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ARTICLE

# Fibronectin 1 activates WNT/β-catenin signaling to induce osteogenic differentiation via integrin β1 interaction

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## Abstract

Osteoporosis (OP) is a systemic skeletal disease leading to fragility fractures and is a major health issue globally. WNT/β-catenin signaling regulates bone-remodeling processes and plays vital roles in OP development. However, the underlying regulatory mechanisms behind WNT/β-catenin signaling in OP requires clarification, as further studies are required to identify novel alternate therapeutic agents to improve OP. Here we report that fibronectin 1 (FN-1) promoted differentiation and mineralization of osteoblasts by activating WNT/β-catenin pathway, in cultured pre-osteoblasts. With isobaric tags for relative and absolute quantitation labeling proteomics analysis, we investigated protein changes in bone samples from OP patients and normal controls. FN-1 accumulated in osteoblasts in bone samples from OP patients and age-related OP mice compared to control group. In addition, we observed that integrin β1 (ITGB1) acts as an indispensable signaling molecule for the interplay between FN-1 and β-catenin, and that FN-1 expression increased, but ITGB1 expression decreased in osteoblasts during OP progression. Therefore, our study reveals a novel explanation for WNT/β-catenin pathway inactivation in OP pathology. Supplying of FN-1 and ITGB1 may provide a potential therapeutic strategy in improving bone formation during OP.

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## Introduction

Osteoporosis (OP) is a common skeletal disease characterized by systemic impairment of bone mass and destruction of bone structures, leading to increased bone fragility and fracture risk and decreased bone strength [1–3]. Osteoporotic fractures in women after the age of 55 years and men after the age of 65 years are become increasingly common, which result in substantial bone-associated morbidities, increased mortality, and health-care costs [4]. OP results from an imbalance between bone formation and bone resorption, causing abnormal bone remodeling [5, 6]. Although recent studies have revealed bone formation signaling pathways, the specific molecular basis of osteoblast differentiation during OP pathogenesis remains unclear, indicating OP prevention and onset remain an unmet clinical need.

WNT/β-catenin signaling pathway is a critical regulatory component of bone homeostasis in OP [7, 8]. Mouse genetics studies have confirmed the importance of WNT/β-catenin signaling in regulating bone-remodeling processes [9, 10]. Activation of WNT/β-catenin signaling results in increased bone mass thanks to excess bone formation, compared to bone resorption. Conversely, WNT/β-catenin signaling impairments induce reduced bone mass with

excess bone resorption over bone formation [11, 12]. In recent clinical trials, an anti-sclerostin antibody, used to inhibit sclerostin to activate WNT/β-catenin signaling, resulted in marked increases in bone mineral density and rapid and substantial reductions in fracture risks, proposing it as an attractive strategy in treating OP [13]. Thus, while the WNT/β-catenin signaling pathway is now a promising target for OP therapeutic intervention, underlying regulatory mechanisms of WNT/β-catenin signaling in osteoblasts during OP pathogenesis remain unclear.

Fibronectin (FN) is a member of the glycoprotein family of ligands, widely expressed in multiple cell types that bind cell surfaces and compounds including collagen, fibrin, heparin, DNA, and actin [14]. Fibronectin 1 (FN-1) is a member of this family and functions in different biological processes, such as cell migration, cell adhesion, and cytoskeleton organization [15, 16]. Equally, aberrant FN-1 expression is also implicated in several diseases such as cancer, atherosclerosis, and arthritis [17–19]. Previous studies have shown that osteoblasts produce FN-1 during proliferation and differentiation, concomitant with collagen type I production, suggesting that osteoblasts generate FN-1 during active bone formation [20]. Moreover, FN-1 is involved in osteoblast compaction through FN-fibrillogenesis cell-mediated matrix assembly processes, essential for osteoblast mineralization [21, 22]. However, FN-1 signaling mechanisms in osteogenesis and OP have not been fully elucidated.

In this study, we identified FN-1 as a molecular regulator in promoting osteogenic differentiation in vitro. FN-1 interacts with and regulates β-catenin signaling, whereas FN-1 overexpression activates WNT/β-catenin signaling and promotes pre-osteoblast differentiation and mineralization. Accumulated FN-1 expression was observed in osteoblasts in bone samples from OP patients and age-related OP mice. Moreover, we discovered that integrin β1 (ITGB1) interacts with FN-1 and was indispensable for FN-1 mediated activation of WNT/β-catenin signaling. ITGB1 expression was gradually decreased as FN-1 expression increased in osteoblasts during OP. Taken together, these data demonstrate that FN-1 interplays with ITGB1 and activates WNT/β-catenin signaling, promoting osteogenesis in OP. Our findings reveal a novel mechanism in WNT/β-catenin signaling regulation, and may provide a new therapeutic direction for future OP treatments.

## Results

### FN-1 modulates osteogenic differentiation in the pre-osteoblast MC3T3-E1 cell line

Recent studies have indicated a crucial role for FN-1 in osteoblast differentiation, as an extracellular matrix protein [21]. Therefore, we investigated whether FN-1 acted as a regulator of osteogenic differentiation in pre-osteoblast cell line, MC3T3-E1. To exclude proliferation effects, MC3T3-E1 cells were seeded at high densities, with differentiation induced after 24 h. First, FN-1 was knocked down using small interfering RNA (siRNA), and cells were stimulated with osteogenic induction medium. After 3 days, the osteoblastic markers, runt-related transcription factor 2 (Runx2), osteocalcin (OCN), and osterix (OSX) were downregulated in FN-1 knockdown cells, as ascertained by immunoblotting (Fig. 1a), and within 14 days, alizarin red staining confirmed diminished mineralization of the extracellular matrix (Fig. 1b). In a separate experiment, MC3T3-E1 cells were transfected with an FN-1 overexpression plasmid. As shown in Fig. 1c, d, osteoblastic marker expression and mineralization of the extracellular matrix were significantly enhanced after FN-1 overexpression. Both these experiments suggested that FN-1 modulates osteogenic differentiation in vitro.

