Biochemical and Biophysical Research Communications 456 (2015) 598–604

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Zinc finger protein 467 regulates Wnt signaling by modulating the expression of sclerostin in adipose derived stem cells

Li You *, Lin Chen, Ling Pan, Wen-sha Gu, Jin-Yu Chen

Department of Endocrinology and Metabolism, Shanghai First People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, China

ARTICLE INFO

Article history:
Received 18 November 2014
Available online 6 December 2014

Keywords:
Zfp467
Sost
Wnt signaling
ADSCs

ABSTRACT

Osteoporosis is a metabolic disease in which a disruption of the balance between bone formation by osteoblasts and bone resorption by osteoclasts leads to the progressive deterioration of bone density and quality. Tissue engineering approaches to the treatment of osteoporosis depend on the identification of factors that promote the differentiation of progenitor cells towards an osteoblastic phenotype. In the present study, we expanded on prior findings on the role of zinc finger protein 467 (Zfp467) in the osteoblastic differentiation of adipose-derived stem cells (ADSCs) and explored the underlying mechanisms. We showed that Zfp467 binds to and regulates the expression of the *SOST* gene, which encodes a secreted glycoprotein named sclerostin (Sost) that is expressed exclusively by osteocytes and functions as a negative regulator of bone formation through the modulation of Wnt signaling. Overexpression of Zfp467 in ADSCs inhibited Wnt signaling by promoting binding of Sost to the Wnt coreceptors LRP5/6 and disrupting Wnt induced Frizzled-LRP6 complex formation, and siRNA mediated Sost silencing reversed the inhibition of Wnt signaling by Zfp467 in ADSCs. Our results indicate that Zfp467 regulates the differentiation of ADSCs via a mechanism involving Sost-mediated inhibition of Wnt signaling, suggesting potential therapeutic targets for the treatment of osteoporosis.

1. Introduction

Osteoporosis is a skeletal disorder characterized by an imbalance between bone formation and bone resorption, resulting in bone loss and an increased risk of fracture as a consequence of reduced bone density and quality [1,2]. Osteoporosis affects an estimated 200 million people worldwide, and approximately 40% of postmenopausal women with osteoporosis and 15–30% of men with osteoporosis sustain one or more fractures in their lifetime [3]. Stem/progenitor cell defects that favor adipocyte over osteoblastic differentiation have been implicated in osteoporosis development [4,5]. Adipose-derived stem cells (ADSCs) have the capacity to differentiate into adipogenic, osteogenic, myogenic and chondrogenic lineages and their use in the treatment of skeletal defects including osteoporosis has been investigated extensively [6].

Therapies for skeletal disorders such as osteoporosis need to be capable of stimulating bone formation without increasing bone resorption [7] and modify the expression of specific target molecules without affecting other organs. Secreted molecules expressed predominantly in bone tissue meet these requirements. Sclerostin (Sost) is a negative regulator of bone formation produced exclusively by osteocytes that has been explored as a potential target for the treatment for osteoporosis [8,9]. Loss of function or deletion mutations in the *SOST* gene, which are associated with sclerosteosis and van Buchem disease, respectively, result in increased osteoblast activity and an increased rate of bone formation [10]. Sost binds to the Wnt coreceptors lipoprotein receptor related (LRP)5 and LRP6 and inhibits the canonical Wnt pathway, suggesting that the increased bone formation in diseases caused by the downregulation of SOST are the result of the activation of the Wnt pathway.

Wnt signaling is involved in the regulation of several cellular processes and plays a critical role in the commitment of pluripotent mesenchymal cells (MSCs) [11]. Wnt proteins bind to a coreceptor complex formed by proteins of the Frizzled (Fzd) family and LRP 5/6 proteins. In the canonical Wnt pathway, the inactivation of a destruction complex that targets β-catenin for proteasomal degradation results in the translocation of β-catenin to the nucleus, where it displaces Groucho from T cell factor/

* Corresponding author at: Department of Endocrinology and Metabolism, Shanghai First People's Hospital, Shanghai Jiao Tong University School of Medicine, 100 Haining Road, Shanghai 200080, China.
E-mail address: youlisky2002@126.com (L. You).

http://dx.doi.org/10.1016/j.bbrc.2014.11.120
0006-291X/

lymphoid enhancer factor (TCF/LEF) to promote the transcription of Wnt target genes [12].

