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Original Article

Mammalian target of rapamycin mediates the adipogenic differentiation of bone marrow mesenchymal stem cells from aplastic anemia patients through regulation of PPAR-γ

Fang Wei, Weihua Zhang

Department of Hematology, The First Hospital of Shanxi Medical University, Taiyuan, Shanxi 030001, P.R. China

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Abstract: Fatty bone marrow is a characteristic feature of aplastic anemia (AA) and can negatively influence hematopoiesis. Previous findings have shown that in AA patients, fatty bone marrow may be linked to peroxisome proliferator-activated receptor γ (PPAR-γ) overexpression in bone marrow mesenchymal stem cells (BM-MSCs). Mammalian target of rapamycin (mTOR) is believed to play a critical role in adipogenesis of some cell types. However, little is known about the role of mTOR and its regulatory mechanisms in the adipogenic differentiation of BM-MSCs from AA patients. The present study was designed to uncover the potential role of mTOR in the adipogenic differentiation of BM-MSCs from AA patients and to further elucidate the underlying molecular mechanisms. We isolated BM-MSCs from 12 newly diagnosed AA patients and 12 healthy controls, and differentiated these cells into adipocytes *in vitro*. The surface antigen expression of BM-MSCs was identified by a flow cytometer. The capacity of BM-MSCs for adipogenic differentiation was determined by quantifying lipid droplets using Oil Red O staining and by western blot analysis of the expression of FABP4. Localization and protein levels of mTOR and PPAR-γ were examined by immunofluorescence and western blot. mRNA levels of mTOR and PPAR-γ were quantitated by RT-PCR. We observed that AA BM-MSCs displayed an enhanced capacity for differentiating into adipocytes compared to that of control BM-MSCs. We found that mTOR was not only activated, but also upregulated in AA BM-MSCs. Moreover, expression levels of mTOR and PPAR-γ in AA BM-MSCs showed a parallel differentiation-dependent increase during adipogenic differentiation and were significantly higher than those in control BM-MSCs at the same time point of adipogenic differentiation. mTOR inhibition not only blocked PPAR-γ mRNA and protein expression, but also disrupted adipogenic differentiation of AA BM-MSCs. Therefore, mTOR signaling may play a critical role in the adipogenic differentiation of BM-MSCs from AA patients by positively regulating PPAR-γ.

Keywords: mTOR, PPAR-γ, adipogenic differentiation, bone marrow mesenchymal stem cells, aplastic anemia

Introduction

Aplastic anemia (AA) is a bone marrow failure syndrome characterized by peripheral pancytopenia and bone marrow hypoplasia with fatty bone marrow. The pathogenesis of AA is complex and has not been completely elucidated. At present, AA is generally considered as an immune-mediated disease for which immunosuppressive therapy (IST) is a key treatment strategy [1, 2]. However, treatment failures or relapses after IST are frequent, and studies aimed at resolving these problems have been unsuccessful. Therefore, other mechanisms likely exist. It is well established that normal hematopoiesis is dependent on an intact and functional bone marrow microenvironment. However, bone marrow in AA patients typically exhibits reduction in hematopoietic stem cells and increase in adipocytes, a condition referred to as fatty bone marrow. In recent years, attention has been paid to increased bone marrow adipocytes, which can negatively influence hematopoiesis [3, 4]. Evidence indicates that increased bone marrow adipocytes can suppress the maturation and differentiation of hematopoietic stem cells in co-culture systems [3, 5], thereby exacerbating the hematopoietic failure of bone marrow [5]. In light of these findings, fatty bone marrow in AA patients may, at

least in part, be involved in AA bone marrow failure. Therefore, a better understanding of mechanisms underlying fatty bone marrow in AA patients is required, and it should ultimately enable clinicians to devise therapies that are more effective. As key precursor cells of bone marrow microenvironment, BM-MSCs are common progenitor cells of osteoblasts and adipocytes. Data from some studies have suggested that under conditions of adipogenic induction, BM-MSCs from AA patients were prone to differentiation into adipocytes but not osteoblasts, which may explain the phenomenon of increased bone marrow adipocytes [6-8]. However, the mechanisms underlying adipogenic differentiation of BM-MSCs in AA are poorly understood.

It has been previously demonstrated that PPAR-γ is both necessary and sufficient for adipogenesis [9-11]. In a cellular model of murine BM-MSC differentiation, loss of PPAR-γ expression impairs the ability of murine BM-MSCs to differentiate into adipocytes [12, 13]. Moreover, PPAR-γ induces phenotypes of mature adipocytes, as indicated by the expression of fatty acid-binding protein 4 (FABP4) [14]. Further studies show that PPAR-γ expression is significantly higher in AA patients, and fatty bone marrow in AA patients may be explained by PPAR-γ overexpression in BM-MSCs [15].

Commitment of BM-MSC differentiation to adipocytes requires a network of transcription factors. PPAR-γ expression in BM-MSCs from AA patients is likely regulated by default to promote differentiation into adipocytes. Mammalian target of rapamycin (mTOR) is a conserved Ser/Thr kinase and a central mediator of several signal transduction pathways that regulate cell growth and proliferation by modulating protein synthesis [16, 17]. The two most well-characterized substrates of mTOR are ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP1). mTOR phosphorylates S6K1 and 4EBP1 [18, 19], thereby promoting protein translation by inactivating 4EBP1 and activating S6K1. The phosphorylation status of S6K1 is commonly used as a marker of mTOR activity [20]. Rapamycin is an mTOR-specific inhibitor that can inhibit mTOR by binding to the FK506-binding protein FKBP12, which in turn physically interacts with the complex and suppresses mTOR activity to hinder the phosphorylation of S6K1 and 4EBP1 [21]. Through the use of rapamycin, several studies have demonstrated that mTOR signaling is required for the adipogenesis of 3T3-L1 cells, mouse embryonic fibroblasts (MEFs) and human pre-adipocytes in primary culture [22-25], and that rapamycin treatment correlates with a decrease in PPAR-γ expression at both the mRNA and the protein level [23, 24]. Thus, these data indicate that mTOR, like PPAR-γ, is both necessary and sufficient to drive adipogenesis in some cell types. However, little is known about the effect of mTOR signaling on the adipogenic differentiation of BM-MSCs from AA patients.

