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Cellular retinol-binding protein 1 (CRBP-1) regulates osteogenenesis and adipogenesis of mesenchymal stem cells through inhibiting RXRα-induced β-catenin degradation

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can differentiate into osteoblasts, chondrocytes and adipocytes, providing a potential source for musculoskeletal tissue engineering. Retinoid signaling plays very important roles in skeletal development. CRBP1 (cellular retinol binding protein 1), a key component of retinoid signaling pathway, is known to take part in vitamin A metabolism and intracellular transporting of retinoids. However, the role of CRBP1 in MSCs remains still obscure. In this study, we investigated the cellular effects of CRBP1 on osteogenic and adipogenic differentiation of bone marrow derived MSCs in vitro and in vivo. Our results showed that CRBP1 overexpression promoted osteogenic differentiation of bone marrow derived MSCs, while inhibited their adipogenic differentiation. We also demonstrated that the possible underlying mechanism for CRBP1 promoting osteogenic differentiation of MSCs was by inhibiting RXRα-induced β-catenin degradation, maintaining β-catenin and pERK1/2 at higher levels. These findings reveal a potential role of CRBP1 in the regulation of β-catenin turnover which can greatly affect the process of osteogenesis and adipogenesis of MSCs.

1. Introduction

Mesenchymal stem cells (MSCs) have multi-potent capacity to differentiate into a variety of cell types, including osteoblasts, adipocytes, chondrocytes, myoblasts and neurons (Jiang et al., 2002; Pittenger et al., 1999). The use of MSCs for tissue repair has been reported with success, in particular MSCs are promising cell source for bone tissue engineering (Devine et al., 2002; Taguchi et al., 2005). Stimulating the osteogenic differentiation and bone forming properties is one of the strategies for improving bone tissue engineering (Yi et al., 2010). Increasing reports showed that certain genes, cytokines, chemicals could enhance osteogenesis of MSCs. For example, Integrinα5 was up-regulated during dexamethasone-induced osteogenesis of human MSCs, and forced expression of Integrinα5 in human MSCs markedly increased de novo osteogenesis in vivo (Hamidouche et al., 2009). All-trans retinoic acid (ATRA) and sodium butyrate have been shown to enhance osteogenesis of mouse and human MSCs (Chen et al., 2007; James et al., 2010). Oncostatin M is a multifunctional cytokine of the interleukin-6 family and has been demonstrated to stimulate osteogenic differentiation and suppress adipogenic differentiation of human adipose-derived MSCs (Song et al., 2007). Recently, Yi et al. (2010) reported that gold nano-particles could promote osteogenic differentiation of MSCs through activating p38 MAPK signaling pathway. In addition, mechanical stimulation alone has been shown to promote osteogenic differentiation of MSCs (Kearney et al., 2010; Kim et al., 2010; Rui et al., 2010).

Several signaling pathways have been shown to be involved in the process of osteogenesis. The Wnt/β-catenin and ERK1/2 signaling pathways have been intensively investigated. The Wnt/β-catenin signaling activity promotes bone formation and inhibits chondrocyte differentiation during skeletal development (Day et al., 2005). In addition, the Wnt/β-catenin signaling pathway has been shown to promote osteogenesis by directly stimulating Runx2 gene expression which is a master transcriptional factor of osteoblast differentiation (Gaur et al., 2005). ERK1/2 is constantly

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Table 1
Sequences of primers for RT-PCR.

Gene Name	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
Alkaline phosphatase (ALP)	TCCGTGGGTCGGATTCCT	GCCGGCCCAAGAGAGAA
C/EBPα	AAGGCCAAGAAGTCGGTGGA	CAGTTCGCGGCTCAGCTGTT
PPARγ	GAGCATGGTGCCTTCGCTGA	AGCAAGGCACTTCTGAAACCGA
Osteopontin (OPN)	TCCAAGGAGTATAAGCAGCGGGCCA	CTCTTAGGGTCTAGGACTAGCTTCT
Osteocalcin (OCN)	GAGCTGCCCTGCACTGGGTG	TGGCCCCAGACCTCTTCCCG
Runx2	CCGATGGGACCGTGGTT	CAGCAGAGGCATTTCGTAGCT
Type I collagen (Col1α2)	CATCGGTGGTACTAAC	CTGGATCATATTGCACA
β-Actin	CGTAAAGACCTCTATGCCAACA	CGGACTCATCGTACTCCTGCT
CRBP1	TACCTGCGTGCGCTCGAT	GAAGTTCATCACCCTCAATCCA

activated during osteogenic differentiation, the study by Jaiswal et al. (2000) has suggested that the commitment of human MSCs into osteogenic or adipogenic lineages is governed by activation or inhibition of ERK1/2 respectively.

Retinoid signaling also plays very important roles in skeletal development (Weston et al., 2003). Retinoid signaling involves many kinds of nuclear receptors, cytoplasmic retinoic acid (RA) related enzymes and RA-binding proteins. Cellular retinol binding protein 1 (CRBP1), as its name depicts, is a cytoplasmic retinoid binding protein and has been linked to retinoid signaling pathway. CRBP1 mainly functions in intracellular transporting of retinoids and vitamin A metabolism (Kuppumbatti et al., 2000). Other than that, some new roles of CRBP1 have also been reported. CRBP1 may involve in wound healing as it was transiently expressed by a significant proportion of fibroblasts during a full-thickness rat skin wound process (Xu et al., 1997). It may also function as a tumor suppressor gene because CRBP1 down-regulation was associated with breast and ovarian cancer development (Cvetkovic et al., 2003; Kuppumbatti et al., 2000). Recent study in CRBP1 knockout mice showed that CRBP1 deficiency was associated with increased adiposity, the embryonic fibroblasts derived from CRBP1 knockout mice had increased adipocyte differentiation and triglyceride accumulation (Zizola et al., 2010). However, little is known about the role of CRBP1 in MSCs in terms of osteogenic and adipogenic differentiation.

