Simvastatin inhibits the adipogenesis of bone marrow-derived mesenchymal stem cells through the downregulation of chemerin/CMKLR1 signaling

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Abstract. Simvastatin is effective in the treatment of osteoporosis, partly through the inhibition of the adipogenesis of bone-marrow derived mesenchymal stem cells (BMSCs). The present study focused on the mechanisms responsible for the inhibitory effects of simvastatin on adipogenesis and examined the effects of simvastatin on the expression of peroxisome proliferator-activated receptor γ (PPARγ), chemerin, chemokine-like receptor 1 (CMKLR1), G protein-coupled receptor 1 (GPR1) and the adipocyte marker gene, adiponectin. BMSCs were isolated from 4-week-old female Sprague-Dawley (SD) rats, and adipogenesis was measured by the absorbance values at 490 nm of Oil Red O dye. The expression of each gene was evaluated by western blot analysis or reverse transcription-quantitative PCR (RT-qPCR). The expression of chemerin increased during adipogenesis, while CMKLR1 exhibited a trend towards a decreased expression. On days 7 and 14, the simvastatin-treated cells exhibited a downregulated expression of chemerin, whereas the upregulated expression of its receptor, CMKLR1 was observed. The results also revealed that CMKLR1 is required for adipogenesis and the simvastatin-mediated inhibitory effect on adipogenesis. Simvastatin regulated adipogenesis by negatively modulating chemerin-CMKLR1 signaling. Importantly, simvastatin stimulation inhibited the upregulation of PPARγ and PPARγ-mediated chemerin expression to prevent adipogenesis. Treatment with the PPARγ agonist, rosiglitazone, partially reversed the negative regulatory effects of simvastatin. On the whole, the findings of the present study demonstrate that simvastatin inhibits the adipogenesis of BMSCs through the downregulation of PPARγ and subsequently prevents the PPARγ-mediated induction of chemerin/CMKLR1 signaling.

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Abbreviations: BMSCs, bone marrow-derived mesenchymal stem cells; HSCs, hematopoietic stem cells; PPAR γ, peroxisome proliferator-activated receptor γ; CMKLR1, chemokine-like receptor 1; GPR1, G protein-coupled receptor 1; HMG-CoA reductase, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl diphosphate; GGOH, geranylgeraniol

Key words: adipogenesis, bone marrow-derived mesenchymal stem cell, chemerin, CMKLR1, PPARγ, simvastatin, osteoporosis, rosiglitazone

Introduction

Osteoporosis is a systemic disease associated with decreased bone mass, leading to reduced bone quality and an increased risk of fracture (1) due to the functional imbalance of osteoclasts and osteoblasts (2). Osteoclasts and osteoblasts are derived from hematopoietic stem cells (HSCs) and bone marrow-derived mesenchymal stem cells (BMSCs) (3). In the bone marrow of patients with osteoporosis, the adipogenic differentiation of BMSCs prevails over osteogenic differentiation, leading to lipid accumulation (4,5). An excessive amount of adipocytes in bone marrow may inhibit bone formation and stimulate bone resorption (6). Adipocytes may produce several adipokines such as leptin, adiponectin and chemerin (7) that alter bone matrix composition (8). Hence, inhibiting the adipogenesis of BMSCs may have the potential to increase osteogenesis.

Statins are normally used to lower cholesterol in patients with hyperlipidemia and cardiovascular diseases (9); however, they have also exhibited efficacy in the treatment of bone loss-related diseases (10-12). The liposoluble simvastatin exhibits anabolic effects on bones by stimulating osteogenesis, inhibiting osteoblasts apoptosis (13), and by suppressing the differentiation and activities of osteoclasts (13). Statins also increase the expression of the bone morphogenic protein-2, enhancing bone formation (14). An animal study using ovariectomized rats suggested that the use of low-dose statin increased bone resorption, while high-dose statin increased

bone formation (15). Simvastatin has also been shown to negatively regulate the adipogenic differentiation of BMSCs and preadipocytes (16) through the downregulation of the peroxisome proliferator-activated receptor γ (PPARγ) (16,17).

PPARγ activation favors BMSC differentiation into adipocytes, and the PPARγ blockade promotes osteogenesis (18). The overexpression of PPARγ and lipid accumulation have been found in the bone marrow from osteoporosis models (19). During adipocyte differentiation, PPARγ directly stimulates the expression and secretion of chemerin (20,21). Chemerin binds to at least 3 receptors: Chemokine-like receptor 1 (CMKLR1), G protein-coupled receptor 1 (GPR1) and chemokine (C-C motif) receptor-like 2 (CCRL2) (22-24). CCRL2 is an atypical receptor and was not investigated in the present study. CMKLR1 and GPR1 are similar in sequence and structure; thus, GPR1 may partially mediate the function of chemerin (22-24). An increased level of chemerin or the deficiency of CMKLR1 leads to bone loss in mice (25,26). Chemerin/CMKLR1 signaling is essential for adipogenesis (20,27-29). On the other hand, the role of GPR1 in adipogenesis upon simvastatin treatment has been less extensively studied, despite a link between GPR1 and bone metabolism (30).

Nevertheless, the mechansims through which simvastatin inhibit adipogenesis remain unclear. In the present study, it was demonstrated that chemerin/CMKLR1 signaling drives the adipogenesis of BMSCs. Simvastatin downregulated the expression of chemerin, thereby inhibiting chemerin/CMKLR1 signaling and adipogenesis. It was also founde that PPARγ induced the expression of chemerin to activate downstream chemerin/CMKLR1 signaling. Simvastatin thus exerts its inhibitory effects by inhibiting PPARγ expression.

Materials and methods

Isolation, culture and identification of BMSCs. All animal experiments were performed in compliance with the research guidelines and were approved by the Ethics Committee of the Shanxi Medical University (no. 2017005). The rats were provided with free access to food and water. They were housed in a facility at 25˚C, 75% humidity, with a 12-12 h light/dark cycle. BMSCs were isolated from one 4-week-old specific pathogen-free (SPF) female Sprague-Dawley (SD) rat (Vital River Laboratory), as previously described (30). The primary cells were cultured in minimum essential medium, α-modification (α-MEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution in 75-cm^2 flasks. An atmosphere of 5% CO_2 and 37˚C was maintained. The medium was changed every 72 h until 80% confluency was achieved. The BMSCs were trypsinized by treatment with 0.05% trypsin-0.02% EDTA solution and passaged at a split ratio of 1:3. Once the same level of confluence was obtained, the cells at passage 3 were trypsinized and seeded at a density of $1.0x10^4$ cells/cm^2 in 6-cm dishes or 6-well plates pre-coated with 0.1% gelatin solution.

