Phactr1 negatively regulates bone mass by inhibiting osteogenesis and promoting adipogenesis of BMSCs via RhoA/ROCK2

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Abstract

The imbalance between osteogenic and adipogenic differentiation of Bone marrow-derived mesenchymal stem cells (BMSCs) is involved in the occurrence and development of osteoporosis (OP). Previous studies have indicated the potential of phosphatase and actin regulator 1 (Phactr1) in regulating osteogenic and adipogenic differentiation of BMSCs. The present study aims to investigate the function and mechanism of Phactr1 in regulating osteogenic and adipogenic differentiation of BMSCs. Herein, the expression of Phactr1 in bone and adipose tissue of OP rats was determined by immunohistochemical. BMSCs were subjected to osteogenic and adipogenic differentiation, and transfected with Phactr1 overexpression lentivirus, small interference RNA (siRNA) and KD025 (selective ROCK2 inhibitor). The relationship between Phactr1 and ROCK2 was detected by Co-IP experiment. The expression of Phactr1, Runx2, C/EBPα, RhoA and ROCK2 was detected by Western blot. Calcium nodule and lipid droplets were determined by alizarin red and Oil red O staining. Interestingly, Phactr1 increased in both bone and adipose tissue of OP rats. During osteogenic differentiation, Phactr1 decreased and active RhoA, ROCK2 increased, while overexpression Phactr1 inhibits the increase of Runx2. Phactr1 increased and active RhoA decreased, ROCK2 did not changed during adipogenic differentiation. While, Knockdown Phactr1 inhibits the increase of C/EBPα. Phactr1 and ROCK2 were combined in osteogenic differentiation, but not in adipogenic differentiation. By using KD025, the decrease of Phactr1 and increase of Runx2 were inhibited respectively in osteogenic differentiation. Meanwhile, when ROCK2 was inhibited, Phactr1, C/EBPα were significantly increased in adipogenic differentiation. These findings indicated that Phactr1 negatively regulates bone mass by inhibiting osteogenesis and promoting adipogenesis of BMSCs by activating RhoA/ROCK2.

Keywords Osteoporosis · Phactr1 · BMSCs · RhoA/ROCK2

Introduction

Osteoporosis (OP) is a common systemic bone metabolism disease, its main characteristics are the reduction of bone mass, decreased bone strength, and damage to bone microstructure, which are prone to fragility fractures (Riggs and Melton 1995). With the advent of the aging society, the incidence of OP and it caused fractures are increasing, which increases the disability and mortality and brings great harm. For example, with a hip fracture, the mortality rate within 1 year is as high as 20% (Johnell and Kanis 2005). Even if they survive, more than 50% of patients need to be taken care of, which brings a heavy burden to individuals, families and society (Martin et al. 2002). Therefore, the research on exploring and developing novel mechanism of anti-Osteoporosis drugs has always been a hot.
In recent years, many studies have shown that there is a close relationship between fat and bone metabolism (Justesen et al. 2001). Once the physiological process of bone and fat formation is disrupted, it will cause some abnormal metabolism diseases, such as OP and Obesity (Zhang et al. 2020). The study also showed that the imbalance between osteogenic and adipogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) is significantly related to the occurrence and development of osteoporosis (Chen et al. 2016). BMSCs are adult stem cells with self-renewal, replication and multidirectional differentiation potential, which can differentiate into osteoblasts and adipocytes under certain conditions. However, the differentiation between them is mutually restrictive (Titorencu et al. 2007).

