

# Upregulated serum sclerostin level in the T2DM patients with femur fracture inhibits the expression of bone formation/remodeling-associated biomarkers via antagonizing Wnt signaling

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**Abstract.** – **OBJECTIVE:** Bone formation/remodeling-associated biomarkers, such as osteocalcin, amino pro-peptide of type 1 collagen (P1NP) and CrossLaps (CTX) have been deregulated in type 2 diabetes mellitus (T2DM) patients. In particular, the T2DM-associated sclerostin markedly inhibits the bone formation, suppresses the osteoblast activity and downregulates the bone turnover.

**PATIENTS AND METHODS:** In the present study, we examined the serum levels of sclerostin, osteocalcin, P1NP and CTX in the T2DM patients. We evaluated the regulation on osteocalcin, P1NP and CTX by sclerostin treatment in osteoblast hFOB 1.19 cells. Finally, we determined the mediation of Wnt signaling in the regulation by sclerostin on osteocalcin, P1NP and CTX in human osteoblast hFOB 1.19 cells.

**RESULTS:** It was demonstrated that osteocalcin, P1NP and CTX were downregulated in the femur fracture of patients with T2DM, whereas the serum level of the sclerostin was markedly higher in the femur fracture of patients with T2DM. Moreover, the downregulated osteocalcin, P1NP or CTX was negatively associated with the upregulated sclerostin. In vitro results confirmed that sclerostin downregulated the expression of osteocalcin, P1NP and CTX in hFOB 1.19 cells. Also, our results demonstrated that Wnt/ $\beta$ -catenin inhibition was associated with the sclerostin-mediated inhibition of osteocalcin, P1NP and CTX in hFOB 1.19 cells. The Wnt/ $\beta$ -catenin level was markedly inhibited by sclerostin treatment, and the siRNA-mediated downregulation of  $\beta$ -catenin reduced the levels of osteocalcin, P1NP and CTX.

**CONCLUSIONS:** Our study demonstrated that the upregulated serum sclerostin level in the T2DM patients with fracture inhibited the ex-

pression of the bone formation/remodeling-associated biomarkers via antagonizing Wnt signaling. It suggests that sclerostin might be an effective target for T2DM-associated bone fracture and delayed fracture healing.

Key Words:

Bone fracture, T2DM, Sclerostin, Osteoblast cells, Wnt signaling.

## Introduction

Increased fracture risk, traditionally conceived to be associated with type 1 diabetes, has recently been of great concern in patients with type 2 diabetes. Type 2 diabetes mellitus (T2DM) is usually complicated with a decreased bone mineral density<sup>1,2</sup>, which promotes the risk of fractures<sup>3,4</sup>, and delays the fracture healing<sup>5</sup>. A variable increase ranging from 20% to 3-fold in fracture risk has been reported in T2DM, depending on the skeletal site, diabetes duration and study design<sup>6,7</sup>. Also, there are significant local inflammation responses to bone fracture. Furthermore, there are increased levels in the local fracture site of inflammatory cytokines, which coordinate the balance between cartilage production and removal, the balance between bone formation and remodeling<sup>8</sup>. In molecular levels, bone formation/remodeling-associated biomarkers have been deregulated in T2DM patients. Osteocalcin, an osteoblast-produced calcium-binding substance, can be taken as a negative biomarker for osteoporosis<sup>9,10</sup> and significant

inverse association between serum osteocalcin and T2DM occurred<sup>9,10</sup>. Amino pro-peptide of type 1 collagen (PINP) is also a serum biomarker of bone formation<sup>9,10</sup>. CrossLaps (CTX), as a predictor of changes in bone mineral density<sup>13</sup>, can also be used as biomarkers in osteoporosis patients. Sclerostin is a 213-amino acid residues long secreted glycoprotein, with a C-terminal cysteine knot-like (CTCK) domain and posing antagonizing activity against the bone morphogenetic protein (BMP)<sup>14</sup>. Sclerostin is produced by the osteocyte and has anti-anabolic effects on bone formation<sup>15</sup>, and suppresses osteoblast activity and downregulates bone turnover<sup>14</sup>. Circulating sclerostin is increased in T2DM independently of gender and age, and is also correlated with duration of T2DM<sup>16</sup>. The increased circulating sclerostin is also associated with the atherosclerotic lesions in T2DM patients, via modulating Wnt signaling<sup>17</sup>. Moreover, the increased sclerostin production in men with T2DM may be involved in the pathogenesis of increased skeletal fragility<sup>18</sup>.

