Phactr1 Negatively Regulates Bone Mass By Inhibiting Osteogenesis And Promoting Adipogenesis of BMSCs Via RhoA/ROCK2

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Abstract

Background: 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 regulatory factor 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.

Results: 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 change during adipogenic differentiation, knockdown Phactr1 inhibits the increase of C/EBP α . Phactr1 and ROCK2 were combined in osteogenic differentiation, but not in adipogenic differentiation. By using KD025, the decrease of Phactr1 and the increase of Runx2 were inhibited respectively in osteogenic differentiation. Meanwhile, when ROCK2 was inhibited, Phactr1, C/EBP α were significantly increased in adipogenic differentiation.

Conclusions: These findings indicated that Phactr1 negatively regulates bone mass by inhibiting osteogenesis and promoting adipogenesis of BMSCs by activating RhoA/ROCK2.

Background

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 [1]. With the advent of the aging society, the incidence of osteoporosis 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% [2]. 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 [3]. Therefore, the research on exploring and developing effective and safe 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 [4]. Once the physiological process of bone and fat formation is disrupted, it will cause some abnormal metabolism related diseases, such as Osteoporosis and Obesity [5]. 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 [6]. BMSCs are adult stem cells with self-renewal, replication and multidirectional differentiation potential, which can differentiate into osteoblasts and adipocytes under certain conditions, the differentiation between them is mutually restrictive [7].

The osteogenic and adipogenic differentiation of BMSCs is regulated by many factors. More and more evidence showed that cytoskeletal proteins play a key role in regulating the osteogenic and adipogenic

differentiation of BMSCs [8]. The results of recent literature showed that 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 [9]. Using Protein Data Bank (PDB) analysis, we found that phosphatase and actin regulatory factor 1 (Phactr1) directly combined with G-Actin, and Nadège [10] found that knocking down Phactr1 in breast cancer cells caused serious rearrangement and increased polymerization of cytoskeleton. It is suggested that phactr1 regulate the cytoskeleton, thereby regulating osteogenesis and adipogenesis of BMSCs, but this effect and specific mechanism need to be further verified and clarified.

In 2004, Patrick [11] systematically and comprehensively reported Phactr1 for the first time. It can bind with actin and regulated the reorganization of actin cytoskeleton. As a typical member of Phactr family, Phactr1 is involved in the occurrence and development of many diseases. Hidenori [12] found that Phactr1 is widely expressed in the central nervous system, regulating various neuronal processes by modulating protein phosphorylation and actin cytoskeleton, and participating in the development of mouse brain. Zhang [13] Showed that Silencing Phactr1 can reduce the nuclear accumulation of p65 and NF- κ B through interaction with MRTF-A, thereby reducing the oxidative stress and inflammatory response of coronary artery endothelial cells. Kiando [14] showed that fibromuscular dysplasia is related to Phactr1 gene defect. However, whether Phactr1 is involved in bone and fat metabolism, and whether it is involved in regulating the osteogenic and adipogenic differentiation of BMSCs has not yet been reported.

The mechanism of osteogenesis and adipogenesis in BMSCs involves many signaling pathways, such as Wnt and TGF- β /BMPs, notch, hippo and hedgehogs, etc [15]. Among them, Rho/ROCK signaling pathway plays an key role in determining the differentiation fate of BMSCs [16]. Rho/ROCK inhibits PPAR γ expression by delaying activation of the typical wnt signaling pathway based on Wnt10b expression and β -catenin induction [17]. At the same time, some studies have shown that 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 [18]. Therefore, we believe that there may be an interaction between Rho/ROCK and Phactr1, which jointly participate in the regulation of osteogenic and adipogenic differentiation of BMSCs, but the specific role and mechanism are not clear and need to be further confirmed and explored.

Based on the abovementioned findings, in the present study, it was hypothesized that Phactr1 Regulates Bone Mass by modulating Osteogenesis and Adipogenesis of BMSCs through RhoA/ROCK2 signal pathway. Therefore, in order to further 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 were investigated.

Methods

Construction of ovariectomized osteoporosis 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 numbered and randomly divided into ovariectomy group(24) and sham operation group(8). The ovariectomy group were performed bilateral oophorectomy, The sham operation group preserved the complete structure and function of ovary and fallopian tube, and excised the same volume of adipose tissue around the ovary. Penicillin 20000 U/kg was injected intramuscularly for 3 days. Three months after operation, the rats in each group were sacrificed by cervical dislocation in accordance with the guidelines of ARRIVE, lumbar spine bone mineral density (BMD) was measured by Micro-CT. The lumbar bone tissue and adipose tissue stored in liquid nitrogen for further experimentation. 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.

