All-Trans Retinoic Acid Inhibits Bone Marrow Mesenchymal Stem Cell Commitment to Adipocytes via Upregulating FRA1 Signaling

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Obesity, caused by an increased number and volume of adipocytes, is a global epidemic that seriously threatens human health. Bone marrow mesenchymal stem cells (BMSCs) can differentiate into adipocytes. All-trans retinoic acid (atRA, the active form of vitamin A) inhibits the adipogenic differentiation of BMSCs through its receptor RARG. The expression level of FRA1 (FOS like 1, AP-1 transcription factor subunit) in atRA-treated BMSCs increased, suggesting that atRA-mediated inhibition of BMSCs adipogenesis involves FRA1. BMSCs were transfected with adenovirus overexpressing Fra1 (ad-fra1) or silenced for Fra1 (si-fra1) and then treated with atRA. BMSCs treated with atRA and treated with ad-fra1 showed decreased mRNA and protein levels of key adipogenic genes (Pparg2, Cebpa) and adipogenesis-associated genes (Cd36, Fabp, Lpl, and Plin); atRA had a stronger inhibitory effect on adipogenesis compared with that in the ad-fra1 group. Adipogenic gene expression in Fra1-silenced BMSCs was significantly upregulated. Compared with that in the atRA group, the si-fra1 + atRA also upregulated adipogenic gene expression. However, compared with si-fra1, si-fra1 + atRA significantly inhibited adipogenic differentiation. Chromatin immunoprecipitation showed that RARG directly regulates Fra1 and FRA1 directly regulates Pparg2 and Cebpa. The results supported the conclusion that atRA inhibits BMSC adipogenesis partially through the RARG-FRA1-PPARG2 or the CEBPA axis or both. Thus, vitamin A might be used to treat obesity and its related diseases.

1. Introduction

Currently, obesity is considered a major health hazard and a global epidemic. The World Health Organization reported that in 2016, there were over 19 billion adults who were overweight, and 6.5 billion adults were obese (http://www.who.int/topics/obesity/en/). Obesity is a major risk in many diseases, including type 2 diabetes, cardiovascular dysfunction, musculoskeletal disorders, Alzheimer’s disease, and cancers (prostate, colorectal, endometrial, and breast) [1–3], even impairing quality of life and leading to depression [4]. Mesenchymal stem cells (MSCs) exist in many tissues and have the potential for multidirectional differentiation. They can differentiate into adipocytes, osteoblasts, chondrocytes, nerve cells, or cardiomyocytes [5]. Bone marrow-derived MSCs (BMSCs) are considered the main type of MSCs for laboratory research and clinical application [6]. The important pathophysiological mechanism of obesity is the increase in the number and volume of adipocytes. The increase in adipocytes is caused by the recruitment of new preadipocytes from MSCs [7]. The differentiation of MSCs is regulated by many factors, among which the most critical molecular interactions are the members of the CCAAT/enhancer binding protein (CEBP) and peroxisome proliferator-activated receptors (PPARs) families [8, 9]. Exploring the molecular mechanism of
2. Materials and Methods

2.1. Plasmid Construct. The plasmids used in this study were designed to function in rat species. An adenovirus plasmid for Fra1 overexpression (ad-fra1) and an adenovirus short hairpin RNA (shRNA) plasmid to silence Fra1 (si-fra1) were designed and produced by Obio Technology Corp. (Shanghai, China). The Fra1 gene sequence was synthesized and inserted into vector pAdeno-MCMV-3Flag-PA2-EGFP, to obtain adenovirus vector pAdeno-MCMV-Fra1-3Flag-PA2-EGFP ad-fra1. The si-fra1 shRNA sequences were inserted into vector pDKD-CMV-eGFP-U6, taking advantage of the Agel and EcoRl enzymes to construct the shuttle plasmid and skeleton plasmid of the target gene in HEK293 cells. Three si-fra1 target sequences were used: Y7339 ATCCACTGCAATTCTGGCC, Y7340 TTCTTGCTTT- CTTCTGGGA, and Y7341 TGCTACTCTTTCGATGGGC.