### FN-1 interacts with and promotes the nuclear translocation of β-catenin in osteoblasts

Previous studies have referred to the connection between FN-1 and β-catenin [17, 23], therefore, to explore underlying FN-1 mechanisms in regulating osteogenic differentiation, we analyzed the relationship between FN-1 and β-catenin signaling in MC3T3-E1 cells and primary osteoblasts. Co-immunoprecipitation (IP) analyses in these cells, with FN-1 or β-catenin primary antibodies, confirmed the interaction between FN-1 and β-catenin (Fig. 2a, b). Importantly, β-catenin expression was diminished by FN-1 knockdown, but was enhanced by FN-1 overexpression (Fig. 2c, d). Using double staining of FN-1 and β-catenin, we observed that FN-1 co-localized with β-catenin in MC3T3-E1 cells under normal, non-osteogenic differentiation condition. Moreover, with the osteogenic differentiation medium treatment, we noticed an enhanced expression of FN-1 and β-catenin in nuclear (Fig. 2e). Similar results were obtained in the cultured primary osteoblasts with double staining of FN-1 and β-catenin (Supplementary Fig. 1). Taken together, these data demonstrate that FN-1 interacts with and promotes the nuclear translocation of β-catenin in osteoblasts.

### FN-1 expression is increased in osteoblasts in bone samples from OP patients

To identify changes in protein expression in OP, we conducted a proteome-wide screen with bone samples from OP patient and controls. Among the 2119 proteins identified, 249 proteins were upregulated and 183 proteins were downregulated (>1.5-fold) in samples from OP patients,

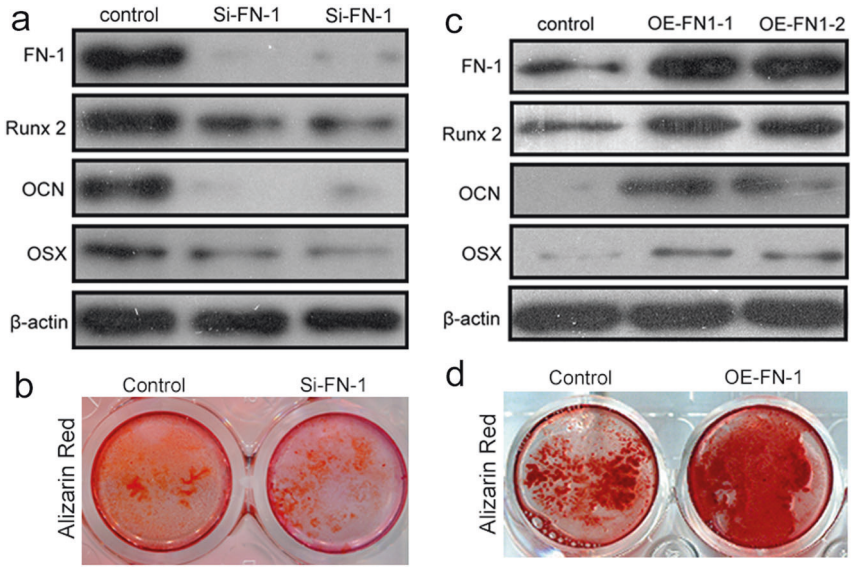


**Fig. 1 FN-1 modulates osteogenic differentiation in vitro.** **a** MC3T3-E1 cells were transfected with controls or two FN-1-specific small interfering RNAs (siRNAs) and stimulated with osteogenic induction medium for 72 h. Expression levels of FN-1, Runx2, OCN, and OSX were detected by immunoblotting. β-actin served as an internal control. Alizarin red staining was performed in cells treated with siRNA and osteogenic induction medium after 14 days (**b**). **c** MC3T3-E1 cells were transfected with controls or two FN-1 encoding plasmids and stimulated with osteogenic induction medium for 72 h. Expression levels of FN-1, Runx2, OCN, and OSX were detected by immunoblotting. β-actin served as an internal control. Alizarin red staining was performed in cells treated with control or plasmids and osteogenic induction medium after 14 days (**d**). All experiments were repeated three times for reproducibility.

when compared to the control group. Notably, we observed that FN-1 expression was upregulated 5.54-fold in OP samples (Fig. 3a). Markedly elevated FN-1 levels were observed in osteoblasts with immunofluorescence (IF) analysis in bone samples from OP patients, when compared to controls (Fig. 3b). In addition, immunoblotting analyses of two bone samples from OP patients also confirmed elevated FN-1 expression (Fig. 3c). These results suggest FN-1 accumulates in osteoblasts in OP patients, and that FN-1 may participate in OP development.

## FN-1 accumulates in osteoblasts in age-related OP mice

To confirm the dysregulation of bone remodeling with age advancing, bone samples from mice aged 2, 12, and 24 months were conducted with haematoxylin and eosin staining. Results showed a gradually loss in bone mass as aging in mice (Fig. 4a). To determine whether FN-1 accumulation occurs in mouse osteoblasts, we double stained FN-1 and OCN in bone samples from mice aged 2, 12, and 24 months. OCN expression was decreased in parallel with aging, which confirmed the OP developed along with aging in mice, but FN-1 levels in OCN positive cells were conversely increased (Fig. 4b). Similarly, by immunoblotting these same bone samples, we observed FN-1 expression increments with age-related OP (Fig. 4c). Thus, FN-1 expression increases in osteoblasts in line with age-related OP in mice.