In the present study, we examined the serum levels of sclerostin and other bone formation and remodeling-associated biomarkers, such as osteocalcin, PINP and CTX in the T2DM patients; then, we evaluated the promotion to osteocalcin, PINP and CTX by sclerostin treatment in osteoblast hFOB 1.19 cells. Finally, we determined the mediation of Wnt signaling in the regulation by sclerostin on osteocalcin, PINP and CTX in hFOB 1.19 cells. Our study implies the regulation by sclerostin on bone formation in osteoblast cells.

## Patients and Methods

### *Serum Samples from Femur Fracture of Patients with or without T2DM*

32 T2DM patients with femur fracture were implicated in this study. 27 cases of non-T2DM patients with hip fracture were taken as control. All serum samples from venous blood were collected from subjects with hip fracture when patients registered at the Emergency Department. Detailed characteristics of these subjects were described in Table I. Written consent was obtained from each subject before the study. The study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University.

### *Cells Medium and Reagents*

Human osteoblastic hFOB 1.19 cell line was purchased from the cell resource center of Chinese

Academy of Medical Sciences (Beijing, China) and was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA), which was supplemented with 1% Non-Essential Amino Acids (NEAA, Gibco, Rockville, MD, USA), 2 mM glutamine (Sigma-Aldrich Co., St. Louis, MO, USA), and 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), at 37°C in humidified incubator with 5% CO<sub>2</sub>. Cells with more than 80% confluence were harvested with 0.25% trypsin-EDTA solution (Ameresco, Framingham, MA, USA) and then seeded in dishes or in plates. Recombinant human sclerostin was purchased from ACROBiosystems (Newark, DE, USA) and was dissolved in dimethyl sulfoxide (DMSO) (Gibco, Rockville, MD, USA) with a storage concentration of 1 mg/ml. Wnt agonist (BML-284) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was also dissolved in DMSO at a concentration of 100 μM before use. siRNA-β-catenin (NM\_001098209) or siRNA-control (Scramble RNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and were transfected with hFOB 1.19 cells by HiPerfect Transfection Reagent (QIAGEN, Valencia, CA, USA), with a concentration of 25 or 50 nM.

### *Enzyme-linked Immunosorbent Assay (ELISA) for Sclerostin and Other Cytokines*

Solid phase enzyme-linked immunosorbent assay (ELISA) was performed to quantify serum or supernatant levels of osteocalcin, PINP, CrossLaps (CTX) or sclerostin with the ELISA kit for each marker (osteocalcin, PINP, CTX or sclerostin) (Abnova, Walnut, CA, USA) according to the kit's manual. In brief, the antibody-precoated microplates (monoclonal mouse anti-human antibody against osteocalcin, PINP, CTX or sclerostin) were firstly blocked with 1% Bovine Serum Albumin (BSA) (Ameresco, Framingham, MA, USA) at 4°C overnight, then were inoculated with serially-diluted standards or samples, and finally were incubated with the horseradish peroxidase-conjugated polyclonal antibody against osteocalcin, PINP, CTX or sclerostin). Four-time washing with 1x Tris-buffered saline containing 0.05% Tween 20 (TBS-T) was performed before each incubation. The optical density of each well was determined immediately at 450 nm.

### *Quantitatively Real-time PCR (qRT-PCR) Analysis*

mRNA samples were isolated with the Magnetic mRNA Isolation Kit (New England Biolabs,

**Table I.** Characteristics of patients with tibial fracture (T2DM/Non-T2DM).

| Characteristics                       | T2DM (n=32)  | Non-T2DM (n=27) | p-value |
|---------------------------------------|--------------|-----------------|---------|
| Age (years)                           | 47.46 ± 6.28 | 44.75 ± 4.80    | 0.4361  |
| Gender (M/F) <sup>a</sup>             | 21/11        | 17/10           | 0.8315  |
| BMI (kg/m <sup>2</sup> ) <sup>b</sup> | 28.26 ± 0.92 | 21.54 ± 0.75    | <0.001  |
| LDL (mg/dL)                           | 2.76 ± 0.98  | 2.12 ± 0.63     | <0.001  |
| HDL (mg/dL)                           | 1.11 ± 0.28  | 1.29 ± 0.29     | <0.001  |
| Fasting glucose (mM)                  | 8.10 ± 2.57  | 4.92 ± 0.64     | <0.001  |
| DM duration (years) <sup>c</sup>      | 14.52 ± 2.91 | /               | /       |
| HbA1c (%) <sup>d</sup>                | 8.94 ± 2.73  | 5.24 ± 0.55     | <0.001  |

<sup>a</sup>M: Male, F: Female; <sup>b</sup>BMI: Body Mass Index; <sup>c</sup>DM: Diabetes mellitus; <sup>d</sup>Glycated hemoglobin A1c.