Immunohistochemistry

The decalcification of bone tissue and adipose tissue was used for Immunohistochemistry. First, Copy: paraffin section, 37 °C oven overnight, Bake in 60 °C oven for 2h before dewaxing. Dewaxing and hydration: put the slices into xylene I and II for 10min respectively, Ethanol gradient (100%, 95%, 80%, 70%) for 5min, Wash with water for 5min, TBS 3min×3, then filter paper to dry around the tissue and nail polish. Antigen repair: hyaluronidase+pepsin repair, hyaluronidase 37 °C, 30min, TBS 3min×3, Pepsin 37 °C, 30min, TBS 3min×3. Sealing: 5% BSA sealing solution at 37 °C for 30min, dry without washing. Incubate the primary antibody: dilute the primary antibody with TBS or PBS and wet box at 4 °C overnight. The next day, incubate the secondary antibody: TBS for 5min×5. Add 3% H₂O₂ dropwise on the slices, keep away from light at room temperature for 10min, add polymerized HRP labeled secondary antibody dropwise, 37 °C, 30min, TBS for 5min×5. Counterstaining: counterstaining with hematoxylin, blue returning with saturated sodium monohydrogen phosphate solution. Seal: dehydration: 70%, 80%, 95% and 100% ethanol for 3min respectively. Transparent: xylene I and II for 5min respectively. Neutral gum seal. Photographing: low magnification first and then high magnification, determine the exposure and contrast satisfactory for dyeing, make a unified ruler, and take photos from different visual fields for preservation.

Isolation and culturing of BMSCs

A total of 10 female Sprague-Dawley rats (4-6 weeks old; 100-120 g) were purchased from the Experimental Animal Center of Sun Yat-Sen University for use in the present study. The rats were allowed to adapt to the housing conditions for 7 days, during which the rats were maintained under a 12-h light/dark cycle at 22°C with free access to food and water (23,24). Afterward, the animals were sacrificed by cervical dislocation in accordance with the guidelines of ARRIVE, 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 (DMEM/F12) growth medium (GM) with 10% FBS(Gibco, cat.no.10099141C,Australia). The cells were cultured in a humidified incubator at 37°C under an atmosphere with 5% CO₂ and the third generation of BMSCs were used in the experiments. 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.

Flow cytometry

BMSCs were isolated from the bone marrow, and sorted by conducting CytoFLEX flow cytometry (Beckman Coulter, USA). In details, the obtained cells were then re-suspended in PBS supplemented with 0.1% FBS and 0.5% NaN₃, 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. The centrifugation was 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 flow 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 fusion reached 60%-70%, add 2 ml osteogenic induction medium (Glutamine, ascorbic acid β-Sodium glycerophosphate, dexamethasone, [Cyagen, USA](#)) into the culture dish. Fresh osteogenic induction medium is used every 3 days. After 2-4 weeks of induction, calcium nodules were stained with Alizarin Red S dye.

Adipogenic differentiation

When BMSCs fusion reached 100% or over fusion, add 2 ml of adipogenic induction medium solution A (Glutamine, insulin, 3-isobutyl-1-methylxanthine, rosiglitazone, dexamethasone, [Cyagen, USA](#)) to the culture dish. After 3 days of induction, suck out solution A and add 2 ml of adipogenic differentiation medium solution B (insulin, [Cyagen, USA](#)), after 24h, suck out liquid B and replace it with liquid A for induction. After the alternating of solution A and solution B for 3-5 times (12-20 days), continue to maintain the cells with solution B for 4-7 days until the lipid droplets become large and round enough.