The differentiation fate of BMSCs is determined by many factors. More and more evidence showed that cytoskeletal proteins play a key role in regulating the osteogenic and adipogenic differentiation of BMSCs (Chen et al. 2021). When the cytoskeleton assembly dynamics changes, the transformation from monomer G-actin to polymer F-actin in microfilaments increases, resulting in the increase of actin polymerization level, which is conducive to promoting the osteogenic differentiation of BMSCs. On the contrary, if the actin network is disordered, BMSCs is conducive to adipogenesis (Khan et al. 2020). Using Protein Data Bank (PDB) analysis, we found that phosphatase and actin regulator 1 (Phactr1) directly combined with G-Actin, and Nadège (Fils-Aimé et al. 2013) found that Knockdown Phactr1 in breast cancer cells caused serious rearrangement and increased polymerization of cytoskeleton. Those findings indicated Phactr1 may participate in the osteogenic and adipogenic differentiation of BMSCs by regulating the function of the cytoskeleton, thereby contributing to the occurrence and development of OP. However, this effect and specific mechanism need to be further verified and clarified.

The mechanism of osteogenesis and adipogenesis in BMSCs involve many signaling pathways, such as Wnt and TGF-β/BMPs, Notch, Hippo and Hedgehogs, etc. (James 2010). Among them, Rho/ROCK signaling pathway plays an key role in determining the differentiation fate of BMSCs (Ke et al. 2019). Rho/ROCK inhibits PPARγ expression by delaying activation of the typical Wnt signaling pathway based on Wnt10b expression and β-catenin induction (Li et al. 2011). At the same time, Rho/ROCK participates in the reorganization of actin cytoskeleton and the phosphorylation of myosin light chain, and plays an important role in regulating cytoskeleton mediated cell deformation (Wang et al. 2016). However, there is still an incomplete understanding of Phactr1 and Rho/ROCK interact to regulate the osteogenic and adipogenic differentiation of BMSCs and thus involve in the occurrence and development of OP.

Therefore, this study aims to elucidate the cellular and molecular mechanisms through which Phactr1 affects bone mass, the effects of Phactr1 on osteogenic and adipogenic differentiation of BMSCs, and the potential role of RhoA/ROCK2 signaling in mediating these effects.

Materials and methods

Ovariectomized rat model

A total of 32 female Sprague–Dawley rats (3 months old) were purchased from the Experimental Animal Center of Sun Yat-Sen University. All rats were housed at 23 ± 1 °C under a 12-h light and dark cycle and free access to food and water. After adaptation to circumstances for a week, rats were numbered and randomly divided into ovariectomy group (24) and sham operated group (8). All rats were anesthetized with 2% pentobarbital sodium (30 mg/kg) through intraperitoneal injection and they underwent either posterior incision without ovariectomy (Sham) or bilateral ovariectomy (OVX) operation. Penicillin potassium (20,000 U/kg, Keda Animal Pharmaceutical, China) was injected intramuscularly for 3 days. Three months after operation, the rats in each group were sacrificed by cervical dislocation, lumbar spine bone mineral density (BMD) was measured by Micro-CT (Aloka Latheta LCT-200, Japan). The lumbar vertebra and adipose tissue in both groups were prepared for immunohistochemistry staining. This study was approved by the ICE for Clinical Research and Animal Trials of The First Affiliated Hospital of Sun Yat-sen University [no. (2019)196] and was performed in accordance with the guidelines for the care and use of laboratory animals.

Immunohistochemistry

Adipose tissue and decalcified sections of the lumbar vertebra were probed with primary antibodies against Phactr1 (cat. no. PA5-98056; Cell Signaling Technology, USA; 1:300) overnight at 4 °C. Next, the sections were then incubated with a goat anti-rabbit biotinylated secondary antibody (cat. no. 31232; Invitrogen, USA) at room temperature for 30 min, and they were reacted with DAB solution (cat. no. ab64238; Abcam, UK). An optical microscope (Olympus IX 71, Japan) was implemented for the staining result observation and photographing. Then, we analyzed the stained images with Image J software (NIH, USA) in every section for determining the staining intensity.