Ipswich, MA, USA) following the kit's manual and were added with the SUPERase•In™ RNase Inhibitor (Thermo Scientific, Rockford, IL, USA). qRT-PCR was performed with the SYBR green One-Step RT-PCR Kit (Takara, Tokyo, Japan) accordingly. Primers for osteocalcin (Primer Forward (PF): 5'-GGCAGATCCCCCTAGACC-3', Primer Reverse (PR): 5'-CGATGAGGAGGGGCATGCCt-3'), P1NP (PF: 5'-GCTGGC-CCCAAAGGATCTCCT-3', PR: 5'-GCAGAC-CAGCTTACC GGACG-3'), Primers for CTX (PF: 5'-GAAGCTGGTCTGCCTGGTG-3', PR: 5'-ATCAGGACCAGGGCTGCCAG-3'), Primers for β-catenin (PF: 5'-AAGGAGCTAAAATGG-CAGTGC-3', PR: 5'-TGTTGAGCAAGGCAAC-CATT-3'), or for β-actin (PF: 5'-GTA CGC CTC TGG CCG TAC C-3', PR: 5'-TGG GCA CAG TGT GGG TGA-3') were synthesized by Invitrogen China (Shanghai, China). qRT-PCR was performed at 42°C for 5 minutes, at 95°C for 10 seconds, and then was performed at 95°C for 5 seconds, 60°C for 20 seconds (for 40 cycles). Relative mRNA level was calculated by ΔΔCt method, with β-actin as control<sup>19</sup>.

#### Western Blotting Assay

Cytosolic protein samples were prepared with Nuclear/Cytosol Fractionation Kit (BioVision, San Diego, CA, USA) following the kit's manual and were supplemented with a protease inhibitor cocktail (Abcam, Cambridge, UK). Cellular or nuclear protein samples were firstly separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%); then, were transferred onto the polyvinylidene fluoride hydrophobic membrane (Millipore, Bedford, MA, USA). The non-specific binding sites were blocked overnight with 2% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA); specific binding to β-catenin (cytosolic) or

to phospho-β-catenin (Ser675) (cytosolic) were examined post the incubation with rabbit-anti-human polyclone antibody against β-catenin or against phospho-β-catenin (Ser675) overnight at 4°C and post the incubation with the incubation with goat-anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA). Four-time washing of the membrane was performed with 1x TBS-T, the specific binding band was visualized with enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL, USA), with β-actin as control.