## ITGB1 is essential for FN-1-mediated osteogenesis, via β-catenin signaling

It is accepted that WNT/β-catenin signaling is the main regulatory pathway of osteoblast differentiation, as pathway inactivation leads to bone-related disorders, i.e., OP [8]. We noticed a decreased expression level of β-catenin in the osteoblasts along with aging in mice (Fig. 5a). In consideration of inconsistencies in FN-1 accumulation with β-catenin signaling inhibition in OP development, we speculated there maybe another modulator between FN-1 and β-catenin. FN-1 is reported to be a ligand of ITGB1, involved in the maintenance of ITGB1 FN receptors. We observed a direct interaction between FN-1 and ITGB1 in MC3T3-E1 cells (Fig. 5b) by co-IP assay. Double staining against FN-1 and ITGB1 also showed that FN-1 co-localized with ITGB1 (Fig. 5c and Supplementary Fig. 2). Surprisingly, IF staining of ITGB1 and FN-1 showed that ITGB1 expression gradually decreased in line with FN-1 accumulation in the osteoblasts of aged mice (Fig. 5d). Next, we observed that FN-1 silencing downregulated β-catenin and OCN expression levels, and that ITGB1 knockdown significantly inhibited already depressed β-catenin and OCN expression in MC3T3-E1 cells. In addition, we observed diminished mineralization in the extracellular matrix of FN-1 knocked down MC3T3-E1 cells, but this was reversed by ITGB1 downregulation (Fig. 5e). In contrast, enhanced β-catenin and OCN

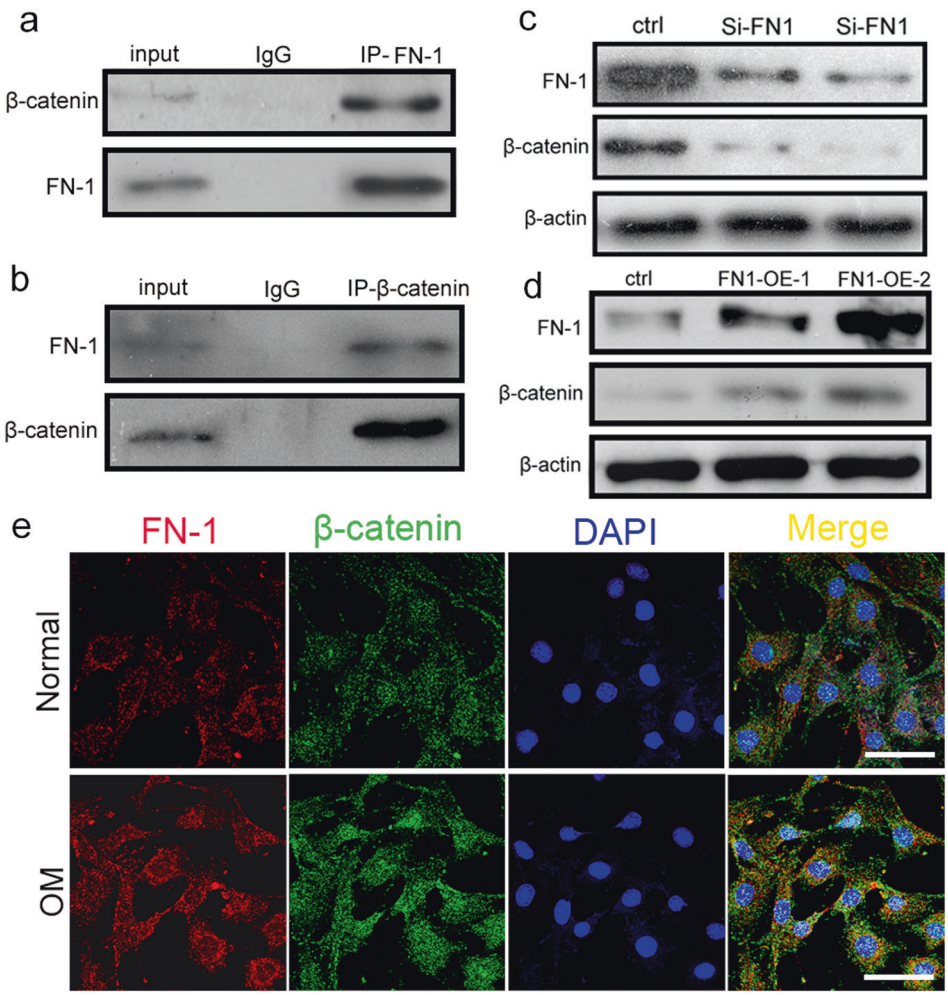


**Fig. 2 FN-1 interacts with and promotes the nuclear translocation of β-catenin in osteoblasts. a** FN-1 was immunoprecipitated from MC3T3-E1 cells using an anti-FN-1 antibody. β-catenin and FN-1 in these immunoprecipitates were evaluated by immunoblotting. **b** β-catenin was immunoprecipitated from MC3T3-E1 cells using an anti-β-catenin antibody. FN-1 and β-catenin in these immunoprecipitates were evaluated by immunoblotting. **c** MC3T3-E1 cells were transfected with a control or two FN-1-specific small interfering RNAs (siRNAs) or two FN-1 encoding plasmids, **d** FN-1 and β-catenin expression levels were detected by immunoblotting. β-actin served as an internal control. All experiments were repeated three times for reproducibility. **e** MC3T3-E1 cells in normal condition and osteogenic differentiation medium (OM) were fixed for IF. FN-1 was detected using a primary anti-FN-1 antibody and a secondary Alexa Fluor 594-conjugated goat anti-mouse IgG antibody. β-catenin was detected using a primary anti-β-catenin antibody, and a secondary Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody. Scale bar: 20 μm.

expression induced by FN-1 overexpression were restrained by ITGB1 silencing, and increased mineralization was also recovered by ITGB1 knockdown in these cells (Fig. 5f). Moreover, nuclear translocation of β-catenin was enhanced by FN-1 overexpression and can be suppressed by ITGB1 silencing (Fig. 5g) in MC3T3-E1 cells.