2.2. Cell Culture. BMSCs were obtained from the bone marrow of 2-week-old male Sprague-Dawley (SD) rats (n = 16, obtained from Chongqing Medical University Animal Care Centre, Chongqing, China) as described previously [22]. In detail, bone marrow was rinsed from femurs and tibia using culture medium that contained Dulbecco’s modified Eagle’s medium (DMEM)/F-12 ( Gibco, Grand Island, NY, USA), 10% fetal bovine serum (FBS) (AusGeneX, Brisbane, Australia), and 1% penicillin and streptomycin. BMSCs were obtained by whole bone marrow differential adherence method. In the first 24, 48, and 72 hours, unadhered cells could be effectively exchanged using total medium exchange. Thereafter, the medium was changed every 3 days. When the density of cells reached 95%, adherent BMSCs were digested with trypsin (Gibco) and passaged on. Third-generation BMSCs were stored in a 90% FBS and 10% dimethyl sulfoxide (DMSO) (Dingguo Biotech, Beijing, China) mixture in liquid nitrogen. If necessary, BMSCs were resuscitated by rapid thawing in a 37°C water bath, plated in culture medium, and cultured at 37°C in humid air with 5% CO₂. Third-generation BMSCs were used for all experiments [23]. BMSCs were maintained in 37°C and 5% CO₂ incubators (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Multiplicity of Infection (MOI) Value Exploration and Efficiency of Adenovirus Transfection. BMSCs were seeded into a 24-well plate (6 × 10⁵ cells well⁻¹). After overnight culture, the culture medium was exchanged and BMSCs were transduced with si-fra1 adenovirus (Y7340) at various MOI (100, 110, 120, 130, 150, 160, 180, and 200) at 37°C in a humidified 5% CO₂, 95% air incubator for 12 h. The supernatant was then removed, fresh medium was added, and the cells were incubated for 72 h. The amount of fluorescence in the BMSCs was observed under a microscope (Nikon, Tokyo, Japan) under green fluorescence excitation. BMSCs were seeded into a 6-well plate (2 × 10⁶ cells well⁻¹), the adenovirus (MOI 120) was into each well, grouped as follows: ad-fra1, si-fra1 (Y7339, Y7340, and Y7341) vector, and blank. The Fra1 overexpression and knockdown efficiency were determined using quantitative real-time reverse transcription PCR (qRT-PCR).

2.4. In Vitro Transduction of BMSCs with Adenoviral Vector and Adipocyte Differentiation. BMSCs were seeded into a 6-well plate (2 × 10⁵ cells well⁻¹); 6–8 h later, the medium was replaced at 2 ml per well. Adenoviral transient infection was carried out when the fusion degree of BMSCs reached more than 95%. The groups were set as follows: ad-fra1, si-fra1 (Y7340), vector + atRA, si-fra1 + atRA, and vector. Adenoviruses were added to the BMSCs and cultured at 37°C with 5% CO₂ for 12 h. Thereafter, the BMSCs were washed with phosphate-buffered saline (PBS) (Dingguo Biotech, Beijing, China) and fed with adipogenic differentiation medium A.
(Cyagen, Jiangsu, China). In addition, atRA (Sigma, St.
Louis, MO, USA), dissolved in pure ethanol, was added to
vector + atRA and si-fra1 + atRA groups to achieve a con-
centration of 5 µmol·L⁻¹. Seventy-two hours later, we
replaced medium A with fresh medium B and cultured the
cells for 24 h. Fresh atRA was added to both medium A and B
for the vector + atRA and si-fra1 + atRA groups. Medium A
contained 87.5% BMSCs adipogenic differentiation basal
medium A, 10% FBS, 1% penicillin-streptomycin, 1% glu-
tamime, 0.2% insulin, 0.1% 3-isobutyl-1-methylxanthine
(IBMX), 0.1% rosiglitazone, and 0.1% dexamethasone.
Medium B (Cyagen) comprised 87.8% BMSCs adipogenic
differentiation basal medium B, 10% FBS, 1% penicillin-
streptomycin, 1% glutamime, and 0.2% insulin. In general,
the adipogenic process comprised culturing BMSCs in
medium A (2 ml per well) for 72 h. Old medium A was
replaced with medium B (2 ml per well) for another 24 h.
After that, the old medium B was replaced with fresh me-
dium A at the same volume. This cycle was repeated three
to five times until lipid droplets formed.

2.5. Oil Red O (ORO) Staining. BMSCs were fixed with 4%
neutral formaldehyde for 30 min in room temperature on the
22nd day of adipogenic induction and then washed with
1 × PBS twice. Oil red O dye solution (Cyagen) was formu-
lated with 60% oil red O storage solution and 40% distilled
water and filtered by neutral filter paper. Then, the BMSCs
were stained with oil red O solution in a biosafety cabinet
(Thermo Fisher Scientific, Waltham, MA, USA) for 60 min.
After the oil red O dye solution was absorbed, the cells were
washed three times with 1 × PBS. Next, adipogenic staining
was observed under a microscope (n = 3 per group). Finally,
the oil red O stained images were analyzed using ImageJ
software (National Institutes of Health, Bethesda, MD, USA),
following the methods described by Xia et al. [24].