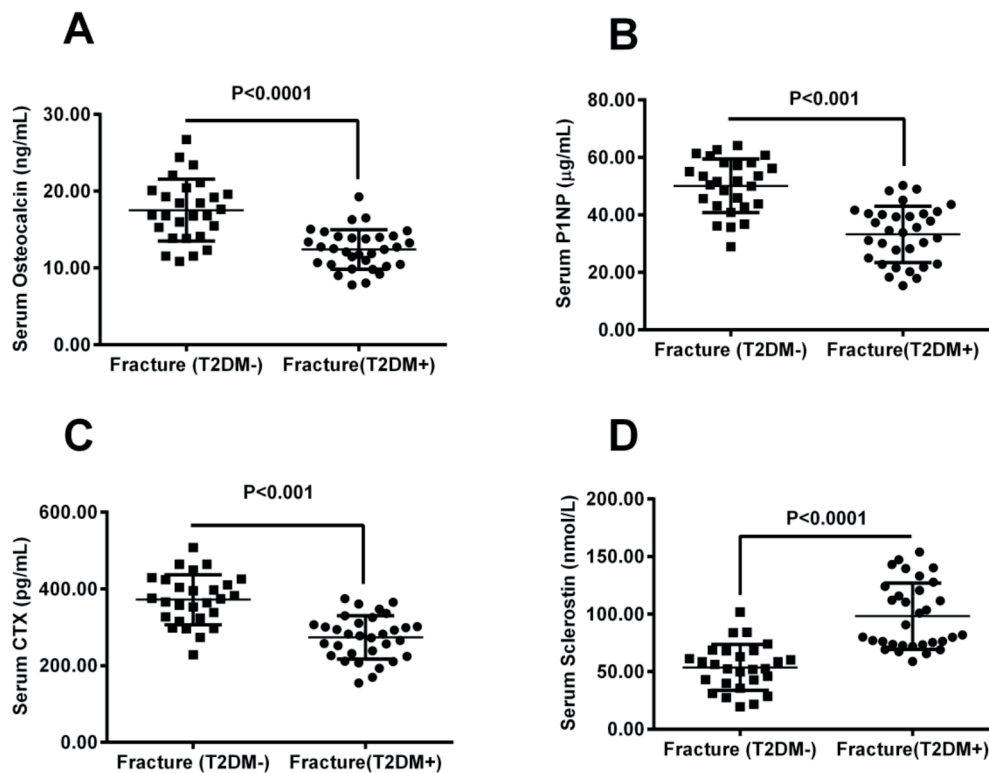
#### Statistical Analysis

Data was presented as mean ± standard error of the mean (SEM) and was analyzed using Student's *t*-test or using one-way ANOVA test on GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). A *p*-value=0.05 or less was considered statistically significant.

## Results

### Deregulated Bone Formation and Remodeling-associated Biomarkers in T2DM Patients with Femur Fracture

We examined serum levels of bone formation/remodeling-associated biomarkers and sclerostin, such as osteocalcin, P1NP and CTX in the T2DM patients. Detailed clinicopathological characteristics of the femur fracture of T2DM patients or of non-T2DM patients were indicated in Table I. The serum levels of LDL, fasting glucose and HbA1c were significantly higher in the T2DM patients. However, there was no difference in age and gender between the two groups. As shown in Figure 1A-C, the levels of osteocalcin, P1NP and CTX were significantly lower in the T2DM group of patients (*p*<0.001 or *p*<0.0001). Notably,



**Figure 1.** Serum levels of osteocalcin, P1NP, CTX and sclerostin in the femur bone fracture of patients with or without T2DM. Serum levels of osteocalcin (A), P1NP (B), CTX (C) and sclerostin (D) were examined with enzyme-linked immunosorbent assay (ELISA) in femur bone fracture of patients with (n = 32) or without (n = 27) T2DM. Data were presented as means  $\pm$  SEM. Statistical significance was considered when  $p=0.05$  or less.

the serum level of sclerostin was markedly higher in these T2DM samples ( $p < 0.0001$ , Figure 1D). Furthermore, to evaluate the association of down-regulated osteocalcin, P1NP and CTX with the upregulated sclerostin, linear regression analysis was performed. Figure 2A demonstrated that the serum osteocalcin was negatively associated with serum sclerostin level ( $R^2=0.4707$ ,  $p < 0.0001$ ). And such negative association was also found between the downregulated P1NP ( $R^2=0.6746$ ,  $p < 0.0001$ , Figure 2B) or CTX ( $R^2=0.4106$ ,  $p < 0.0001$ , Figure 2C) with the upregulated sclerostin. Taken together, we found the upregulation of sclerostin in association with the downregulated osteocalcin, P1NP and CTX in the femur fracture of patients with T2DM.

#### **Sclerostin Downregulates the Expression of Osteocalcin, P1NP and CTX in hFOB 1.19 Cells**

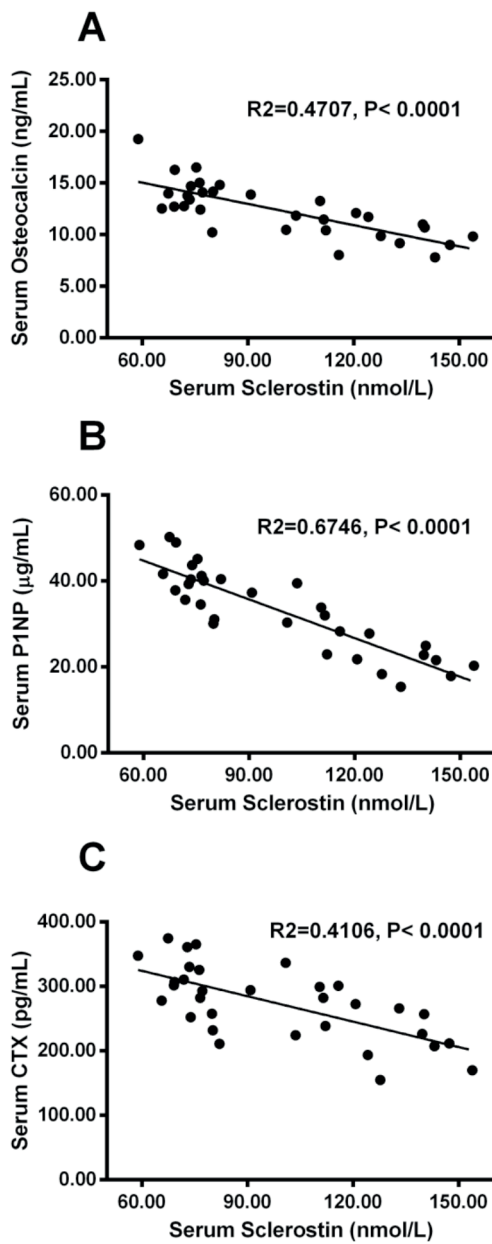
To further determine the possible regulation by sclerostin on the expression of bone formation/remodeling-associated biomarkers, we treated