Collectively, these data suggest that ITGB1 is essential for FN-1 in promoting osteogenesis via β-catenin signaling in OP.

## Discussion

During OP, osteoblasts are characterized by reduced proliferation and differentiation, ultimately leading to defections and imbalances in bone formation and bone-remodeling processes, respectively [5]. In this study, we defined the essential role of FN-1 in osteogenesis. We observed that FN-1 interacted with and regulated β-catenin signaling in vitro. FN-1 overexpression activated the WNT/β-catenin signaling pathway, and promoted pre-osteoblast differentiation and mineralization. FN-1 accumulated in osteoblasts in bone samples from OP patients, and its expression was increased in osteoblasts from mice with age-related OP. Moreover, we found that ITGB1 interacted with FN-1 and was essential for the FN-1 activation of WNT/β-catenin signaling. ITGB1 expression was gradually decreased as FN-1 expression increased in osteoblasts during mouse aging. These data suggest that FN-1 interacts

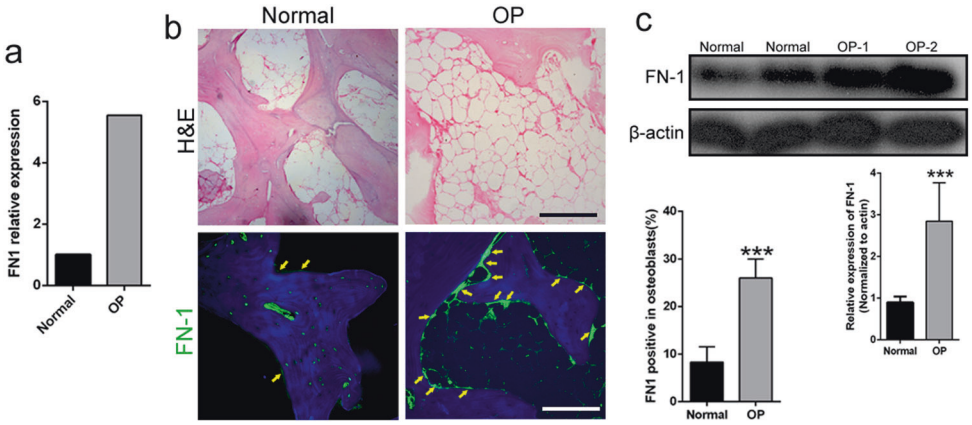


**Fig. 3 FN-1 expression in OP patient bone samples is significantly higher than the normal group. a** Quantitative FN-1 expression analysis using a proteome-wide screen for the proteins by analyzing bone samples from OP patients and controls. **b** Hematoxylin and eosin (H&E) staining and IF of FN-1 (green) in bone samples from OP patients and controls. FN-1 expression in osteoblasts was counted and normalized. Scale bars: 50 μm. **c** Immunoblotting analysis and densitometry quantification of FN-1 in bone samples from OP patients and controls. β-actin served as an internal control. All experiments were performed three times for reproducibility. ***$P < 0.001$. All data are shown as the mean ± SD.