The present study was designed to determine the potential role of mTOR in the adipogenic differentiation of BM-MSCs from AA patients, and to further elucidate the underlying molecular mechanisms.

Materials and methods

Materials and reagents

Dexamethasone (DEX), isobutyl methyl xanthine (IBMX), insulin, and 0.25% trypsin-EDTA were purchased from Sigma (St. Louis, Missouri, USA). Antibodies against PPAR-γ, mTOR, and p-mTOR (Ser2448) were purchased from Cell Signaling Technology (Carlsbad, California, USA). Rapamycin (an mTOR inhibitor) and antibodies against S6K1, p-S6K1 (Thr389), and FABP4 were purchased from Sangon Biotech (Shanghai, China). Oil Red O staining kits, Cy3-conjugated secondary sheep/goat anti-mouse IgG antibodies, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Boster Biological Technology (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (South Logan, Utah, USA). Fetal bovine serum (FBS) was purchased from Gibco (Carlsbad, California, USA).

Patients and controls

Bone marrow samples were derived from 12 newly diagnosed AA patients (4 men and 8 women) and 12 healthy controls (4 men and 8 women). The median age was 42 (range: 22-67) years for the AA patients and 44 (range: 29-60) years for the healthy controls. AA diagnosis was made on the basis of morphological examinations of bone marrow and blood after other dis-

eases involving pancytopenia were excluded, such as congenital AA, myelodysplastic syndrome, and paroxysmal nocturnal hemoglobinuria. In addition, patients with secondary AA arising from infectious disease, pregnancy, connective tissue disease, drug use, and tumors were not included in this study, according to international criteria [26]. All patients were newly diagnosed and had not received any AA-specific therapy prior to enrollment, including androgen, cyclosporine A, or anti-thymocyte globulin. Controls were healthy volunteers, based on morphological examinations of bone marrow and blood.

Isolation and culture of primary human BM-MSCs

Bone marrow cells from healthy controls and AA patients were collected from marrow aspirates. Bone marrow cells were cultured in basal medium consisted of DMEM supplemented with 10% FBS and 100 units/mL penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The BM-MSCs preferentially attached to the polystyrene surface after 48-72 h in culture, after which the non-adherent cells were discarded. The basal medium was replaced with fresh medium every 3 or 4 days thereafter. When the BM-MSCs reached 90% confluency, they were detached using 0.25% trypsin-EDTA solution and then subcultured. The cells used for experiments were from passage 3.

Morphology and immunophenotype of BM-MSCs

At passage 3, morphology of BM-MSCs was assessed using an inverted light microscope, and surface antigen expression of adherent cells was identified by a FACScan flow cytometer (BD Biosciences, Mountain View, CA, USA). The following antibodies were used: CD29, CD34, CD45, CD14, CD44, CD105, and HLA-DR (BD Pharmingen, San Jose, CA, USA). The nonspecific mouse IgG (BD Pharmingen, San Jose, CA, USA) served as the isotype control. Ten thousand labeled cells were acquired and analyzed.

Immunofluorescence analysis

BM-MSCs were cultured on glass slides. Cells were fixed for 15 min with 4% paraformaldehyde and then washed three times with phosphate buffer saline (PBS) (5 min/wash). The cells were then permeabilized with 0.2% Triton X-100 in PBS for 8 min and washed three times with PBS (5 min/wash). Next, the cells were blocked in 5% BSA for 30 min at 25°C and then incubated overnight in a humidified chamber at 4°C with a primary antibody solution (1% BSA in PBS) containing monoclonal rabbit antibodies against PPAR-γ and phospho-mTOR (p-mTOR; Ser2448). After three 5-min washes with PBS, the cells were subsequently incubated with a Cy3-conjugated secondary sheep/goat anti-mouse IgG antibody at room temperature for 30 min, followed by six 5-min washes with PBS. Nuclei were stained with DAPI (1 μg/mL) for 10 min. Slides were observed and imaged by laser scanning confocal microscopy.

Induction of adipogenic differentiation

To stimulate adipogenesis, cultures were exposed to adipogenic differentiation medium (defined as day 1) consisted of basal medium supplemented with 1 μmol/L DEX, 0.5 mmol/L IBMX, and 10 μg/mL insulin for 3 days, 7 days, 14 days, and 21 days, respectively. At these indicated time points, the cells were harvested for analysis by western blot, real-time polymerase chain reaction (RT-PCR), and staining with Oil Red O. In experiments involving rapamycin treatment, cells were treated with rapamycin at a final concentration of 100 nmol/L in adipogenic differentiation medium during days 0-14 or days 15-21 of adipogenic differentiation.

Oil Red O staining

To monitor adipogenic differentiation of BM-MSCs, cells were washed with PBS, fixed in 4% paraformaldehyde for 1 h, and then stained using a filtered solution of 0.2% Oil Red O in 60% isopropanol for 1 h. The stained cells were observed under an inverted light microscope. Oil Red O staining was performed to visualize fat-containing cells, and adipogenesis was quantified by counting the percentage of cells showing lipid droplet accumulation (red) in 10 random fields under the light microscope.

Western blot analysis

BM-MSCs from control subjects and AA patients were isolated as described above. Next, the levels of PPAR-γ, mTOR, p-mTOR, S6K1, p-S6K1, and FABP4 protein expression were quantified

Table 1. Clinical features of aplastic anemia patients

Patient	Age/Sex	Diagnosis	Hb (g/L)	Neutrophil (10^9/L)	PLT (10^9/L)	Ret (10^9/L)
1	33/F	NSAA	51	0.85	32	8.5
2	29/F	SAA	46	0.39	11	5.2
3	67/M	NSAA	65	0.78	33	9.0
4	62/M	NSAA	67	0.85	24	9.2
5	22/F	NSAA	43	0.55	27	8.8
6	49/M	NSAA	66	0.61	29	9.6
7	46/F	SAA	48	0.41	12	4.8
8	36/F	SAA	39	0.38	9	3.6
9	32/F	NSAA	65	0.71	21	9.0
10	49/F	SAA	69	0.46	10	5.0
11	56/F	NSAA	90	0.65	27	11.6
12	26/M	SAA	68	0.2	2	4.2

M, male; F, female.