Lentivirus-Phactr1 transfection

Phactr1 lentivirus were procured from Shanghai GeneChem Co. 1ml transfection enhancer Polybrene fresh culture medium (8 µg/ml) was added to the well plate, and then the diluted and blown Phactr1 lentivirus and positive control virus suspension were 100 µl respectively add to the well plate with gently mixing. After 48 hours, the medium containing virus was replaced with fresh medium (no Polybrene). On the third day, the virus with fluorescent label began to express the fluorescent signal observed by fluorescence microscope. On the third day after BMSCs transfection, G418 culture with screening

concentration was added for one week, the remaining cell population was digested with 0.25% enzyme and divided into two groups. One was screened and cultured in normal whole medium, Another cell was subcultured. The transfection efficiency was determined by western blot analysis of the control group and Lenti-Phactr1 group.

siRNA-Phactr1 Interference

siRNA Phactr1 was procured from Shanghai GeneChem Co. The day before interference, 3×10^5 cells/cm², 2.5ml growth medium without antibiotics was added to each well. Remove the growth medium from the cells, Add 2.5 ml of fresh serum-free growth medium. Sterile ddH₂O configuration concentration is 20 μ mol siRNA mother liquor, sub packed, frozen at -20°C for standby. Mix an selected amount of interference chain with transfection reagent, gently blowed at room temperature for 15-20 minutes. Add the mixture to the cell culture dish containing serum-free growth medium and shake it gently. After 5-6 hours, The medium containing serum was added for 48 hours. 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. Following 0, 3, 7 days of osteogenic differentiation in control, Lenti-Phactr1 and KD025 groups, Following 0, 3, 7 days of adipogenic differentiation in control, siRNA-Phactr1 and KD025 groups, BMSCs were lysed in 50 μ l of protein extraction reagent (M-PER) (BestBio) and protease inhibitor cocktail. The protein samples were harvested following centrifugation for 15 min (12,000 $\times$ g, 4°C) and boiled for 5 min, total protein concentration was determined using a NanoDrop 2000 spectrophotometer. Equal volumes (20 μ l) of the samples were separated via 10% SDS-PAGE and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk and then incubated overnight at 4°C with primary antibodies specific for rabbit anti-Phosphatase and Actin Regulator 1 (Phactr1, 1:1000, cat. no. 23446-1-AP, Cell Signaling Technology, Inc.), Mouse anti-Runx2-related transcription factor 2 (Runx2, 1:500, ab76956), rabbit anti-CCAAT/Enhancer-Binding Protein (C/EBP α , 1:500, ab140479), rabbit anti-Rho-associated coiled-coil-containing protein kinase 2 (ROCK2, 1:500, ab66320), and anti- β -tubulin (1:1000, cat. no. 2128, Cell Signaling Technology, Inc.). The samples were rinsed with tris-buffered saline tween 20 (TBST) three times (10 min each time). After that incubation with a alkaline phosphatase-conjugated goat anti-IgG (1:2,000, cat. no. 7054; Cell Signaling Technology, Inc.) for 1 h at room temperature. The immunoreactive proteins were visualized using a chemiluminescence kit (EMD Millipore), and The gray value of target band was quantified by ImageJ software.

Alizarin red staining

After osteogenic differentiation, use 1 $\times$ PBS flushing 1-2 times. Add 2 ml of 4% neutral formaldehyde solution to each well and fix for 30 min. Absorb neutral formaldehyde solution and use 1 $\times$ PBS flushing twice. Add 1 ml alizarin red dye solution to each well for 3-5 min. Absorb alizarin red dye and use 1 $\times$ PBS flushing 2-3 times. The culture plate was placed under the microscope to observe the calcium nodules.

Oil red O staining

After adipogenic differentiation, used 1×PBS flushing 1-2 times. Add 2 ml of 4% neutral formaldehyde solution to each well and fix for 30 min. Absorb neutral formaldehyde solution and use 1×PBS flushing twice. Add 1 ml of oil red O dye working solution into each well for 30min (working solution preparation method: oil red O storage solution: distilled water = 3:2, mix well and filter with neutral filter paper). Absorb the oil red O dye solution and use 1×PBS flushing 2-3 times. The culture plate was placed under the microscope to observe the effect of lipid droplets staining.