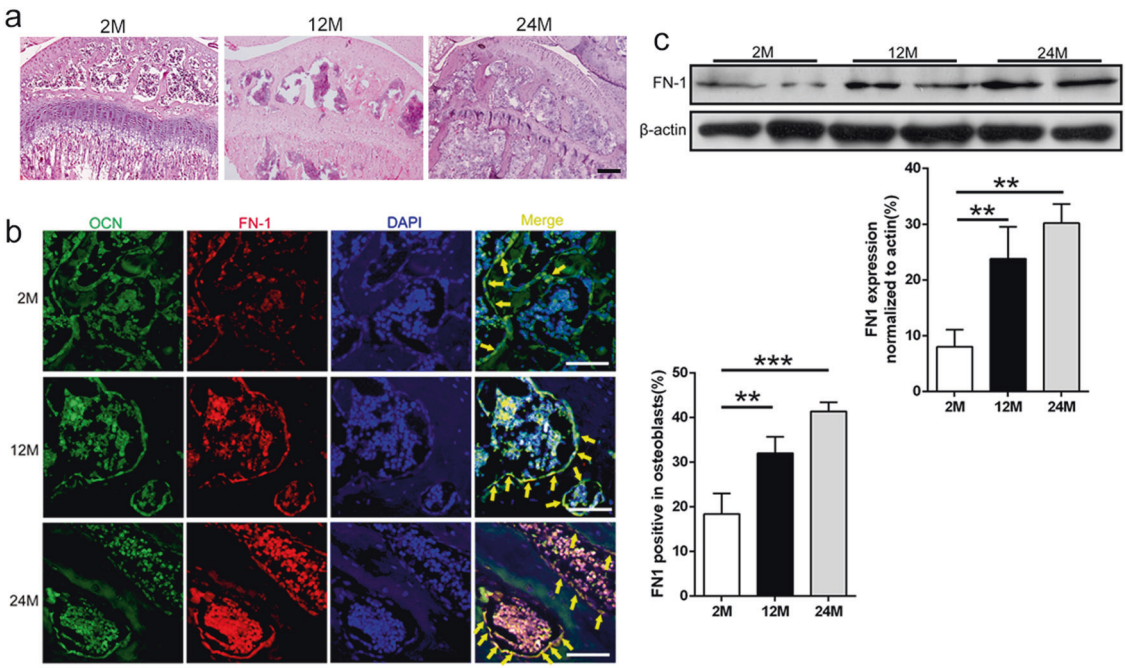


**Fig. 4 FN-1 expression accumulates in osteoblasts in aging mice. a** Hematoxylin and eosin staining of bone samples from mice at 2, 12, and 24 months old. $n = 5$; scale bars: 50 μm. **b** Representative IF images (left) for OCN (green) and FN-1 (red) and the quantification (right) of FN-1 positives osteoblasts in bone samples from mice at 2, 12, and 24 months old. Scale bars: 50 μm. **c** Immunoblotting and densitometry quantification of FN-1 in bone samples from mice at 2, 12, and 24 months old. β-actin served as an internal control. All experiments were performed three times for reproducibility. **$P < 0.01$, ***$P < 0.001$. All data are shown as the mean ± SD.

with and activates WNT/β-catenin signaling to promote osteogenic processes, and that FN-1 interaction with β-catenin relies on ITGB1 in osteoblasts.

Osteoblasts represent a unique cell type, which initially lays down the extracellular matrix and then mineralizes it to form bone [24]. FN-1 is a ubiquitous and large extracellular matrix protein that binds to itself and collagen to form intricate networks [25]. The importance of FN-1 to collagen matrix assembly suggests its absence results in serious matrix defects resembling those seen in osteogenesis imperfecta [26, 27]. FN-1 is also produced by osteoblasts and forms mineralized nodules in vitro [28]. We confirmed the effects of FN-1 on osteoblast differentiation and mineralization in vitro. The in vitro knockdown of FN-1