by western blot analysis. Briefly, BM-MSCs were washed three times with sterile, cold PBS, and were harvested in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). The lysates were collected by scraping cells from the plates and then centrifuging at 14,000 ×*g* for 15 min at 4°C. Lysates were mixed 1:1 with Laemmli sample buffer and boiled before separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel, after which the proteins were transferred to a 0.22 μm polyvinylidene fluoride (PVDF) membrane and incubated with antibodies against PPAR-γ, S6K1, p-S6K1 (Thr389), FABP4, and β-actin. For mTOR and p-mTOR (Ser2448) detection, the proteins were resolved by 6% SDS-PAGE and transferred to 0.45 μm PVDF membranes. β-actin expression was used as an internal control to normalize expression levels. Membranes were incubated overnight with primary antibodies at 4°C on a shaker, followed by incubation with appropriate secondary antibodies. Horseradish peroxidase-conjugated IgG and enhanced chemiluminescence detection (GE Healthcare, UK) were used for detection.

Quantitative real-time PCR analysis

To measure transcript levels, total RNA was isolated from cells with TRIzol reagent (Invitrogen, Carlsbad, USA). First-strand complementary DNA (cDNA) was synthesized with First Strand cDNA Synthesis Kit (Fermentas, Burlington, CA). RT-PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Burlington, CA) and analyzed using the manufacturer's complimentary software. *GAPDH* was used as an internal control to normalize the expression levels. The following primers were used: mTOR forward: 5'-ccaacagttcaccctcaggt-3', reverse: 5'-gctgccactctccaagtttc-3'; PPAR-γ forward: 5'-tgcaggtgatcaagaagacg-3', reverse: 5'-tggaagaagggaaatgttgg-3'.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Statistical analysis

All data in the present study were expressed as the mean ± SD. Student's unpaired *t*-test was used for comparing two sets of data generated in the experiments. ANOVA was used for comparing more than two sets of data. Correlations between the expression level of PPAR-γ and the expression level of mTOR or p-mTOR were tested by Spearman's correlation. *P* value < 0.05 was considered statistically significant.

Results

Morphology and phenotype analysis of AA BM-MSCs

BM-MSCs cultures were successfully established from 12 AA patients and 12 healthy controls. **Table 1** presents the diagnosis and main hematologic features of AA patients. BM-MSCs from both the AA group and the control group formed a monolayer of bipolar spindle-like cells in a whirlpool-like array (**Figure 1**). BM-MSCs from both the AA group and the control group expressed CD105, CD29, and CD44, but lacked expression of CD34, CD45, CD14, and HLA-DR.

Enhanced capacity of adipogenic differentiation of AA BM-MSCs in vitro

To evaluate the capacity of adipogenic differentiation of AA BM-MSCs, BM-MSCs from the AA group and the control group were incubated in adipogenic differentiation medium, and adipocyte formation was determined based on the appearance of lipid droplets by Oil Red O stain-

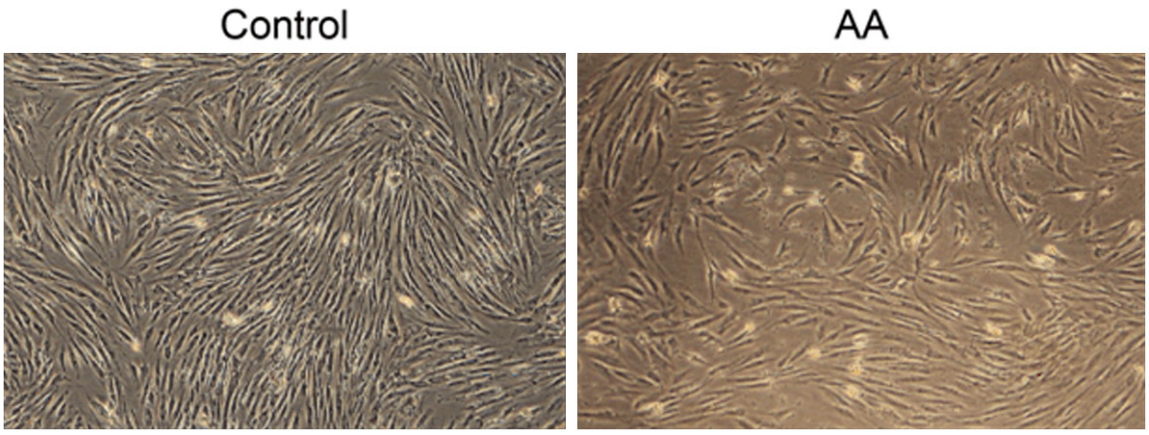

Figure 1. Representative morphology of BM-MSCs (3rd passage) from AA patients and healthy controls. In culture, BM-MSCs of AA patients and healthy controls shared a similar spindle-like morphology in a whirlpool-like array. Original magnification, 40×.