Co-Immunoprecipitation

Generally, The proteins of BMSCs on the 7th day of osteogenic and adipogenic differentiation were extracted respectively, add specific antibody 2 µg, gently incubate at 4 °C for 12h on a shaking bed, add protein A/G agarose beads 40 µg, continue to incubate at 4 °C for 6h, centrifuge at 2500 rpm for 15min, remove the supernatant, wash with PBS buffer for 3 times (2500 rpm, 5min 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. (San Diego, USA). In brief, the cells were collected and washed with cold PBS, and lysed in a Lysis/Binding/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 (SPSS, Inc.). The data are presented as the means ± standard deviation. 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

The expression of Phactr1 increased in both bone and adipose tissue of OP rats

In order to clarify the expression of the target gene Phactr1 in bone and adipose tissue, The ovariectomized osteoporosis rat model was successfully established, and the bone mineral density (BMD, mg/cm³) was significantly lower than control group (Fig.1e, **** $P < 0.0001$). The bone and adipose

tissue of osteoporosis rats and control rats were collected for immunohistochemical staining, The expression of Phactr1 in bone tissue of osteoporotic rats was higher than control group (Fig.1a,b,f, * $P \leq 0.05$). Meanwhile, The expression of Phactr1 in adipose tissue of osteoporosis rats was higher than control group (Fig.1c,d,f, *** $P \leq 0.001$).

Identification of BMSCs

The BMSCs showed 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 cultured cells had similar morphological and immunophenotypical characteristics of BMSCs.

Phactr1 inhibits osteogenic differentiation of BMSCs

In vitro, after osteogenic differentiation, Western blot showed (Fig.3a, * $P \leq 0.05$, ** $P \leq 0.01$): The expression of Runx2, a related index of osteogenesis, gradually increased with the induction time 0, 3, 7 days, while the expression of phactr1 gradually decreased. Alizarin red staining showed (Fig.3b): With the increase of induction time 0, 3, 7, 10, 14 days, calcium nodules gradually increased and the staining deepened. 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, *** $P \leq 0.001$). Western blot showed (Fig.3d, * $P \leq 0.05$): overexpression of Phactr1 inhibited the increase of Runx2 On the 7th day of osteogenic differentiation.

Phactr1 promotes adipogenic differentiation of BMSCs

In vitro, after adipogenic differentiation. Western blot showed (Fig.4a, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$): The expression of C/EBP α , a related index of adipogenic, gradually increased with the induction time 0, 3, 7 days, Meanwhile, the expression of phactr1 gradually increased. Oil red O staining showed (Fig.4b): with the increase of 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 deepened significantly. Further, We found that the expression level of Phactr1 was decreased by Phactr1 siRNA transfection (Fig. 4c, * $P \leq 0.05$). Western blot showed (Fig.4d, **** $P \leq 0.0001$): knockdown of Phactr1 inhibited the increase of C/EBP α On the 7th day of adipogenic differentiation.

Proteins bond with Phactr1 On the 7th day of Osteogenic/Adipogenic differentiation and KEGG PATHWAY analysis

The 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 genes may bind to phactr1, while on the 7th day of adipogenic differentiation, 91 genes may bind to phactr1, 19 genes that bind to phactr1 in osteogenic and adipogenic differentiation, The specific binding proteins are shown in (Table 1). Among them, we found Phactr1 can bind to ROCK2

in osteogenic differentiation, but not in adipogenic differentiation. The enrichment signal pathways related to these proteins are shown in (Fig.5b,c,d).

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

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 of osteogenesis induction compared with that on the 0th day. Otherwise on the 7th day of adipogenic differentiation, ROCK2 did not changed significantly, and active RhoA decreased (Fig. 6b, c, $*P<0.05$). After inhibition of ROCK2 (KD025), The decrease of Phactr1 was inhibited, and the increase of Runx2 was inhibited in osteogenic differentiation. Meanwhile, when ROCK2 was inhibited, Phactr1, C/EBP α were significantly increased in adipogenic differentiation (Fig. 6d-h, $*P<0.05$ or $**P<0.01$).

Discussion

The 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. Previous studies have shown that in postmenopausal women, OP is likely to occur in obese people, and OP is also likely to cause fat metabolism diseases such as fatty liver and hyperlipidemia [19]. In addition, diseases such as osteopenia and osteoarthritis also belong to bone metabolism diseases, often combined with abnormal fat metabolism [20]. In diseases such as osteoporosis and senile bone loss, the reduction of bone mass is often accompanied by obvious accumulation of adipose tissue [4]. 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 [21]. The above studies fully show that there is a close relationship between fat and bone metabolism. Osteoblasts and adipocytes originate from BMSCs, the differentiation between them is considered to be a mutually restrictive [7]. However, The imbalance between osteogenic and adipogenic differentiation of BMSCs is involved in the occurrence and development of osteoporosis . Further, in order to verify the role of Phactr1 in regulating osteogenic and adipogenic differentiation, we successfully isolated BMSCs by whole bone marrow adhesion method. In the process of osteogenic differentiation, the expression of Phactr1 decreased, while in the process of adipogenic differentiation, the expression of Phactr1 decreased first and then increased. The decrease of Phactr1 in the early stage of osteogenic and adipogenic differentiation may due to the common start process in the early stage of directional differentiation of BMSCs, In the early stage of osteogenesis and adipogenesis, the cytoskeleton rearranged violently, and the expression of Phactr1 decreased. In the later stage of osteogenesis and adipogenesis, the expression of Phactr1 were different. 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. Overexpression of Phactr1 during osteogenic differentiation inhibits the increase of Runx2, indicating that Phactr1 inhibits osteogenic differentiation. In the process of adipogenic