Previous work from our group showed that zinc finger protein 467 (Zfp467), a regulator of osteoblast and adipocyte commitment, plays an important role in the differentiation of ADSCs [13]. Silencing of Zfp467 favored osteoblast lineage commitment *in vitro* and restored bone function and structure in a mouse model of osteoporosis. Zfp467, also known as EZI, belongs to the Krüppel-like family of transcription factors, a group of zinc finger DNA-binding proteins that regulate gene expression and are implicated in cell proliferation, differentiation and development [14]. Zfp467 expression is suppressed by bone formation factors such as parathyroid hormone (PTH) and the cytokines OSM and CT-1, and Zfp467 overexpression stimulates the differentiation of precursor cells towards the adipocyte lineage. Zfp467 interacts with and enhances the activity of the transcription factor signal transducer and activator of transcription 3 [15].

In the present study, we explored the role of Zfp467 in the differentiation of ADSCs and elucidated a potential mechanism involving the regulation of Wnt signaling through the modulation of the expression of Sost in ADSCs. Our findings support the potential application of Zfp467 silencing for the treatment of bone disorders and as a target for cell therapy.

2. Materials and methods

2.1. Isolation and culture of ADSCs

Mouse abdominal adipose tissues were obtained following the Animal Research Guidelines of Shang Jiao Tong University School of Medicine. ADSCs were isolated from lipoaspirate and then cultured in DMEM (HyClone, Logan, UT) supplemented with 10% FBS (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin as described previously [16]. Differentiation of ADSCs into adipocytes and osteoblasts was monitored by oil red O staining of lipid droplets and von Kossa staining of calcium deposits in the extracellular matrix, respectively.

2.2. Vector construction and subcloning

Subcloning was performed using a previously described method with modifications [17]. Briefly, fragments of the Sost promoter region were generated by PCR and subcloned into the XhoI and MluI sites of the pGL-3 vector (Promega, Madison, WI). Primer sequences were designed based on the published sequence of the Sost promoter and were as follows: Sost, (1) SOST-Xho-3: 5′GCG CCT CGA GTG TCC AGC CTA GAT ACG GTT G, (2) SOST-Mlu-1K-5:

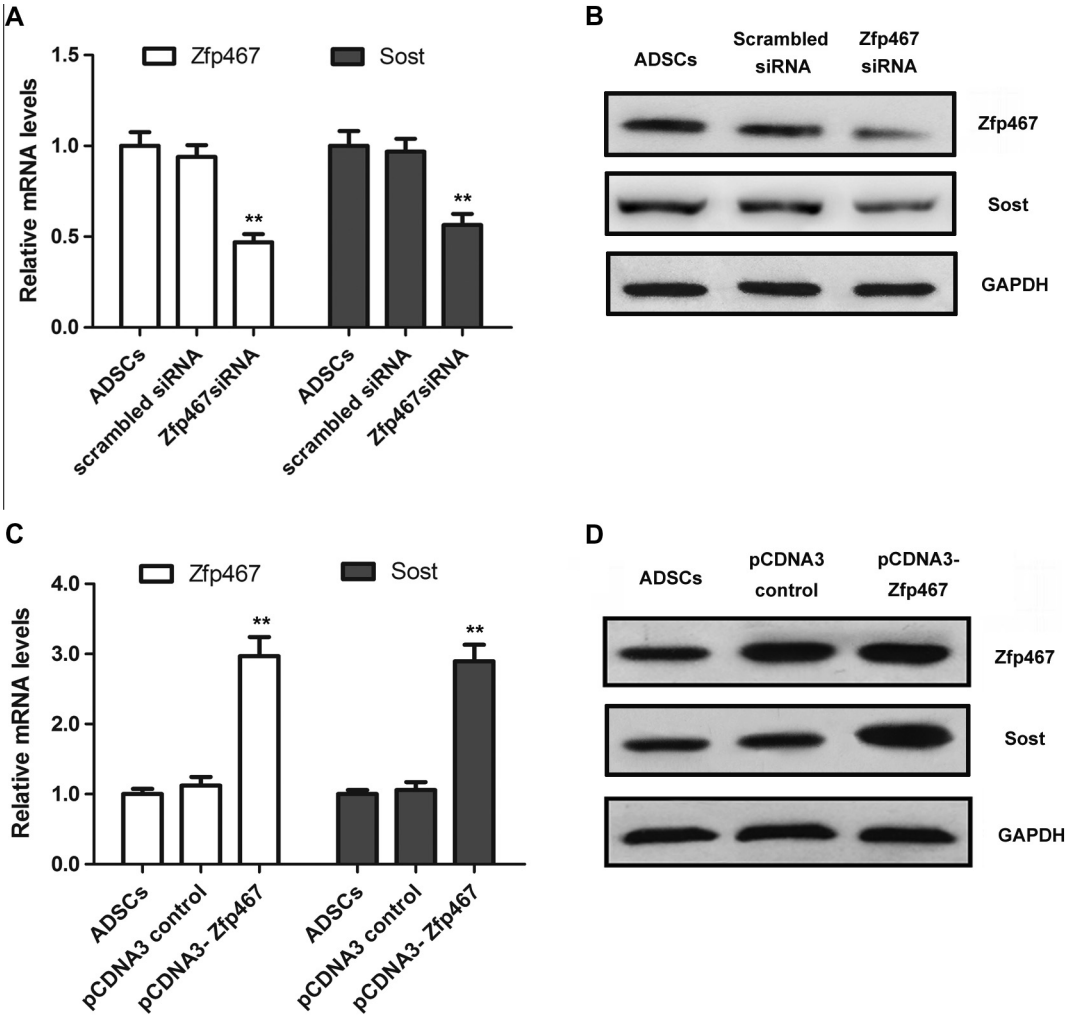

Fig. 1. Effect of Zfp467 on Sost gene expression. The mRNA (A and C) and protein (B and D) levels of Zfp467 and Sost were measured in ADSCs transfected with Zfp467 siRNA, scrambled siRNA, pCDNA3-Zfp467 or pCDNA3 control vectors by qRT-PCR and western blotting. Data are representative of three independent experiments and are expressed as the mean ± SD. $^{**}p < 0.01$.