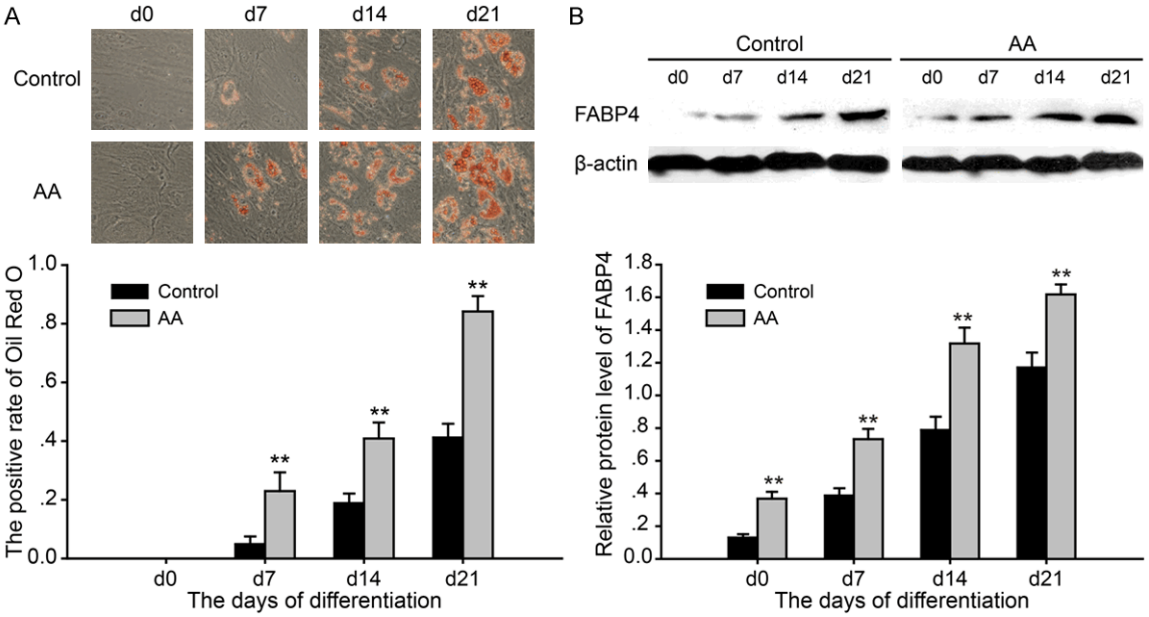

Figure 2. Adipogenic differentiation of BM-MSCs from AA patients and healthy controls *in vitro*. A. Adipogenic differentiation of BM-MSCs from AA patients and healthy controls was induced in differentiation medium as described in the Materials and Methods section. On days 0, 7, 14, and 21 of adipogenic differentiation, cells were stained with Oil Red O and visualized by inverted light microscopy. Original magnification, 40×. The number of stained cells in 10 random fields were counted at an original magnification 10×. B. Protein expression level of FABP4 was analyzed by western blot. β-actin expression was measured as an internal control. (control group: n = 12; AA group: n = 12). $^{**}P < 0.01$; control vs. AA.

ing and by the expression of a marker of terminal adipogenesis, FABP4 (**Figure 2**).

After one week of growth in adipogenic differentiation medium, approximately 22.9% of AA BM-MSCs became rounded, with perinuclear lipid droplets within the cytoplasm. In contrast, the morphology of control BM-MSCs was not noticeably changed, with few and small perinuclear granules observed within the cytoplasm ($P < 0.01$; **Figure 2A**). At the end of the second week, AA BM-MSCs acquired the morphology of mature differentiated adipocytes, showing an accumulation of big and small lipid droplets within the cytoplasm. Small perinuclear lipid droplets within the cytoplasm of control BM-MSCs expanded slightly in size (**Figure 2A**). The adipogenic differentiation rate of BM-MSCs

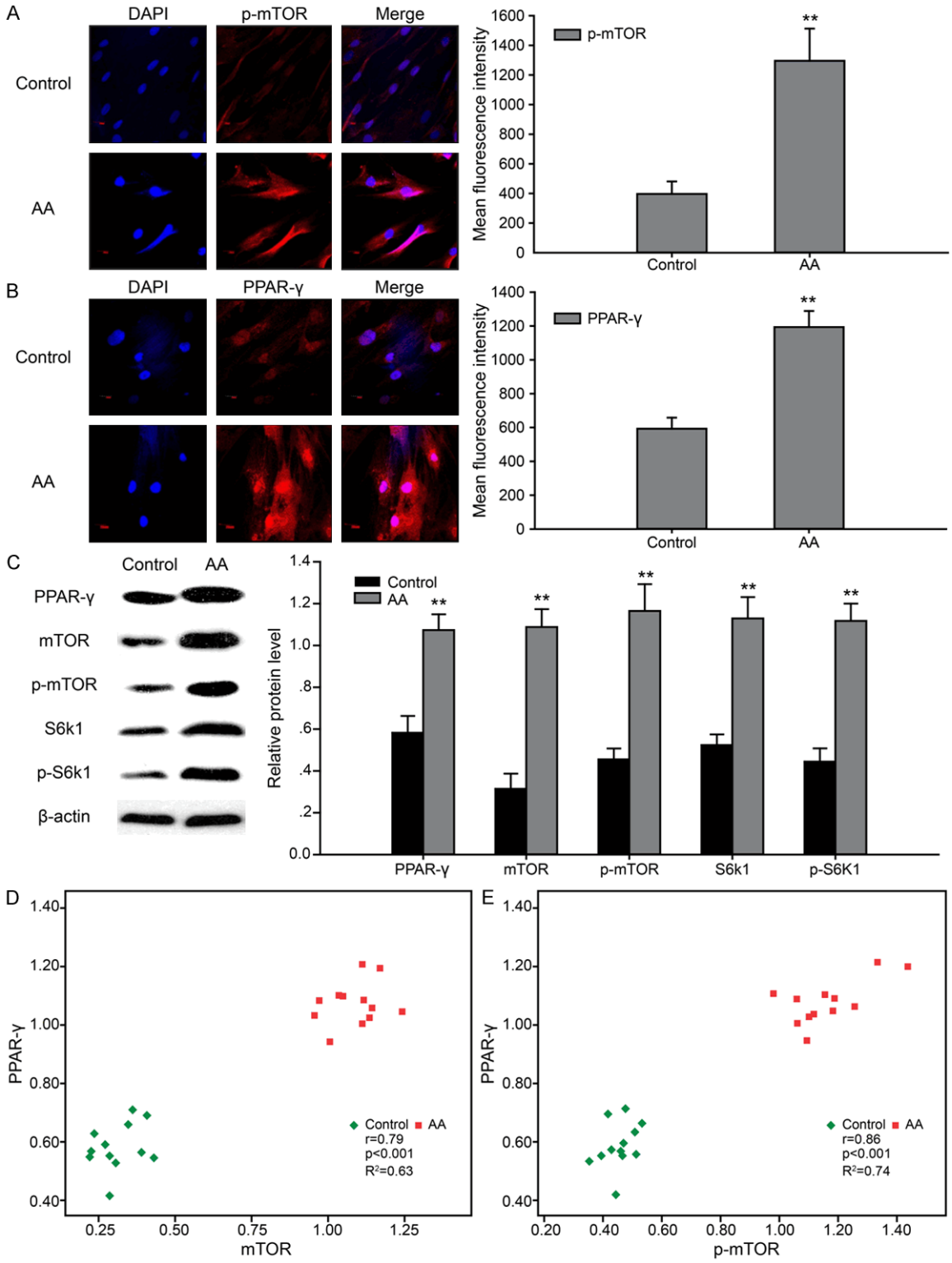

Figure 3. Localization and expression of mTOR signaling and PPAR-γ in BM-MSCs from AA patients and healthy controls. Localization and expression of p-mTOR (A) and PPAR-γ (B) were assessed by immunofluorescence confocal microscopy. Cells were immunostained with an appropriate Cy3-conjugated mAb (red). Nuclear counterstaining was visualized with DAPI (blue). Left: representative image, original magnification, 600×; right: semi-quantitative analysis of expression (Control group: n = 6; AA group: n = 6). $^{**}P < 0.01$; control vs. AA. (C) Protein expression levels of PPAR-γ, mTOR, p-mTOR, S6K1, and p-S6K1 in BM-MSCs from AA patients and healthy controls were analyzed by