hFOB 1.19 cells with recombinant sclerostin and re-examined the mRNA levels of osteocalcin, P1NP and CTX. As shown in Figure 3A, 5 or 10  $\mu\text{g/ml}$  of sclerostin markedly reduced the mRNA level of osteocalcin in hFOB 1.19 cells ( $p < 0.01$  respectively). Figure 3B indicated that P1NP was also significantly downregulated by 2, 5 or 10  $\mu\text{g/ml}$  sclerostin ( $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ ), dose-dependently. And the CTX mRNA level was also markedly lower in the sclerostin-treated hFOB 1.19 cells ( $p < 0.05$  or  $p < 0.01$ , Figure 3C). Thus, we confirmed the downregulation by sclerostin on the expression of osteocalcin, P1NP and CTX in hFOB 1.19 cells.

#### **Wnt Signaling Involves in the Sclerostin-mediated Downregulation on the Expression of Osteocalcin, P1NP and CTX in hFOB 1.19 Cells**

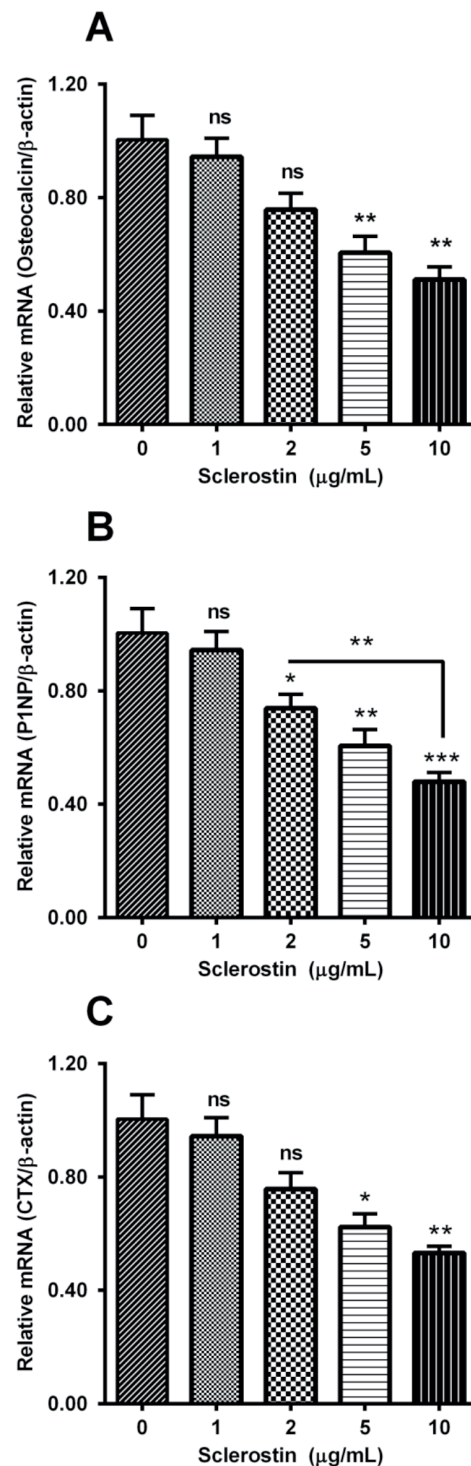
The activation of the Wnt signaling by the stabilization of  $\beta$ -catenin<sup>20</sup>, which was accumulated in the cytoplasm, then, was translocated to the nucleus to activate the transcription of target genes