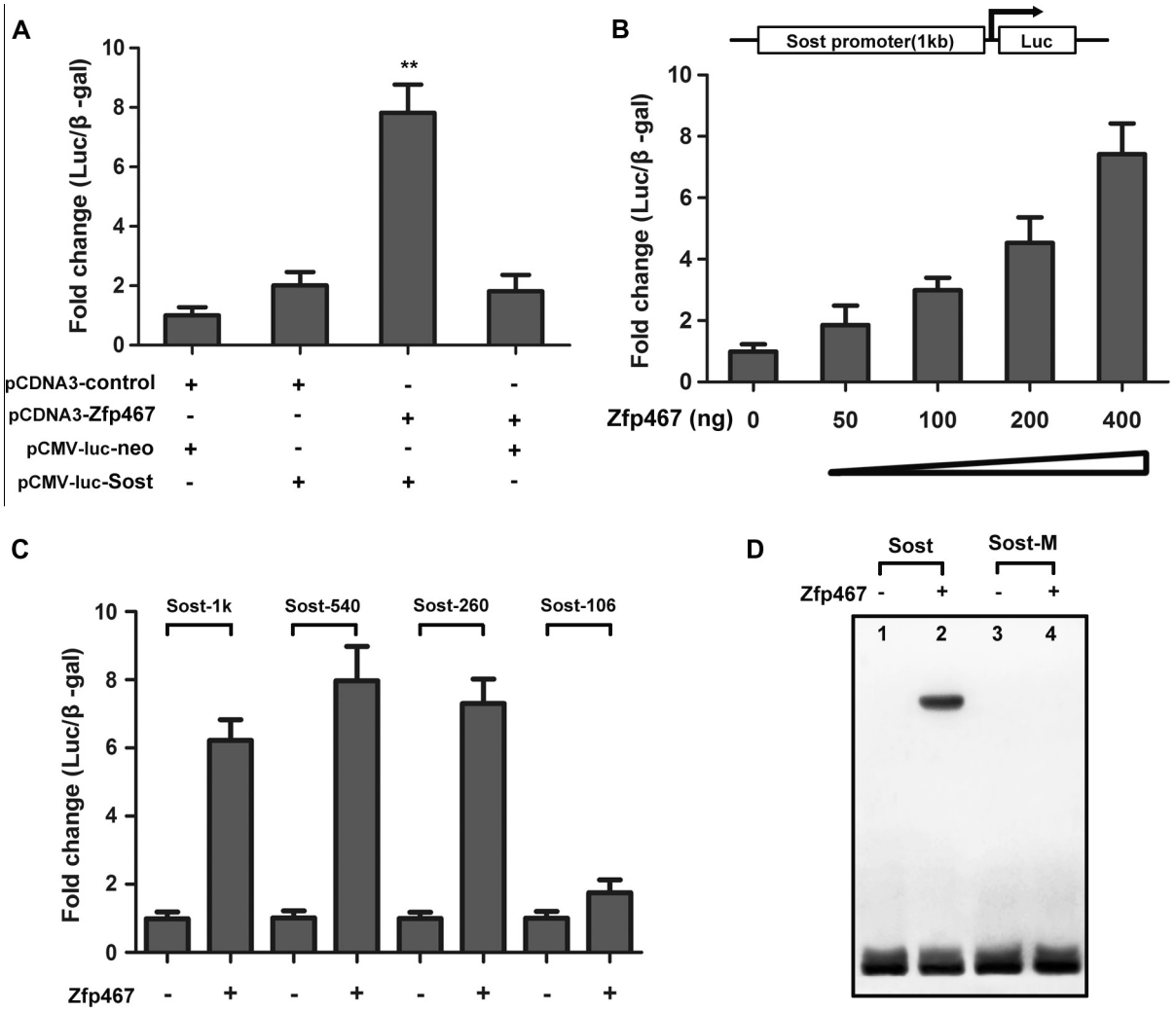

Fig. 2. Effect of Zfp467 on Sost promoter activity. (A) ADSCs were transiently co-transfected with pCDNA3-Zfp467, pCDNA-3 control, pGL3-Sost or pGL3-neo and firefly luciferase activity was measured and normalized to that of Renilla luciferase. Values are presented as the mean ± SD. $^{**}p < 0.01$. (B) Zfp467 stimulates Sost promoter activity in a dose-dependent manner. ADSCs were transfected with a 1 kb Sost promoter-luciferase reporter gene with or without increasing amounts of pCDNA3-Zfp467 plasmid as indicated. Luciferase activity was normalized by β-galactosidase activity. Values are presented as the mean ± SD. (C) Deletion analysis of the Sost promoter-reporter constructs. ADSCs were cotransfected with luciferase reporter vectors containing fragments of the Sost promoter of different length as indicated together with 200 ng of a Zfp467 expression plasmid, and luciferase activity was measured after 24 h. Data are expressed as fold change after normalization to β-galactosidase activity and represent the mean ± SD. (D) Zfp467 directly binds to a GC-rich sequence in the Sost promoter. A P^{32}-labeled oligo corresponding to a Sost GC-rich sequence within 260 bp of the Sost promoter region or a point mutant (Sost-M) was incubated with Zfp467 and binding was analyzed by gel shift assay. Nuclear extracts isolated from ADSCs were used as the protein source.

5′GCG CAC GCG TGA AAG ACA CCT CCT CAG GTC, (3) Sost-Mlu-540: 5′GCG CAC GCG TAA GGC ATC CTT CTG, (4) Sost-Mlu-260: 5′GCG CAC GCG TTG TGT CCC TGC CTC, (5) Sost-Mlu-106: 5′GCG CAC GCG TTG AGG AGG AGG GTG A. For examining the Wnt signaling, pGL3-TCF/LEF reporter plasmid was generated by inserting six multimerized oligonucleotides containing LEF-1 binding sites into the XhoI and MluI sites of the pGL-3 vector [18]. Zfp467 expression plasmid was generated by insertion of mouse Zfp467 cDNA (BC029859, MGC: 35888, IMAGE: 4457967) into pCDNA3 (Invitrogen, Carlsbad, CA).

2.3. Transient transfection assay

ADSCs were cultured as described above, plated in 12-well plates and grown to 60–80% confluence before transfection using FuGENE6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cells were co-transfected with 300 ng of Sost or TCE/LEF promoter luciferase reporter, the Zfp467 and/or Wnt-1 expression plasmid and 25 ng of pSV2-β-gal and incubated for 24 h. Luciferase activity was measured using a BD Monolight system (BD Biosciences, San Jose, CA) and normalized to β-galactosidase activity. All experiments were repeated at least three times and data are expressed as the mean ± standard deviation (SD).

2.4. Small interfering RNA vector construction

The Zfp467-SiRNA vector was generated in the pBShH1 plasmid DNA as described previously [13]. For silencing of Sost, ADSCs were transfected with siRNA against mouse Sost using Lipofectamine 2000 (Invitrogen) as previously described [19].