2.6. RNA Isolation and qRT-PCR. Total RNA was extracted
from cells using an RNA-Quick Purification kit (YiShan
Biotech, Shanghai, China) according to manufacturer’s in-
stuctions. The RNA concentration was measured using
spectrophotometer (Nanodrop Technologies, Wilmington,
DE, USA). Then reverse transcription of RNA into cDNA
was performed following the PrimeScript Buffer kit in-
structions. qRT-PCR was carried out using the SYBR-
Green mix and a Bio-Rad CFX Connect Real-Time system
(Bio-Rad) (n = 6 per group). Primers for the rat Fra1, Pparg2,
and Cebp genes were as follows: Fra1 primer sense TCAGGAGTT-
CAAGGCCCCAGTC, antisense 5'-CTCTGGAAGGATGT-
TGAGG-3'; Pparg2 primer sense 5'-CACTGGAAGTT-
TGGAGAAGGAA-3', antisense 5'-TCTGGGATTTGT-
GATGTTGAA-3'; Cebp primer sense 5'-ATAAG-
GACGCACAATCTCAGGACTCT-3', anti-sense 5'-
GATCACCCTTACGCGACCAC-3'. The qRT-PCR sam-
ple was subjected to 1% agarose gel electrophoresis to
confirm and detect the amplified fragments under a UV light
(Syngene G:BOX, Cambridge, UK) [28]. Fold enrichment
was calculated over IgG using 2ΔΔCT, where ΔΔCT =
normalized CtPparg2-CtGapdh).

2.7. Isolation of Total Protein and Western Blotting. We
followed the methods of Lai et al. [26]. The radio immuno-
precipitation assay (RIPA) lysis buffer (KeyGEN Biotech,
Nanjing, China) which contained 0.1% protease inhibitor
cocktail (KeyGEN) was used to extract total protein from
BMSCs. The concentration of proteins was determined using
a BCA kit according to the manufacturer’s instructions (ATGene,
Chongqing, China) and a microtiter plate reader (Thermo
Fisher Scientific). 5 × SDS-PAGE sample buffer was added to
equal amounts of protein, which were boiled at 100°C in a water
bath for 10 min before being subjected to 8% SDS-PAGE.

Western blotting was performed as previously described
[27]. Some adjustments were made, we used anti-RARG
(Abcam, Cambridge, UK) (1:1000), anti-FRA1 (Abcam) (1 :
100), anti-CEBPA (Abcam) (1 :100), and anti-PPARG2
(Abcam) (1 :500) antibodies according to the manufacturer’s
instructions (n = 6 per group). An ECL Prime kit (Millipore
Corp, Billerica, MA, USA) and an ECL Imaging system
(Syngene G:BOX, Cambridge, UK) were used to analyze the
levels of immunoreactive proteins. ImageJ software was used
to analysis the intensity of the protein bands.

2.8. Chromatin Immunoprecipitation (ChIP). ChIP was
performed using a Millipore company a ChIP kit (EZ-ChIP,
Millipore Corp) following its manufacturer’s protocol and
according to the details from Lai’s report [26]. In brief,
BMSC chromatim was sheared by sonication (medium
power, 20 cycles of 30 s between pulses) to 200–1000 bp.
The chromatin was incubated overnight at 4°C with primary
antibodies recognizing FRA1 (Santa Cruz, CA, USA) or
RARG (Santa Cruz) or IgG antibody (the negative control).
QRT-PCR was carried out using the SYBR-Green mix and a
Bio-Rad CFX Connect Real-Time system (Bio-Rad) (n = 6
per group). Primers for the rat Fra1, Pparg2, and Cebp
genres were as follows: Fra1 primer sense TCAGGAGTT-
CAAGGCCCCAGTC, antisense 5'-CTCTGGAAGGATGT-
TGAGG-3'; Pparg2 primer sense 5'-CACTGGAAGTT-
TGGAGAAGGAA-3', antisense 5'-TCTGGGATTTGT-
GATGTTGAA-3'; Cebp primer sense 5'-ATAAG-
GACGCACAATCTCAGGACTCT-3', anti-sense 5'-
GATCACCCTTACGCGACCAC-3'. The qRT-PCR sam-
ple was subjected to 1% agarose gel electrophoresis to
confirm and detect the amplified fragments under a UV light
(Syngene G:BOX, Cambridge, UK) [28]. Fold enrichment
was calculated over IgG using 2ΔΔCT, where ΔΔCT =
normalized CtPparg2-CtIgG).

2.9. Statistical Analysis. All data were represented by the
mean ± SEM. GraphPad Prism 5.0 software (GraphPad
Software, Inc. La Jolla, CA, USA) was used for this analysis.
The data from the oil red O staining, qRT-PCR, western
blotting, and ChIP-qPCR were analyzed using an unpaired
Student’s t test (two sets of data) or one-way analysis of
variance (ANOVA) (>2 sets of data) as data. Adjusted
p values were calculated using Tukey’s HSD post hoc an-
alyses. The significance level was set at p < 0.05.