Here, we showed that over-expression of CRBP1 in MSCs promoted osteogenic differentiation and inhibited adipogenic differentiation. This phenomenon was reversed by CRBP1 silencing using shRNA. Furthermore, we demonstrated that CRBP1 promoted osteogenic differentiation of MSCs by inhibiting RXRα-induced β-catenin degradation, thus maintaining β-catenin and pERK1/2 at higher levels, and eventually lead to elevated osteogenesis-related genes expression.

2. Materials and methods

2.1. Chemicals

All the chemicals used were purchased from Sigma, except where specified.

2.2. Isolation and culture of BM-MSCs

All experiments were approved by the Animal Research Ethics Committee of the authors' institution. Bone marrow was flushed out from the bone cavity of the Sprague-Dawley rats and subject to density gradient centrifugation over Lymphoprep™ (1.077 g/ml; AXIS-SHIELD, Norway) to obtain the mononuclear cells (MNCs). The MNCs were cultured in α-MEM, 10% fetal bovine serum, 2 mM L-glutamine (Invitrogen) at 37 with 5% CO_2. When colonies were confluent, the cells were trypsinized and re-plated for further expansion and examination. The BM-MSCs used in this study were between passage 3 and 8.

2.3. RNA extraction and real-time PCR

Genes associated with osteogenesis and adipogenesis were assayed by quantitative real time PCR (RT-PCR). Total cellular RNA was isolated with RNeasy (Qiagen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized with MMLV reverse transcriptase (Promega, USA). PCR amplification was performed using the primer sets outlined in Table 1. Primer sequences were determined through established GenBank sequences. β-Actin was used as an internal control to evaluate the relative expression.

2.4. Plasmid construction, transfection, production of lentivirus and infection

The gene encoding rat CRBP1 (GenBank number: NM_012733) was amplified and cloned into pLenti-MCS-dsRed vector by in vitro recombination. The gene encoding rat RXRα (GenBank number: NM_012805) was cloned into pCMV-Flag plasmid. Two different shRNAs were chosen from rat CRBP1 mRNA sequence, which target nucleotides 420–439 and 620–639 respectively, and one nonspecific shRNA was designed as control. The two CRBP1 shRNA target sequences are: 5′-GTGGATTGAGGGTGATGAA-3′ and 5′-GTCTGTCTCATTGCCTTGT-3′. CRBP1 shRNA and scrambled shRNA sequence templates were inserted into pLL3.7 plasmid as described (Rubinson et al., 2003). Pseudo-lentiviruses were produced by transient transfection of 293FT packaging cells (Invitrogen, USA) using the calcium phosphate method. Culture supernatants were harvested at 48 and 72 h after transfection and lentiviral particles were concentrated using PEG6000 (Kutner et al., 2009). For transduction, 1×10^5 cells were seeded into 6-well plate and incubated with lentiviruses and 8 μg/mL polybrene in the incubator for 24 h. The plasmids encoding RXRα and CRBP1 were co-transfected into HEK293T cells using lipofactamine 2000 (Invitrogen, USA).

2.5. Osteogenic differentiation

The MSCs were trypsinized and replated in 6-well plate at a concentration of 1×10^5 cells per well. These cells were incubated in the α-MEM for two or three days. The medium was then replaced by osteogenic induction medium (OIM) containing 100 nmol/L dexamethasone, 10 mmol/L beta-glycerophosphate, and 0.05 mmol/L L-ascorbic acid-2-phosphate. The OIM was changed every three days. The osteogenic differentiation was evaluated by real-time PCR and Alizarin Red S staining.

2.6. Adipogenic differentiation

The MSCs were trypsinized and replated in 6-well plate at a concentration of 1×10^5 cells per well. These cells were incubated in the α-MEM for two or three days. The medium was then replaced by adipogenic induction medium (AIM) containing