Isolation and culturing of BMSCs

BMSCs were isolated as described previously (Zhu et al. 2010). In details, the rats were sacrificed by cervical dislocation and sterilized in 70% (vol/vol) ethanol (cat. no. E111989; Aladdin, China). Later, the skin of the inguinal...
The region was incised, the muscles were disassociated and the femurs were cut off below the femoral head. Cleaned the muscles and tendons from tibiae and femurs, removed the epiphyses just below the end of the marrow cavity, BMSCs were harvested from the femoral and tibial medullary cavities by flushing with a 5 ml syringe and suspended in Dulbecco's modified Eagle medium F-12 (Gibco, USA) with 10% FBS (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA), and maintained with 5% CO₂ at 37 °C. Cells were trypsinized and passaged until they reached about 80–90%. BMSCs at early passages (passage numbers 2–4) were used for subsequent experiments. This study was approved by the ICE for Clinical Research and Animal Trials of The First Affiliated Hospital of Sun Yat-sen University [no. (2019)196] and was performed in accordance with the guidelines for the care and use of laboratory animals.

**Flow cytometry**

BMSCs were sorted by conducting CytoFLEX flow cytometry (Beckman Coulter, USA). In details, the obtained cells were then re-suspended in PBS (Gibco, USA) supplemented with 0.1% FBS (Gibco, USA) and 0.5% NaN₃ (Sigma, USA), centrifuged, and rinsed. After re-suspension, the cells were incubated with antibodies specific to CD29, CD34, CD45, and CD105 (Abcam, UK) at room temperature in conditions devoid of light for 30 min. The supernatant was discarded and the precipitate was rinsed in PBS. After that, the precipitate was centrifuged at 1000 rpm for 5 min and the supernatant was removed after centrifugation, repeated for three times. The harvested precipitate was re-suspended in 300 μl PBS. Before testing, the instrument was sterilized under 30 min ultraviolet irradiation, while the few tubes were sterilized by hypochlorous acid. Sorting parameters were set based on the forward scatter (FSC), side scatter (SSC), and size of the cells. The separator was installed where the cells were sorted. Cells with the largest volume and quantity and most intra-cellular granules were selected as target cells for further experimentation.

**Osteogenic differentiation**

When BMSCs reached 60–70% in the 6-well plate, replaced the medium with 2 ml osteogenic induction medium solution A (cat. No. RASMX-90021; Cyagen, USA). After 3 days of induction, sucked out solution A and added 2 ml of osteogenic differentiation medium solution B (cat. No. RASMX-90021; Cyagen, USA), 24 h later, replaced solution B with solution A. After the alternating of solution A and solution B for 3–5 times, continued to maintain the cells with solution B for 4–7 days until the lipid droplets become large and round enough. Finally, the cells were stained with Oil red O (Cyagen, USA) at room temperature for 30 min, followed by use of an optical microscope (Olympus IX 71, Japan) to assess the lipid droplets.

**Adipogenic differentiation**

When BMSCs reached 100% or over fusion in the 6-well plate, replaced the medium with 2 ml adipogenic induction medium solution A (cat. No. RASMX-90021; Cyagen, USA). After 3 days of induction, sucked out solution A and added 2 ml of adipogenic differentiation medium solution B (cat. No. RASMX-90021; Cyagen, USA), 24 h later, replaced solution B with solution A. After the alternating of solution A and solution B for 3–5 times, continued to maintain the cells with solution B for 4–7 days until the lipid droplets become large and round enough. Finally, the cells were stained with Oil red O (Cyagen, USA) at room temperature for 30 min, followed by use of an optical microscope (Olympus IX 71, Japan) to assess the lipid droplets.

**Lentivirus-Phactr1 transfection**

Phactr1 overexpression lentivirus were designed and procured from GeneChem Company (ShangHai, China). BMSCs treated with 1 ml grown medium contained 8 μg polybrene (cat. No. sc-134220; Santa Cruz, USA), and then added the Phactr1 lentivirus and negative control virus at a multiplicity of infection (MOI) of 100 according to the manufacturer’s protocol. After 48 h, the medium containing virus was replaced with fresh grown medium. On the third day, the virus with fluorescent label began to express the fluorescent signal observed by fluorescence microscope. The transected cells were selected and incubated in puro- mycin (cat. No. P8833; Sigma, USA) at 3 μg/ml for 12-24 h. The transfection efficiency was determined by western blot analysis of the control group and Lenti-Phactr1 group.