3. Results

3.1. Efficiency of Adenovirus Transfection into BMSCs.
BMSCs were transduced with adenoviral si-fra1 (Y7339),
which also expressed the enhanced green fluorescent protein
(EGFP). The intracellular fluorescence intensity was used to evaluate transfection efficiency of adenovirus. Si-fra1 was infected at a MOI 100, 110, 120, 130, 150, 160, 180, and 200, and the intracellular fluorescence intensities were detected after 72 h of adenovirus transfection. The transfection efficiency, as determined by percentage of BMSCs containing green fluorescence, were 8.2%, 18.2%, 51.4%, 58.8%, 59.3%, and the intracellular fluorescence intensities were detected inflected at a MOI 100, 110, 120, 130, 150, 160, 180, and 200, respectively. There was a significant difference between the result for a MOI 120 and that for 110. However, above a MOI 120, the difference was not significant. Therefore, we chose adenovirus at a MOI of 120 for subsequent experiments.

Expression of untransduced (blank), vector, ad-fra1, and si-fra1 (Y7339, Y7340, and Y7341) mRNA was detected using qRT-PCR to confirm the level of Fra1 mRNA expression. As expected, compared with the blank and vector, adenovirus ad-fra1 upregulated Fra1 mRNA expression (p < 0.001), and adenovirus si-fra1 could markedly reduce the expression of the Fra1 mRNA (p < 0.001). Y7340 had the most significant silencing effect and thus was chosen for future experiments. Fra1 expression was not significantly different between the blank and vector. Figures are shown in Supplementary Figure 1. These data suggested that the Fra1 gene overexpression and silencing were successful. In addition, the adenoviral vector itself would not affect the expression level of Fra1. In the latter experiment, we used the vector as the control group and the Fra1 expression levels were maintained over 3 weeks.

3.2. ATRA Treatment Could Upregulate the Expression of RARG during the Adipogenic Differentiation of BMSCs. ATRA binds to three retinoic acid nuclear receptors (RARα/β/γ) via noncovalent bonds. RARY plays an important role in the retinoic acid signaling pathway [18]. BMSCs were treated with 5 μmol/L aTRA and then induced for adipogenesis according to the previously mentioned method. After 8 days of treatment, qRT-PCR was used to analyze the expression of Rarg in the mRNA level. Using the vector group as the control group, Rarg mRNA expression was significantly increased in the vector + aTRA group (p < 0.001) (Figure 1(a)). Western blotting showed the same results for the RARG protein level (p < 0.001) (Figures 1(b) and 1(c)). These data showed that aTRA could increase the expression of RARG at both the mRNA and protein levels.

3.3. Overexpression of Fra1 and aTRA Treatment Could Block BMSC Differentiation into Adipocytes. The inhibitory effect of aTRA on adipogenesis has been confirmed in many studies [18, 19, 29, 30]. We repeated a previous experiment with 5 μmol/L aTRA in BMSCs. After 22 days of adipogenic differentiation treatment, oil red O staining was performed to reveal the accumulation of fat-characterized adipocytes. The number of lipid droplets in the BMSCs treated with aTRA was significantly fewer than those in the vector group (p < 0.001). Similarly, inhibition of differentiation to mature adipocytes and accumulation of lipid droplets were observed in the BMSCs overexpressing Fra1 (p < 0.001), as indicated by a decreased number of Oil-Red-O-positive cells. ImageJ was used to analyze the pictures of Oil-Red-O staining, which was significantly inhibited compared with that in the vector only group (Figure 2(a)). The inhibition of BMSCs’ adipogenic differentiation was stronger in the aTRA group than in the ad-fra1 group.

PPARG is the motive force of adipocyte differentiation and comprises two different subsets, namely, PPARG1 and PPARG2. The expression of PPARG1 in adipocytes is very low, while PPARG2 has a high fat-selective function and is highly expressed in adipocytes [9]. When Cebpa was coexpressed with PPARG2, myoblastic cell lines also had the ability to convert to adipocytes [31]. Therefore, both PPARG2 and Cebpa play important roles in adipogenesis. In addition, the induction of several genes characterizing functional mature adipocytes, namely, Cd36 (CD36 molecule), Fabp (fatty acid binding protein), Lpl (lipoprotein lipase), and Plin (Perilipin) are adipogenesis-associated genes. Proteins and mRNA were extracted from BMSCs after 8 days of adipogenesis induction. QRT-PCR demonstrated markedly increased Fra1 expression in the ad-fra1 group compared with that in the vector group (p < 0.001). Using the vector group as the control group, Fra1 mRNA expression was increased in the vector + aTRA group (p < 0.05). Conversely, both in the ad-fra1 and vector + aTRA groups, the mRNA expression levels of PPARG2, Cebpa and the adipogenesis associated genes (Cd36, Fabp, Lpl, and Plin) decreased strongly (Figures 2(b) and 2(d)). Furthermore, the mRNA expression