According to the International Society for Cellular Therapy position statement (31), the identification of the cells was performed by examining the morphological features and flow cytometry, as previously described (30). The differentiation capacity of the BMSCs was also detected using SD rat bone mesenchymal stem cell osteogenic differentiation medium kit (RASMX-90021) and adipogenic differentiation medium kit (RASMX-90031), according to the manufacturer's instructions [Cyagen Biosciences (Guangzhou) Co., Ltd.].

Flow cytometry. Flow cytometry was performed according to a previously published method (30). BMSCs at passage 3 were trypsinized according to the method described above, and were then centrifuged at 179 x g for 4 min at room temperature. The cell concentration was then adjusted to $1.0x10^7$ cells/ml. Subsequently, the cells were incubated with antibodies (BD Biosciences) against CD44 (FITC mouse anti-rat CD44, cat. no. 550974), CD45 (PE-Cy™5 mouse anti-rat CD45, cat. no. 559135), CD90 (APC mouse anti-rat CD90, cat. no. 561409) and CD11b (PE mouse anti-rat CD11b, cat. no. 562105) at a concentration of 10 μl of a 1/50 dilution for 10^6 cells in 100 μl. Following incubation for 30 min at 4˚C protected from light, the samples were washed twice with wash buffer and resuspended in flow cytometry buffer. Fluorescence was measured using a FACSCalibur (BD Biosciences).

Proliferation analysis of BMSCs. The proliferation assay was performed using Cell Counting kit-8 (CCK-8) according to the manufacturer's instructions (AR1160, Wuhan Boster Biological Technology, Ltd.). Briefly, BMSCs were seeded in 96-well plates (1,000 cells/well) and cultured in the maintenance medium, which was changed every 3 days, with simvastatin (HY-17502, MedChemExpress) at various concentrations (10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M). PBS was added to the control group. The CCK-8 solution (10 μl/well) was added at 1 h before absorbance values at 450 nm were read using a SPECTRA max PLUS microplate reader (Molecular Devices, LLC) to draw the growth curves.

Adipocyte differentiation. The conditioned media included basal media A and B. Basal medium A contained α-MEM, 1% penicillin-streptomycin solution (Wuhan Boster Biological Technology, Ltd.), 10% FBS (ScienCell Research Laboratories, Inc.), 10 μM insulin, 200 μM indomethacin, 500 μM 3-isobutyl-1-methylxanthine and 1 μM dexamethasone (Sigma-Aldrich; Merck KGaA). Basal medium B contained α-MEM, FBS, penicillin-streptomycin solution and insulin at the same concentrations as in basal medium A. The adipogenic induction of BMSCs followed a protocol for 3 cycles involving incubation in basal medium A for 3 days and then in basal medium B for 1 day. Subsequently, the cells were incubated in basal medium B until day 14 of differentiation. Moreover, to examine the role of PPARγ, 0.5 μM rosiglitazone (HY-17386, MedChemExpress) was used as an agonist; thus, it was not included in the adipogenic induction medium.

Simvastatin was diluted beforehand with PBS and was added to the medium at a final concentration of 10^{-7} M throughout the differentiation process. The conditioning medium of the control group contained the same amount of PBS. Oil Red O staining was performed at 14 days after induction to verify the effects of different intervention schemes of simvastatin (simvastatin incubation for 14 days; for the first 3 days; after 3 days) on adipogenesis. The adipogenic differentiated cells were washed twice with PBS, and then fixed with 2 ml of 4% neutral formaldehyde (Boster Biological Technology)

for 30 min. The cells were washed again, and subsequently stained with filtered Oil Red O solution (60% Oil Red O dye and 40% water) (Cyagen Biosciences, Inc.) at room temperature for 30 min. The Oil Red O dye was further extracted from the adipogenic cells with 1 ml isopropanol before absorbance values at 490 nm were read.

Adenovirus generation and transduction. CCX832, the only small-molecule antagonist for CMKLR1, is not commercially available (24). Therefore, the present study sought to knock down CMKLR1 (NM_008153) expression by RNA interference (RNAi). Virus packaging requires two types of plasmids. One is the packaging plasmid or auxiliary plasmid, which is used to express the capsid elements of the virus and to form the virus itself. The other is the shuttle vector, which is used to carry the shRNA expression system and is wrapped in the virus shell to form functional viral particles. An adenovirus backbone vector containing the target sequence 5'-GCAATG GCCTGGTGATTGTCA-3' was synthesized by GenePharma and co-transfected into 293A cells (Cell Bank of the Chinese Academy of Sciences) along with a shuttle vector, according to the manufacturer's instructions. The negative control carried a nonsense shRNA sequence (5'-GTT CTC CGA ACG TGT CAC GT-3'). This sequence does not target any gene in human, rat, or mouse. Adenovirus solution with a titer of 10^8 TU/ml was prepared, and a multiplicity of infection of 100 was selected for subsequent BMSC transfection. The BMSCs were seeded onto 6-cm dishes (for western blot analysis) or 6-well plates [for reverse transcription-quantitative polymerase chain reaction (RT-qPCR)] 24 h prior to transfection. The adenovirus solution was then diluted using the transfection medium, which was 10% FBS and 5 μg/ml polybrene (Sigma-Aldrich; Merck KGaA) containing α-MEM, and added into the culture dishes or plates. The transfection medium was replaced with culture medium after 24 h. After a further 24 h, the cell culture medium was replaced with the induction medium for differentiation. The differentiated cells were transfected using the adenovirus at 8 days post-differentiation with the same protocol to maintain interference efficiency. Adenovirus transfection efficiency was evaluated using an inverted fluorescence microscope (LX70; Olympus Corporation) by quantifying GFP expression. The interference efficiency was verified by the analysis of the mRNA and protein expression levels of CMKLR1.