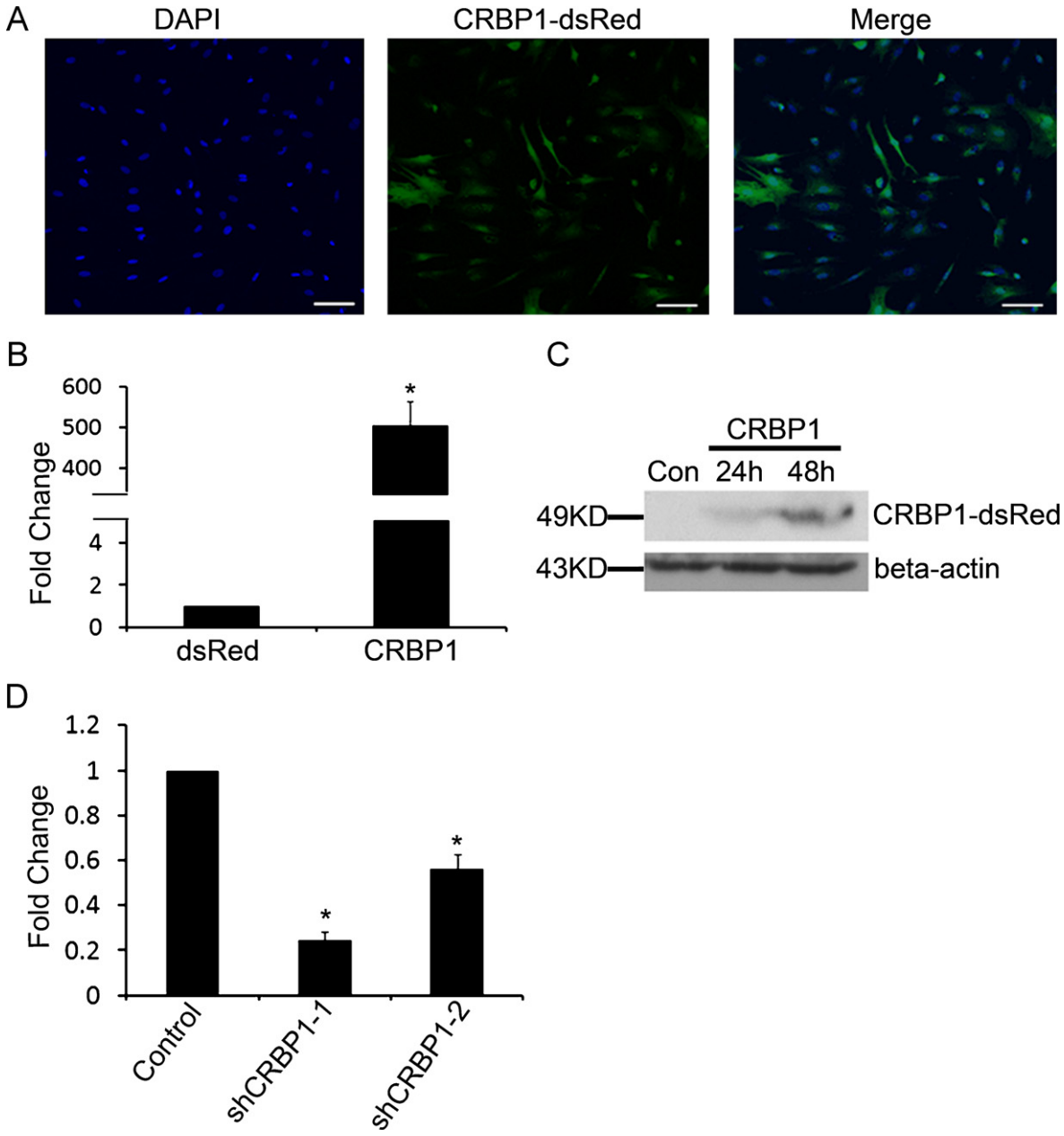

Fig. 1. Infection of BM-MSCs with pseudo-lentivirus carrying CRBP1 or shRNAs. (A) MSCs were infected with lentivirus expressing CRBP1. The expression of CRBP1 was confirmed by immunofluorescence labeling with anti-dsRed polyclonal antibody and detected by goat anti-mouse FITC conjugated secondary antibody. Nuclei were counterstained with DAPI. Scale bar = 100 μm. (B) Total RNA used for real time PCR was extracted from MSC-dsRed and MSC-CRBP1. The mRNA level of CRBP1 in MSC-dsRed and MSC-CRBP1 were detected using real time PCR. β-Actin was used as an internal control. The mRNA level of CRBP1 in MSC-dsRed was arbitrarily given as 1.0. The data are expressed as mean ± SD ($n = 3$). (C) Total proteins extracted from MSC-dsRed and MSC-CRBP1 were analyzed by western blot using anti-CRBP1 antibody. β-Actin was used as loading control. (D) Total RNA was extracted from MSCs transduced with CRBP1 shRNA or scrambled shRNA (control). The relative expression level of CRBP1 was evaluated using real time PCR. The data are expressed as mean ± SD ($n = 3$).

10% fetal bovine serum, 1 μM dexamethasone, 10 μg/ml insulin, 50 μM indomethacin, and 0.5 mM isobuthyl-methylxanthine. Prior to staining, the cells were rinsed with PBS and fixed in 70% ethanol for 10 min. They were then incubated in 2% (wt/vol) Oil Red O reagent for 5 min at room temperature and excess stain was removed by washing with distilled water.

2.7. Western blot

The cells were washed with cold PBS twice, and harvested by scraping in cold cell extraction buffer (Invitrogen, Cat. no. FNN0011). Protein concentration was determined by Bradford method (Biorad, USA). Equal proteins were loaded onto 10% Tris/glycine gels for electrophoresis and then transferred to a PVDF membrane (Millipore, Bedford, MA) and blocked in 5% non-fat milk (Biorad, USA) for 1 h at room temperature with rocking. Then, the primary antibody, anti-osteopontin (OPN) (1:1000, Novus Biologicals, USA), anti-β-catenin (1:1000, BD Biosciences, USA), anti-p-ERK (1:1000, BD Biosciences, USA), anti-CRBP1 (1:1000, Santa Cruz, USA) anti-GAPDH (1:1000, Santa Cruz, USA) or anti-actin (1:1000, Santa Cruz, USA) was added and incubated for 2 h at room temperature or at 4 °C overnight. After washing in TBST for three times (5 min for each time), the membrane was incubated with horseradish peroxidase-linked secondary antibodies

