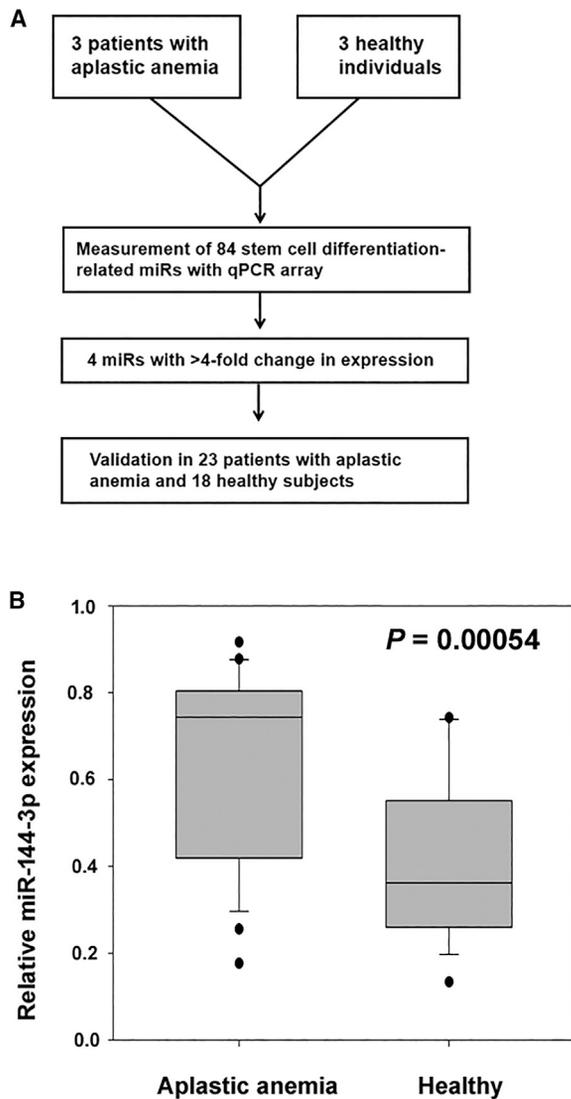


Figure 1. miR-144-3p Is Upregulated in BMSCs from Patients with Aplastic Anemia

(A) Flow chart of the screening for differentially expressed miRNAs between BMSCs from healthy controls and patients with aplastic anemia. (B) Quantitative real-time PCR analysis of miR-144-3p expression in BMSCs from healthy controls ($n = 18$) and patients with aplastic anemia ($n = 23$).

study, published when a revised version of this manuscript was in preparation, has reported that miR-144-3p can inhibit osteogenic differentiation of BMSCs from patients with steroid-associated osteonecrosis.²¹ In contrast, miR-144-3p facilitates adipogenesis in 3T3-L1 pre-adipocytes.²² These findings suggest that miR-144-3p is a regulator of osteogenic and adipogenic differentiation.

Despite these advances, the pivotal miRNA regulators coordinating differentiation of BMSCs from patients with aplastic anemia are largely unknown. In the present study, we performed miRNA quan-

titative real-time PCR arrays to screen for differentially expressed miRNAs between BMSCs from patients with aplastic anemia and healthy controls. The role of miRNA candidates in regulating osteogenic differentiation of BMSCs was explored.

RESULTS

miR-144-3p Is Upregulated in BMSCs from Patients with Aplastic Anemia

To identify novel regulators involved in the pathogenesis of aplastic anemia, we performed miRNA quantitative real-time PCR arrays in 3 independent samples to search for differentially expressed miRNAs between BMSCs from healthy controls and patients with aplastic anemia (Figure 1A). Among the 84 stem cell differentiation-related miRNAs tested, 4 miRNAs (i.e., miR-9, miR-125b, miR-144-3p, and miR-302) exhibited changes of greater than 4-fold (Table S1). To validate the results of miRNA quantitative real-time PCR arrays, we measured the expression of the 4 candidate miRNAs by quantitative real-time PCR analysis in 23 patients with aplastic anemia and 18 healthy controls. As a result, we confirmed that miR-144-3p was significantly upregulated in BMSCs from patients with aplastic anemia relative to healthy equivalents (Figure 1B; $p = 0.00054$). However, no significant changes were noted in the expression of the other 3 candidate miRNAs (data not shown). These results suggest that miR-144-3p plays a major role in the biology of BMSCs from patients with aplastic anemia.

miR-144-3p Inhibits the Osteogenic Potential of BMSCs from Patients with Aplastic Anemia

To determine the function of miR-144-3p in osteogenic differentiation of BMSCs, we knocked down miR-144-3p in BMSCs from patients with aplastic anemia. The knockdown efficiency was verified by quantitative real-time PCR analysis (Figure 2A). The miR-144-3p-depleted cells were cultured in osteogenic medium, and osteogenic differentiation was evaluated. Notably, the alkaline phosphatase (ALP) activity was significantly elevated in miR-144-3p-depleted cells (Figure 2B). Moreover, miR-144-3p deficiency markedly increased ALP and Alizarin Red staining (Figures 2C and 2D). miR-144-3p knockdown was associated with increased expression of osteoblast-specific genes *ALP* and *osteocalcin* (Figure 2E). However, miR-144-3p did not affect the adipogenic potential of BMSCs from patients with aplastic anemia (data not shown).