Western blot analysis. For western blot analysis, proteins from the 14-days cultured cells of different groups were extracted using cell lysis buffer containing 1% phenylmethanesulfonyl fluoride and were centrifuged at 12,000 x g for 5 min at 4˚C. A BCA Protein Concentration Assay kit (AR0146, Wuhan Boster Biological Technology, Ltd.) was used to determine the protein concentration, according to the manufacturer's instructions. 10% SDS-PAGE gel was used to separate the proteins, 50 μg/lane of which was transferred onto a polyvinylidene difluoride membrane. The membrane was then incubated with primary antibodies for adiponectin (ab22554, mouse monoclonal antibody, 1:1,000 dilution, Abcam), CMKLR1 (ab64881, rabbit polyclonal antibody, 1:500 dilution, Abcam), β-actin (A5441, mouse monoclonal antibody, 1:10,000 dilution, Sigma-Aldrich; Merck KGaA) or β-tubulin (AF1216, rabbit monoclonal antibody, 1:10,000 dilution, Beyotime Institute of Biotechnology) overnight at 4˚C. The membrane was incubated with the appropriate secondary antibodies (adiponectin, A0216, horseradish peroxidase-conjugated goat anti-mouse IgG, 1:1,000 dilution, Beyotime Institute of Biotechnology; β-tubulin, A0208, horseradish peroxidase-conjugated goat anti-Rabbit IgG, 1:1,000 dilution, Beyotime Institute of Biotechnology; CMKLR1, 33101ES60, horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:2,000 dilution, Yeasen Biotechnology (Shanghai) Co., Ltd.; β-actin: 33201ES60, horseradish peroxidase-conjugated goat anti-mouse IgG, 1:2,000 dilution, Yeasen Biotechnology (Shanghai) Co., Ltd.) for 2 h at room temperature. The specific blotting bands were detected by electrogenerated chemiluminescence and analyzed using a Gel-Pro Analyzer (Media Cybernetics). Protein expression was normalized to that of β-tubulin (Fig. 1) or β-actin (Fig. S4). Densitometry was performed using Gelpro32 4.0 software (Media Cybernetics).

Gene expression analysis. Gene expression analysis was performed using RT-qPCR. According to the manufacturer's instructions, RNAiso Plus (9108, Takara Bio, Inc.) was used to extract total RNA from the cultured cells of the different groups. The absorbance values at 260 nm were analyzed using a biospectrometer (Eppendorf AG) to determine the concentrations of extracted RNA, 0.4 μg of which were subsequently mixed with 5X PrimeScript RT Master Mix (RR036A, Takara Bio, Inc.) and RNase-free dH_2O. The mixed solution (10 μl) was used in reverse transcription reactions in a Veriti 96-Well Fast Thermal Cycler (Applied Biosystems) to generate cDNA. The cDNA solution was then mixed with SYBR Premix Ex Taq II (2X) (RR820A, Takara Bio, Inc.), primers (0.4 μM), ROX Reference Dye, and RNase Free dH_2O prior to quantitative polymerase chain reaction. mRNA expression analysis was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) in a two-step protocol according to the following conditions: Initial denaturation for 30 sec at 95˚C, followed by 40 cycles with denaturation for 5 sec at 95˚C, and annealing and extension for 30 sec at 60˚C. All primer sequences are provided in Table I. The expression levels of PPARγ, chemerin, CMKLR1, GPR1 and adiponectin were calculated relative to those of GAPDH by the $2^{-\Delta\Delta Cq}$ method (32) for the 3, 7 and 14-day-differentiated cell samples. The melting curves obtained were examined for the specificity of the products prior to analysis.

Statistical analysis. Statistical analyses were performed using SPSS 22.0 (IBM Corp.). The results are expressed as the means ± SD. A two-tailed Student's t-test was used to compare the mean values between the 2 groups. One-way analysis of variance (ANOVA) followed by the Dunnett or LSD post hoc tests was performed in the case of multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of BMSCs. To identify the BMSCs of SD rats, flow cytometry was performed and the presence of the BMSC marker proteins, CD44 and CD90, and the absence of the

Table I. Primer sequences used for RT-qPCR.

Gene	Primer sequence (5'→3')	Product size
GAPDH	Fw: GATGCTGGTGCTGAGTATGT	104 bp
	Rv: GCGGAGATGATGACCCTTT	
PPARG	Fw: TCCCGTTCACAAGAGCTGAC	107 bp
	Rv: ATAATAAGGCGGGGACGCAG	
RARRES2	Fw: GGTGTGGACAGTGCTGATGA	169 bp
	Rv: TGGGGTCCAGTTTGATGCAG	
CMKLR1	Fw: AGTGACTGATCAGCCGAGGA	141 bp
	Rv: GATGTAGTCCGAGCCGTCAG	
GPR1	Fw: CCGGACCCTGAAGAACTCAC	86 bp
	Rv: CCCGGAAGTACAGGGTAGGA	
ADIPOQ	Fw: CAGCATTCAGCGTAGGGC	191 bp
	Rv: GAAGAGGCTCACTTTCACATCC	

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *PPARG*, peroxisome proliferator-activated receptor γ gene; *RARRES2*, chemerin gene; *CMKLR1*, chemokine-like receptor 1; *GPR1*, G protein-coupled receptor 1; *ADIPOQ*, adiponectin gene; Fw, forward; Rv, reverse.

monocyte marker, CD11b, or hematopoietic cell marker, CD45, were observed on the cell surface (Fig. 1A). The morphological features and differentiation capacity of the cultured cells were also examined and it was found that cultured BMSCs from passage 3 maintained a fibroblast-like morphology (Fig. 1B). The cells were incubated in a specific induction medium to induce their differentiation into osteoblasts or adipocytes. At 28 days post-osteogenic differentiation, calcium deposition was observed (Fig. 1B). Oil Red O staining at 21 days post-adipogenic differentiation revealed lipid accumulation within the cells (Fig. 1B). Taken together, these lines of evidence suggest that these cells were BMSCs and could be used for further experiments.

Determination of the concentration and intervention scheme of simvastatin. To determine whether various concentrations of simvastatin exert differential effects on cell growth, cell growth was monitored following simvastatin stimulation for 5 days. The results revealed that simvastatin slightly enhanced the proliferation of BMSCs at a low concentration (10^{-8} M), but inhibited cell growth at higher concentrations (10^{-5} M and 10^{-6} M) (Fig. 1C). Cells treated with 10^{-7} M simvastatin exhibited a similar growth pattern compared to the control group (Fig. 1C).

To verify the intervention scheme of simvastatin on adipogenesis, Oil Red O staining of adipogenic BMSCs treated with simvastatin for 14 days (SIM group) was performed. In addition, BMSCs were examined during the first 3 days (group A, early stage of adipogenesis) and after 3 days (group B) of adipogenesis; BMSCs treated with PBS (CON group) were also examined. The protein expression of adiponectin in the simvastatin-treated group was markedly decreased compared to that of the control group, and the lipid accumulation within the cells exhibited a similar simvastatin-dependent lipid loss (Fig. 1D and E), indicating that simvastatin inhibited the adipogenic differentiation of BMSCs during the whole process of adipogenesis. Moreover, the simvastatin-treated group (SIM group) exhibited a lower absorbance at 490 nm compared with the control group or group A (Fig. 1F). The SIM group exhibited a comparable level of absorbance with group B (Fig. 1F). In addition, the fact that the inhibitory effect was more potent in group B and the SIM group than in group A (Fig. 1F) indicated that longer stimulation with simvastatin enhanced its inhibitory effects. The P-values obtained for the data in Fig. 1 are provided in detail in Tables SI and SII.