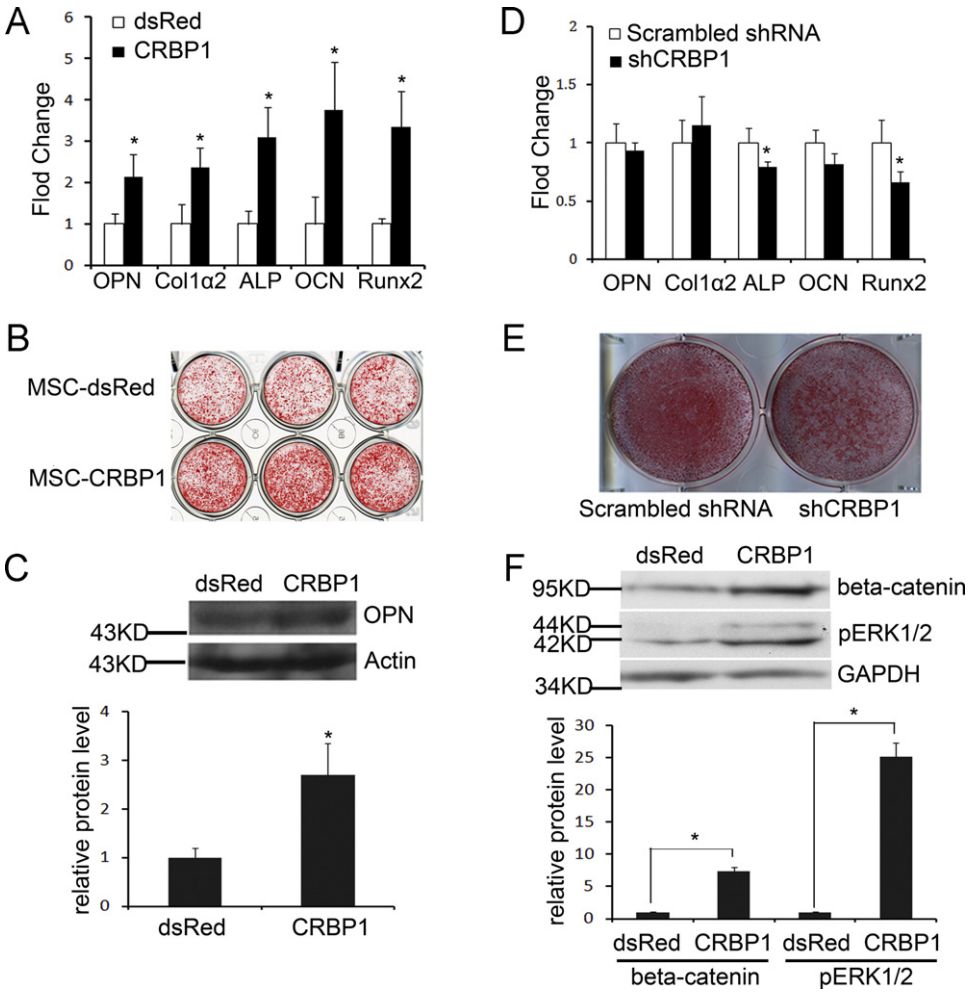

Fig. 2. CRBP1 promoted osteogenic differentiation of BM-MSCs through regulating β-catenin and ERK1/2 signaling pathways. (A) Relative expression of OPN, Col1α2, ALP, OCN and Runx2 in MSC-CRBP1. Total RNA used for real time PCR was extracted from MSC-dsRed and MSC-CRBP1 which were subjected for osteogenic induction for 7 days. The data are expressed as mean ± SD ($n = 3$). (B and C) MSC-dsRed and MSC-CRBP1 were treated with OIM for 19 days, then mineralization of MSCs was stained by Alizarin Red S (B); or total proteins were extracted and analyzed by western blot to detect the expression level of OPN. β-Actin was used as loading control. Quantification of the band intensity showed that the expression of OPN was significantly upregulated. The protein level was normalized to β-actin (C). (D and E) CRBP1 knockdown impaired osteogenesis of MSCs. Relative expression of OPN, Col1α2, ALP, OCN and Runx2 in MSC-CRBP1 was checked using real time PCR (D); MSCs transduced with shCRBP1-1 or scrambled shRNA were treated with OIM for 19 days, then calcium nodules were stained by Alizarin Red S (E). (F) MSC-dsRed and MSC-CRBP1 were treated with OIM for 7 days, and then protein extracts were prepared for western blot analysis with anti-β-catenin and anti-pERK1/2 antibodies. The blot was probed with anti-GAPDH antibody to confirm equal loading. Quantification of the band intensity showed that the level of β-catenin and pERK1/2 was significantly upregulated. The protein levels were normalized to GAPDH. The experiments were repeated three times.

(anti-mouse or anti-goat) for 1 h at room temperature. Following three TBST washes, protein was detected with the enhanced chemiluminescence (ECL) blotting reagents (Amersham Biosciences, USA) according to the manufacturer's instruction. The intensity of bands was quantified with Image J software (Image J, National Institutes of Health, USA) and compared statistically.

2.8. Immunofluorescence labeling and fluorescence microscopy

BM-MSCs were fixed in 4% paraformaldehyde for 15 min at 4 °C, and permeabilized with 0.3% Triton X-100 for 15 min, and blocked with 10% FBS for 1 h. Then the cells were labeled with anti-dsRed (1:200, BD Biosciences, USA) for 2 h at room temperature or overnight at 4 °C. Cultures were then washed three times with PBS and incubated with a FITC-conjugated anti-mouse IgG (Invitrogen, USA) for 1 h at room temperature. Nuclear counterstaining was performed with DAPI Vectashield mounting medium (Vector Laboratories, USA). Immunostaining was observed under Olympus FV1000 confocal microscope.