### Table 1: Specific primer sequences used for qRT-PCR analysis.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (forward)</th>
<th>Primer sequence (reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rarg</td>
<td>5′-CTGCACCTGAACGAACCGC-3′</td>
<td>5′-CACAGATAGGGCAGATAGCA-3′</td>
</tr>
<tr>
<td>Fra1</td>
<td>5′-GCCAGCATGGTAGAGG-3′</td>
<td>5′-GGAGATCACAGGGGTTGTG-3′</td>
</tr>
<tr>
<td>Cebpa</td>
<td>5′-GCTGGAAAGACAGAGCA-3′</td>
<td>5′-TCACTCAGATCCCTTGCT-3′</td>
</tr>
<tr>
<td>Pparg2</td>
<td>5′-CTCCCTCAGTAAAGATGG-3′</td>
<td>5′-CAGACAAACTCAAATGAGC-3′</td>
</tr>
<tr>
<td>Cd36</td>
<td>5′-CTCTGACATTGTCAGTC-3′</td>
<td>5′-AGTTGCTGGTTGTGTC-3′</td>
</tr>
<tr>
<td>Fabp</td>
<td>5′-AGCATGTAACCTCCTGGAG-3′</td>
<td>5′-GGCTTTCTGACATTCCTCCA-3′</td>
</tr>
<tr>
<td>Lpl</td>
<td>5′-CTGGTCTGACATTCCTCA-3′</td>
<td>5′-GGCCACTGGTCCATACAGA-3′</td>
</tr>
<tr>
<td>Plin</td>
<td>5′-AGCTCACCCTCACTGGTCAT-3′</td>
<td>5′-CCTGACCCAGATCTCTTCTC-3′</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5′-CTCCCGGAAAACCTGCCAAG-3′</td>
<td>5′-CACAGGAGACAACCTGGTCC-3′</td>
</tr>
</tbody>
</table>

Rarg, retinoic acid receptor gamma; Fra1, FOS like 1, AP-1 transcription factor subunit; Cebpa, CCAAT enhancer binding protein alpha; Pparg2, peroxisome proliferator activated receptor gamma; Cd36, Cd36 molecule; Fabp, fatty acid binding protein; Lpl, lipoprotein lipase; Plin, Perilipin; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.
Figure 1: BMSCs treated with vector and vector + atRA for adipogenic differentiation for 8 days. (a) The mRNA expression of the target gene (Rarg), (n = 7/group). (b, c) The protein levels of RARG in the late stage of BMSC adipogenic differentiation (n = 6/group). The values are the mean ± SEM. *** p < 0.001. BMSC, bone marrow mesenchymal stem cell; RARG, retinoic acid receptor gamma.

Figure 2: BMSCs treated with vector, ad-fra1, and vector + atRA for 22 days: (a) the effect of ad-fra1 and atRA on BMSCs adipogenic differentiation (oil red O staining ×100 and ×400), (n = 3/group). The mRNA expression in BMSCs treated with vector, ad-fra1, and vector + atRA for 8 days; (b) target gene (Fra1) and two adipocyte key genes (Pparg2 and Cebpa); (d) adipogenesis-associated genes (Cd36, Fabp, Lpl, and Plin), (n = 7/group). (c) The protein levels of FRA1, PPARG2, and CEBPA in the late stage of BMSCs adipogenic differentiation (n = 6/group). The values are the mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant in multiple comparisons. Both the ad-fra1 group and the vector + atRA group compared with the vector group. BMSC, bone marrow mesenchymal stem cell; FRA1, FOS like 1, AP-1 transcription factor subunit; atRA, all-trans retinoic acid; PPARG2, peroxisome proliferator-activated receptor gamma; CEBPA, CCAAT enhancer binding protein alpha; Cd36, CD36 molecule; Fabp, fatty acid binding protein; Lpl, lipoprotein lipase; Plin, perilipin.
levels of these genes were even lower in the vector + atRA group. Western blotting showed the same results for FRA1, Pparg2, and CEBPA protein levels (Figure 2(c)). These data strongly indicated that FRA1 upregulation can partly imitate the effects of atRA and is associated negatively with BMSC adipocyte differentiation.