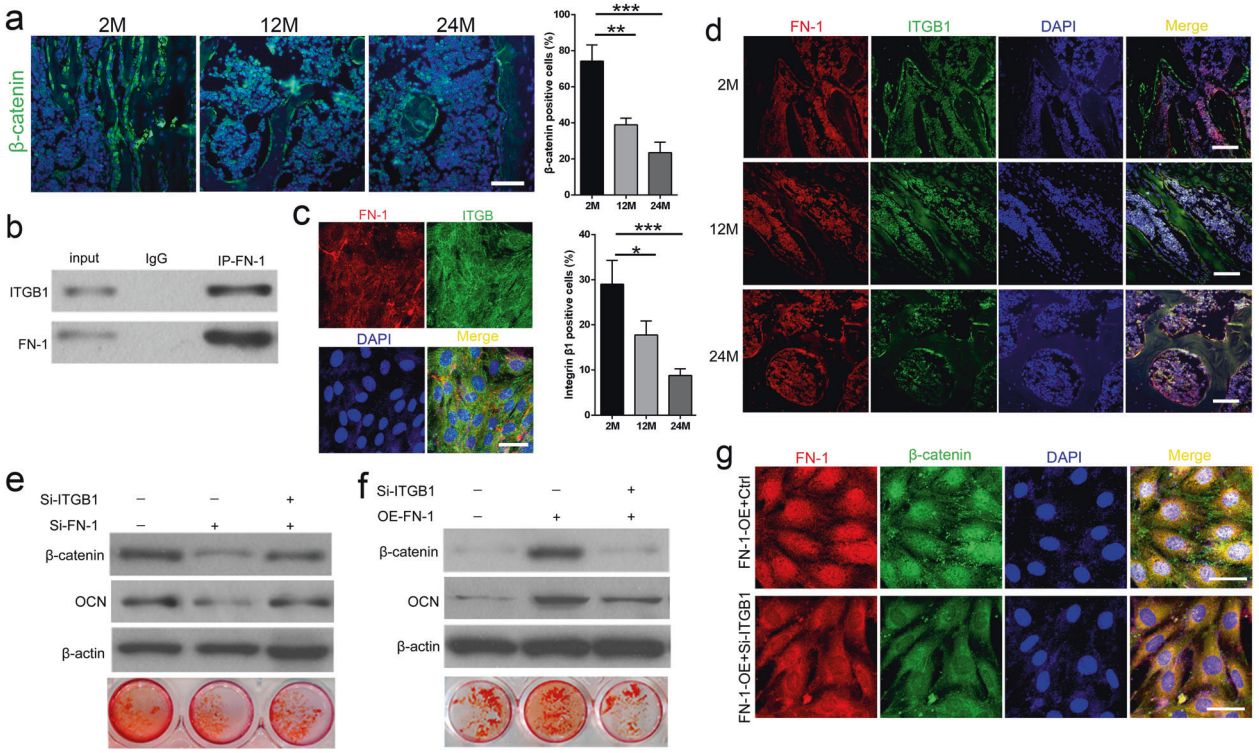


**Fig. 5 ITGB1 is essential for FN-1 in promoting osteogenesis via β-catenin signaling. a** Representative IF images for β-catenin (left) the quantification (right) of β-catenin positives osteoblasts in bone samples from mice at 2, 12, and 24 months old. $n = 5$; scale bars: 50 μm. **b** FN-1 was immunoprecipitated from MC3T3-E1 cells using an anti-FN-1 antibody. The presence of both ITGB1 and FN-1 in these immunoprecipitates was evaluated by immunoblotting. **c** MC3T3-E1 cells were fixed for IF. FN-1 was detected using an anti-FN-1 primary antibody, and an Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibody, and ITGB1 was detected using a primary anti-ITGB1 antibody, and an Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody. Scale bar: 20 μm. **d** Representative IF images for ITGB1 (green) and FN-1 (red) in bone samples from mice at 2, 12, and 24 months old. $n = 5$; scale bars: 50 μm. **e** MC3T3-E1 cells were transfected with a control, FN-1 siRNA or FN-1 plus ITGB1 siRNA. Expression levels of β-catenin and OCN were detected by immunoblotting. β-actin served as an internal control. Alizarin red staining was performed in MC3T3-E1 cells treated with siRNA and osteogenic induction medium after 14 days. All experiments were performed three times for reproducibility. **f** MC3T3-E1 cells were transfected with a control, FN-1-encoding plasmid or FN-1-encoding plasmid plus ITGB1 siRNA, expression levels of β-catenin and OCN were detected by immunoblotting. β-actin served as an internal control. Alizarin red staining was performed in cells transfected with plasmids or siRNA and osteogenic induction medium after 14 days. All experiments were performed three times for reproducibility. **g** MC3T3-E1 cells were transfected with FN-1 plasmid to overexpression FN-1 or transfected with FN-1 plasmid plus ITGB1 siRNA. Then, FN-1 was detected using a primary anti-FN-1 antibody and a secondary Alexa Fluor 594-conjugated goat anti-mouse IgG antibody. β-catenin was detected using a primary anti-β-catenin antibody, and a secondary Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody. Scale bar: 20 μm. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All data are shown as the mean ± SD.

decreased β-catenin expression and inhibited WNT/β-catenin signaling to obstruct osteoblast differentiation and mineralization, while FN-1 overexpression led to the opposite. To the best of our knowledge, this study is the first to report FN-1 interactions with WNT/β-catenin signaling in osteoblasts.

The vital role of the WNT/β-catenin pathway in OP pathogenesis has been frequently discussed. This pathway is inhibited in OP, and the underlying mechanism is still unclear. In using isobaric tags for relative and absolute quantitation (iTRAQ) labeling and LC–MS/MS proteomics, we observed increased FN-1 levels in bone samples from OP patients, when compared to normal controls. FN-1 accumulation was mainly detected in osteoblasts. In mice, FN-1 expression gradually increased in osteoblasts along with age-related OP. As described, FN-1 directly interacts with and promotes the nuclear translocation of β-catenin in osteoblasts under osteogenic differentiation condition, activates WNT/β-catenin pathway in vitro. Interestingly, FN-1 expression increases in OP and age-related OP osteoblasts, but the WNT/β-catenin pathway is inhibited during OP. Thus, we speculate there must be other regulators responsible for this discrepancy in vivo.

Integrins are a large family of heterodimeric surface molecules that are key regulators in multiple signaling pathways to affect the survival, migration, and differentiation in diverse cell lines [29, 30]. Studies have shown that ITGB1 increases Runx2 and Colα1 expression, while increasing mineralization in mesenchymal stem cells, and that ITGB1 is a potential primary mediator of osteogenesis

in response to mechanical stimulation [31, 32]. Importantly, ITGB1 mediates FN signaling, with FN-1 being a ligand of ITGB1 [33–35]. As expected, we observed that FN-1 directly interacted with ITGB1 in osteoblasts. With ITGB1 silencing, FN-1 activation on the WNT/β-catenin pathway was prevented in osteoblasts, while osteoblast differentiation and mineralization were limited. We also noted that ITGB1 expression was deceased in osteoblasts, in line with mouse aging. Therefore, we propose that ITGB1 is essential for FN-1 activation of the WNT/β-catenin pathway in osteoblasts. Due to reduced ITGB1 expression in osteoblast during OP, FN-1 accumulation could not promote the nuclear translocation of β-catenin and the bone formation process is retarded.

Activation of canonical Wnt signaling stimulates the generation of osteoblasts and production of bone, but also attenuates osteoclast differentiation and function by stimulating production of osteoprotegerin [10, 36]. Thus, inhibitors of the antagonists of WNT/β-catenin signaling, such as sclerostin and Dickkopf-related protein 1, are potential candidates for the prevention and treatment of bone loss in OP [37]. In our study, we report that FN-1 promotes osteoblast differentiation and mineralization by activating the WNT/β-catenin pathway in vitro. Similarly, ITGB1 is indispensable for the interaction of FN-1 with β-catenin. FN-1 expression increases, but ITGB1 expression decreases in osteoblasts during OP progression, which may provide a novel explanation for WNT/β-catenin pathway inactivation in OP. Supplying of FN-1 and ITGB1 could provide a viable therapeutic strategy to improve bone formation during OP.

## Materials and methods

### Human samples

Samples were collected from male patients with hip fractures, requiring surgical intervention, at the Affiliated Hospital of Youjiang Medical University for Nationalities. Fresh Bone samples were obtained during surgery and were kept in liquid nitrogen. Next, the obtained bone samples were subjected to proteomics or immunostaining analysis. Patient consent and approval from the Ethics Committee of the Affiliated Hospital of Youjiang Medical University for Nationalities were obtained before sampling.

### Immunohistochemistry (IHC)/immunofluorescence (IF) staining

After deparaffinization, sections were immerged in 10 mM citric acid buffer at 60 °C overnight to unmask the antigen. Next, the sections were washed with phosphate-buffered saline (PBS), blocked with 1% sheep serum for 1 h at room temperature (RT), and stained with diluted primary antibodies (Cell Signaling Technology, MA, USA) at 4 °C overnight. For IHC, sections were next incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research, USA) for 1 h at RT, and visualized with diaminobenzidine solution (Zsbio, Beijing, China). For IF, sections were next stained with Alexa Fluor 488- and Alexa Fluor 594-labeled secondary antibodies (Life Technologies, Carlsbad, CA, USA) for 1 h at RT, and nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies, Carlsbad, CA, USA). Olympus BX51 microscope or FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) was used to obtain images.