2.9. Ectopic bone formation assay

2×10^5 MSCs overexpressing CRBP1 or dsRed were loaded onto sterilized Skelite® resorbable HA-TCP bone graft substitute, and incubated at 37 °C for 3 h to allow attachment. Four nude mice were under general anesthesia and the substitutes with cells were then implanted subcutaneously at the dorsal sides. The transplants were harvested 8 weeks later and subjected to histological examination. The sections were subjected to hematoxylin and eosin or Alcian blue/Sirius red staining. Anti-OPN antibody was used for immunohistochemical analysis. Staining was detected with DAB detection system (DAKO). The osteoid matrix areas were measured using Image J software, five microscopic fields were chosen from each sample and measured.

2.10. Statistical analysis

All experiments were performed at least three times. The data were analyzed by nonparametric test using SPSS (version 16.0; SPSS Inc., Chicago, IL, USA). $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Transduced BM-MSCs with pseudo-lentivirus carrying CRBP1 or shRNAs

The BM-MSCs were characterized for their surface markers, they were found to express CD90 (over 98%), CD44 (over 50%) and CD73 (over 50%), while did not express CD34 and CD45 (both lower than 2%) (data not shown). The gene encoding CRBP1 was cloned into a lentivirus vector (pLenti-MCS-dsRed) under the control of CMV promoter. The pseudo-lentivirus carrying CRBP1 gene was transduced into BM-MSCs, and above 80% of MSCs were positive for CRBP1 expression as confirmed by immunofluorescence (Fig. 1A); the CRBP1 proteins were located both in the cytoplasm and nuclear (Fig. 1A). Total RNA or protein was extracted from MSCs-overexpressing CRBP1 (MSC-CRBP1) or dsRed (MSC-dsRed), and the increase of CRBP1 mRNA and protein was confirmed by real-time PCR (Fig. 1B) and western blot (Fig. 1C).

Next, we selected two different shRNAs from rat CRBP1 mRNA, one is located in the 3′ noncoding region (shCRBP1-1: 620–639), the other is located in the coding region (shCRBP1-2: 420–439). The expression level of CRBP1 mRNA was detected using real-time PCR after MSCs were infected with pseudo-lentivirus carrying different shRNA sequences. As shown in Fig. 1D, the two selected shRNAs targeting different sequences of CRBP1 mRNA both significantly inhibited CRBP1 expression, of which CRBP1 mRNA level was knocked down nearly 80% by shCRBP1-1, which was more effective than shCRBP1-2.

3.2. CRBP1 accelerated osteogenesis of BM-MSCs via ERK1/2 and β-catenin pathways

To evaluate the effect of CRBP1 on osteogenesis of BM-MSCs, the cells were transduced with lentivirus carrying CRBP1 or dsRed and then were treated with OIM for 7 days. Various osteogenic differentiation-related genes were checked using real-time PCR analysis, including ALP, Runx2, OCN, OPN, collagen type I α2. As compared with control, significant increase in all of these genes was observed in MSC-CRBP1 after OIM treatment for 7days (Fig. 2A). When the cells were treated with OIM for 19 days, much more calcium deposits appeared in the MSC-CRBP1 group (Fig. 2B). In addition, we also checked the expression of OPN which is a prominent bone matrix protein produced by osteoblastic cells by western blot, confirmed that the OPN expression was up-regulated in the MSC-CRBP1 (Fig. 2C).

On the other hand, the osteogenesis of MSCs was impaired when endogenous CRBP1 was knocked down by shCRBP1-1. As shown in Fig. 2D, the mRNA levels of two important osteogenic differentiation related markers, ALP and Runx2, were significantly downregulated in MSCs transduced with shCRBP1-1, while the

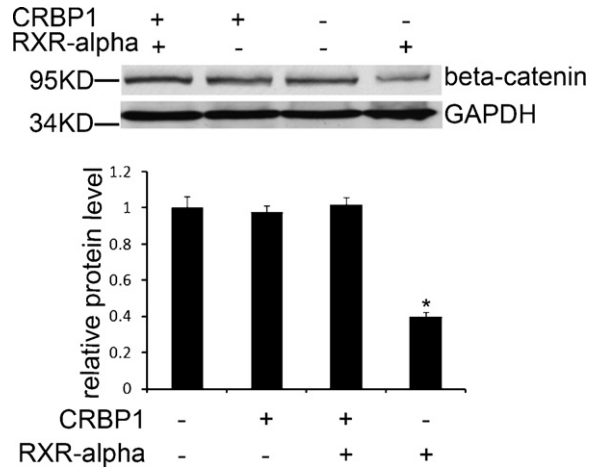

Fig. 3. CRBP1 inhibited RXRα-induced β-catenin degradation. HEK293T cells were cotransfected with 1.0 μg of the indicated plasmids, the total proteins were collected 48hs after transfection. The level of β-catenin was probed with β-catenin antibody. GAPDH was used as loading control. Quantification of β-catenin showed that RXRα-induced β-catenin degradation was suppressed by CRBP1. The protein level was normalized to GAPDH. The experiments were repeated three times.