We also overexpressed miR-144-3p in BMSCs (Figure 3A) and tested their osteogenic potential. The results showed that ectopic expression of miR-144-3p led to reduced ALP and Alizarin Red staining after culturing in osteogenic medium (Figure 3B). Consistently, ALP activity (Figure 3C) and osteoblast marker gene expression (Figure 3D) were diminished in miR-144-3p-overexpressing BMSCs. Collectively, miR-144-3p serves as a repressor of osteogenic differentiation of BMSCs.

miR-144-3p Negatively Regulates the Expression of TET2 in BMSCs

To identify direct target genes of miR-144-3p, we conducted an *in silico* analysis using the TargetScan tool. The results showed that

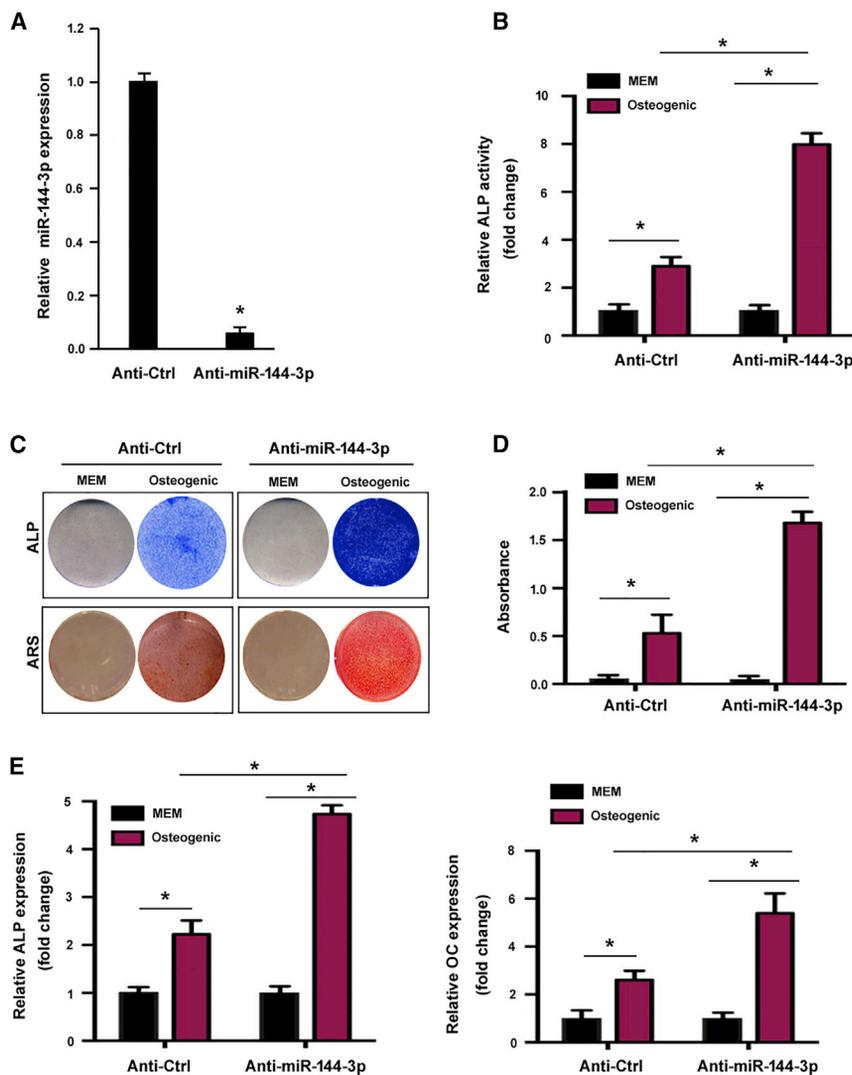


Figure 2. Knockdown of miR-144-3p Increases the Osteogenic Potential of BMSCs from Patients with Aplastic Anemia

(A) Quantitative real-time PCR analysis of miR-144-3p expression in BMSCs transfected with anti-Ctrl or anti-miR-144-3p. (B) Measurement of ALP activity in BMSCs transfected with anti-Ctrl or anti-miR-144-3p after culturing in osteogenic medium. (C) ALP and Alizarin Red staining in BMSCs treated as in (B). Representative images are shown. (D) Quantification of Alizarin Red staining results. (E) Quantitative real-time PCR analysis of ALP and osteocalcin (OC) mRNA levels. $n = 3$ for each group in all experiments. * $p < 0.05$.

When the luciferase reporter containing wild-type *TET2* 3' UTR was cotransfected with the miR-144-3p-expressing plasmid, the luciferase activity was reduced by 75% (Figure 4E). Elimination of the miR-144-3p responsive element impaired the suppressive effect of miR-144-3p (Figure 4E). Taken together, miR-144-3p shows the ability to repress the expression of *TET2* in BMSCs from patients with aplastic anemia.

TET2 Mediates the Suppressive Effects of miR-144-3p on Osteogenesis

Next, we tested whether miR-144-3p exerts anti-osteogenic effects through repression of *TET2*. Knockdown of *TET2* (Figure 5A) remarkably prevented the osteogenic differentiation of BMSCs cultured in osteogenic medium, as evidenced by decreased ALP activity (Figure 5B) and *ALP* and osteocalcin expression (Figure 5C). We also performed rescue experiments by overexpressing *TET2* cDNA lacking the 3' UTR (Figure 5D). miR-144-3p-mediated inhibition of osteogenesis was relieved by ectopic expression of *TET2* (Figures 5E and 5F). The results identify *TET2* as a functional target of miR-144-3p in BMSCs from patients with aplastic anemia.