Effects of simvastatin on PPARγ, chemerin/CMKLR1 signaling and adiponectin expression. In agreement with previous studies, the expression of chemerin increased after 3, 7 and 14 days of adipogenesis, while one of the receptors for chemerin, CMKLR1, exhibited a trend towards a decreased expression (data not shown).

Simvastatin treatment did not affect the overall kinetics of the expression patterns and exerted no marked effects on the expression of chemerin and CMKLR, compared to the control group on day 3 (Fig. 2A and B). On days 7 and 14, the simvastatin-treated cells exhibited a downregulated expression of chemerin, whereas they exhibited an upregulated expression of its receptor, CMKLR1 (Fig. 2A and B). Notably, the expression of the adipocyte marker, adiponectin, was decreased compared with the control at day 14 (Fig. 2C), suggesting that simvastatin exerted an inhibitory effect on adipogenesis. PPARγ expression increased during adipogenesis while the expression of the other chemerin receptor, GPR1, exhibited a decreasing trend (data not shown). Simvastatin treatment decreased PPARγ expression on day 3 (Fig. S1). Simvastatin treatment did not affect the expression of GPR1 on days 3 and 7, but induced GPR1 expression on day 14 (Fig. S2A). The P-values for the data in Fig. 2 are provided in detail Tables SIII-SV.

CMKLR1 is required for adipogenesis and the simvastatin-mediated inhibitory effect on adipogenesis. Since simvastatin was found to differentially regulate the expression of chemerin and its receptor CMKLR1 in BMSCs, the present study directly examined the role of chemerin/CMKLR1 signaling during the simvastatin-mediated inhibition of adipogenesis by knocking down CMKLR1 in BMSCs using

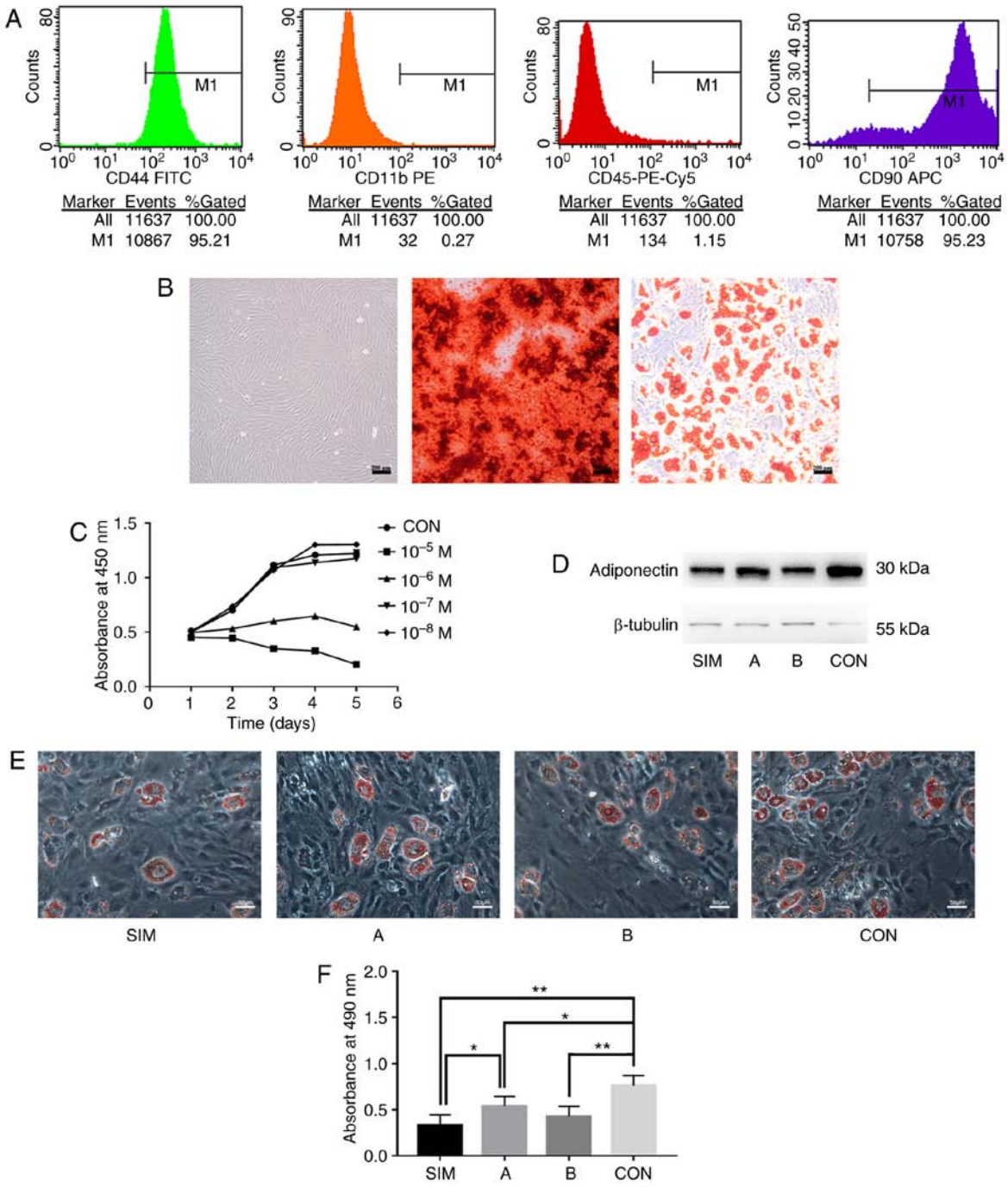

Figure 1. Determination of the concentration and scheme of simvastatin stimulation. (A) Prevalence and number of cells expressing the cell surface marker CD44, CD90, CD11b, or CD45 examined by flow cytometry. (B) Representative images of BMSCs from passage 3 under a light microscope (left panel), Alizarin Red staining after osteogenic induction (middle panel) and Oil Red O staining following adipogenic induction (right panel) (magnification, x100). (C) Growth curves of BMSCs treated with simvastatin at various concentrations or PBS (n=5). Data were examined by analysis of variance followed by Dunnett's post hoc test. (D) Western blot analysis of adiponectin levels. (E) Representative images of Oil Red O staining on differentiated BMSCs from different groups (magnification, x200). (F) Absorbance values (n=3) at 490 nm of cells as in (E). The data are expressed as the means ± SD. Data were examined by analysis of variance followed by the LSD post hoc test. *P<0.05 and **P<0.01. BMSCs, bone marrow-derived mesenchymal stem cells; SIM, BMSCs treated with simvastatin for 14 days; A, for the first 3 days; B, after 3 days; and CON, with PBS for 14 days.