differentiation, after interference with siRNA-Phactr1, the increase of C/EBP α was inhibited, indicating that Phactr1 promotes adipogenic differentiation. Based on our finds, we believe that Phactr1 is involved in osteogenic and adipogenic differentiation of BMSCs, and its specific role lies in inhibiting osteogenic differentiation and promoting adipogenic differentiation.

In recent years, more and more studies have shown that cytoskeleton plays a key role in the directional differentiation of BMSCs into different lineages [8]. Cytoskeleton refers to the protein fiber network structure in eukaryotic cells, including microtubules, microfilaments and intermediate fibers, which plays an important role in maintaining cell homeostasis and normal physiological function [22]. As an important molecule regulating cytoskeleton proteins, Phactr1 plays a key role in maintaining cytoskeleton stability and rearrangement. Therefore, we believe that the role of Phactr1 in promoting adipogenic differentiation and inhibiting osteogenic differentiation may be realized by regulating the function of cytoskeleton. Actin in cytoskeleton microfilaments is the medium of internal movement of cytoskeleton and participates in the morphological change process of cytoskeleton [23]. Actin is mainly divided into three subtypes, namely α , β and γ , α -Actin is mainly distributed in muscle cells and plays a role in contraction, and β -Actin and γ -Actin mainly exists in non-muscle cells and participates in the internal movement changes of cytoskeleton [24]. Actin mainly exists in two forms: monomeric form of globular actin (G-actin) and polymer form of fibrillary actin (F-actin), which can be transformed into each other under certain physiological conditions. The process of actin transformation from G-actin to F-actin is called actin assembly, Microfilaments gradually form polymers from monomers, and the degree of polymerization increases, which leads to changes in the size of the cytoskeleton and an increase in the area of the cytoskeleton, In this situation, it can provide favorable conditions for the osteogenic differentiation and inhibit the adipogenic differentiation of BMSCs [25,26]. Phactr1 binds to actin and regulates the reorganization of cytoskeleton. In the osteogenic differentiation of BMSCs, the decrease of Phactr1 causes serious rearrangement of actin, promotes the conversion of actin from G-actin to F-actin, increases the polymerization of cytoskeleton, and promotes Osteogenic differentiation and inhibition of adipogenic differentiation. On the contrary, in the adipogenic differentiation of BMSCs, the expression of Phactr1 increased, which inhibited the rearrangement of actin, reduced the conversion of G-actin to F-actin, disordered cytoskeleton arrangement, promoted adipogenic differentiation and inhibited osteogenic differentiation.

In order to explore the mechanism of Phactr1 in regulating the Osteogenic and adipogenic differentiation of BMSCs. Co-IP-MS showed that on the 7th day of osteogenic differentiation, 145 proteins bind to Phactr1, while on the 7th day of adipogenic differentiation, 91 proteins bind to Phactr1, 19 proteins that bind to Phactr1 in osteogenic and adipogenic differentiation. In details, in adipogenic differentiation, it can bind to MYH9, MYL9 and RAC2. While in osteogenic differentiation, it can bind to MYH10 and ROCK2, which can regulate the function of cytoskeleton. This result shows that Phactr1 is indeed involved in the regulation of cytoskeleton, and then regulate the osteogenic and adipogenic differentiation of BMSCs. In addition, we found that Phactr1 binds 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. The serine/threonine kinase family includes ROCK1 and ROCK2 [27], and ROCK2 is mainly concentrated in the cytoplasm and also located on the plasma membrane through the C-terminal region, which is related to vimentin and actin tension fibers [28,29]. ROCK2 participates in a variety of physiological activities of cells and plays an important physiological role in regulating cell movement, morphology, polarity, division and gene expression by regulating actin [30, 31]. Relevant studies have shown that [32], 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 [33]. RhoA/ROCK signaling pathway is involved in the formation of cytoskeleton and can promote osteogenic differentiation by changing different contact areas between cells [34]. Shi [35] Found that the destruction of ROCK1 and ROCK2 genes in cardiomyocytes promoted autophagy and reduced myocardial fibrosis during aging. 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 [36]. Based on the above results, ROCK2 and Phactr1 may act together on the cytoskeleton to regulate the osteogenic and adipogenic differentiation of BMSCs. This effect and related mechanism need to be further verified.