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Correlation of the Expression of miR-144-3p and TET2 in BMSCs from Patients with Aplastic Anemia

Finally, we explored the correlation in the expression of miR-144-3p and *TET2* in aplastic anemia. Of note, there was a significant negative correlation between the expression of miR-144-3p and *TET2* in BMSCs from patients with aplastic anemia ($r = -0.5571$, $p = 0.0058$; Figure 6A). These results suggest that the miR-144-3p/*TET2* axis plays an important role in the biology of BMSCs from patients with aplastic anemia.

several osteogenesis-related genes, including *TET2*, *HOXA10*, *EZH2*, *FZD4*, *FOXO1*, and *PAX3*, are predicted as miR-144-3p targets (data not shown). Overexpression of miR-144-3p led to a marked reduction of *TET2* protein expression in BMSCs from patients with aplastic anemia (Figure 4A). Furthermore, *TET2* mRNA levels were suppressed by overexpression of miR-144-3p (Figure 4B). However, the expression of *HOXA10*, *EZH2*, *FZD4*, *FOXO1*, and *PAX3* remained unchanged after miR-144-3p overexpression (Figure S1). In agreement with reduced *TET2* expression, the total 5hmC level was significantly diminished by miR-144-3p overexpression (Figure 4C). In addition, depletion of miR-144-3p significantly increased the expression of *TET2* (Figures 4A and 4B) and total 5hmC levels (Figure 4C) in BMSCs from patients with aplastic anemia.

Next, we performed luciferase reporter assays to check whether miR-144-3p could directly target *TET2* mRNA. The 3' UTR of *TET2* was predicted to contain putative miR-144-3p binding sites (Figure 4D).

DISCUSSION

Several lines of evidence have established a link between the reduced osteogenic potential of BMSCs and aplastic anemia.^{5,6,23} However,

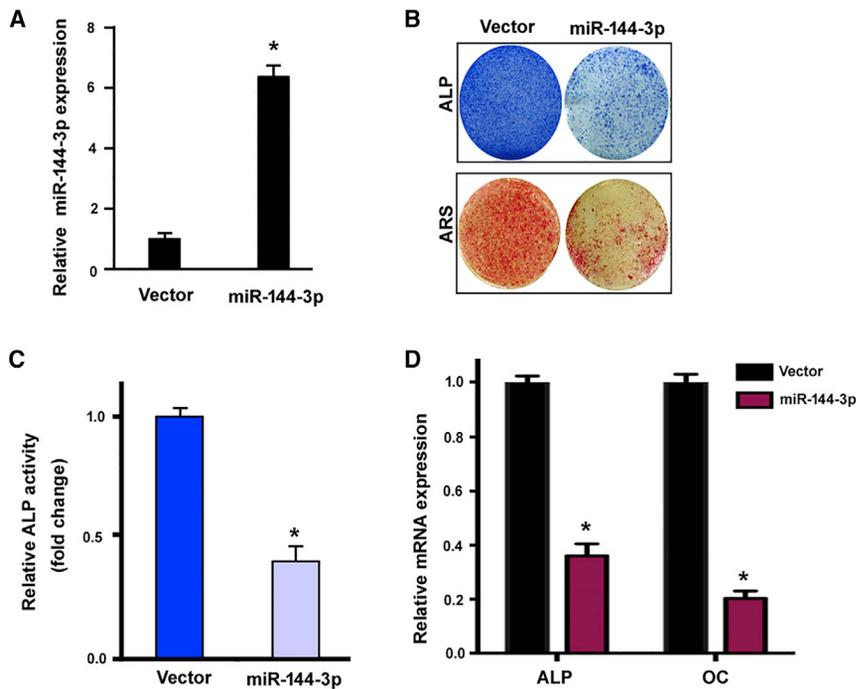


Figure 3. Overexpression of miR-144-3p Inhibits the Osteogenic Potential of BMSCs from Patients with Aplastic Anemia

(A) Quantitative real-time PCR analysis of miR-144-3p expression in BMSCs transfected with miR-144-3p-expressing plasmid or empty vector. (B) ALP and Alizarin Red staining in BMSCs transfected with miR-144-3p-expressing plasmid or empty vector after culturing in osteogenic medium. (C) Measurement of ALP activity in BMSCs treated as in (B). (D) Quantitative real-time PCR analysis of ALP and osteocalcin mRNA levels. $n = 3$ for each group in all experiments. $*p < 0.05$.

the underlying molecular mechanism remains unclear. In this work, we demonstrate that BMSCs from patients with aplastic anemia have a significantly higher level of miR-144-3p than those from healthy individuals. miR-144-3p is implicated in multiple biological processes, including tumorigenesis,^{24,25} osteogenesis,^{20,26} and adipogenesis.²² In particular, overexpression of miR-144-3p can block osteogenic differentiation and proliferation of murine MSCs by repressing Smad4.²⁰ Wang et al.²⁶ reported that miR-144-3p regulates bone homeostasis in patients with osteoporosis. Consistently, our results show that knockdown of miR-144-3p leads to increased osteogenesis of BMSCs from patients with aplastic anemia. We suggest that the increased expression of miR-144-3p may account for the impaired, reduced osteogenic potential of BMSCs in patients with aplastic anemia. Although miR-144-3p exhibits an ability to promote adipogenesis in 3T3-L1 mouse adipocyte precursors,²² we did not observe the regulation of adipogenesis of BMSCs from patients with aplastic anemia by miR-144-3p. The unexpected results may reflect that miR-144-3p effects depend on the cellular contexts. Despite these findings, an unbiased, systematic approach will be utilized to identify more miR regulators of osteogenic differentiation of BMSCs in aplastic anemia.