3.4. Knockdown of Fra1 In Vitro Promoted the Differentiation of BMSCs into Adipocytes. BMSCs were plated in 6-well plates at a density of 2 × 10^6 cells per well. BMSCs were treated with adenoviruses silencing Fra1 for 12 h, after which the cells were induced to differentiate to adipocytes for 8 days. QRT-PCR detected that the mRNA expression level of Fra1 was significantly lower in si-fra1 group compared with that in the vector group (p < 0.01). The mRNA expression levels of Cebpa and Pparg2 in the si-fra1 group were significantly upregulated compared with those in the vector group (p < 0.05 and p < 0.001, respectively) (Figure 3(a)). Western blotting showed similar results at the protein level (Figure 3(b)). The mRNA levels of adipocyte associated genes, which are markers of terminal adipogenesis, were upregulated in the si-fra1 group during adipogenesis differentiation 8th day (Figure 3(c)). Compared with the vector group, si-fra1 group showed distinct lipid accumulation on the 22nd day, as indicated by the amount of lipid droplets that could be seen using oil red O staining (Figure 3(d)).

Vector + atRA and si-fra1 + atRA groups were treated with empty adenoviral vector and the adenoviral vector silencing Fra1 for 12 hours, after which they were treated with atRA and placed in adipogenic differentiation medium for 8 days. Using the vector group as the control, the Fra1 mRNA was strongly silenced in si-fra1 + atRA group and there was no significant difference between si-fra1 group and si-fra1 + atRA group. QRT-PCR data showed that Cebpa and Pparg2 expression levels were significantly upregulated in the si-fra1 + atRA group compared with those in the vector + atRA group (p < 0.01 and p < 0.05, respectively) (Figure 3(a)). Western blotting showed similar results for the protein levels (Figure 3(b)). The mRNA expression levels of the adipocyte associated genes were also significantly upregulated (Figure 3(c)). The increased adipogenesis was confirmed by oil red O staining (Figure 3(d)). Thus, the results confirmed that silencing Fra1 promoted the differentiation of BMSCs into mature fat cells.

3.5. RARG Upregulates Transcription of Fra1 and FRA1 Downregulates the Transcription of Pparg2 or Cebpa by Binding to its Proximal Promoter after atRA Treatment. Previous research in our laboratory identified that atRA inhibits BMSC adipogenic differentiation by activating RARG [18]. The data in the present study showed that both atRA and FRA1 can downregulate the expression of adipocyte-associated genes. To further investigate the interaction between RARG and Fra1, we conducted a ChIP-qPCR experiment using anti-RARG antibodies. The ChIP-qPCR analysis clearly showed that the RARG protein was present on the Fra1 promoter in BMSCs, with statistically significant enrichment after atRA treatment (p < 0.001) (Figure 4(a)).

Agarose gel electrophoresis detection confirmed this finding (Figure 4(d)). The Genomatix software predicted that the RARG binding site in the promoter of Fra1 is in the following sequence: TCCATCTCAATTGACCTTCCTCCTC. This site is 823 bp 5' to the transcription initiation site (Figure 4(g)). Therefore, we concluded that RARG enters the nucleus after treatment with atRA to regulate Fra1 expression by binding to its promoter region.

Our experiments showed that overexpression of Fra1 downregulated Pparg2 and Cebpa expression. Conversely, Fra1 silencing upregulated Pparg2 and Cebpa expression. To clarify the relationship between FRA1 and Pparg2 and Cebpa, we conducted ChIP-qPCR tests using anti-FRA1 antibodies. The results showed that FRA1 protein was present at the Pparg2 promoter in BMSCs and was significantly enriched after atRA treatment (p < 0.05) (Figure 4(b)). Agarose gel electrophoresis showed the same result (Figure 4(e)). The Promo software predicted that the FRA1 bind site in the promoter of Pparg2 is in the following sequence: AGATGACTCAAAG. This site is 404 bp 5' to the transcription initiation site (Figure 4(h)). The ChIP-qPCR data also showed that FRA1 protein was present at the Cebpa promoter in BMSCs; however, there was no statistically significant enrichment after atRA treatment (Figure 4(c)). Agarose gel electrophoresis showed that FRA1 was obviously enriched in the Cebpa sample (Figure 4(f)). The Genomatix software predicted a FRA1 binding site in the Cebpa promoter in the following sequence: AGACGCACAATC-TCAGCAGTCTCGGG. This site is 602 bp 5' to the transcription initiation site (Figure 4(i)). Overall, we confirmed that FRA1 enters the nucleus after treatment with atRA, where it regulates Pparg2 and Cebpa expression by binding to its promoter regions.