### iTRAQ® labeling and LC–MS/MS proteomics

OP patient bone samples and bone from normal controls were washed in PBS. Total protein was extracted in lysis buffer containing 7 M urea, 1 mM PMSF, 1 mM $Na_3VO_4$, and 1 mg/ml DNase I. Samples were centrifuged at 12,000 × *g* for 30 min at 4 °C. After this, 100 μg each protein sample was used for enzymolysis and iTRAQ® labeling. This labeling was performed using a previously described method. Peptide samples were labeled with the iTRAQ® Reagent Multiplex Kit (Applied Biosystems, USA). LC–MS/MS (Sciex TripleTOF 5600, Applied Biosystems) was performed by Fitgene Biological Technology Co. Ltd (Fitgene, Guangzhou, China). Protein Pilot software v4.0 (Applied Biosystems) was used to convert raw data (.wiff files) into peak lists (.mgf files). Average relative expression (fold change in expression between labeled protein samples), *P* values, error factors, lower and upper confidence intervals were calculated using Protein Pilot, and then exported into Excel. Proteins with fold change ≥ 1.5 or ≤0.5, and *P* values ≤ 0.05 were considered significantly differentially expressed.

### Cell culture

Primary osteoblasts were obtained from the calvaria of newborn mice. MC3T3-E1 and primary osteoblasts were both cultured in α-MEM medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco) at 37 °C with 5% CO2. The addition of 100 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO. Germany) and 10 mM β-glycerol phosphate (Sigma-Aldrich) to induced osteogenesis. Then, the cells were stained with alizarin red according to standard techniques to confirm osteogenesis. MC3T3-E1 cells were transfected with an FN CRISPR/cas9 knockout (KO) plasmid (sc-420394, Santa Cruz, CA), integrin β1 siRNA (sc-29372, Santa Cruz, CA), and control siRNA (sc-37007, Santa Cruz, CA), or with an FN

activation plasmid (sc-420394-Act, Santa Cruz, CA) using Lipo3000 transfection reagent (Invitrogen, Carlsbad, Canada) according to manufacturer's instructions.

### Co-immunoprecipitation assay

MC3T3-E1 cells were washed twice with PBS and lysed in ice-cold lysis buffer (40 mM HEPES (pH 7.4), 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 0.3% CHAPS and EDTA-free protease inhibitors (Roche, Basel, Switzerland)). The cell lysates were centrifugated and incubated with primary antibodies for 2 h at 4 °C, followed by the addition of 30 ml of a 50% protein G Sepharose bead slurry for a further hour. Beads were washed three times with low salt wash buffer, transferred into sodium dodecyl sulfate lysis buffer at 96 °C for 5 min and supernatants were subjected to immunoblotting.

### Immunoblotting

Cells and bone sample tissues were lysed at 96 °C for 10 min with lysis buffer (10 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 10 mM dithiothreitol, 0.01% bromophenol blue). Protein samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Berkeley, USA) with the wet transfer method. Then, the membrane was incubated with specific antibodies and the signals were detected by enhanced chemiluminescence (ECL Kit, Amersham Biosciences, Piscataway, NJ, USA).

### Statistical analysis

All experiments were performed at least three times and observed by independent observations. All data are presented as the mean ± SD, using SPSS software version 20.0. Graphs were generated by GraphPad Prism 6.0. Student's *t* tests assessed statistical significance between groups. $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

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**Author contributions** YT, KL, and JL conceived the project, designed and supervised the experiments. KL and JL wrote the paper. CY, CW, and JZ performed the experiments and analyzed the data. QL, FH, and FL helped with the cell-cultured experiments and immunoblotting analysis. YL, JC, FZ, and CH helped prepare the figures and finished the IHC/IF analysis.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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## References

1. NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy. Osteoporosis prevention, diagnosis, and therapy. JAMA. 2001;285:785–95.
2. Sambrook P, Cooper C. Osteoporosis. Lancet. 2006;367:2010–18.
3. Rachner TD, Khosla S, Hofbauer LC. Osteoporosis: now and the future. Lancet. 2011;377:87.
4. Compston JE, McClung MR, Leslie WD. Osteoporosis. Lancet. 2019;393:364–76.
5. Corrado A, Sanpaolo ER, Di Bello S, Cantatore FP. Osteoblast as a target of anti-osteoporotic treatment. Postgrad Med. 2017;129: 858–65.
6. An J, Yang H, Zhang Q, Liu C, Zhao J, Zhang L, et al. Natural products for treatment of osteoporosis: the effects and mechanisms on promoting osteoblast-mediated bone formation. Life Sci. 2016;147:46–58.
7. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. Nat Med. 2013;19: 179–92.
8. Clevers H. Wnt/beta-catenin signaling in development and disease. Cell. 2006;127:469–80.
9. Morvan F, Boulukos K, Clement-Lacroix P, Roman Roman S, Suc-Royer I, Vayssiere B, et al. Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. J Bone Miner Res. 2006;21:934–45.
10. Glass DA 2nd, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, et al. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell. 2005;8:751–64.
11. Ge L, Cui Y, Liu B, Yin X, Pang J, Han J. ERalpha and Wnt/betacatenin signaling pathways are involved in angelicindependent promotion of osteogenesis. Mol Med Rep.2019;19:3469–76.
12. Liu SL, Zhou YM, Tang DB, Zhou N, Zheng WW, Tang ZH, et al. LGR6 promotes osteogenesis by activating the Wnt/beta-catenin signaling pathway. Biochem Biophys Res Commun. 2019;519:1–7.
13. Inoue D. Sequential treatment of osteoporosis with anti-sclerostin. Clin Calcium. 2019;29:363–9.
14. Maurer LM, Ma W, Mosher DF. Dynamic structure of plasma fibronectin. Crit Rev Biochem Mol Biol. 2015;51:213–27.
15. Schwarzbauer JE, DeSimone DW. Fibronectins, their fibrillogenesis, and in vivo functions. Cold Spring Harb Perspect Biol. 2011;3:a005041.
16. Zollinger AJ, Smith ML. Fibronectin, the extracellular glue. Matrix Biol. 2017;60-61:27–37.
17. Gao W, Liu Y, Qin R, Liu D, Feng Q. Silence of fibronectin 1 increases cisplatin sensitivity of non-small cell lung cancer cell line. Biochem Biophys Res Commun. 2016;476:35–41.
18. Kimura E, Kanzaki T, Tahara K, Hayashi H, Hashimoto S, Suzuki A, et al. Identification of citrullinated cellular fibronectin in synovial fluid from patients with rheumatoid arthritis. Mod Rheumatol. 2014;24:766–9.