**siRNA-Phactr1 interference**

Phactr1 siRNA were designed and procured from RIBOBIO Company (GuangZhou, China). Firstly, mixed an selected amount of Phactr1 siRNA with 6 μl HiPerFect Transfection Reagent (cat. no. 301704; QIAGEN, Germany) gently at room temperature for 15–20 min. BMSCs treated with mixture and FBS-free growth medium. After 5–6 h, the medium containing 10% FBS was replaced for 48 h. The interference efficiency was determined by western blot analysis of the control group and siRNA-Phactr1 group.

**Western blot analysis**

Western blot analysis was performed using standard techniques. BMSCs were lysed in 50 μl of protein extraction reagent (M-PER) (cat. no. 78501; ThermoFisher, USA) for 15 min on the ice. The protein samples were harvested following centrifugation for 15 min (12,000×g, 4 °C) and then boiled for 5 min, total protein concentration was determined.
using a NanoDrop 2000 spectrophotometer (ThermoFisher, USA). Equal volumes (20 µl) of the samples were separated via 10% SDS-PAGE and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 1 h and then incubated overnight at 4 °C with primary antibodies, followed by incubation with HRP-linked secondary antibodies. The primary antibodies used for western blot were as follows: rabbit anti-Phactr1 (cat. no. 23446-1-AP; Cell Signaling Technology, USA; 1:1000), Mouse anti-Runx2 (cat. no. ab76956; Abcam, UK; 1:500), rabbit anti-C/EBPα (cat. no. ab140479; Abcam, UK; 1:500), rabbit anti-ROCK2 (cat. no. ab66320; Abcam, UK; 1:500), and anti-β-tublin (cat. no. 2128; Cell Signaling Technology, USA; 1:1000). All of the secondary antibodies (1:2000 dilution) were purchased from Cell Signaling Technology company. The immunoreactive proteins were visualized using a chemiluminescence kit (cat. no. WBKLS0500; Merck millipore, Germany), and the gray value of target band was quantified by Image J software (NIH, USA).

**Co-immunoprecipitation**

Generally, the proteins of BMSCs on the 7th day of osteogenic and adipogenic differentiation were extracted respectively, added specific antibody Phactr1 2 µg, gently incubated at 4 °C for 12 h on a shaking bed, add protein A/G agarose beads 40 µg, continue to incubate at 4 °C for 6 h, centrifuge at 2500 rpm for 15 min, remove the supernatant, wash with PBS buffer for 3 times (2500 rpm, 5 min each time), and finally lyse the agarose beads and immunoprecipitation complex with lysate, Collect the lysate, The rest steps (electrophoresis and transfer, immunoblotting and detection) are the same as Western blot analysis.

**Active RhoA detection**

Active RhoA detection was carried out using a kit from Cell Biolabs, Inc. (cat. no. STA-403-A-T; San Diego, USA). In brief, the cells were collected and washed with cold PBS, and lyzed in a lysis/bindin/wash buffer. The cell lysates were incubated with glutathione resin and GST-Rhotekin-RBD to allow GTP-bound GTPase binding to the glutathione resin through GST-linked binding protein. The bound proteins were eluted with SDS buffer containing DTT, then run on SDS-PAGE gel and analyzed by Western blotting to detect the presence of active RhoA using a rabbit anti-RhoA antibody. HRP-linked anti-rabbit IgG was used as the secondary antibody.

**Statistical analysis**

Statistical analyses were performed using SPSS 22.0 software. The data are presented as the means ± standard deviation (SD). Students t-test was performed to compare the differences between two groups. Differences between the quantitative values among multiple groups were analyzed with one-way analysis of variance (ANOVA) with the Tukey’s test. P < 0.05 was considered to indicate a statistically significant difference.