4. Discussion

Vitamin A was the first identified fat-soluble vitamin. Its physiological functions include maintaining visual function [32], promoting immunoglobulin synthesis [33], and maintaining the stability of reproductive epithelial cells [34]. Recent research has focused on the physiological functions of vitamin A, and its metabolite atRA, such as regulating energy metabolism, insulin sensitivity, and lipid metabolism [35, 36]. An epidemiological investigation reported that a low serum retinol concentration was a risk factor for obesity. According to the data of the Third American Health and Nutrition Survey, the level of serum beta-carotene in obese people (P_{95} < BMI) was significantly lower than that in the normal weight group [37]. A similar survey was performed by Aasheim and his colleagues. They compared the serum levels of vitamin A in 110 patients with severe obesity with that in 58 healthy people and found that the levels of vitamin A in obese patients were significantly lower than those in the healthy controls [38]. Studies found that feeding fat rats with a vitamin A-supplemented diet could correct insulin resistance, plasma high-density lipoprotein level, and hypercholesterolemia in obese rats [39, 40]. In addition, the role of retinoic acid in inhibiting adipogenesis in vitro was confirmed in 3T3-L1 cells [41]. These studies demonstrated that
vitamin A and atRA play important roles in inhibiting adipocyte differentiation, which provided a new focus for the clinical prevention and treatment of obesity.

BMSCs can be directed to differentiate into mature adipocytes in vitro. Our previous work found that atRA has a significant potential to inhibit the BMSCs’ differentiation.
into adipocytes [18]. During this process, the level of RARG is upregulated, while the level of PPARG2 is downregulated. Co-IP analysis proved the connection between RARG and PPARG2 [18]. However, the result of ChIP-qPCR demonstrated that RARG could not regulate PPARG2 directly [19]. The results of the present study reconfirmed the potential of adipogenic differentiation of BMSCs and the significant inhibition of atRA such adipogenic differentiations. Oil red O staining showed that the number of fat droplets in BMSCs after atRA treatment was significantly less than that in the control group. After atRA treatment, the expression levels of PPARG2 and CEBPA and adipocyte associated genes decreased significantly.

FRA1 is a member of the AP-1 family. A previous study found that Fra1 mRNA was highly expressed in the atRA intervention group [19]. Luther et al. showed that elevated Fra1 expression in mice can cause severe lipodystrophy [42]. Therefore, we aimed to determine how atRA inhibited BMSCs adipogenic differentiation through FRA1. BMSCs overexpressing Fra1 showed few lipid droplets after oil red O staining and showed a significant decrease in adipogenic gene expression. The results were validated at the mRNA and protein levels. By contrast, BMSCs silenced for Fra1 showed increased amounts of lipid droplets after oil red O staining. Furthermore, key adipogenic genes and adipogenesis-related genes showed higher expression in the si-fra1 group. This confirmed that FRA1 has an inhibitory effect on the induced adipogenic differentiation of BMSCs.

Although both atRA and FRA1 play a negative role in BMSCs' adipogenic differentiation, they have different efficiencies. Both ad-fra1 and atRA could increase the expression of Fra1. The mRNA and protein levels of Fra1 in the ad-fra1 group were almost 40 times and 10 times higher, respectively, than those in the atRA intervention group. However, treatment with atRA caused stronger inhibition of adipogenesis. This was specifically reflected in the expression of key adipogenic genes (PPARG2 and CEBPA) and adipocyte-associated genes. The PPARG2 and CEBPA protein levels also confirmed this result. In addition, after oil red O staining, a small amount of lipid droplets could be seen in the ad-fra1 group, but there were almost no lipid droplets in the atRA group. We hypothesized that silencing Fra1 could promote BMSCs' adipogenic differentiation. Results from qRT-PCR, western blotting, and oil red O staining supported this hypothesis. We also found that although there were no statistical differences in the mRNA expression of Fra1 between si-fra1 group and the si-fra1 + atRA group. Because of the role of atRA, the adipogenic ability in BMSCs was further inhibited in the si-fra1 + atRA group.