A number of target genes of miR-144-3p, such as Smad4 and FZD4, have been identified to mediate the inhibitory effects on osteogenic differentiation.^{20,21} However, we did not observe the negative regulation of the 2 target genes in miR-144-3p-overexpressing BMSCs from patients with aplastic anemia (Figure S2). We speculated that a novel mechanism is likely involved in the anti-osteogenic role of miR-144-3p in aplastic anemia. Intriguingly, we revealed that miR-144-3p selectively targets TET2, leading to a reduction of TET2 mRNA and protein expression in BMSCs from patients with aplastic anemia.

TET2 functions as an epigenetic modifier and has the ability to regulate osteogenic lineage-associated genes.¹² Aplastic anemia is associated with genetic instability, with frequent mutations in multiple genes, including *TET2*.^{13,27} Huang et al.¹³ showed that there is a significant correlation between TET2 mutations and good response to immunosuppressive therapy in patients with aplastic anemia. Our data provide evidence for the post-transcriptional regulation of TET2 in aplastic anemia. We demonstrate

that TET2 serves as a direct target of miR-144-3p in BMSCs from patients with aplastic anemia. Moreover, depletion of TET2 suppresses the osteogenic differentiation of BMSCs from patients with aplastic anemia. Rescue experiments further confirm that overexpression of TET2 reverses miR-144-3p-mediated inhibitory effects on osteogenesis. There is an inverse correlation between miR-144-3p and TET2 expression in aplastic anemia. These data indicate that TET2 plays an essential role in modulating the differentiation capacity of BMSCs, which may represent a mechanism for the involvement of TET2 in aplastic anemia.

The osteogenic markers ALP and osteocalcin can be epigenetically regulated during osteogenesis.^{28,29} Liu et al.²⁸ reported that treatment with 5-aza-2'-deoxycytidine, a DNA methyltransferase inhibitor, significantly increases the expression of osteogenesis-related genes in human periodontal ligament stem cells, resulting in enhanced osteogenic differentiation. Similarly, Delgado-Calle et al.²⁹ found that the degree of methylation in the promoter of *ALP* is inversely associated with the transcriptional levels of ALP in the osteoblastic cell line MG-63. Our data show that miR-144-3p overexpression significantly diminishes the total 5hmC level in BMSCs from patients with aplastic anemia, which is accompanied by reduced expression of ALP and osteocalcin. In contrast, miR-144-3p knockdown increases total 5hmC levels and promotes the expression of ALP and osteocalcin. Taken together, miR-144-3p contributes to epigenetic regulation of osteogenic genes, likely mediated through repression of TET2 (Figure 6B).

In summary, our data indicate that miR-144-3p negatively regulates osteogenic differentiation of BMSCs from patients with aplastic anemia. The anti-osteogenic activity is causally linked to repression of

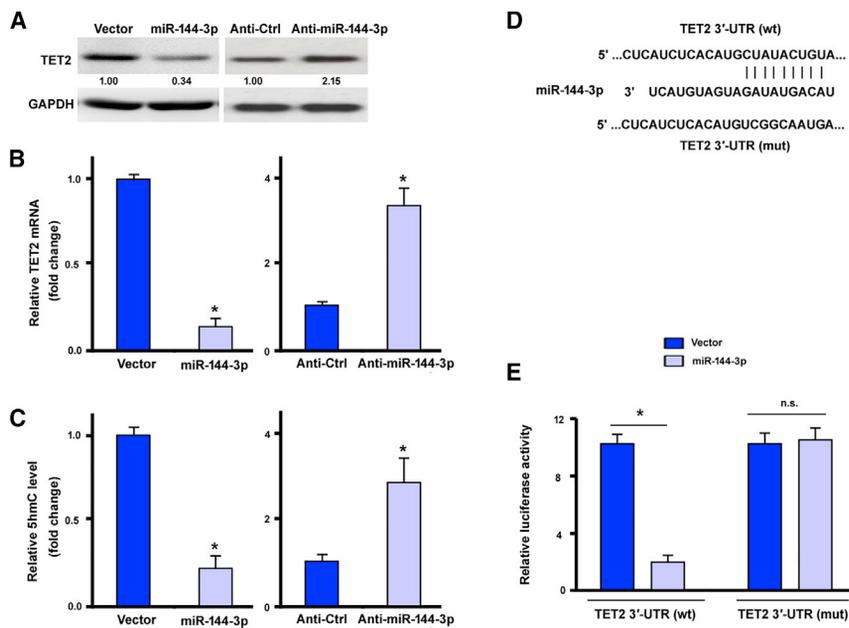


Figure 4. miR-144-3p Negatively Regulates the Expression of TET2 in BMSCs

(A) Western blot analysis of TET2 protein in BMSCs transfected with miR-144-3p-expressing plasmid or empty vector. (B) Quantitative real-time PCR analysis of TET2 mRNA levels in BMSCs treated as in (A). (C) Measurement of total 5hmC levels. (D) Bioinformatic prediction of miR-144-3p binding sites in the 3' UTR of *TET2* mRNA. (E) Luciferase reporter assay showing that the luciferase reporter containing wild-type *TET2* 3' UTR was repressed by miR-144-3p. Mutation of the miR-144-3p binding site impaired the miR-144-3p-mediated repression of the luciferase reporter. $n = 3$ for each group in all experiments. * $p < 0.05$. n.s. indicates no significance.

TET2 and epigenetic modulation of osteogenic markers. Therefore, we suggest that the miR-144-3p/TET2 axis may be engaged in the progression of aplastic anemia.