**Results**

**Phactr1 increased in both bone and adipose tissue of OP rats**

In order to clarify the expression of Phactr1 in bone and adipose tissue in different groups. The ovariectomized OP rats model was established successfully, and the lumbar bone mineral density (BMD, mg/cm³) of OP rats was significantly lower than control group (Fig. 1e, ****P<0.0001). The bone and adipose tissue of OP rats and control rats were preserved for immunohistochemical staining. The expression of Phactr1 in bone tissue of OP rats was higher than control group (Fig. 1a, b, f, *P<0.05). Meanwhile, the expression of Phactr1 in adipose tissue of OP rats was higher than control group either (Fig. 1c, d, f, ***P<0.001).

**Identification of BMSCs**

The isolated and cultured BMSCs showed a typical spindle-shaped cell morphology (Fig. 2a). Flow cytometry analysis confirmed that these cells were positive for mesenchymal cell markers CD29 (99.9%) and CD105 (95.63%), but negative for hematopoietic cell markers CD34 (1.15%) and CD45 (1.01%) (Fig. 2b), suggesting that the cells we obtained had similar morphological and immunophenotypical characteristics of BMSCs.

**Phactr1 inhibits osteogenic differentiation of BMSCs**

In vitro, after osteogenic differentiation, Western blot showed (Fig. 3a, *P<0.05, **P<0.01): The expression of Runx2, a related index of osteogenesis, gradually increased with the osteogenic induction time (0, 3, 7 days), while the expression of Phactr1 gradually decreased. Alizarin red S staining showed (Fig. 3b): with the increase of osteogenic induction time (0, 3, 7, 10, 14 days), calcium nodules numbers gradually increased and the staining became more red. At 14 days, a large number of calcium nodules were obviously formed and the staining was significantly deepened. Further, we found that the expression level of Phactr1 was increased by Phactr1 overexpression lentivirus transfection (Fig. 3c, ***P<0.001). Western blot showed (Fig. 3d, *P<0.05): overexpression of
Phactr1 inhibited the increase of Runx2 on the 7th day of osteogenic differentiation. ARS staining in con-7 days and Lenti-Phactr1-7 days groups showed (Fig. 3e): overexpression of Phactr1 made alizarin red staining became lighter on the 7th day of osteogenic differentiation.

**Phactr1 promotes adipogenic differentiation of BMSCs**

In vitro, after adipogenic differentiation, Western blot showed (Fig. 4a, *P < 0.05, **P < 0.01, ***P < 0.001): the expression of C/EBPα, a related index of adipogenesis,
gradually increased with the adipogenic induction time (0, 3, 7 days). Meanwhile, the expression of Phactr1 decreased first then gradually increased. Oil red O staining showed (Fig. 4b): with the increase of adipogenic induction time (0, 3, 7, 10, 14, 21 days), the formation of lipid droplets increased, and the staining deepened gradually. At 21 days, a large number of lipid droplets were formed in the cells, and the staining became more red. Further, we found that the expression level of Phactr1 was decreased by Phactr1 siRNA interference (Fig. 4c, *P < 0.05). Western blot showed (Fig. 4d, ****P < 0.0001): knockdown Phactr1 inhibited the increase of C/EBPα on the 7th day of adipogenic differentiation. Oil red O staining in con-7days and Lenti-Phactr1-7days groups showed (Fig. 4e): knockdown Phactr1 made it no much difference on the 7th day of adipogenic differentiation.

**Proteins bond with Phactr1 On the 7th day of osteogenic/adipogenic differentiation and their KEGG PATHWAY analysis**

To explore the underlying mechanism of Phactr1 in regulating the osteogenic and adipogenic differentiation of BMSCs. Co-immunoprecipitation mass spectrometry
Co-IP-MS showed: (Fig. 5a) on the 7th day of osteogenic differentiation, 145 proteins bond to Phactr1, while on the 7th day of adipogenic differentiation, 91 proteins bond to Phactr1, 19 proteins bond to Phactr1 in both osteogenic and adipogenic differentiation. The specific bond proteins are shown in (Table 1). Among them, we found Phactr1 can bond to ROCK2 in osteogenic differentiation, but not in adipogenic differentiation. The enrichment signal pathways related to these proteins were showed in (Fig. 5b–d).