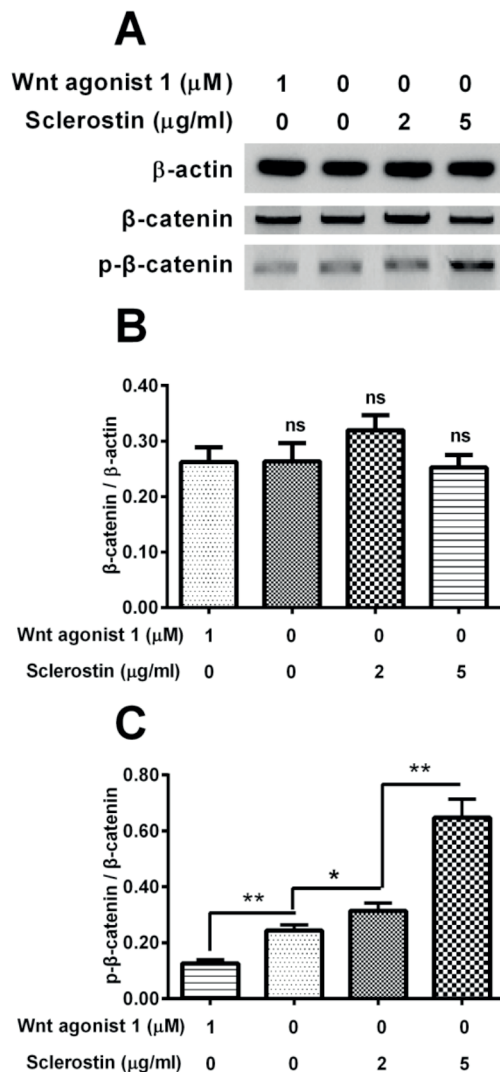


**Figure 2.** Association of the reduced osteocalcin, P1NP or CTX level with the promoted sclerostin level in the femur bone fracture of patients with T2DM. Linear-regression analysis between the level of osteocalcin (**A**), P1NP (**B**) or CTX (**C**) and the level of sclerostin in the femur bone fracture of patients with T2DM (n = 32). Statistical significance was considered with a *p*-value=0.05 or less.

by the binding of T-cell factor/lymphoid enhancer factor<sup>21</sup>. On the other side, the phosphorylation of  $\beta$ -catenin enables the recognition by ubiquitin and, then, leads to the degradation of  $\beta$ -catenin in the proteasome<sup>22</sup>. We supposed that Wnt/ $\beta$ -catenin signaling pathway might involve in the scleros-



**Figure 3.** mRNA levels of osteocalcin, P1NP and CTX in the sclerostin-treated hFOB 1.19 cells. hFOB 1.19 cells were treated with 0, 1, 2, 5 or 10  $\mu$ g/ml sclerostin for 12 h; then, the mRNA levels of osteocalcin (**A**), P1NP (**B**) or CTX (**C**) was quantified with specific primers for each biomarker. Data are presented as means  $\pm$  SEM for triple independent assays. Statistical significance was shown \*: *p*<0.05, \*\*: *p*<0.01, \*\*\*: *p*<0.001, ns: no significance.



**Figure 4.** Promotion of Wnt/ $\beta$ -catenin signaling in sclerostin-treated hFOB 1.19 cells. **A**, Western blot analysis of  $\beta$ -catenin with or without phosphorylation (Ser675) in hFOB 1.19 cells, post the treatment with 2 or 5  $\mu\text{g/ml}$  sclerostin or with 1  $\mu\text{M}$  Wnt agonist (BML-284) for 24 h; **B** and **C**, Relative level of  $\beta$ -catenin without phosphorylation (**B**) or with phosphorylation (**C**) in the sclerostin or Wnt agonist-treated hFOB 1.19 cells, with  $\beta$ -actin as internal control. Each experiment was performed independently in triplicate. Statistical significance was shown as \* $p < 0.05$  or \*\* $p < 0.01$ , ns: no significance.