MATERIALS AND METHODS

Patients

To screen for differentially expressed miRNAs, we enrolled 3 patients with aplastic anemia and 3 age- and sex-matched healthy individuals who had normal bone marrow findings. In the validation cohort, we included 23 patients with aplastic anemia (9 women, 14 men; ages 33 ± 4 years) and 18 healthy subjects (8 women, 10 men; ages 31 ± 3 years). For both cohorts, eligibility criteria were age ≥ 18 years and diagnosis of aplastic anemia (based on pancytopenia and hypocellular bone marrow). The patients who had other causes of hematological disorders were excluded. The study was approved by the Institutional Review Board at Henan Cancer Hospital (Zhengzhou, China). An informed consent was obtained from each subject.

BMSC Isolation and Cell Lines

As described previously,¹⁹ mononuclear cells were isolated from bone marrow aspirates by Ficoll-Paque density gradient centrifugation (1.077 g/mL) and cultured in Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; Invitrogen). After 72 h, nonadherent cells were discarded. Adherent cells were subcultured and used for further experiments. For immunophenotypic characterization, BMSCs were examined for the expression of CD45, CD34, CD105, CD73, and CD44 by flow cytometry.¹⁹

HEK293T cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM, supplemented with 10% FBS.

kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR was performed with the SYBR Premix Ex TaqII (Takara, Dalian, China), as per the manufacturer's instructions. The PCR primers are as follows: ALP: forward 5'-ACACCTTGACTGTGGTTACTG-3' and reverse 5'-CCATATAGGATGGCCGTGAAG-3'; osteocalcin: forward 5'-AATAGCCCTGGCAGATTCCC-3' and reverse 5'-CTCTCATGGTGTCTCGGTGG-3'; TET2: forward 5'-ACGCAAGCCAGGCTAAACA-3' and reverse 5'-GCTGGGACTGCTGCATGA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward 5'-GGATTGGTTCGATTGGG-3' and reverse 5'-GGAAGATGGTGATGGGATT-3'.^{30,31} For detection of miR-144-3p, total RNA was isolated using the miRNeasy Mini Kit (QIAGEN, Hilden, Germany). Quantitative real-time PCR was performed using the miScript SYBR PCR Kit (QIAGEN) with the MystiCq microRNA qPCR Assay Primer (Sigma-Aldrich, St. Louis, MO, USA). U6 was used as an internal control. The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method.³²

For profiling of mature miRNAs, cDNA was added to the TaqMan Human Stem Cell miRNA PCR Array (Hanyu Biomed, Beijing, China), which contains specific primers for 84 stem cell-related miRNAs. The relative miRNA expression was determined after normalization to U6.

Plasmids and Oligonucleotides

miR-144-3p inhibitor (anti-miR-144-3p) and negative control inhibitor (anti-Ctrl) were purchased from Thermo Fisher Scientific. TET2-targeting small interfering RNA (siRNA) was purchased from Sigma-Aldrich. The plasmids expressing mature miR-144-3p and TET2 were obtained from Hanyu Biomed. Wild-type 3' UTR of *TET2* or its mutant version was cloned downstream of the firefly luciferase cDNA of pGL3 vector.