Phactr1 inhibits osteogenic differentiation and promotes adipogenic differentiation of BMSCs through RhoA/ROCK2 signaling pathway

In this study, Co-IP showed Phactr1 and ROCK2 were combined in osteogenic differentiation, but they were not combined in adipogenic differentiation (Fig. 6a). Western blot showed during osteogenic differentiation, active RhoA and ROCK2 increased significantly on the 7th day compared
Fig. 5  Venn diagram and KEGG PATHWAY analysis of the proteins bond with Phactrl On the 7th day of osteogenic and adipogenic differentiation in BMSCs. a Co-IP-MS was used to analyse the number and name of proteins bond with Phactrl On the 7th day of osteogenic, adipogenic differentiation and in both groups. b KEGG PATHWAY analysis of the proteins bond with Phactrl On the 7th day of osteogenic and adipogenic differentiation. c KEGG PATHWAY analysis of the proteins bond with Phactrl On the 7th day of adipogenic differentiation. d KEGG PATHWAY analysis of the Proteins bond with Phactrl On the 7th day of osteogenic differentiation. Co-IP-MS co-immuno-precipitation mass spectrometry, AD adipogenic differentiation, OD osteogenic differentiation, KEGG Kyoto Encyclopedia of Genes and Genomes
with 0th day. Otherwise on the 7th day of adipogenic differentiation, ROCK2 did not change significantly, and active RhoA decreased (Fig. 6b, c, *P < 0.05). After inhibition of ROCK2 by using KD025, the decrease of Phactr1 was inhibited, and the increase of Runx2 was inhibited in osteogenic differentiation (Fig. 6d, f, g, *P < 0.05 or **P < 0.01). ARS staining in con-7 days and KD025-7 days groups showed (Fig. 6i): inhibition of ROCK2 made alizarin red staining become lighter on the 7th day of osteogenic differentiation. Meanwhile, when ROCK2 was inhibited, Phactr1, C/EBPα were significantly increased in adipogenic differentiation (Fig. 6e, f, h *P < 0.05 or **P < 0.01). Oil red O staining in con-7 days and KD025-7 days groups showed (Fig. 6j): inhibition of ROCK2 formed much lipid droplets in BMSCs and staining became more red on the 7th day of adipogenic differentiation.

### Discussion

Previous studies have shown that in postmenopausal women, OP was likely to occur in obese people, and easily caused some fat metabolism diseases such as fatty liver and hyperlipidemia (Cui et al. 2005). In addition, diseases such as osteopenia and osteoarthritis always combined with abnormal fat metabolism (Oliveira et al. 2020). In diseases such as OP and senile bone loss, the reduction of bone mass is often accompanied by obvious accumulation of adipose tissue (Justesen et al. 2001). On the contrary, in some diseases characterized by high bone mass, the main mechanism is to promote osteogenic differentiation and inhibit adipogenic differentiation of BMSCs (Qiu et al. 2007). In this steady, immunohistochemical results showed Phactr1 was highly expressed in bone and adipose tissue of OP rats, indicating that Phactr1 played a certain role in regulating bone mass, and may participated in the occurrence and development of OP. Osteoblasts and adipocytes were originated from BMSCs, the differentiation between them considered to be mutually restricted (Titorencu et al. 2007). However, the imbalance between osteogenic and adipogenic differentiation of BMSCs is involved in the occurrence and development of OP. Further, in order to explore the role of Phactr1 in regulating osteogenic and adipogenic differentiation, we successfully isolated BMSCs with high purity by whole bone marrow adhesion method (Zhu et al. 2010). In osteogenic differentiation, Phactr1 kept decreasing, while in adipogenic differentiation, Phactr1 decreased first and then increased. The results may due to the common start process in the early stage of directional differentiation of BMSCs, in details, In the early stage, the cytoskeleton rearranged violently, and the expression of Phactr1 decreased, However, in the later stage, the difference expression of Phactr1 influenced the degree of cytoskeleton rearrangement. These results suggest that Phactr1 may involved in regulating the balance of osteogenic and adipogenic differentiation of BMSCs, and then cause the change of bone mass. Over-expression of Phactr1 inhibits the increase of Runx2 and alizarin red staining became lighter, indicating that Phactr1 inhibits osteogenic differentiation. In adipogenic differentiation, after interference with siRNA-Phactr1, the increase of C/EBPα was inhibited. However, there is no much difference in Oil red O staining, it may be that lipid droplets were formed in the later stage, indicating that Phactr1 promotes adipogenic differentiation. Based on our finds, we believe