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, while the expression of active RhoA decreased and ROCK2 was not significantly changed on the 7th day of adipogenic differentiation. It further confirmed that RhoA/ROCK2 was involved in the regulation of osteogenic and adipogenic differentiation of BMSCs by Phactr1. Co-IP results showed that ROCK2 binds to phactr1 on the 7th day of osteogenic differentiation, but they did not bind on the 7th day of adipogenic differentiation, indicating that ROCK2 could promote osteogenic differentiation and inhibit adipogenic differentiation by binding with Phactr1. However, the specific interaction mechanism between the Phactr1 and ROCK2 is not very clear. Therefore, after KD025 was used to inhibit ROCK2, the decrease of Phactr1 during osteogenic differentiation was inhibited, and the indirect increase of Phactr1 resulted in the inhibition of Runx2 expression and osteogenic differentiation. After inhibition of ROCK2, the expression of Phactr1 was up-regulated and C/EBPα was increased either during adipogenic differentiation, indicating that the expression of Phactr1 may be regulated by RhoA/ROCK2, and involved in regulating osteogenic and adipogenic differentiation of BMSCs. The specific mechanism may lie in regulating osteogenic and adipogenic differentiation by mediating the deformation of cytoskeleton. Generally, on the one hand, the increase of RhoA will promote the expression of ROCK2, trigger the assembly of actin, promote the conversion from G-actin to F-actin in polymer state, increase the polymerization level of actin and increase the area of cytoskeleton, which is conducive to the osteogenic differentiation and inhibit adipogenic differentiation of BMSCs. Our study is consistent with the conclusions of Tong [37], The increase of cytoskeleton F-actin promotes osteogenic differentiation. On the other hand, the combination of ROCK2 and Phactr1 reduces the free Phactr1. At the same time, the combination of ROCK2 and Phactr1 may promote the degradation of Phactr1, thereby

reducing the combination of Phactr1 and actin, causing actin rearrangement, increasing the polymerization level of actin, promoting the osteogenic differentiation and inhibiting the adipogenic differentiation of BMSCs. The combination of Phactr1 and ROCK2 can promote osteogenic differentiation and inhibit 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 Phcatr1 on BMSCs, thorough investigations in the future are still required to reveal the potential implications of these findings in a clinical setting.

Declarations

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Authors' 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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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

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.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

The expression of Phactr1 in bone and adipose tissue in OP and control rats. a,b Immunohistochemical staining detected The expression of Phactr1 in the bone tissue of OP (b) and Control group(a). c,d Immunohistochemical staining detected The expression of Phactr1 in the adipose tissue of OP (d) and Control group (c). e The difference Lumbar BMD of OP and control rats. **** $P < 0.0001$ vs. Control (Sham-operation rats). f The difference expression of Phactr1 in bone tissue and adipose tissue between osteoporosis and control rats. * $P < 0.05$ or *** $P < 0.001$ vs. Control (bone and adipose tissue from Sham-operation rats). Phactr1 Phosphatase and Actin Regulator 1, OP osteoporosis, BMD Bone Mineral Density. Continuous data were expressed as mean $\pm$ standard deviation

Figure 2

Identification of BMSCs. a BMSCs adopted a uniformly spindle-shaped population. b Flow cytometry revealed that BMSCs expressed the surface markers CD29 and CD105, but not CD34 and CD45. BMSCs Bone Marrow-Derived Mesenchymal Stem Cells.