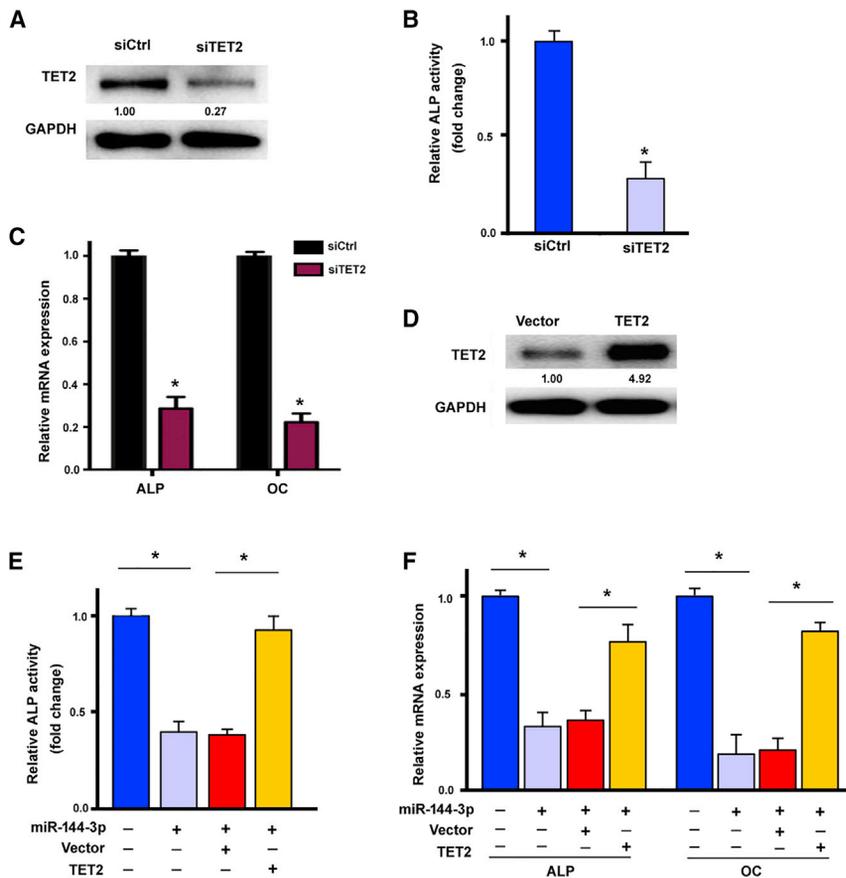


Figure 5. TET2 Mediates the Suppressive Effects of miR-144-3p on Osteogenesis

(A) Western blot analysis of TET2 protein in BMSCs transfected with TET2-targeting siRNA (siTET2) or control siRNA (siCtrl). (B) Measurement of ALP activity in BMSCs transfected with siTET2 or siCtrl after culturing in osteogenic medium. (C) Quantitative real-time PCR analysis of ALP and osteocalcin mRNA levels. (D) Western blot analysis of TET2 protein in BMSCs transfected with TET2-expressing plasmid or empty vector. (E) Measurement of ALP activity in BMSCs transfected with indicated constructs after culturing in osteogenic medium. (F) Quantitative real-time PCR analysis of ALP and osteocalcin mRNA levels. $n = 3$ for each group in all experiments. * $p < 0.05$.

ALP Activity Assay

ALP activity was determined using the Colorimetric Alkaline Phosphatase Assay Kit (Abcam, Cambridge, MA, USA), as per the manufacturer's instructions. Absorbance was measured at 405 nm.

Western Blot Analysis

Cells were lysed with radioimmunoprecipitation (RIPA) buffer (Sigma-Aldrich) on ice. Total protein concentration was determined using the Pierce Coomassie Plus Bradford assay kit, following the manufacturer's instructions (Thermo Fisher Scientific). Proteins were resolved by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. After

blocking with 5% bovine serum albumin, the membrane was incubated with rabbit anti-TET2 polyclonal antibody (1:300 dilution; Sigma-Aldrich) or anti-GAPDH polyclonal antibody (1:2,000 dilution; Abcam) overnight at 4°C. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) for 1 h at room temperature. Signals were visualized with an Amersham enhanced chemiluminescence (ECL) detection system (GE Healthcare, Cambridge, MA, USA). Densitometry was performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Dual-Luciferase Reporter Assay

A luciferase reporter assay was conducted, as described previously.³³ HEK293T cells were cotransfected with TET2 3' UTR luciferase reporters, together with miR-144-3p-expressing plasmid or empty vector. 48 hours later, the cells were harvested and tested for firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Firefly luciferase activity was normalized to that of *Renilla* luciferase.

Quantification of Total 5hmC Levels

Total 5hmC levels were measured using the Quest 5hmC DNA ELISA kit (Zymo Research, Irvine, CA, USA), as described previously.³⁴ In

Cell Transfection

BMSCs were seeded at 2×10^6 cells per well in 6-well plates and incubated overnight at 37°C. Cell transfection was performed with indicated constructs (2 μ g) using HiPerFect Transfection Agent (QIAGEN), according to the manufacturer's instruction. Twenty-four hours later, transfected cells were subjected to osteogenic differentiation and gene-expression analysis.

In Vitro Osteogenic Differentiation

As described previously,¹⁹ BMSCs were seeded in 6-well plates and cultured in DMEM/F12, supplemented with 10 mM β -glycerophosphate, 0.1 μ M dexamethasone, and 0.2 mM ascorbic acid (Sigma-Aldrich) for 3 weeks.

ALP and Alizarin Red Staining

For ALP staining, cells were fixed in 4% paraformaldehyde and stained with the ALP Staining Kit (Cell Biolabs, San Diego, CA, USA), following the manufacturer's protocol. For Alizarin Red staining, cells were fixed and stained with 1% Alizarin Red S (pH 4.2; Sigma-Aldrich). The Alizarin Red staining results were quantified by a spectrophotometer at 595 nm.

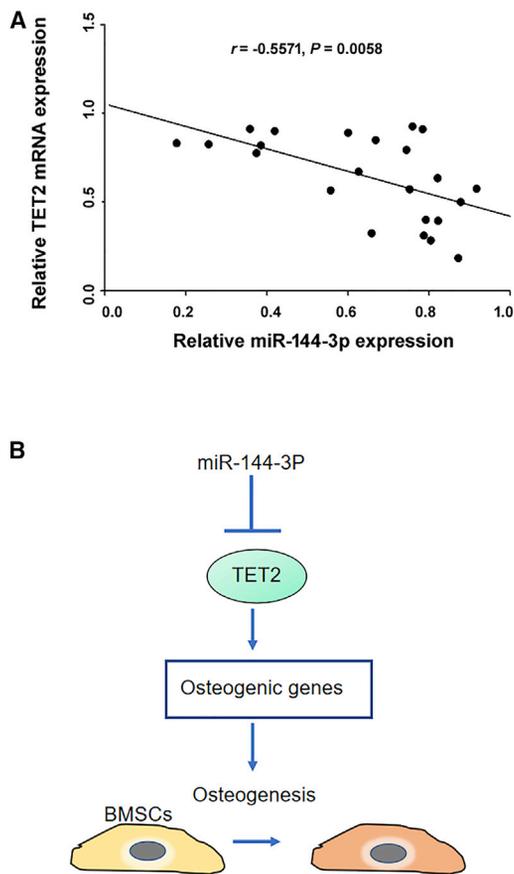


Figure 6. Correlation of the Expression of miR-144-3p and TET2

(A) A significant negative correlation was noted between the expression of miR-144-3p and TET2 in BMSCs from patients with aplastic anemia ($n = 23$). (B) Schematic model of miR-144-3p-mediated suppression of osteogenesis in BMSCs.

brief, genomic DNA was extracted from cells using the Universal Genomic DNA Extraction Kit (Takara). An anti-5hmC polyclonal antibody was diluted and added to the wells of assay plates. Genomic DNA was denatured and added to the coated wells at a final concentration of 100 ng/well. The mixture was incubated at 37°C for 1 h. After incubation with HRP-conjugated secondary antibody, substrate solution was added and colors developed. Absorbance was recorded at 405 nm.