RNAi. BMSCs were transfected with adenovirus at 48 h prior to adipogenic differentiation, and the immunofluorescence of each group was confirmed (Fig. S3). In comparison with the control group, a markedly lower expression of CMKLR1 at the protein (Fig. S4) and mRNA (Fig. 3A) level was detected on day 3 post-differentiation. The expression of GPR1, which shares sequence and structural similarities with CMKLR1, was also markedly decreased on days 3 and 14 post-differentiation (Fig. S2B), while a compensatory increase was not observed. Of note, the knockdown of CMKLR1 markedly inhibited adipogenesis, as evidenced by the decreased expression of adiponectin at 14 days post-differentiation (Fig. 3B). Consistently, decreased Oil Red O staining was observed in the cells in which CMKLR1 was knocked down (Fig. 3C),

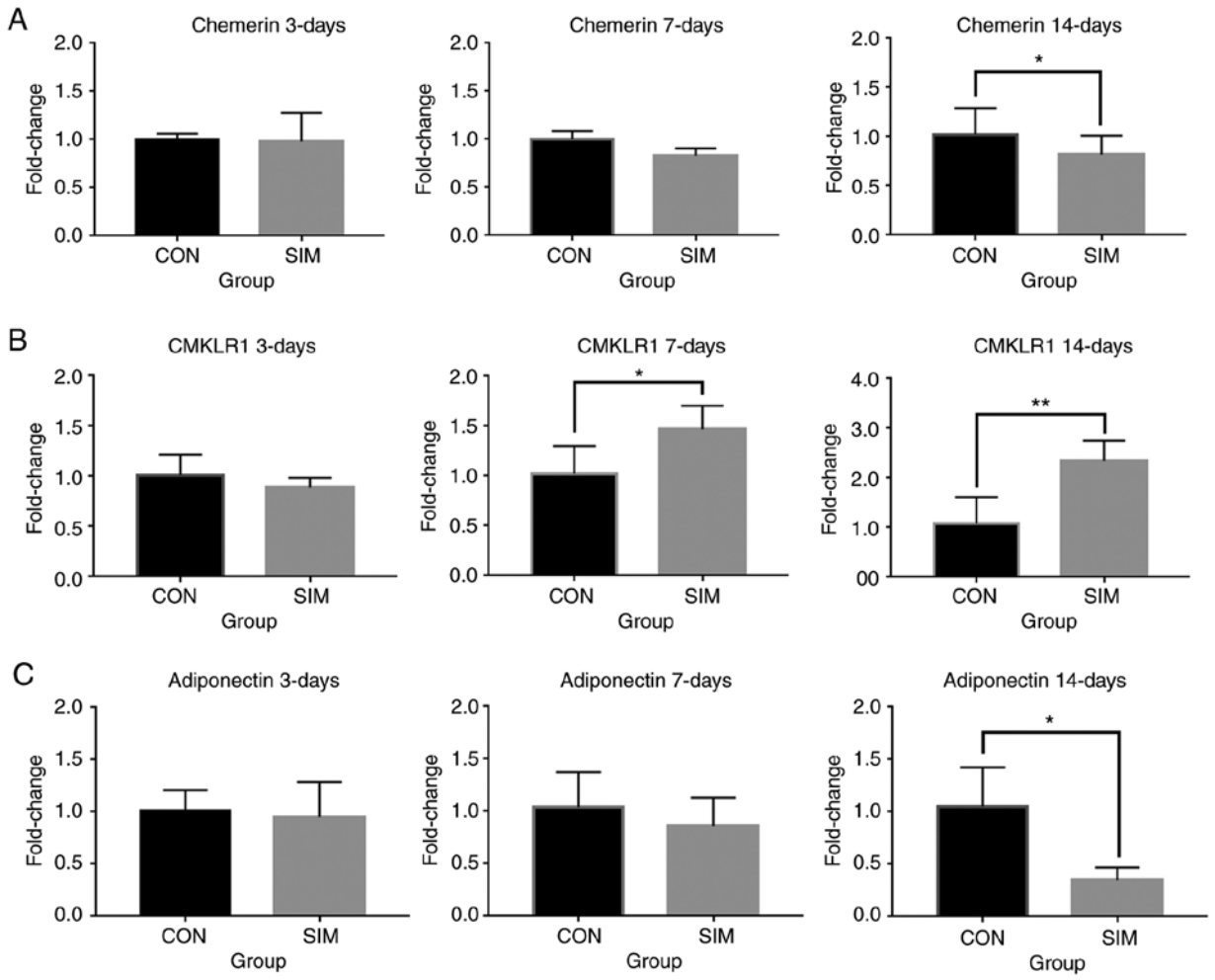

Figure 2. Effects of simvastatin on gene expression in BMSCs during adipogenic differentiation. Expression levels of (A) chemerin, (B) CMKLR1, and (C) adiponectin after simvastatin (SIM) or PBS (CON) treatment at days 3, 7 and 14 post differentiation analyzed by RT-qPCR (n=3). All RT-qPCR assays were performed 3 times and in triplicate. Fold change was expressed relative to the CON group, and the data are expressed as the means ± SD. Data were analyzed by a Student's t-test. *P<0.05 and **P<0.01. BMSCs, bone marrow-derived mesenchymal stem cells; CMKLR1, chemokine-like receptor 1.

which was further confirmed by quantitative analysis (Fig. 3D), suggesting that the inhibition of chemerin/CMKLR1 signaling prevented the adipogenesis of BMSCs. Notably, simvastatin stimulation did not further decrease the expression of adiponectin, or Oil red O staining in the cells in which CMKLR1 was knocked down (Fig. 3C and D), suggesting that the inhibitory effects of simvastatin on adipogenesis of BMSCs are dependent on chemerin/CMKLR1 signaling. This is consistent with the negative regulation of chemerin expression by simvastatin stimulation observed above (Fig. 2), further supporting the notion that simvastatin negatively regulates adipogenesis by modulating chemerin/CMKLR1 signaling. The P-values for the data presented in Fig. 3 are provided in detail in Tables SVI-SVIII.