western blot. β-actin expression was measured as an internal control. Data are presented as the mean ± SD (control group: n = 12; AA group: n = 12). **P < 0.01; control vs. AA. (D) Correlation between protein expression levels of PPAR-γ and mTOR in BM-MSCs from both groups (control group: n = 12; AA group: n = 12). (E) Correlation between protein expression levels of PPAR-γ and mTOR in BM-MSCs from both groups (control group: n = 12; AA group: n = 12).

from the AA group was 40.89 ± 5.43%, which was significantly higher than that of the control group (18.86 ± 3.27%) (P < 0.01; **Figure 2A**). At the end of the third week, adipocytes of the AA group were fully differentiated. The differentiated adipocytes continued to produce larger and more numerous lipid droplets within the cytoplasm (**Figure 2A**). The adipogenic differentiation rate of BM-MSCs from the AA group was 84.18 ± 5.22%, which was significantly higher than that of the control group (41.23 ± 4.68%) (P < 0.01; **Figure 2A**). To determine whether the increase in lipid accumulation in the BM-MSCs reflected an increase in adipogenesis, we measured the protein expression of FABP4 (a molecular marker of adipogenesis) on days 7, 14, and 21 of adipogenic differentiation by western blot analysis. The protein level of FABP4 was significantly higher in BM-MSCs from the AA group than in BM-MSCs from the control group at the same time points of adipogenic differentiation (P < 0.01; **Figure 2B**). Compared to control BM-MSCs, AA BM-MSCs were readily differentiated into adipocytes.

mTOR was activated and upregulated in AA BM-MSCs

To determine the localization and expression of mTOR and PPAR-γ in AA BM-MSCs, we first performed immunofluorescence analysis. We found that p-mTOR and PPAR-γ were expressed in both cytoplasmic and nuclear compartments of BM-MSCs from the AA group and the control group (**Figure 3A**, **3B**). Compared with control BM-MSCs, AA BM-MSCs had significantly elevated levels of both p-mTOR and PPAR-γ in the cytoplasm and nucleus (P < 0.01), as shown in **Figure 3A** and **3B**.

To confirm the immunofluorescence analysis results, we determined the protein levels of mTOR, p-mTOR, and PPAR-γ in BM-MSCs from both groups by western blot analysis. The protein levels of mTOR and p-mTOR in BM-MSCs from the AA group were significantly higher (2-3-fold) compared to those in BM-MSCs from the control group (P < 0.01; **Figure 3C**). Similarly, protein level of PPAR-γ in BM-MSCs from the AA group was also significantly higher than that in BM-MSCs from the control group (P < 0.01; **Figure 3C**). Thus, both immunofluorescence and western blot analysis demonstrated that mTOR was not only activated, but also upregulated in BM-MSCs from AA patients. Moreover, the protein level of PPAR-γ was strongly correlated with the protein levels of mTOR (r = 0.79, P < 0.001) and p-mTOR (r = 0.86, P < 0.001) in BM-MSCs from both groups (**Figure 3D**, **3E**).

Parallel expression of mTOR and PPAR-γ during adipogenic differentiation of AA BM-MSCs

To investigate the relationship between mTOR and PPAR-γ, we examined their temporal expression during the 21-day adipogenic differentiation of BM-MSCs from the AA group and the control group. mTOR protein level in control BM-MSCs peaked on day 14 and then gradually decreased, whereas PPAR-γ protein level increased continuously throughout the differentiation period (**Figure 4A**). mTOR protein level in AA BM-MSCs started to become elevated on day 3 and continued to increase throughout the adipogenic differentiation process (**Figure 4A**). PPAR-γ protein level in AA BM-MSCs followed a chang similar to that in control BM-MSCs during the adipogenic differentiation (**Figure 4A**). Moreover, protein levels of both mTOR and PPAR-γ from the AA group were significantly higher than those of the control group at the same time points of adipogenic differentiation (P < 0.01; **Figure 4A**). Meanwhile, protein levels of S6K1 (a well-known downstream target of mTOR) and p-S6K1 underwent the same changes as that of mTOR observed in the respective groups (**Figure 4A**). mRNA levels of mTOR and PPAR-γ in BM-MSCs from both groups corresponded with their respective protein levels (**Figure 4B**).

Inhibition of mTOR by rapamycin disrupted adipogenic differentiation of AA BM-MSCs

To elucidate whether mTOR was essential for adipogenic differentiation of AA BM-MSCs, we next used the mTOR inhibitor rapamycin to sup-