Statistical Analysis

Data are expressed as the mean \pm standard deviation. Differences between groups were analyzed using the Student's *t* test, one-way analysis of variance, or Mann-Whitney *U* test. The correlation between miR-144-3p and TET2 expression was assessed with Spearman's correlation analysis. Values of $p < 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2019.12.017>.

AUTHOR CONTRIBUTIONS

N.L., Y.S., and B.F. designed the research and analyzed the data. N.L., L.L., Y.L., and S.L. conducted the experiments and collected the data. N.L. and B.F. wrote the paper. All authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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REFERENCES

- Dong, X., Han, Y., Abeysekera, I.R., Shao, Z., and Wang, H. (2019). GDF11 is increased in patients with aplastic anemia. *Hematology* 24, 331–336.
- Zhu, N., Wu, D., and Ye, B. (2018). The Progress of Traditional Chinese Medicine in the Treatment of Aplastic Anemia. *J. Transl. Int. Med.* 6, 159–164.
- Medinger, M., Drexler, B., Lengerke, C., and Passweg, J. (2018). Pathogenesis of Acquired Aplastic Anemia and the Role of the Bone Marrow Microenvironment. *Front. Oncol.* 8, 587.
- Park, M., Park, C.J., Jang, S., Kim, D.Y., Lee, J.H., Lee, J.H., Lee, K.H., Hwang, K., and Lee, Y.H. (2015). Reduced expression of osteonectin and increased natural killer cells may contribute to the pathophysiology of aplastic anemia. *Appl. Immunohistochem. Mol. Morphol.* 23, 139–145.
- Hamzic, E., Whiting, K., Gordon Smith, E., and Pettengell, R. (2015). Characterization of bone marrow mesenchymal stromal cells in aplastic anaemia. *Br. J. Haematol.* 169, 804–813.
- Xu, Y., Takahashi, Y., Wang, Y., Hama, A., Nishio, N., Muramatsu, H., Tanaka, M., Yoshida, N., Villalobos, I.B., Yagasaki, H., and Kojima, S. (2009). Downregulation of GATA-2 and overexpression of adipogenic gene-PPARgamma in mesenchymal stem cells from patients with aplastic anemia. *Exp. Hematol.* 37, 1393–1399.
- Tripathy, N.K., Singh, S.P., and Nityanand, S. (2014). Enhanced adipogenicity of bone marrow mesenchymal stem cells in aplastic anemia. *Stem Cells Int.* 2014, 276862.
- Arab, K., Karaulanov, E., Musheev, M., Trnka, P., Schäfer, A., Grummt, I., and Niehrs, C. (2019). GADD45A binds R-loops and recruits TET1 to CpG island promoters. *Nat. Genet.* 51, 217–223.
- Su, P.H., Hsu, Y.W., Huang, R.L., Chen, L.Y., Chao, T.K., Liao, C.C., Chen, C.W., Wu, T.I., Mao, S.P., Balch, C., and Lai, H.C. (2019). TET1 promotes 5hmC-dependent stemness, and inhibits a 5hmC-independent epithelial-mesenchymal transition, in cervical precancerous lesions. *Cancer Lett.* 450, 53–62.
- Dawlaty, M.M., Breiling, A., Le, T., Barrasa, M.I., Raddatz, G., Gao, Q., Powell, B.E., Cheng, A.W., Faull, K.F., Lyko, F., and Jaenisch, R. (2014). Loss of Tet enzymes compromises proper differentiation of embryonic stem cells. *Dev. Cell* 29, 102–111.
- Yang, R., Yu, T., Kou, X., Gao, X., Chen, C., Liu, D., Zhou, Y., and Shi, S. (2018). Tet1 and Tet2 maintain mesenchymal stem cell homeostasis via demethylation of the P2rx7 promoter. *Nat. Commun.* 9, 2143.
- Cakouros, D., Hemming, S., Gronthos, K., Liu, R., Zannettino, A., Shi, S., and Gronthos, S. (2019). Specific functions of TET1 and TET2 in regulating mesenchymal cell lineage determination. *Epigenetics Chromatin* 12, 3.
- Huang, J., Ge, M., Lu, S., Shi, J., Li, X., Zhang, J., Wang, M., Yu, W., Shao, Y., Huang, Z., et al. (2015). Mutations of ASXL1 and TET2 in aplastic anemia. *Haematologica* 100, e172–e175.
- Bhaskaran, V., Nowicki, M.O., Idriss, M., Jimenez, M.A., Lugli, G., Hayes, J.L., Mahmoud, A.B., Zane, R.E., Passaro, C., Ligon, K.L., et al. (2019). The functional synergism of microRNA clustering provides therapeutically relevant epigenetic interference in glioblastoma. *Nat. Commun.* 10, 442.
- Kertesz, M., Iovino, N., Unnerstall, U., Gaul, U., and Segal, E. (2007). The role of site accessibility in microRNA target recognition. *Nat. Genet.* 39, 1278–1284.