2.5. Electrophoretic mobility shift assay (EMSA)

To identify transcription factor binding sites, DNA binding proteins were detected using a Gel Shift Assay System (Promega). P^{32}-labeled oligos corresponding to a Sost GC-rich sequence

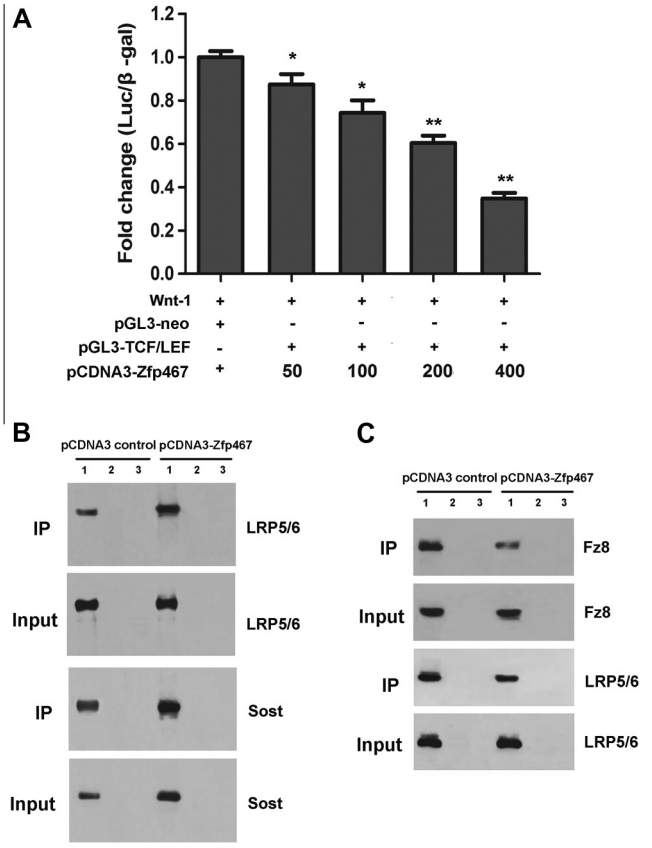

Fig. 3. Effect of Zfp467 on TCF/LEF-dependent Wnt signaling in ADSCs. (A) ADSCs were transiently transfected with pGL3-TCF/LEF or pGL3-neo reporter gene expression vector together with expression plasmids encoding Wnt-1 and increasing amounts of Zfp467. Luciferase activity was normalized to β-galactosidase activity. Values are presented as the mean ± SD. $^{**}p < 0.01$; $^{*}p < 0.05$. (B) Zfp467 promotes the complex formation of Sost and Wnt coreceptors LRP5/6 in ADSCs. Cell lysates from ADSCs transfected with pCDNA3-Zfp467 or control vector were precipitated with anti-Sost antibody and lysates (Input) or precipitates (IP) were immunoblotted with anti-Sost and anti-LRP5/6 antibodies. Lane 1, Sost precipitates; lane 2, cell lysates precipitated with isotype control antibody; lane 3, cell lysates precipitated with PBS. (C) Zfp467 disrupts Wnt induced Frizzled-LRP6 complex formation. Cell lysates from ADSCs transfected with pCDNA3-Zfp467 or control vector were precipitated with anti-LRP5/6 antibody and lysates (Input) or precipitates (IP) were immunoblotted with anti-Fz8 and anti-LRP5/6 antibodies. Lane 1, Frizzled precipitates; lane 2, cell lysates precipitated with isotype control antibody; lane 3, cell lysates precipitated with PBS.

identified within 260 bp of the Sost promoter region [20] were incubated with Zfp467 with nuclear extracts from ADSCs isolated using the CellLytic NuCLEAR extraction kit (Sigma–Aldrich, St. Louis, MO) as the protein source. Protein–DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by autoradiography.

2.6. Immunoprecipitations and western blotting

Cell lysates for immunoblotting were prepared in lysate buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA, 0.1% SDS, protease inhibitors (Roche), 1 mM Na_3VO_4, and 1 mM NaF. Cell lysates for immunoprecipitation were prepared as described previously [21]. For immunoprecipitation (IP) experiments, lysates were precleared with 2 μg of the appropriate antibodies at 4 °C for 8 h, and then incubated with protein A-agarose and antibodies at 4 °C overnight. For IP experiments using anti-Sost or anti-LRP5/6 antibody, the cell lysates contained 1% SDS and received prior heat treatment. The precipitates were pelleted, washed three times with lysis buffer and analyzed by western blotting using standard protocols. The antibodies and concentrations used were as follows: anti-Zfp467 (1:500; R&D Systems Inc., Minneapolis, USA), anti-Sost (1:100; R&D Systems Inc.), anti-LRP5/6 (1:1000; Abcam, Cambridge, UK), anti Fz8 (1:500; Abcam), anti-GAPDH (1:2500; Abcam). Bands were visualized by detection with horseradish peroxidase conjugated protein A and the ECL system (Amersham, Little Chalfort, UK).