Figure 3

Phactr1 inhibits osteogenic differentiation of BMSCs. a Western blot detected the expressions of Phactr1 and Runx2 in BMSCs at 0, 3, 7 days after osteogenic induction. b (40×) Calcium nodules in BMSCs at 0, 3, 7, 10 and 14 days after osteogenic induction were determined by Alizarin Red S (ARS) staining. * $P < 0.05$ or ** $P < 0.01$ vs. 0 day (BMSCs without osteogenic induction). c Western blot detected the expression of Phactr1 in Lenti-Phactr1 and control groups. d Western blot detected the expression of Runx2 in con-0day,

con-7days and Lenti-Phactr1-7days groups (the latter two groups treated with osteogenic induction). ***P < 0.001 vs. control (BMSCs without Phactr1 overexpression lentivirus), *P < 0.05 vs. con-7day(BMSCs without Phactr1 overexpression lentivirus). Phactr1 Phosphatase and Actin Regulator 1, BMSCs Bone Marrow-Derived Mesenchymal Stem Cells, Runx2 Runt-Related Transcription Factor 2. Tublin was used as an internal control. Continuous data were expressed as mean ± standard deviation

Figure 4

Phactr1 promotes adipogenic differentiation of BMSCs. a Western blot detected the expressions of Phactr1 and C/EBPα in BMSCs at 0, 3, 7 days after adipogenic induction. b Lipid droplets in BMSCs at 0, 3, 7, 14 and 21 days after adipogenic induction were determined by oil red O staining. * $P \leq 0.05$ or ** $P \leq 0.01$ or *** $P \leq 0.001$ vs. 0 day (BMSCs without adipogenic induction). c Western blot detected the expressions of Phactr1 in si-Phactr1 and control groups. d Western blot detected the expression of C/EBPα in con-0day, con-7days and Si-Phactr1-7days groups (the later two groups treated with adipogenic induction). * $P \leq 0.05$ vs. control (BMSCs without Phactr1 siRNA), **** $P \leq 0.0001$ vs. con-7day(BMSCs without Phactr1 siRNA). Phactr1 Phosphatase and Actin Regulator 1, BMSCs Bone Marrow-Derived Mesenchymal Stem Cells, C/EBPα CCAAT/Enhancer-Binding Protein. Tublin was used as an internal control. Continuous data were expressed as mean $\pm$ standard deviation

Figure 5

Venn Diagram and KEGG PATHWAY analysis of the Proteins bond with Phactr1 On the 7th day of osteogenic and adipogenic differentiation in BMSCs. a Co-IP-MS analyse Number and name of Proteins

bond with Phactr1 On the 7th day of osteogenic, adipogenic differentiation and in both groups. b KEGG PATHWAY analysis of the Proteins bond with Phactr1 On the 7th day of osteogenic and adipogenic differentiation. c KEGG PATHWAY analysis of the Proteins bond with Phactr1 On the 7th day of adipogenic differentiation. d KEGG PATHWAY analysis of the Proteins bond with Phactr1 On the 7th day of osteogenic differentiation. Co-IP-MS Co-Immunoprecipitation Mass Spectrometry, AD adipogenic differentiation, OD osteogenic differentiation, KEGG Kyoto Encyclopedia of Genes and Genomes

Figure 6

Phactr1 inhibits osteogenic differentiation and promotes adipogenic differentiation of BMSCs through RhoA/ROCK2 signaling. a Co-Immunoprecipitation confirm Phactr1 bind to ROCK2 on the 7th day of osteogenic differentiation, but not bind to ROCK2 on the 7th day of adipogenic differentiation . b, c Western blot detected the expressions of Active RhoA and ROCK2 On the 7th day of adipogenic differentiation and osteogenic differentiation. * $P \leq 0.05$ vs. 0days. d, f, g Western blot detected the expressions of Phactr1, Runx2 in Con-0d, Con-7d and KD025-7d groups (the latter two groups treated with osteogenic induction). * $P \leq 0.05$ or ** $P \leq 0.01$ vs. Con-7d (BMSCs without KD025). e, f, h Western blot detected the expressions of Phactr1, C/EBP α in Con-0d, Con-7d and KD025-7d groups (the latter two groups treated with adipogenic induction). * $P \leq 0.05$ or ** $P \leq 0.01$ vs. Con-7d (BMSCs without KD025). RhoA Ras homolog gene family member A, ROCK2 Rho-associated coiled-coil-containing protein kinase 2, KD025 Selective ROCK2 Inhibitor. Tublin was used as an internal control. Continuous data were expressed as mean $\pm$ standard deviation

Supplementary Files

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