PPARγ agonist, rosiglitazone, partially reverses the effects of simvastatin on the expression of specific genes. To further determine whether PPARγ targets chemerin/CMKLR1 signaling during the simvastatin-mediated inhibition, the cells were treated with the PPARγ agonist, rosiglitazone. On days 3 and 14, rosiglitazone significantly increased the expression of PPARγ (Fig. 4A) and chemerin (Fig. 4B), suggesting that PPARγ indeed activates chemerin/CMKLR1 signaling downstream. Moreover, rosiglitazone also partially reversed the downregulation of PPARγ and chemerin induced by simvastatin on days 3 and 14 (Fig. 4A and B). The expression of CMKLR1 and the other receptor GPR1 were decreased by treatment with rosiglitazone (Figs. 4C and S2C). Consistent with an increase in the expression of chemerin, an increase in adipogenesis was observed in the rosiglitazone-treated BMSCs following simvastatin stimulation, as evidenced by the higher expression of adiponectin at 14 days compared to the control group (Fig. 4D). The P-values for the data presented in Fig. 4 are provided in detail in Tables SIX-SX.

Taken together, these data suggest that simvastatin inhibits PPARγ-mediated chemerin signaling to prevent adipogenesis in BMSCs and that the activation of PPARγ signaling by rosiglitazone is able to reverse, at least partially, such an inhibitory effect.

Discussion

In addition to clinical applications for the treatment of hyperlipidemia and cardiovascular diseases, statins reduce the production of mevalproic acid and subsequent downstream products involved in various physiological and pathological

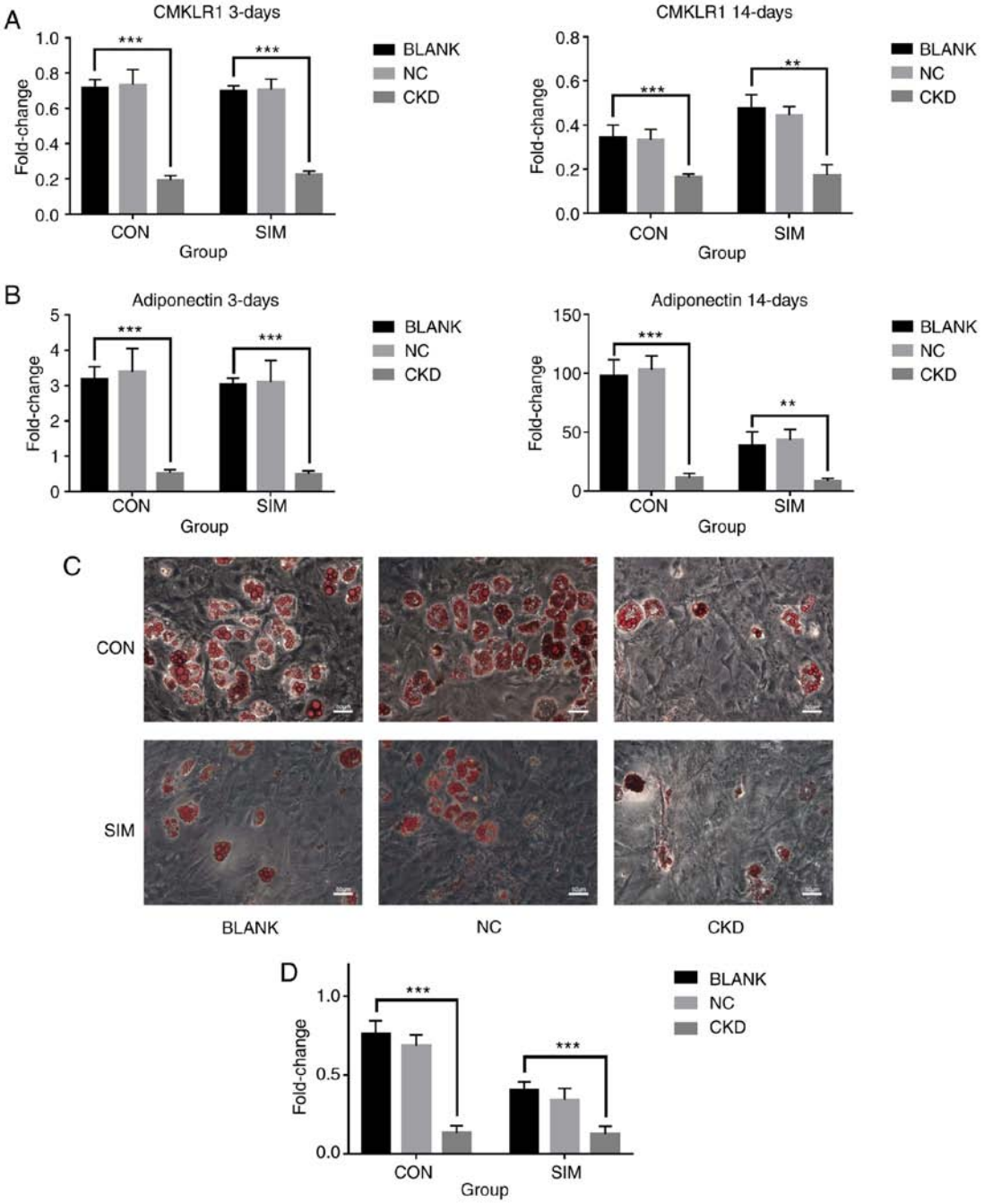

Figure 3. CMKLR1 is required for driving adipogenesis. BMSCs were transfected with or without adenovirus 48 h before differentiation. At days 3 and 14 post-differentiation, cells that exhibited adipogenic differentiation with PBS (CON) or simvastatin (SIM) treatment were analyzed by RT-qPCR for (A) CMKLR1, and (B) adiponectin expression (n=3). Fold change was expressed relative to undifferentiated BMSCs. (C) Representative images of Oil red O staining on BMSCs at days 14 post-differentiation from different groups (magnification, x200). (D) Absorbance values (n=3) at 490 nm of cells as in (C). The data are expressed as the means ± SD. Data were examined by analysis of variance followed by the LSD post hoc test. **P<0.01 and ***P<0.001. BMSCs, bone marrow-derived mesenchymal stem cells; CMKLR1, chemokine-like receptor 1; BLANK, untransfected cells; NC, negative control; CKD, CMKLR1 knockdown.

processes (33). These are the so-called statin pleiotropic effects, one of which is on adipogenesis through the chemerin signaling (29,34). Other examples are that statins may activate Wnt/β-catenin signaling (35,36) and inhibit Rho (37,38) in different biological processes, and coincidentally, both CMKLR1 and GPR1 mediate chemerin signaling through the RhoA/ROCK pathway (39), and the former has been proven to be a Wnt target gene (27). Thus, the regulation of chemerin signaling via multiple mechanisms may, at least partly, account for the pleiotropic effects of statins. Nevertheless, although this coincidence indicates a possible link between chemerin signaling and statins, no direct evidence has been found to date. To the best of our knowledge, the present study is the first to bridge the inhibitory effect of simvastatin on adipogenesis of BMSCs and chemerin signaling through the regulation of PPARγ (Fig. 5).