2.7. Real-time quantitative PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Approximately 2 μg of total RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) with oligo DT at 42 °C for 1 h. PCR conditions were as described previously [13]. Primers used were as follows: Zfp467 forward, 5′-GCA CTC CTG CTC AGG GCA-3′; reverse, 5′- CTT GTC GCA CTC TGA GCA-3′; Sost forward, 5′-CCA GGG CTT GGA GAC TAC C-3′; reverse, 5′-GCA GCT GTA CTC GGA CAC ATC-3′.

2.8. Statistical analysis

All experiments were repeated a minimum of 3 times. Data was reported as the mean ± SD. Comparisons between groups were made by Student's t test with $p < 0.05$ considered statistically significant.

3. Results

3.1. Zfp467 modulates Sost gene expression

To examine the effect of Zfp467 on the expression of Sost, Zfp467 was overexpressed or silenced and Sost levels were assessed by qRT-PCR and western blotting. SiRNA mediated silencing of Zfp467 significantly downregulated Sost expression, whereas overexpression of Zfp467 by transfection with the pCDNA3-Zfp467 vector significantly upregulated Sost expression at the mRNA (Fig. 1A and C) and protein (Fig. 1B and D) levels compared to scrambled siRNA or untransfected control ADSCs ($p < 0.01$). These results indicate that Zfp467 is a positive regulator of Sost expression.

3.2. Zfp467 interacts with and stimulates Sost promoter activity

To further examine the regulation of Sost expression by Zfp467, a luciferase reporter construct was generated and ADSCs were co-transfected with a Sost promoter reporter plasmid and pCDNA3-Zfp467 or pCDNA3-control vector and luciferase activity was measured. ADSCs co-transfected with pCDNA3-Zfp467 and pCMV-luc-Sost showed a significant increase in luciferase activity compared to cells transfected with empty vector control, indicating that Zfp467 interacts with the Sost promoter to modulate gene expression (Fig. 2A). To confirm binding of Zfp467 to the Sost promoter, a luciferase reporter construct driven by 1 kb of the Sost promoter was co-transfected with increasing amounts of pCDNA3-Zfp467. As shown in Fig. 2B, luciferase activity increased in correlation with the amounts of Zfp467, indicating that Zfp467 dose-dependently stimulates Sost promoter activity. To identify the Zfp467 binding site, deletion analysis was performed by generating Sost luciferase reporter constructs driven by different lengths of the Sost promoter (Fig. 2C). The results of luciferase assays showed that Zfp467 stimulated Sost promoter reporter activity at 1k, 540 and 260 bp, whereas the Sost-106 bp reporter showed a