19. Doddapattar P, Gandhi C, Prakash P, Dhanesha N, Grumbach IM, Dailey ME, et al. Fibronectin splicing variants containing extra domain A promote atherosclerosis in mice through toll-like receptor 4. Arterioscler Thromb Vasc Biol. 2015;35:2391–400.
20. Bentmann A, Kawelke N, Moss D, Zentgraf H, Bala Y, Berger I, et al. Circulating fibronectin affects bone matrix, whereas osteoblast fibronectin modulates osteoblast function. J Bone Miner Res. 2010;25:706–15.
21. Sens C, Huck K, Pettera S, Uebel S, Wabnitz G, Moser M, et al. Fibronectins containing extradomain A or B enhance osteoblast differentiation via distinct integrins. J Biol Chem. 2017;292:7745–60.
22. Brunner M, Millon-Fremillon A, Chevalier G, Nakchbandi IA, Mosher D, Block MR, et al. Osteoblast mineralization requires beta1 integrin/ICAP-1-dependent fibronectin deposition. J Cell Biol. 2011;194:307–22.
23. Bielefeld KA, Amini-Nik S, Whetstone H, Poon R, Youn A, Wang J, et al. Fibronectin and beta-catenin act in a regulatory loop in dermal fibroblasts to modulate cutaneous healing. J Biol Chem. 2011;286:27687–97.
24. Ecarot-Charrier B, Glorieux FH, van der Rest M, Pereira G. Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. J Cell Biol. 1983;96:639–43.
25. Kadler KE, Hill A, Canty-Laird EG. Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators. Curr Opin Cell Biol. 2008;20:495–501.
26. Grzesik WJ, Frazier CR, Shapiro JR, Sponseller PD, Robey PG, Fedarko NS. Age-related changes in human bone proteoglycan structure. Impact of osteogenesis imperfecta. J Biol Chem. 2002; 277:43638–47.
27. Marini JC, Forlino A, Cabral WA, Barnes AM, San Antonio JD, Milgrom S, et al. Consortium for osteogenesis imperfecta mutations in the helical domain of type I collagen: regions rich in lethal mutations align with collagen binding sites for integrins and proteoglycans. Hum Mutat. 2007;28:209–21.
28. Moursi AM, Damsky CH, Lull J, Zimmerman D, Doty SB, Aota S, et al. Fibronectin regulates calvarial osteoblast differentiation. J Cell Sci. 1996;109:1369–80.
29. Sun M, Chi G, Xu J, Tan Y, Xu J, Lv S, et al. Extracellular matrix stiffness controls osteogenic differentiation of mesenchymal stem cells mediated by integrin alpha5. Stem Cell Res Ther. 2018;9:52.
30. Lai CF, Cheng SL. Alphavbeta integrins play an essential role in BMP-2 induction of osteoblast differentiation. J Bone Miner Res. 2005;20:330–40.
31. Popov C, Radic T, Haasters F, Prall WC, Aszodi A, Gullberg D, et al. Integrins alpha2beta1 and alpha11beta1 regulate the survival of mesenchymal stem cells on collagen I. Cell Death Dis. 2011;2:e186.
32. Hamidouche Z, Fromigue O, Ringe J, Haupl T, Vaudin P, Pages JC, et al. Priming integrin alpha5 promotes human mesenchymal stromal cell osteoblast differentiation and osteogenesis. Proc Natl Acad Sci USA. 2009;106:18587–91.
33. Brown AC, Dysart MM, Clarke KC, Stabenfeldt SE, Barker TH. Integrin alpha3beta1 binding to fibronectin is dependent on the ninth type III repeat. J Biol Chem. 2015;290:25534–47.
34. Robinson EE, Foty RA, Corbett SA. Fibronectin matrix assembly regulates alpha5beta1-mediated cell cohesion. Mol Biol Cell. 2004;15:973–81.
35. Koshida S, Kishimoto Y, Ustumi H, Shimizu T, Furutani-Seiki M, Kondoh H, et al. Integrinalpha5-dependent fibronectin accumulation for maintenance of somite boundaries in zebrafish embryos. Dev Cell. 2005;8:587–98.
36. Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S. Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. J Biol Chem. 2005;280:41342–51.
37. Rossini M, Gatti D, Adami S. Involvement of WNT/beta-catenin signaling in the treatment of osteoporosis. Calcif Tissue Int. 2013;93:121–32.