16. Li, X., Wu, J., Liu, S., Zhang, K., Miao, X., Li, J., Shi, Z., and Gao, Y. (2019). miR-384-5p targets Gli2 and negatively regulates age-related osteogenic differentiation of rat bone marrow mesenchymal stem cells. *Stem Cells Dev.* 28, 791–798.
17. Yang, C., Liu, X., Zhao, K., Zhu, Y., Hu, B., Zhou, Y., Wang, M., Wu, Y., Zhang, C., Xu, J., et al. (2019). miRNA-21 promotes osteogenesis via the PTEN/PI3K/Akt/HIF-1 α pathway and enhances bone regeneration in critical size defects. *Stem Cell Res. Ther.* 10, 65.
18. Xu, Y., Huang, Y., Guo, Y., Xiong, Y., Zhu, S., Xu, L., Lu, J., Li, X., Wan, J., Lu, Y., and Wang, Z. (2019). microRNA-690 regulates induced pluripotent stem cells (iPSCs) differentiation into insulin-producing cells by targeting Sox9. *Stem Cell Res. Ther.* 10, 59.
19. Zhao, J., Wang, C., Song, Y., and Fang, B. (2014). 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)* 46, 885–893.
20. Huang, C., Geng, J., Wei, X., Zhang, R., and Jiang, S. (2016). MiR-144-3p regulates osteogenic differentiation and proliferation of murine mesenchymal stem cells by specifically targeting Smad4. *FEBS Lett.* 590, 795–807.
21. Sun, Z., Wu, F., Yang, Y., Liu, F., Mo, F., Chen, J., Wang, G., and Zhang, B. (2019). MiR-144-3p inhibits BMSC proliferation and osteogenic differentiation via targeting FZD4 in steroid-associated osteonecrosis. *Curr. Pharm. Des.* Published online September 29, 2019. <https://doi.org/10.2174/1381612825666190930094019>.
22. Shen, L., Li, Q., Wang, J., Zhao, Y., Niu, L., Bai, L., Shuai, S., Li, X., Zhang, S., and Zhu, L. (2018). miR-144-3p Promotes Adipogenesis Through Releasing C/EBP α From Klf3 and CtBP2. *Front. Genet.* 9, 677.
23. Shipounova, I.N., Petrova, T.V., Svinareva, D.A., Momotuk, K.S., Mikhailova, E.A., and Drize, N.I. (2009). Alterations in hematopoietic microenvironment in patients with aplastic anemia. *Clin. Transl. Sci.* 2, 67–74.
24. Lan, F., Yu, H., Hu, M., Xia, T., and Yue, X. (2015). miR-144-3p exerts anti-tumor effects in glioblastoma by targeting c-Met. *J. Neurochem.* 135, 274–286.
25. Liu, F., Chen, N., Xiao, R., Wang, W., and Pan, Z. (2016). miR-144-3p serves as a tumor suppressor for renal cell carcinoma and inhibits its invasion and metastasis by targeting MAP3K8. *Biochem. Biophys. Res. Commun.* 480, 87–93.
26. Wang, C., He, H., Wang, L., Jiang, Y., and Xu, Y. (2018). Reduced miR-144-3p expression in serum and bone mediates osteoporosis pathogenesis by targeting RANK. *Biochem. Cell Biol.* 96, 627–635.
27. Kulasekararaj, A.G., Jiang, J., Smith, A.E., Mohamedali, A.M., Mian, S., Gandhi, S., Gaken, J., Czepulkowski, B., Marsh, J.C., and Mufti, G.J. (2014). Somatic mutations identify a subgroup of aplastic anemia patients who progress to myelodysplastic syndrome. *Blood* 124, 2698–2704.
28. Liu, Z., Chen, T., Sun, W., Yuan, Z., Yu, M., Chen, G., Guo, W., Xiao, J., and Tian, W. (2016). DNA Demethylation Rescues the Impaired Osteogenic Differentiation Ability of Human Periodontal Ligament Stem Cells in High Glucose. *Sci. Rep.* 6, 27447.
29. Delgado-Calle, J., Sañudo, C., Sánchez-Verde, L., García-Renedo, R.J., Arozamena, J., and Riancho, J.A. (2011). Epigenetic regulation of alkaline phosphatase in human cells of the osteoblastic lineage. *Bone* 49, 830–838.
30. Wu, R., Ruan, J., Sun, Y., Liu, M., Sha, Z., Fan, C., and Wu, Q. (2018). Long non-coding RNA HIF1A-AS2 facilitates adipose-derived stem cells (ASCs) osteogenic differentiation through miR-665/IL6 axis via PI3K/Akt signaling pathway. *Stem Cell Res. Ther.* 9, 348.
31. Yun, H.M., Park, K.R., Quang, T.H., Oh, H., Hong, J.T., Kim, Y.C., and Kim, E.C. (2015). 2,4,5-Trimethoxydalbergiquinol promotes osteoblastic differentiation and mineralization via the BMP and Wnt/ β -catenin pathway. *Cell Death Dis.* 6, e1819.
32. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods* 25, 402–408.
33. Guo, L., Zhou, L., Gao, Q., Zhang, A., Wei, J., Hong, D., Chu, Y., Duan, X., Zhang, Y., and Xu, G. (2017). MicroRNA-144-3p inhibits autophagy activation and enhances *Bacillus Calmette-Guérin* infection by targeting ATG4a in RAW264.7 macrophage cells. *PLoS ONE* 12, e0179772.
34. Stöger, R., Scaife, P.J., Shephard, F., and Chakrabarti, L. (2017). Elevated 5hmC levels characterize DNA of the cerebellum in Parkinson's disease. *NPJ Parkinsons Dis.* 3, 6.