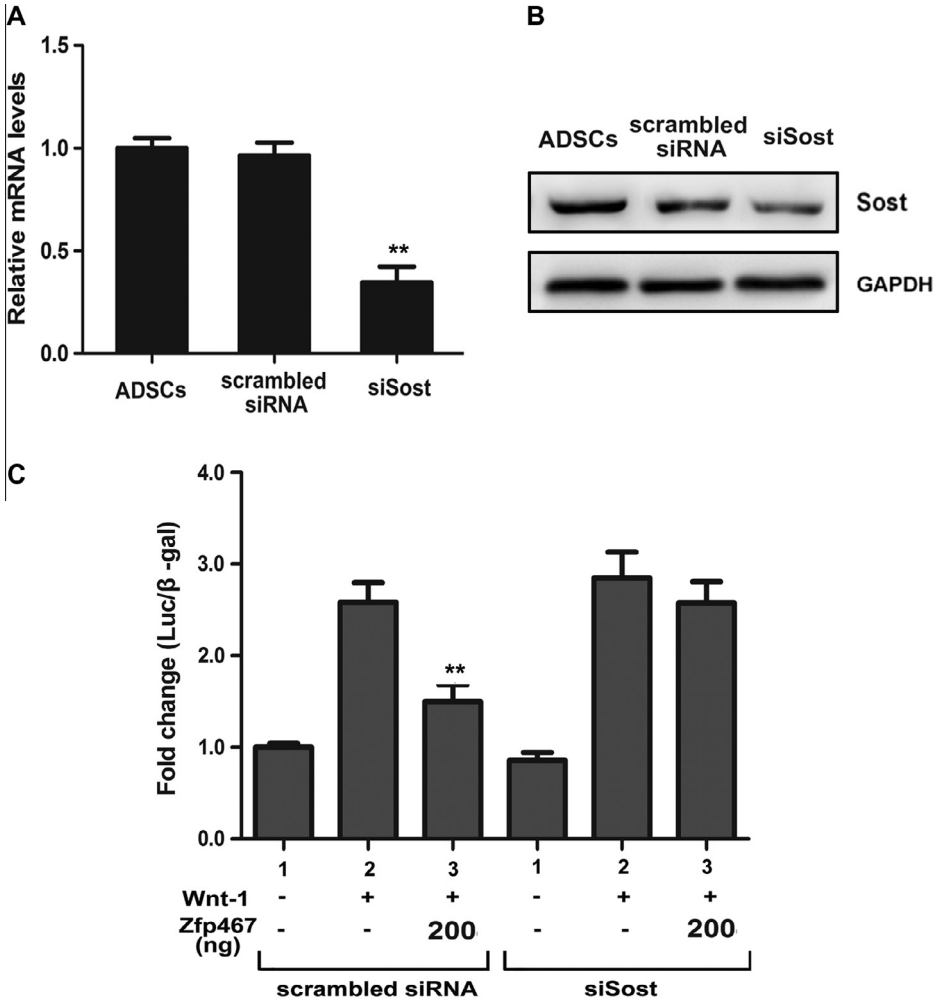

Fig. 4. Effect of Sost knock-down on Zfp467 mediated inhibition of Wnt signaling in ADSCs. (A and B) ADSCs were transfected with Sost siRNA or scrambled siRNA and the relative mRNA (A) and protein (B) levels of Sost were measured by qRT-PCR and western blotting. $^{**}p < 0.01$. (C) ADSCs transfected with Sost siRNA or scrambled siRNA were transiently transfected with pGL3-TCF/LEF reporter gene expression vector together with expression plasmids encoding Wnt-1 and Zfp467. Luciferase activity was normalized to β-galactosidase activity and expressed as fold change with respect to untransfected controls. Values are presented as the means ± SD. $^{**}p < 0.01$ vs. the second group.

marked reduction in activity, suggesting that the region of the Sost promoter between 260 bp and 106 bp contains the Zfp467 binding site. Based on a GC-rich element on the Sost promoter identified in a previous study as critical for promoter activation [20], a point mutant of Sost (Sost-M) was generated and direct binding of Zfp467 to this region was examined by gel shift assay (Fig. 2D). Radiolabeling of an oligo containing the Sost GC-rich sequence within 260 bp of the Sost promoter region and incubation with Zfp467 showed that Zfp467 bound to the GC-rich oligo (lane 2) but not to Sost-M (lane 4), confirming that Zfp467 specifically binds to the GC-rich sequence in the Sost promoter.

3.3. Zfp467 affects Wnt signaling through the modulation of Sost expression in ADSCs

Because Sost inhibits the activity of the Wnt canonical pathway by binding to LRP5/6, we investigated the effect of Zfp467 on Wnt signaling via its target Sost. To examine the effect of Zfp467 on Wnt signaling, ADSCs were transiently transfected with pGL3-TCF/LEF or pGL3-neo together with increasing amounts of an expression plasmid encoding Zfp467 in the presence of Wnt-1. Luciferase assays showed a decrease in the reporter gene activity in response to increasing amounts of exogenous Zfp467 expression, indicating that Zfp467 dose-dependently inhibits Wnt induced TCF/LEF-dependent transcriptional activity in ADSCs (Fig. 3A). To examine whether Sost is involved in the inhibition of Wnt signaling by Zfp467, the effect of Zfp467 overexpression on the interaction of Sost with LRP5/6 was assessed by co-immunoprecipitation. Immunoprecipitation with anti-Sost antibody and immunoblotting against LRP5/6 showed that overexpression of Zfp467 promoted the association of Sost with LRP5/6 (Fig. 3B). Immunoprecipitation with anti-LRP5/6 antibody and immunoblotting against Fz8 showed that co-immunoprecipitation of Fz8 with LRP5/6 was suppressed in ADSCs overexpressing Zfp467 (Fig. 3C). Taken together, these results indicated that Zfp467 inhibits the Wnt signaling pathway by promoting Sost binding to LRP5/6, resulting in the disruption of the Fz8-LRP5/6 complex and inhibition of TCF/LEF-dependent transcriptional activity.