### Table 1

| Names       | Total | Elements |
|-------------|-------|----------|
| OD-7d       | 145   | 91 R13P3 DNML1 CO1A1 GMDS HNRPU TMM43 HECW2 RS23 PREP ARMC9 DJB14 MYZAP RL35 RALA RL10 CF217 |
| AD-7d       | 91    | R13P3 DNML1 CO1A1 GMDS HNRPU TMM43 HECW2 RS23 PREP ARMC9 DJB14 MYZAP RL35 RALA RL10 CF217 |

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< Springer
A

IB:Phactr1

IB:ROCK2

OD

Impact: ROCK2

Input Igg IP:ROCK2

B

OD

AD

Activate RhoA

(22 kDa)

Tubulin

(55 kDa)

C

OD

AD

(161 kDa)

Tubulin

(55 kDa)

D

Con-0d

Con-7d

KD025-7d

Phactr1

(66 kDa)

Runx2

(57 kDa)

E

Con-0d

Con-7d

KD025-7d

Phactr1

(66 kDa)

C/EBPα

(43 kDa)

F

G

H

I

Con-7d (OD)

KD025-7d (AD)

J

Con-7d (OD)

KD025-7d (OD)
Phactr1 inhibits osteogenic differentiation and promotes adipogenic differentiation of BMSCs through RhoA/ROCK2 signaling pathway. a Co-Immunoprecipitation confirmed Phactr1 bond to ROCK2 on the 7th day of osteogenic differentiation, but not bond to ROCK2 on the 7th day of adipogenic differentiation. b, c Western blot detected the expressions of active RhoA and ROCK2 in Con-7d and KD025-7d groups. *P < 0.05 vs. 0 days (n = 3). d, f, g Western blot detected the expressions of Phactr1, Runx2 in Con-0d, Con-7d and KD025-7d groups. *P < 0.05 or **P < 0.01 vs. Con-7d (n = 3). e, f, h Western blot detected the expressions of Phactr1, C/EBPα in Con-0d, Con-7d and KD025-7d groups. *P < 0.05 or **P < 0.01 vs. Con-7d (n = 3). i Comparison of ARS effect between con-7 days and KD025-7 days groups after Osteogenic differentiation (× 40). j Comparison of oil red 0 staining effect between con-7 days and KD025-7 days groups after Adipogenic differentiation (40x). RhoA Ras homolog gene family member A, ROCK2 Ras homolog gene family member A, ROCK2.