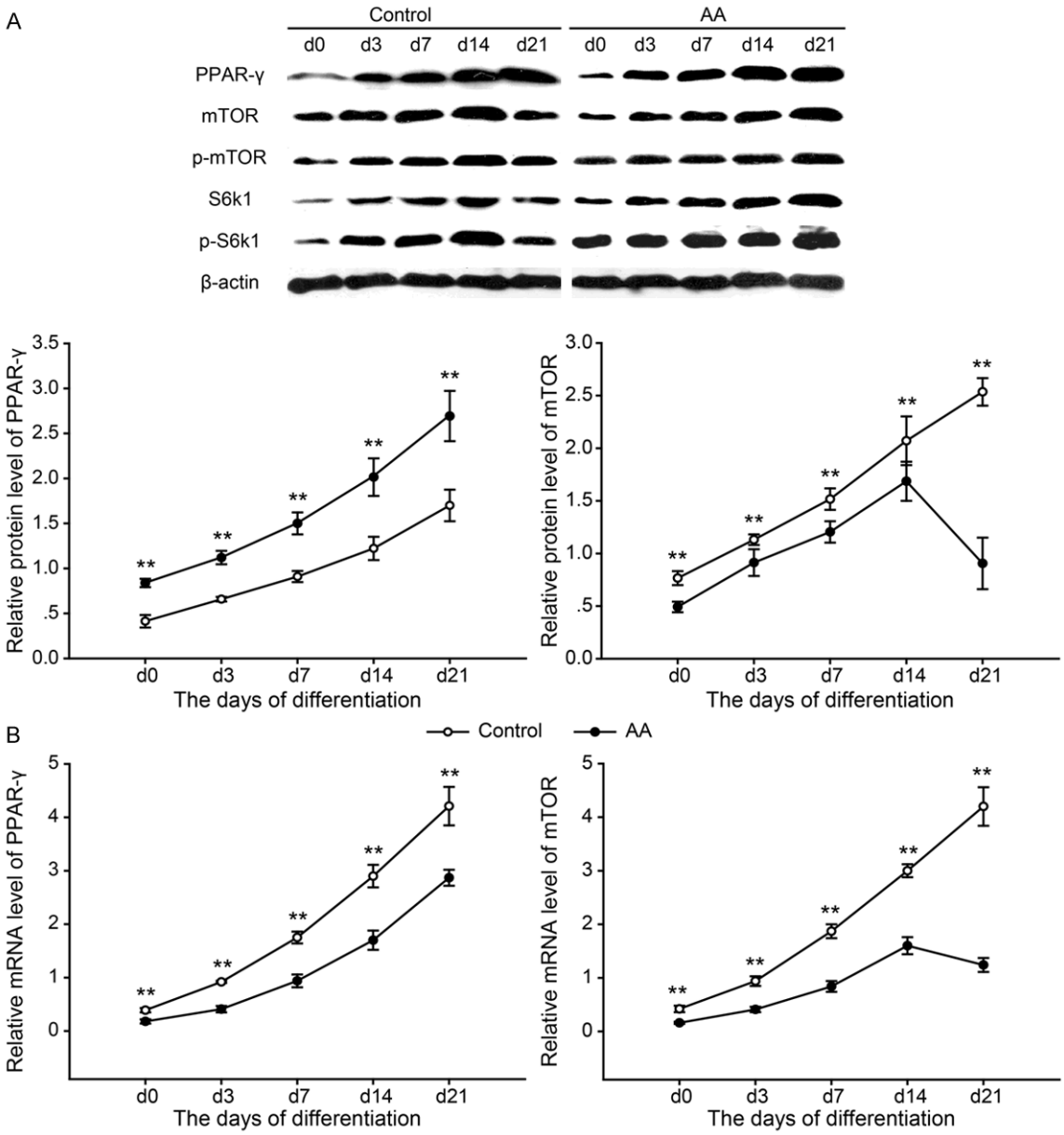

Figure 4. Expression of PPAR-γ and mTOR signaling in BM-MSCs from AA patients and healthy controls during adipogenic differentiation *in vitro*. A. BM-MSCs from AA patients and healthy controls were induced to differentiate into adipocytes in differentiation medium. On days 0, 3, 7, 14, and 21 of adipogenic differentiation, protein expression levels of PPAR-γ, mTOR, p-mTOR, S6K1, and p-S6K1 in BM-MSCs from AA patients and healthy controls were analyzed by western blot. β-actin was used as an internal control. B. mRNA expression levels of PPAR-γ and mTOR were analyzed by RT-PCR. *GAPDH* was used as an internal control. Data are presented as the mean ± SD (control group: n = 6; AA group: n = 6). $^{**}P < 0.01$; control vs. AA.

press mTOR activation and subsequently assessed adipogenic differentiation of BM-MSCs from both groups. We added rapamycin to BM-MSCs from both groups during days 0-14 or days 15-21 of adipogenic differentiation in separate experiments, after which the final rapamycin concentration in the adipocyte differentiation medium was 100 nmol/L. On days 21 of adipogenic differentiation, we evaluated adipogenesis by Oil Red O staining and by measuring FABP4 protein expression. AA BM-MSCs failed to differentiate into adipocytes in the presence of rapamycin during days 0-14 of adipogenic differentiation, exhibiting a near-