3.4. Inhibition of Wnt signaling by Zfp467 is dependent on Sost expression

The involvement of Sost in the Zfp467-mediated inhibition of Wnt signaling was examined by si-RNA silencing of Sost in ADSCs. The result shows a significant downregulation of Sost mRNA (Fig. 4A) and protein (Fig. 4B) expression in ADSCs transfected with

Sost siRNA as determined by qRT-PCR and western blotting. The effect of Zfp467 was then examined in control or Sost knock-down cells transiently transfected with a pGL3-TCF/LEF reporter vector and expression plasmids encoding Wnt-1 and Zfp467. Luciferase activity assays showed that knock-down of Sost reversed the Zfp467 mediated inhibition of Wnt signaling in ADSCs (Fig. 4C), confirming the involvement of Sost in the effect of Zfp467 on the Wnt signaling pathway.

4. Discussion

Defects in bone regeneration caused by low numbers of stem cells and alterations in cell proliferation, migration and differentiation have been associated with osteoporosis [22]. Sclerostin overproduction and high expression of the bone morphogenetic protein (BMP) inhibitor MAB21L2 in MSCs have been identified as factors associated with regeneration defects [23]. MSCs from various sources constitute the basis of tissue engineering, either by ex vivo amplification and direct transplantation or through the use of a pre-differentiation step to enhance the osteogenic commitment before transplantation [24]. The multipotency and relative ease of isolation and culture of ADSCs makes them a valuable and high yield source for cell-based therapeutic applications. Under specific conditions, ADSCs differentiate into an osteoblast lineage, as demonstrated by the expression of the osteoblast-associated proteins osteocalcin and alkaline phosphatase, and the acquisition of bone cell-like functional properties such as responsiveness to fluid shear stress [25,26]. However, the factors that promote the commitment of ADSCs to an osteocytic lineage are not clear. Studies have shown that BMP-2 stimulates osteogenic differentiation of ADSCs [27,28], although the effect of BMP-2 is controversial [29]. The transcription factor Tbx3 and inhibition of histone deacetylase by valproic acid were shown to promote osteogenic differentiation of ADSCs [30,31]. ADSCs grown on collagen scaffolds differentiate into osteoblast-like cells [32]. However, despite extensive research into the therapeutic potential of ADSCs in the treatment of skeletal defects, their application for tissue engineering in bone disorders including osteoporosis remains limited, and the identification of factors that promote or favor the osteoblastic differentiation of progenitor cells is essential. In the present study, we examined the role of Zfp467 in the differentiation of ADSCs and elucidated a potential underlying mechanism by which Zfp467 modulates Wnt signaling by regulating the expression of its target protein Sost.

The results of the present study indicate that Zfp467 binds to a GC-rich region of the Sost promoter and is a positive regulator of Sost expression in ADSCs. Sost is a negative regulator of bone formation associated with the development of sclerostosis and van Buchem disease whose expression is restricted to osteocytes and its mechanism of action is mediated by the binding to LRP5/6 and inhibition of Wnt signaling [7]. Sclerostin has therefore been studied as a potential target for the treatment of osteoporosis and other skeletal diseases based on the hypothesis that inhibition of sclerostin would enhance Wnt signaling and stimulate osteoblastic bone formation [33]. Monoclonal antibodies against sclerostin upregulate bone formation and downregulate bone resorption markers, leading to increased bone mass, and several agents including romosozumab, blosozumab and BPS804 are currently in clinical trials to assess their efficacy in the reduction of fracture risk. Our findings showing the modulation of Sost expression by Zfp467 reveal a novel regulatory mechanism with potential therapeutic applications.

Zfp467 was identified as a co-factor promoting the differentiation of mesenchymal cell progenitors to an adipocyte lineage in a search of genes responsive to PTH, which enhances bone mass by promoting the differentiation of osteoblasts [14]. In the present study, we expanded on prior findings showing that Zfp467 regulates the differentiation of ADSCs and elucidated the underlying mechanism. We showed that the effect of Zfp467 on the differentiation of ADSCs is mediated by its regulation of the Wnt signaling pathway through the modulation of Sost expression. These findings suggest that Zfp467 could serve as a target for the treatment of bone disorders and a potential modulator of the differentiation of ADSCs, which has important implications for tissue engineering.

Acknowledgment

This work was supported by grants from Shanghai National Science Foundation (No. 13ZR1433700).

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