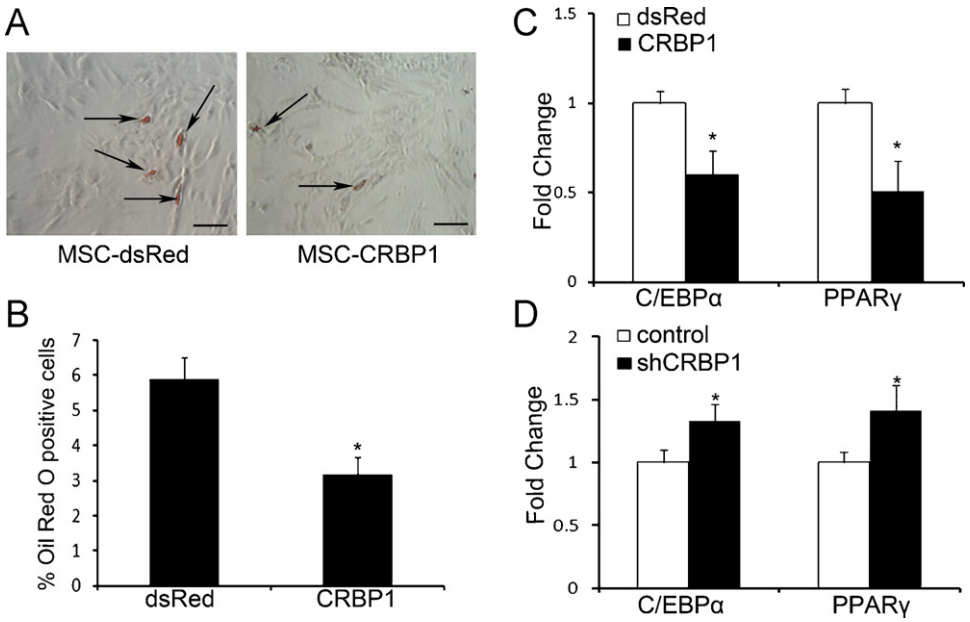

Fig. 4. Effect of CRBP1 on adipogenesis of BM-MSCs. (A and B) CRBP1 overexpression decreased the nubmer of adipocytes differentiated from MSCs. MSC-dsRed and MSC-CRBP1 were subjected to AIM for 14 days, then Oil Red O staining was used to stain adipocytes. The number of Oil Red O positive adipocytes was counted. The experiment was repeated three times, 20 images were randomly taken and about 1000 cells were counted in each experiment. Scale bar = 100 μm. (C) MSC-CRBP1 and MSC-dsRed were subjected to AIM for 7 days, then total RNA was extracted for real-time PCR analysis of adipogenesis-related markers (C/EBPα and PPARγ). (D) CRBP1 knockdown increased the expression level of C/EBPα and PPARγ genes. β-Actin was used as an internal control. The data are expressed as mean ± SD ($n = 3$).

other markers such as OPN and OCN showed slight decrease. Also, the number of calcium nodules formed in the CRBP1 knock-down group were significantly less than that of the control group (Fig. 2E).

As previous studies have demonstrated both ERK1/2 and β-catenin are important regulators of osteogenesis, we investigated whether β-catenin and ERK1/2 signaling pathways were involved in regulating CRBP1-promoted osteogenic differentiation of MSCs. The western blot analysis showed that the level of β-catenin and pERK1/2 was significantly higher in the MSC-CRBP1 after treated with OIM for 7 days (Fig. 2F).

3.3. CRBP1 stabilized β-catenin through inhibiting RXRα-induced degradation

Levels of β-catenin are tightly regulated by adenomatous polyposis coli (APC)-dependent proteasomal degradation pathways, and retinoid X receptor (RXR)-mediated APC-independent pathway. We mainly focused on the latter pathway as both RXR and CRBP1 are related to retinoid. In order to check whether CRBP1 could inhibit RXRα-induced β-catenin degradation, the gene encoding RXRα was cloned and co-transfected with CRBP1 into HEK293T cells. The endogenous β-catenin was evaluated by