ATRA induces bioactivity by activating its nuclear receptors, RARs (RARα/β/γ), and retinoid X receptors (RXRs/β/γ), which combine as a heterodimer to regulate target
genes by binding to specific binding elements [43]. However, the activation of PPARG is related to the inhibition of E2F dimerization partner 1 (E2F/DP, also known as transcription factor Dp-1 (TFDP1)). E2F/DP is a key transcription factor that regulates the growth of many kinds of cells [31]. The expression of PPARG precedes the expression of CEBPA, and CEBPA can up-regulate the expression of PPARG [31]. Based on our research results, we propose the hypothesis that atRA inhibits BMSCs’ adipogenic differentiation via the atRA-RARG-FRA1-PPARG2/CEBPA axis. Before the ChIP-qPCR experiment, we used the software PROMO and Genomatix to predict the binding sites of transcription factors. Interestingly, five potential RAR binding sites were predicted in the promoter of the rat Fra1 gene. ChIP-qPCR analysis identified an 823bp 5’ gene fragments to the promoter region of Pparg2. These results indicated that FRA1 could directly regulate Pparg2 expression by binding to its promoter region. Hasenfuss et al.’s findings also supported this result [21]. D’Ambrosio et al. used human hepatocellular carcinoma cells (HuH7) and human renal epithelial cells (293T) to detect luciferase reporter genes, which showed that FRA1 could significantly inhibit Pparg2 gene transcriptional activity [17]. We used anti-FRA1 antibodies to precipitate Cebpa gene fragments and found that FRA1 was present at the Cebpa promoter in BMSCs. Similarly, Luther et al. detected FRA1 could directly inhibit Cebpa transcription [42].

Our data showed that Fra1 expression was increased in both the vector + atRA group and the ad-fra1 group, compared with that in the vector group. However, compared with that in the vector + atRA group, the expression of Fra1 in the ad-fra1 group was higher. For the key adipogenic genes and adipocyte-associated genes, the vector + atRA group showed stronger expression inhibition than that in the ad-fra1 group. The result was also confirmed using western blotting and oil red O staining. QRT-PCR showed that in both the si-fra1 and si-fra1 + atRA groups, Fra1 was silenced successfully and there was no statistical difference between them. However, the expression of master adipogenic genes, transcription factors, and lipogenic genes were statistically significantly decreased compared with that without atRA treatment, indicating that atRA reduced adipogenesis. Western blotting and oil red O staining confirmed this result. The ChiP-qPCR data proved that atRA could inhibit adipogenic differentiation of BMSCs through the RARG-FRA1-PPARG2 and/or CEBPA axis. Figure 5 shows the inferred relationship between atRA, RARG, and FRA1, and two important downstream molecules PPARG2 and CEBPA.

Compared with the effect of FRA1 alone, atRA has a stronger inhibitory effect on adipogenesis. This suggested that atRA could inhibit adipogenesis via other pathways.

Studies have shown that retinoic acid can stimulate the expression of transforming growth factor β (TGFβ) effector protein SMAD family member 3 (SMAD3). SMAD3 interacts with CCAAT enhancer binding protein beta (CEBPB) to interfere with the binding of CEBP DNA to other transcription factors, thereby interfering with the adipogenic signaling pathway [44]. Ayala-Sumuano et al. demonstrated that retinoic acid inhibits 3T3-F442A cell adipogenesis by modulating CEBPB phosphorylation and downregulating sterol regulatory element binding transcription factor 1 (SREBP1) expression [45]. In addition, studies have shown that retinoic acid inhibits adipogenic differentiation of preadipocytes in vitro by upregulating the gene expression of transcription factors preadipocyte factor 1 (PREF1), SRY-box 9 (SOX9), and Kruppel-Like Factor 2 (KLF2) by activating RARG receptors. Retinoic acid receptor response elements (RAREs) are present in the promoter regions of these transcription factor genes [46–49]. Furthermore, Wang et al. discovered that RARG-C-FOS-PPARG2 signaling is critical for atRA-mediated inhibition of 3T3-L1 cell adipocyte differentiation [50]. Thus, atRA inhibits adipogenesis by affecting different signaling pathways, and the induction of atRA will have therapeutic potential in metabolic diseases such as obesity.

5. Conclusions

Taken together, our results show that atRA inhibits adipogenic differentiation of BMSCs by upregulating FRA1 signaling. We investigated FRA1 and atRA in the adipogenic differentiation of rat BMSCs. Both of them play a negative role in BMSC adipogenic differentiation. The molecular mechanism involves atRA exerting its effect via its nuclear
receptor RARG and downregulating the expression of PPARγ2 and CEBPA in the adipogenic differentiation of BMSCs by upregulating FRA1. In brief, the RARG-FRA1-PPARγ2 or CEBPA axes, or both, are effective in the process of atRA inhibition of adipogenic differentiation of BMSCs. However, there might be other signaling pathways involved in atRA-mediated inhibition of BMSC adipogenesis, which should be explored in the future study. This deeper understanding of the molecular mechanism by which atRA inhibits BMSC adipogenesis supports the use of vitamin A to treat obesity and related diseases.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have declared no conflicts of interest.

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Supplementary Materials

Supplementary Figure 1: the efficiency of adenovirus transfection into BMSCs. The optimal MOI for intervene BMSCs with adenovirus was 120. Frai gene overexpression and silencing were successful in BMSCs. (Supplementary Materials)

References