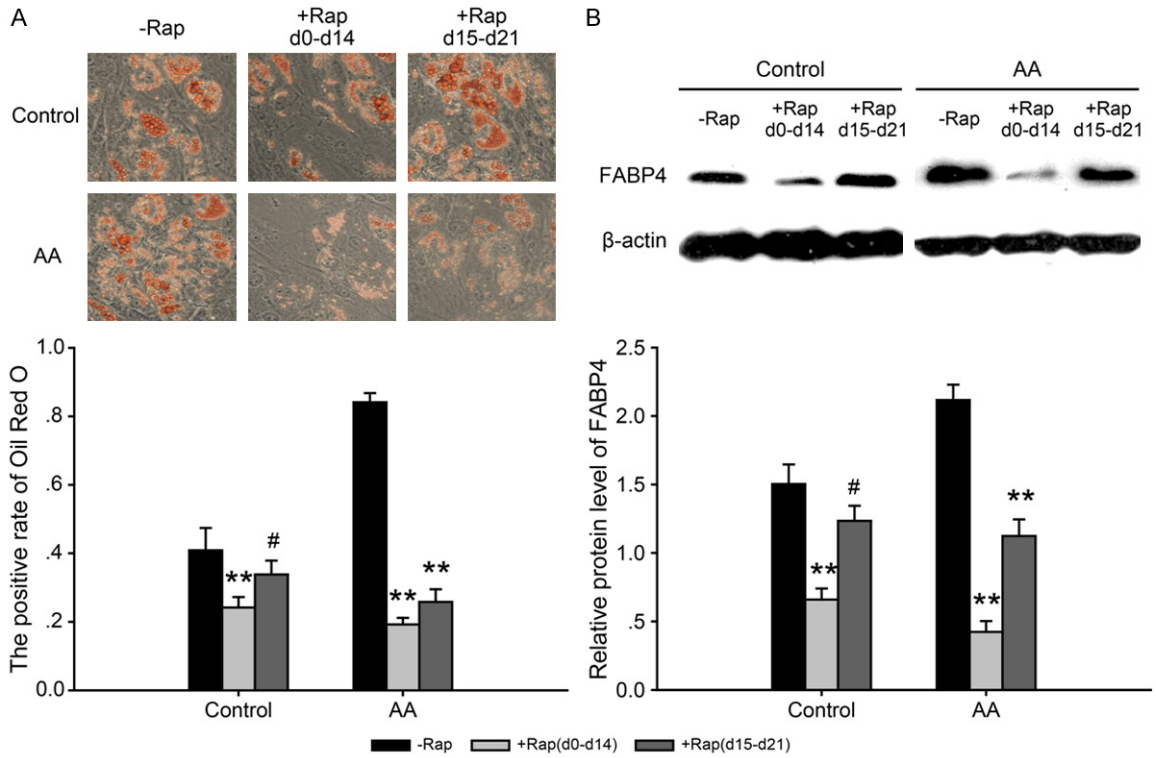

Figure 5. Effect of mTOR inhibitor on the adipogenic differentiation of BM-MSCs from AA patients and healthy controls. A. BM-MSCs from AA patients and healthy controls were either left untreated or treated with 100 nmol/L rapamycin during days 0-14 or days 15-21 of adipogenic differentiation. On days 21 of the adipogenic differentiation, cells were stained with Oil Red O and visualized under an inverted light microscope. Original magnification, 40×. The number of stained cells in 10 random fields was counted at an original magnification of 10×. B. Protein expression level of FABP4 was analyzed by western blot. β-actin expression was measured as an internal control. Data are presented as the mean ± SD (control group: n = 6; AA group: n = 6). #$P > 0.05$, **$P < 0.01$; -Rap vs. +Rap (days 0-14) or -Rap vs. +Rap (days 15-21).

complete loss of Oil Red O staining ($P < 0.01$; **Figure 5A**) and a 77% decrease in FABP4 protein expression ($P < 0.01$; **Figure 5B**). Rapamycin also blocked the terminal adipogenic differentiation of AA BM-MSCs, as evidenced by a significant decrease in Oil Red O staining ($P < 0.01$; **Figure 5A**) and a 68% reduction in FABP4 protein expression ($P < 0.01$; **Figure 5B**) when cells were treated with rapamycin during days 15-21 of adipogenic differentiation. Rapamycin also blocked adipogenic differentiation of control BM-MSCs when added during days 0-14 of adipogenic differentiation, showing a significant decrease in Oil Red O staining ($P < 0.01$; **Figure 5A**) and a 66% decrease in FABP4 protein expression ($P < 0.01$; **Figure 5B**). However, terminal differentiation of control BM-MSCs was not significantly inhibited when rapamycin was added during days 15-21 of adipogenic differentiation, as evidenced by the lack of significant decrease of Oil Red O staining ($P > 0.05$; **Figure 5A**) and FABP4 protein expression ($P > 0.05$; **Figure 5B**).

Inhibition of mTOR by rapamycin significantly reduced PPAR-γ expression in AA BM-MSCs

To elucidate the mechanism by which mTOR regulates the adipogenic differentiation of AA BM-MSCs, we focused on the transcription factor PPAR-γ, which plays a major regulatory role in adipogenesis [4, 7, 8], and revealed an expression pattern that is parallel with mTOR during the adipogenic differentiation of AA BM-MSCs in our study (**Figure 3**). We used rapamycin to suppress mTOR activation, which resulted in near-complete loss of p-S6K1, and subsequently assessed PPAR-γ protein and mRNA levels in BM-MSCs. As demonstrated by western blot analysis (**Figure 6**), treatment of rapamycin during days 0-14 of adipogenic differentiation suppressed PPAR-γ protein expres-

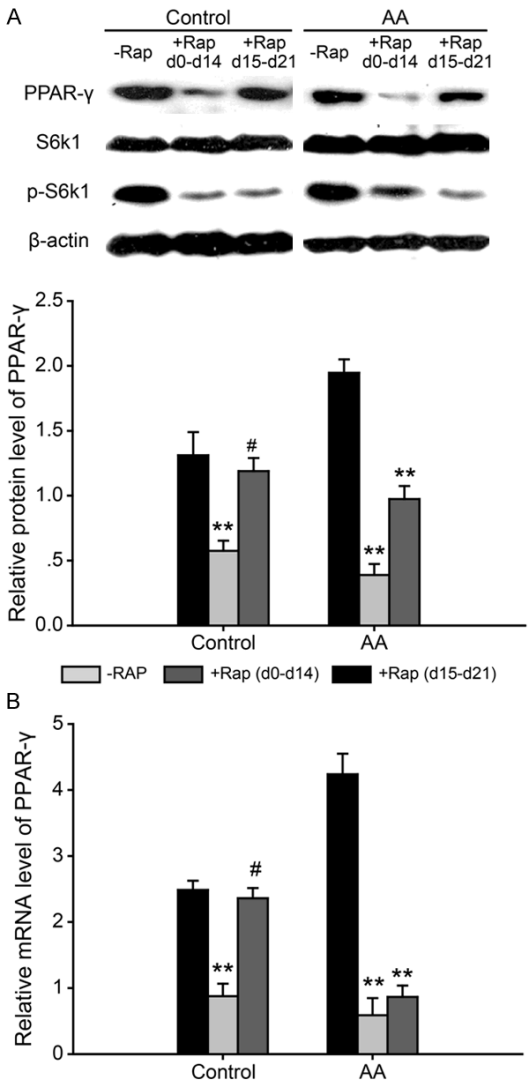

Figure 6. Effect of mTOR inhibition on PPAR-γ expression in BM-MSCs from AA patients and healthy controls. A. BM-MSCs from AA patients and healthy controls were either left untreated or treated with 100 nmol/L rapamycin during days 0-14 or days 15-21 of the adipogenic differentiation process. On days 21 of the adipogenic differentiation, protein expression levels of PPAR-γ, S6K1, and p-S6K1 were assessed by western blot analysis. β-actin expression was measured as an internal control. B. mRNA expression levels of PPAR-γ and mTOR were analyzed by RT-PCR. *GAPDH* was used as an internal control. Data are presented as the mean ± SD (control group: n = 6; AA group: n = 6). #$P > 0.05$, **$P < 0.01$; -Rap vs. +Rap (days 0-14) or -Rap vs. +Rap (days 15-21).

sion in BM-MSCs from both the AA group and the control group ($P < 0.01$; **Figure 6A**). Treatment of rapamycin during days 15-21 of adipogenic differentiation was able to block PPAR-γ protein expression in AA BM-MSCs ($P < 0.01$; **Figure 6A**), but could not significantly inhibit PPAR-γ protein expression in control BM-MSCs ($P > 0.05$; **Figure 6A**). As demonstrated by the RT-PCR analysis shown in **Figure 6B**, the effect of rapamycin on PPAR-γ protein expression was closely mirrored by that on the corresponding mRNA expression (**Figure 6B**).