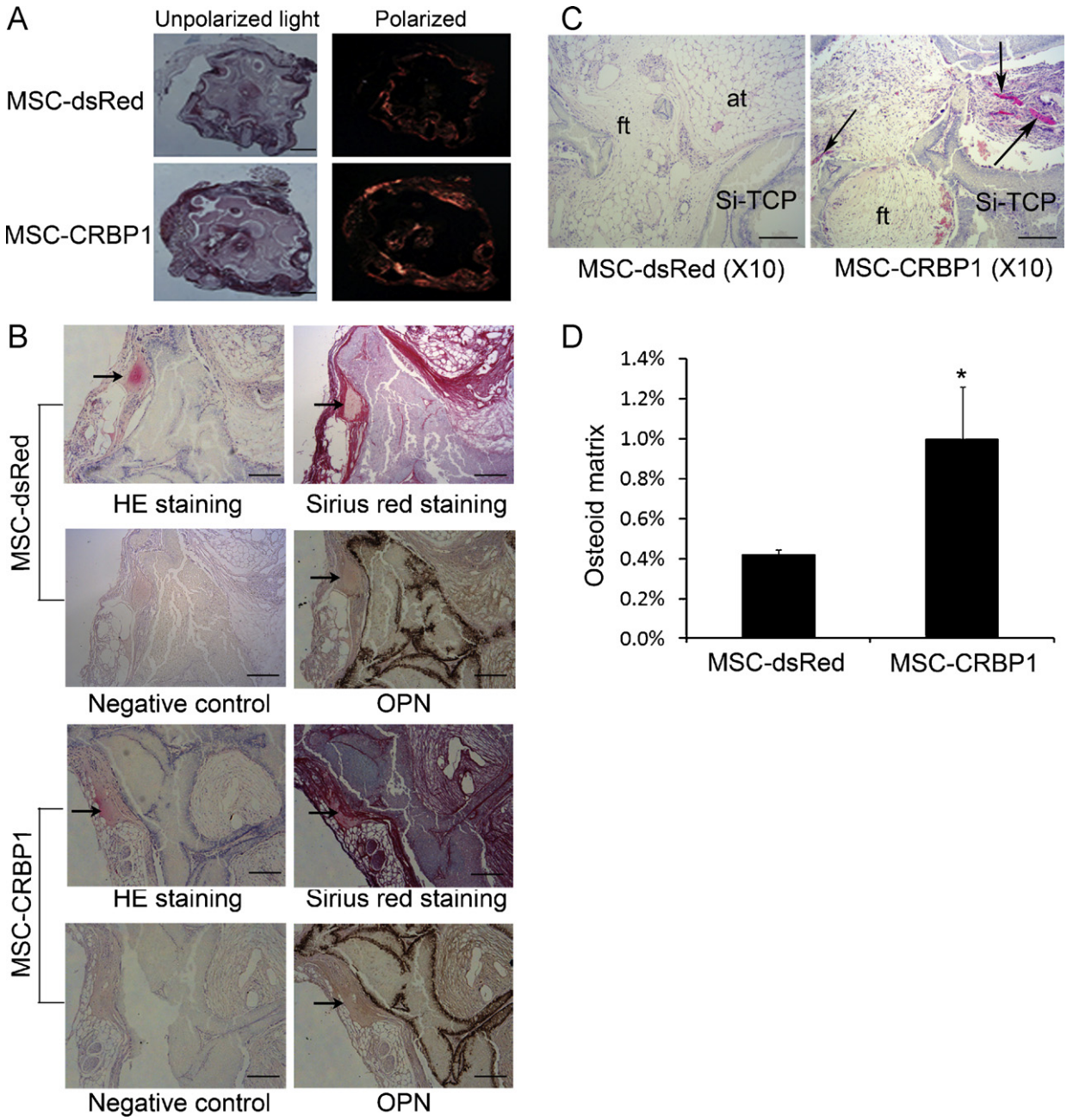

Fig. 5. Overexpression of CRBP1 in MSCs promoted ectopic bone formation. MSC-dsRed and MSC-CRBP1 were loaded onto sterilized porous calcium phosphate restorable granules, and then implanted subcutaneously into the dorsal surfaces. The transplants were harvested 8 weeks later for histological examination. (A) The sections were stained with Alcian blue/Sirius red and then were observed under normal or polarized microscope. Collagen type I presented a yellow, orange or red color. Scale bar = 500 μm. (B) Both collagen fibers and OPN exist in the area of osteoid. Arrow head indicates the osteoid matrix. Scale bar = 200 μm. (C) The sections were stained with routine hematoxylin and eosin, amorphous osteoid matrix could be seen in the pores of transplants (arrow head). at: adipose tissue; ft: fibrous tissue. Scale bar = 200 μm. (D) Quantification of osteoid matrix showed that there was more osteoid matrix in CRBP1 overexpressing group. Five microscopic fields from each sample were used for measurement. Results are presented as mean ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

western blot analysis, the result indicated that RXRα overexpression could induce degradation of β-catenin in HEK293T cells, and this was inhibited by the presence of CRBP1 (Fig. 3).

3.4. CRBP1 inhibited adipogenesis of BM-MSCs

For evaluation the effect of CRBP1 on adipogenesis of BM-MSCs, the MSCs were treated with AIM for 14 days. The Oil Red O positive cells in the MSC-CRBP1 group were significantly reduced than that of control (Fig. 4A and B). The expression levels of adipocyte markers, peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα were greatly decreased in the MSCs overexpressing CRBP1 following 7 days of AIM treatment (Fig. 4C). Moreover, the mRNA expression levels of C/EBPα and PPARγ were significantly up-regulated when the MSCs were transduced with shCRBP1-1 (Fig. 4D).

3.5. CRBP1 promoted osteogenesis in vivo

To establish the role of CRBP1 on promoting the osteogenesis of MSCs, MSC-CRBP1 and MSC-dsRed were loaded onto sterilized Skelite® resorbable HA-TCP bone graft substitutes and implanted subcutaneously at the dorsal sides. The transplants were harvested 8 weeks later and subjected to histological examination. The sections were subjected to HE staining, Alcian blue/Sirius red staining or immunohistochemical analysis to detect the distribution of osteoid, collagens and OPN. The results showed that more mature collagen fibers were observed in MSC-CRBP1 group under the polarized light microscope compared with that of the MSC-dsRed group (Fig. 5A). And both collagen fibers and OPN exist in the area of osteoid (Fig. 5B). And amorphous osteoid matrix was observed in both groups, but the amount of osteoid matrix in the MSC-CRBP1 group was much higher (Fig. 5C and D).

4. Discussion

The present study provides evidence that CRBP1 promotes the expression of osteoblast markers and osteogenic differentiation of MSCs through inhibiting RXRα-induced β-catenin degradation, leading to upregulation of β-catenin and pERK1/2, as summarized by illustration in Fig. 6. On the other hand, overexpression of CRBP1 also inhibits adipogenesis of MSCs.

Usually, osteogenesis and adipogenesis of MSCs maintains a dynamic balance in the bone marrow, and the balance can be regulated by many kinds of agents, such as BMP2 (Mie et al., 2000), dexamethasone (Honda et al., 2011), transforming growth factor TGF-β (Enomoto et al., 2004), etc. It has been observed that cancellous bone apposition rate, osteoid volume, and age were all associated with the increase in the proportion of adipose tissues present in the bone marrow (Verma et al., 2002).