Owing to evidence that the post-confluence clone expansion of BMSCs may influence adipogenesis (40), and

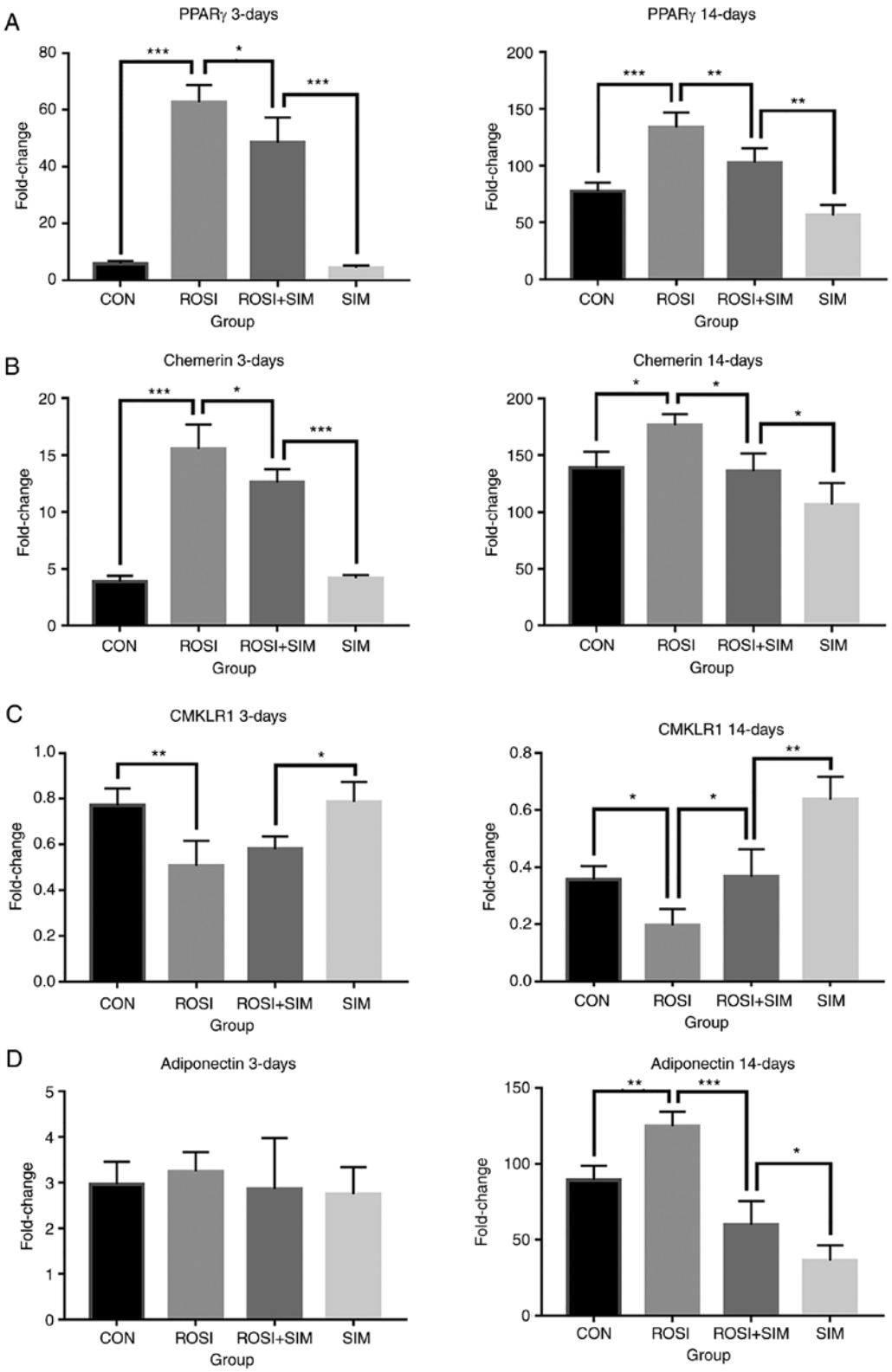

Figure 4. PPARγ agonist rosiglitazone partly reverses the effects of simvastatin on the expression of specific genes. At days 3 and 14 post-differentiation, cells that exhibited adipogenic differentiation with PBS (CON), rosiglitazone (ROSI), rosiglitazone and simvastatin (ROSI + SIM) and simvastatin (SIM) treatment were analyzed by RT-qPCR for expression of (A) PPARγ, (B) chemerin, (C) CMKLR1, and (D) adiponectin (n=3). Fold change was expressed relative to undifferentiated BMSCs, and the data are expressed as the means ± SD. Data were examined by analysis of variance followed by the LSD post hoc test. *P<0.05, **P<0.01 and ***P<0.001. BMSCs, bone marrow-derived mesenchymal stem cells; CMKLR1, chemokine-like receptor 1; PPARγ, peroxisome proliferator-activated receptor γ.

that simvastatin had effects, though with controversy, on BMSC proliferation (41,42), the present study selected the concentration of simvastatin which minimally influenced proliferation to minimize its effect on proliferation-related adipogenesis. Furthermore, although the early stage is of vital importance for adipogenesis (28), simvastatin intervention was