In order to explore the mechanism of Phactr1 in regulating the osteogenic and adipogenic differentiation of BMSCs. Co-IP-MS showed that in adipogenic differentiation, it can bond to MYH9, MYL9 and RAC2. While in osteogenic differentiation, it can bond to MYH10 and ROCK2, which can regulated the function of cytoskeleton. In addition, we found that Phactr1 bond to ROCK2 on the 7th day of osteogenic differentiation, but not in adipogenic differentiation, the results was confirmed by Co-IP. Preliminary analysis, we believe that ROCK2 may involved in the regulation of osteogenic and adipogenic differentiation of BMSC by Phactr1. Relevant studies have shown that (Arnsdorf et al. 2009), ROCK2 is activated under mechanical stress stimulation, maintains cytoskeleton tension by regulating actin, inhibits adipogenic and promotes osteogenic differentiation. RhoA, as an upstream molecule of ROCK2, also plays an important role in regulating cytoskeleton function (Lu et al. 2020). RhoA/ROCK signaling pathway is involved in the formation of cytoskeleton and can promote osteogenic differentiation by changing different contact areas between cells (Seo et al. 2014). In osteoclasts, studies have shown that the role of Gα13 is to inhibit the signal pathway and related gene expression mediated by c-Src, Pyk2 and RhoA/ROCK2, thereby controlling osteoclast fusion, adhesion, and actin cytoskeleton remodeling And absorption, inhibit bone resorption, and prevent bone loss (Nakano et al. 2019). Based on the above results, RhoA/ROCK2 and Phactr1 may act together on the cytoskeleton to regulate the osteogenic and adipogenic differentiation of BMSCs. This effect and related mechanism confirmed by subsequent research.

This study found that during the osteogenic and adipogenic differentiation of BMSCs, RhoA/ROCK2 signaling pathway was activated, and the expression of active RhoA and ROCK2 increased significantly on the 7th day of osteogenic differentiation. However, RhoA/ROCK2 signaling pathway was suppressed and the expression of active RhoA decreased and ROCK2 was not significantly changed on the 7th day of adipogenic differentiation. It was further confirmed that RhoA/ROCK2 was involved in the regulation of osteogenic and adipogenic differentiation of BMSCs. However, the specific interaction mechanism between the Phactr1 and ROCK2 is not very clear. Therefore, after inhibition of ROCK2, the decrease of Phactr1 during osteogenic differentiation was inhibited, and the indirect increase of Phactr1 resulted in the inhibition of Runx2 expression and alizarin red staining became lighter on the 7th day of osteogenic differentiation. After inhibition of ROCK2, the expression of Phactr1 was up-regulated and C/EBPα was increased, Oil red O staining became more red on the 7th day of adipogenic differentiation. The specific mechanism of Phactr1 in regulating osteogenic and adipogenic differentiation may lied in mediating the deformation of cytoskeleton. Generally, on the one hand, the decrease of Phactr1 promoted the expression of RhoA/ROCK2, triggered the assembly of actin, promoted the conversion from G-actin to F-actin in polymer state, increased the polymerization level of actin and increased the area of cytoskeleton, which is conducive...
to the osteogenic differentiation and inhibited adipogenic differentiation of BMSCs. Our study is consistent with the conclusions of Tong (2020). The increased of cytoskeleton F-actin promoted osteogenic differentiation. On the other hand, the combination of ROCK2 and Phactr1 reduced the free Phactr1. However, the combination of ROCK2 and Phactr1 may promoted the degradation of Phactr1, thereby reduced the combination of Phactr1 and actin, caused actin rearrangement, increased the polymerization level of actin, promoted the osteogenic differentiation and inhibited the adipogenic differentiation of BMSCs.

Conclusions

Collectively, our key findings suggested that Phactr1 negatively regulates bone mass by inhibiting osteogenesis and promoting adipogenesis of BMSCs by activating the RhoA/ROCK2 axis. More importantly, although our provisional results display the positive influence of Phactr1 on BMSCs, thorough investigations in the future are still required to reveal the potential implications of these findings in a clinical setting.

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Author contributions

Substantial contributions to conception and design: BC; performed the experiments: WL, ZC, XM, SZ and ZW; data analysis and interpretation and wrote the manuscript: WL, WC, DF; drafting the article or critically revising it for important intellectual content: BC; final approval of the version to be published: all authors.

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Declarations

Conflict of interest

All authors have no conflicts of interest.

Ethical approval

The present study was approved by the Animal Care Committee of Sun Yat-Sen University [No. (2019)196] and was performed in accordance with the guidelines for the use of laboratory animals.

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