Discussion

Bone marrow adipocytes serve a variety of functions, including maintenance of bone marrow microenvironment and bone energy metabolism [27]. However, increased bone marrow adipocytes, a characteristic feature of AA, can reduce the expansion of hematopoietic cells in a co-culture system and may inhibit hematopoiesis *in vivo* [3-5]. It is well-established that BM-MSCs are common progenitors of adipocytes and osteocytes. The dysfunction of BM-MSCs may result in the imbalance of differentiation. In our study, we demonstrated the differentiation defects of AA BM-MSCs. Although BM-MSCs from AA patients exhibited a spindle-like morphology in whirlpool-like array (**Figure 1**) and an immunophenotype similar to those observed in BM-MSCs from healthy controls, BM-MSCs from AA patients showed a higher capability for differentiation into adipocytes compared to that of BM-MSCs from healthy controls when cultured in an adipogenic differentiation medium (**Figure 2**), consistent with previous findings [6-8].

Adipogenic differentiation is a multistep process regulated by complex signaling pathways. In this context, the nuclear receptor PPAR-γ can initiate adipogenesis and play a key role in the adipogenic differentiation process [9-11]. PPAR-γ overexpression in BM-MSCs from AA patients may be culpable for the fatty bone marrow [15]. However, the exact gene expression reprogramming necessary for PPAR-γ to stimulate the adipogenesis of AA BM-MSCs has not been thoroughly investigated [28, 29]. Recently, it has been established that mTOR is a key regulator of adipogenesis [24]. Results from some studies have suggested that by regulating PPAR-γ expression, mTOR signaling controls adipogenic differentiation in MEFs and 3T3-L1 cells [22-25]. In this study, we observed that mTOR in AA BM-MSCs was not only activated, but also upregulated. Moreover, protein levels of both mTOR and p-mTOR correlated

with that of PPAR-γ in BM-MSCs from both groups (**Figure 3**). These data suggest that mTOR signaling may be essential for BM-MSCs to differentiate into adipocytes, and may be associated with the increased capacity of adipogenic differentiation of BM-MSCs from AA patients. In addition, we observed that BM-MSCs from both AA patients and healthy controls were accompanied not only by alterations in cell morphology, but also by persistent elevation in PPAR-γ expression during adipogenic differentiation (**Figure 3**), which is consistent with previous findings [28]. Moreover, we found that protein and mRNA levels of both mTOR and PPAR-γ in BM-MSCs from AA patients were significantly higher than those in BM-MSCs from healthy controls at the same time point of adipogenic differentiation, and that expression levels of mTOR and PPAR-γ in AA BM-MSCs showed a parallel and profound differentiation-dependent increase during adipogenic differentiation (**Figure 4**). These results suggest that both mTOR and PPAR-γ may play important roles in adipogenic differentiation of BM-MSCs from AA patients, and that mTOR-PPAR-γ interaction may persist for the entire course of adipogenic differentiation of BM-MSCs from AA patients, which is consistent with reports that mTOR signaling controls adipogenesis in multiple cell types by regulating PPAR-γ [24, 30]. In contrast, parallel expression between mTOR and PPAR-γ in BM-MSCs from healthy controls was not observed during adipogenic differentiation. Therefore, the effect of mTOR on adipogenic differentiation of BM-MSCs from healthy controls remains unclear.

We revealed that mTOR inhibition by rapamycin blocked PPAR-γ protein and mRNA expression, and disrupted adipogenic differentiation of BM-MSCs from AA patients at both the early-middle and the late stages. These results suggest that mTOR signaling mediates the adipogenic differentiation of BM-MSCs from AA patients by regulating PPAR-γ. Importantly, mTOR inhibition can partially reverse the adipogenic differentiation of BM-MSCs from AA patients *in vitro* (**Figures 5** and **6**). In addition, we observed that mTOR inhibition reduced PPAR-γ protein and mRNA expression, and suppressed adipogenic differentiation of BM-MSCs from healthy controls at the early-middle stage, but did not significantly inhibit PPAR-γ expression or disrupt adipogenic differentiation at the late stage (**Figures 5** and **6**). These findings imply that mTOR signaling mediates the adipogenic differentiation of BM-MSCs from healthy controls only at the early-middle stages, and that adipogenic differentiation of BM-MSCs from healthy controls may be independent of mTOR signaling at the late stage.

In conclusion, our results provide evidence that BM-MSCs of AA patients possess enhanced adipogenic potential and that mTOR signaling may be indispensable for the adipogenic differentiation of BM-MSCs from AA patients. mTOR signaling mediates the adipogenic differentiation of BM-MSCs from AA patients by positively regulating PPAR-γ. Importantly, mTOR inhibition can partially reverse adipogenic differentiation of BM-MSCs from the AA patients *in vitro*. These results help better understand the role of mTOR and underlying mechanisms by which mTOR signaling promotes the development of fatty bone marrow in AA patients. In addition, these data provide a basis for further investigations on employing mTOR inhibitors to reverse or alleviate fatty bone marrow in AA mouse models *in vivo*.

Ethics statement

This study was approved by the Ethics Review Board of Shanxi Medical University. Bone marrow samples were obtained from AA patients and healthy volunteers after obtaining informed consent. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Disclosure of conflict of interest

None.

Address correspondence to: Weihua Zhang, Department of Hematology, The First Hospital of Shanxi

Medical University, Taiyuan, Shanxi 030001, P.R. China. Tel: +860351-4639562; E-mail: zwhlzh@126.com

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