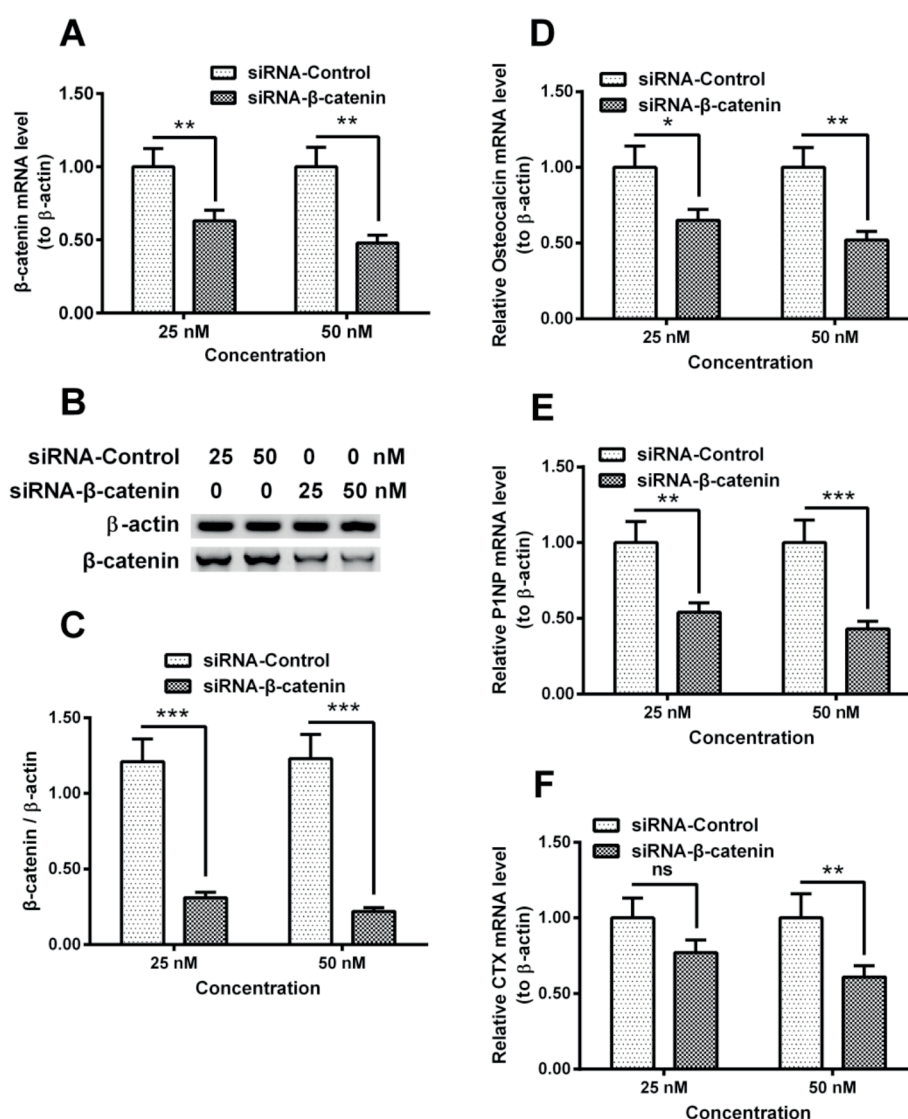
tin-mediated downregulation on the expression of bone formation/remodeling-associated biomarkers and, therefore, we examined the level of  $\beta$ -catenin with or without phosphorylation. Western blotting results (Figure 4A) demonstrated that, though the Wnt agonist (1  $\mu\text{M}$ ) or the sclerostin treatment (2 or 5  $\mu\text{g/ml}$ ) did not regulate the protein level of  $\beta$ -catenin in the cytoplasm (Figure

3B), the phosphorylated  $\beta$ -catenin (Ser675) in the cytoplasm was significantly downregulated by the Wnt agonist ( $p < 0.001$ , Figure 3C), whereas the sclerostin treatment with 5  $\mu\text{g/ml}$  markedly up-regulated the phosphorylated  $\beta$ -catenin (Ser675) (cytoplasm) in hFOB 1.19 cells.

To reconfirm the involvement of Wnt signaling pathway in the sclerostin-mediated downregulation on the expression of osteocalcin, PINP and CTX, we then knocked down  $\beta$ -catenin expression with RNAi technology. As shown in Figure 5A, either 25 or 50 nM siRNA- $\beta$ -catenin markedly reduced the  $\beta$ -catenin mRNA level in hFOB 1.19 cells, compared to siRNA-control. And the Western blotting (Figure 5B) demonstrated that  $\beta$ -catenin (cytoplasm) was significantly downregulated by 25 or 50 nM siRNA- $\beta$ -catenin ( $p < 0.001$  respectively, Figure 5C) than siRNA-control. More interestingly, the transfection with 25 or 50 nM siRNA- $\beta$ -catenin also markedly reduced the expression of osteocalcin in hFOB 1.19 cells ( $p < 0.05$  or  $p < 0.01$ , Figure 5D). The mRNA level of both PINP and CTX was downregulated by siRNA- $\beta$ -catenin ( $p < 0.01$  or  $p < 0.001$ , Figure 5E and 5F). Therefore, we confirmed the involvement of Wnt signaling pathway in the sclerostin-mediated downregulation on the expression of bone formation/remodeling-associated biomarkers in hFOB 1.19 cells.