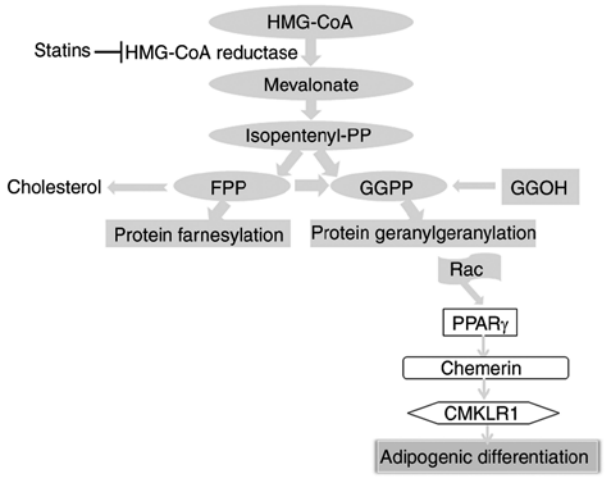

Figure 5. Summary of the effects of statins on adipogenic differentiation. The present study provides an explanation of the bridge between the inhibitory effect of simvastatin and adipogenesis in BMSCs and chemerin signaling through the regulation of PPARγ. Simvastatin cannot affect adipogenesis without functional CMKLR1. Hence, CMKLR1 mediates the downregulated signals of PPARγ and chemerin in the process of simvastatin-mediated inhibition of adipogenesis. HMG-CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl diphosphate; GGOH, geranylgeraniol; PPARγ, peroxisome proliferator-activated receptor γ; CMKLR1, chemerin chemokine-like receptor 1.

performed in the whole adipogenic differentiation process of BMSCs to maintain its continuous role in adipogenesis and its possible role on the expression of PPARγ and chemerin signaling. It has been reported that the decreased expression of PPARγ leads to the simvastatin-mediated inhibition of adipogenesis (43,44), and chemerin, which plays vital roles in adipogenesis, was a target gene of PPARγ in adipogenesis of BMSCs (20). The results of the present study demonstrated that the expression of both genes increased significantly during the adipogenic differentiation process, and simvastatin intervention inhibited such increase, demonstrating that the inhibitory effect of simvastatin may be due to the downregulation of PPARγ and chemerin signals.

It is noteworthy that while the chemerin receptor CMKLR1 expression was upregulated by simvastatin, chemerin-mediated adipogenesis was still inhibited by simvastatin. This may be due to that the decreased expression of chemerin by simvastatin stimulation overrides the elevated expression of CMKLR1, thereby leading to the inhibition of chemerin-CMKLR1 signaling and reduced adipogenesis. Similarly, the PPARγ agonist rosiglitazone partially rescued the inhibitory effect of simvastatin but reduced the levels of CMKLR1. In this case, the upregulated expression of chemerin by PPARγ activation may override CMKLR1 downregulation leading to increased chemerin-CMKLR1 signaling and adipogenesis. Indeed, PPARγ has been shown to decrease β-catenin-dependent CMKLR1 expression by promoting the degradation of β-catenin (29). Moreover, consistent with the findings of the present study, the downregulation of CMKLR1 is associated with an enhancement of chemerin/CMKLR1 signaling leading to bone resorption activity in mature osteoclasts (28).

Apart from the expression of PPARγ, an increase in chemerin expression was also detected by rosiglitazone intervention in the present study, and a possible role of PPARγ in the simvastatin-mediated inhibition of adipogenesis was demonstrated. Moreover, the decreased expression of both chemerin receptors, CMKLR1 and GPR1, along with adipogenesis, was further reduced in the context of rosiglitazone, and such a decrease was partially reversed by simvastatin intervention. To further validate this hypothesis, RNAi was used to knock down the expression of CMKLR1. Simvastatin treatment did not exert a further inhibition of adipogenesis with CMKLR1 knockdown; in other words, simvastatin intervention did not affect adipogenesis without functional CMKLR1. Hence, CMKLR1 mediates the downregulated signals of PPARγ and chemerin in the process of simvastatin-mediated inhibition of adipogenesis (Fig. 5).

Considering the controversies in functional studies of CMKLR1, researchers have proposed that the functional differences of CMKLR1 may partlly be due to the activation of GPR1 by chemerin (28). The results of the present study demonstrated a similar decrease in the gene expression levels of CMKLR1 and GPR1 during adipogenesis, and the decreased expression of both receptors was inhibited by simvastatin, thus suggesting that GPR1 may function similarly as CMKLR1 during this process. Nevertheless, although both receptors can modulate the osteogenic differentiation of BMSCs, only CMKLR1 was proven to be effective in adipogenesis. Surprisingly, it was found that GPR1 expression markedly decreased after CMKLR1 knockdown, and this decrease was not promoted by simvastatin. These results indicate that in the absence of CMKLR1, GPR1 may not significantly affect the inhibitory effect of simvastatin, and the increase in the expression of GPR1 with simvastatin may simply be due to the inhibitory effect of simvastatin on adipogenesis. Moreover, GPR1 showed lower expression than CMKLR1, demonstrating that the function of GPR1 may not be as important as that of CMKLR1 during adipogenesis. This result was in accordance with the findings of the study by Rourke *et al*, that GPR1 did not contribute to adipogenesis (45). Despite the expression data on GPR1 from the present study, the role of this receptor in the adipogenesis of BMSCs remains to be further revealed.

In the present study, the effects of simvastatin on chemerin and CMKLR1 expression were not verified at the protein level, and translation, post-translation processing and modification, and different detection times may lead to inconsistent mRNA and protein levels. It is not known whether the lipid-inhibiting effect of simvastatin is directly achieved through the chemerin and CMKLR1 proteins, or whether it affects proteins downstream of the chemerin signaling pathway. This is a limitation of the present study. Therefore, the expression of chemerin and CMKLR1 at the protein level may or may not be consistent with the changes at the RNA level. To solve the problem of gene expression differences at different levels, well-designed experiments are required to eliminate the interference of various factors in gene translation.

In conclusion, for the first time, to the best of our knowledge, the present study demonstrates that PPARγ bridges the inhibitory effect of statins on adipogenesis and chemerin signaling. Simvastatin inhibits the adipogenesis of BMSCs through the downregulation of PPARγ and subsequently prevents PPARγ-mediated induction of chemerin/CMKLR1 signaling. This partially explains how simvastatin inhibits the

adipogenesis of BMSCs and provides new ideas for the clinical application of statins on osteoporosis treatment. Considering that there may be more possible connections between chemerin signaling and the pleiotropic effects of statins, more information about the relevant downstream events must be obtained to have a better understanding of their interplay.

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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.

Authors' contributions

YG was the main contributor to the conception and design of the present study and in the drafting of the manuscript. JH and DW played an important role in data analysis and contributed to the writing of the manuscript. HH and XJ performed the experiments of cell culture, cell identification and RNAi. EZ and BN performed RT-qPCR and western blot analysis, and analyzed the collected data. QL played an important role in the conception and design of the study, and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed in compliance with the research guidelines and approved by the Ethics Committee of the Shanxi Medical University (no. 2017005).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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