CRBP1 is a key component of retinoid signaling which plays important roles in many developmental processes, especially in limb induction (Helms et al., 1996; Zhao et al., 2009). Previous studies have demonstrated that CRBP1 involves in vitamin A metabolism and intracellular transporting of retinol (vitamin A alcohol). In the present study, we found that CRBP1 overexpression could enhance osteogenic differentiation of BM-MSCs, while inhibited their adipogenic differentiation; when CRBP1 was knocked down in MSCs using shRNA technique, the effects of CRBP1 on osteogenesis and adipogenesis of MSCs was reversed. These data suggested that the dynamic balance between osteogenesis and adipogenesis of MSCs could be regulated by the expression level of CRBP1. The inhibitory effect of CRBP1 on adipogenesis of MSCs was consistent with a recent study by Zizola et al. (2010) in which CRBP1 has been shown to inhibit adipogenesis both in vitro and in vivo through affecting PPARγ activity. The mRNA level of PPARγ and C/EBPα was much lower in MSCs overexpressing CRBP1, but we did not measure whether PPARγ activity was inhibited.

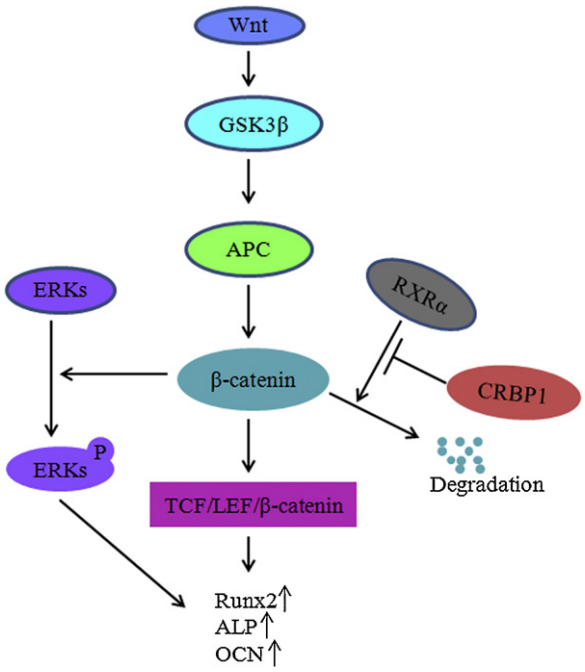

Fig. 6. Schematic of possible mechanisms by which CRBP1 promotes osteogenis of MSCs. RXRα leads to the degredation of β-catenin through direct protein–protein interaction. CRBP1 could block this pathway through unknown mechanism, resulting in accumulation of β-catenin and upregulation of pERK1/2, enventually leading to upregulation of osteogenesis-related markers.

Several signaling pathways are responsible for the commitment of MSCs to osteogenic lineages, such as ERK1/2 (Kim et al., 2007), Wnt/β-catenin (Day et al., 2005), hedgehog signaling pathways (Wu et al., 2004), etc. In our study, we demonstrated that both pERK1/2 and β-catenin were maintained at higher level during osteogenesis in the MSCs overexpressing CRBP1, suggesting both ERK1/2 and β-catenin signaling pathways were involved in this process. ERK1/2 has been reported to play an important role in the regulation of osteogenesis, when a dominant negative ERK1 was used to suppress both ERK1 and ERK2, osteoblast differentiation and mineralization were significantly inhibited (Lai et al., 2001). β-Catenin is a key downstream effector of Wnt signaling pathway which also plays important roles in osteogenesis and bone formation, Wnt/β-catenin signaling pathway also initiate early differentiation of MSCs (Gaur et al., 2005; Gong et al., 2001). Usually, the levels of β-catenin are tightly regulated by three pathways including p53/Siah-1/APC, Wnt/glycogen synthase kinase-3β/APC and retinoid X receptor (RXR)-mediated APC-independent pathway. RXR is a type of nuclear hormone receptor which has α, β and γ three known isoforms. RXRα has recently been found to mediate β-catenin degradation through direct protein–protein interaction (Han et al., 2008; Xiao et al., 2003). However, the biological consequences and its regulation have not been well understood. Here, we showed that CRBP1 was a negative regulator of this pathway. The crosstalk between Wnt/β-catenin and ERK1/2 signaling pathways have recently been identified as β-catenin siRNA reduced ERKs activity (Yun et al., 2005). In the present study, we proved that CRBP1 overexpression could maintain the level of β-catenin through inhibiting RXRα-induced β-catenin degradation, causing prolonged activation of ERKs, and eventually leading to enhanced osteogenesis (as illustrated in Fig. 6). The mechanism of how CRBP1 inhibited RXRα-induced β-catenin degradation remains to be further studied.

In conclusion, we have demonstrated that CRBP1 regulates osteogenesis and adipogenesis of MSCs. CRBP1 promotes osteogenesis at least partially through inhibiting RXRα-induced β-catenin degradation, thus activates ERK1/2 and β-catenin signaling pathways. These data provide evidences that CRBP1 may play roles in MSCs fate determination between osteogenesis and adipogenesis.

Author's contribution

The authors, Liangliang Xu, Ming Ni and Fanbiao Meng took care of collection and/or assembly of data. Both Liangliang Xu and Chao Song were responsible for conception and design together with data analysis and interpretation. Huiqi Xie effected experimental designs and animal experiments. Though Liangliang Xu endeavoured manuscript writing, Gang Li steered conception and design, financial support, manuscript scripting, and final approval of manuscript.

Disclosure

No conflict of interest to declare.

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