## Discussion

T2DM is associated with increased fracture risk and delayed fracture healing. The previous studies<sup>23</sup> recognized the inhibited maturation of primary human osteoblasts and reduced osteoblast function. The current study identified the inhibition by sclerostin on the expression of bone formation/remodeling-related biomarkers, such as osteocalcin, PINP and CTX. Sclerostin is increased in the T2DM patients<sup>16</sup> and inhibits the bone formation<sup>15</sup> or is associated with an increased bone fracture<sup>24</sup>. Increased sclerostin production in men with T2DM may be involved in the pathogenesis of increased skeletal fragility<sup>18</sup>. However, it is not clear the molecular mechanisms. In the present study, we recognized the deregulated bone formation and remodeling-associated biomarkers in the T2DM patients with femur fracture, such as osteocalcin, PINP and CTX. However, the serum level of sclerostin was markedly higher in the femur fracture of patients with T2DM. Moreover, the downregulated osteo-



**Figure 5.** Promotion of Wnt/ $\beta$ -catenin signaling in sclerostin-treated hFOB 1.19 cells. **A**, mRNA level of  $\beta$ -catenin in the hFOB 1.19 cells, which were transfected with 25 or 50 nM siRNA- $\beta$ -catenin or siRNA-control and were inoculated for 12 h. **B and C**, Western blot analysis (B) and relative level (C) of  $\beta$ -catenin to  $\beta$ -actin in the siRNA- $\beta$ -catenin- or siRNA-control-transfected hFOB 1.19 cells post an inoculation for 24 h. **D-F**, Relative mRNA levels of osteocalcin (D), P1NP (E) or CTX (F) in the siRNA- $\beta$ -catenin- or siRNA-control-transfected hFOB 1.19 cells post an inoculation for 24 h. Each experiment was performed independently in triplicate. Statistical significance was shown as \*  $p < 0.05$ , \*\*  $p < 0.01$  or \*\*\*  $p < 0.001$ , ns: no significance.

calcin, P1NP or CTX was negatively associated with the upregulated sclerostin. Therefore, the upregulation of sclerostin was associated with the downregulated osteocalcin, P1NP and CTX in the femur fracture of patients with T2DM. Further *in vitro* results confirmed that sclerostin downregulated the expression of osteocalcin, P1NP and CTX in hFOB 1.19 cells.

Wnt/ $\beta$ -catenin signaling pathway has been recognized to positively regulate the bone formation and maintenance<sup>25</sup>, whereas such signaling path-

way was inhibited by sclerostin<sup>26,27</sup>. The activated Wnt signaling is accumulated in the cytoplasm and then, is translocated to the nucleus to activate the transcription of target genes<sup>21</sup>. The previous studies<sup>28</sup> have already identified the involvement of Wnt signaling pathway in the abnormal metabolism and  $\beta$ -cell biology in diabetes mellitus. Patients with T2DM showed higher levels of circulating sclerostin that were associated with disease duration but inversely related to bone turnover markers (BTMs)<sup>18,29</sup>. However, little is known

about the Wnt-targeted bone formation/remodeling-associated biomarkers in T2DM. Our findings found that Wnt/ $\beta$ -catenin inhibition was associated with the sclerostin-mediated inhibition of bone formation/remodeling-related biomarkers, such as osteocalcin, P1NP and CTX in human osteoblast hFOB 1.19 cells. The Wnt/ $\beta$ -catenin level was markedly inhibited by sclerostin treatment, and the siRNA-mediated downregulation of  $\beta$ -catenin reduced the levels of osteocalcin, P1NP and CTX.

### Conclusions

Our work demonstrated that the upregulated serum sclerostin level in the T2DM patients with fracture inhibited the expression of bone formation/remodeling-associated biomarkers via antagonizing Wnt signaling. It suggests that sclerostin might be an effective target for T2DM-associated bone fracture and delayed fracture healing.

### Ethical Approval

The present study was in accordance with the Ethical Standards of the Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and Ethical Standards. Ethical approval was granted by the Research Ethics Committee in Nanfang Hospital, Southern Medical University. Informed consent was obtained from all individual participants in the study.

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### Conflict of Interest

Authors declared no conflict. The authors agreed to allow the Journal to review and control the data if requested.

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