FRZB affects *Staphylococcus aureus*-induced osteomyelitis in human bone marrow derived stem cells by regulating the Wnt/β-catenin signaling pathway

XIN LI, WENYONG PANG, HONGSONG FAN, HAO WANG and LEIBING ZHANG

Department of Emergency Surgery, Guizhou Provincial People's Hospital, Guiyang, Guizhou 550023, P.R. China

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Abstract. Osteomyelitis is an infectious disease of bone tissue caused by bacterial infection, which can infect through hematogenous, traumatic or secondary ways and then lead to acute or chronic bone injury and relative clinical symptoms, bringing physical injury and economic burden to patients. Frizzled related protein (FRZB) participates in the regulation of various diseases (osteoarthritis, cardiovascular diseases and types of cancer) by regulating cell proliferation, motility, differentiation and inflammation, while its function in osteomyelitis remains to be elucidated. The present study aimed to uncover the role and underlying mechanism of FRZB mediation in *Staphylococcus aureus* (*S. aureus*)-induced osteomyelitis. Human bone marrow derived stem cells (hBMSCs) were treated with *S. aureus* to imitate an inflammatory osteomyelitis micro-environment *in vitro*, then mRNA and protein expression were severally assessed by RT-PCR and western blotting. The activity, apoptosis and differentiation of the cells were characterized via CCK-8, caspase-3 activity and Alizarin red sulfate/alkaline phosphatase staining, respectively. Expression levels of FRZB were upregulated in *S. aureus*-infected hBMSCs. Over-expression of FRZB significantly reduced hBMSC cell viability and differentiation while promoting cell apoptosis with or without *S. aureus* infection. However, FRZB knockdown reversed these effects. Once Wnt was impeded, the effect of FRZB downregulation was impeded to a great extent. Taken together, FRZB participated to regulate the osteomyelitis by activating the Wnt/β-catenin signaling pathway.

Correspondence to: Dr Leibing Zhang, Department of Emergency Surgery, Guizhou Provincial People's Hospital, 83 Zhongshan East Road, Guiyang, Guizhou 550023, P.R. China
E-mail: jakmed@163.com

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Introduction

Osteomyelitis is an inflammatory reaction in which bacteria or inflammatory mediators invade periosteum, sclerotin and bone marrow in various ways, which can result in progressive bone deterioration, bone neoformation and severe inflammatory responses, leading to substantial mortality and morbidity in patients (1). *Staphylococcus aureus* (*S. aureus*) is the commonest bacterial infection (80-90%), followed by *Streptococcus* and *Escherichia coli* (2). Due to the anatomical and physiological characteristics of bone, antimicrobial therapy for bone and joint infections has not achieved a high success rate in most infectious diseases, so osteomyelitis is still considered one of the most difficult to treat infectious diseases (3). Osteomyelitis is featured by progressive devastating of the bone and the forming of sequestra, which acted as the major directors of net bone formation or resorption during normal physiological turnover of bone and following infection. Promoting osteoblast formation is the key to inhibit progressive bone destruction and bone isolation caused by osteomyelitis (4).

Wnt is a secreted L-cysteine rich glycoprotein with an important role in regulating the differentiation of mesenchymal stem cells (MSCs), containing three main receptors including LDL receptor-related protein (LRP-5/6), frizzled receptors (FZDs) and β-catenin protein (5). β-catenin serves an important role in the regulation of bone formation and the osteoblasts differentiation (6). In addition, Runx2 can directly stimulate the Wnt/β-catenin signaling pathway, thus regulate genes transcription such as osteocalcin (OCN), type I collagen (COL1A1), osteopontin (OPN) and collagenase 3 during osteoblast proliferation and differentiation of bone marrow mesenchymal stem cells (BMSCs) (7). The Wnt/β-catenin signal transduction pathway is also involved in regulating the expression of downstream matrix metalloproteinases (MMPs) family, which include the main enzymes destroying the extracellular matrix (ECM) of articular cartilage, such as MMP-2 and MMP-3, which are closely associated with the pathogenesis of osteomyelitis (8,9).

Frizzled related protein (FRZB), as a competitive inhibitor of the Wnt signal pathway, can competitively bind to the transmembrane frizzle receptor and LRP-5/6 complex receptor, thus inhibiting the Wnt signal pathway (10). Secreted frizzled related protein 3 (sFRP3) is an important member of the frizzled related protein family, which is encoded by the FRZB gene (10). FRZB has been reported to be involved in the process

regulation of osteoarthritis, cardiovascular disease and types of cancer (11-13). The high expression of FRZB may be closely associated with bone metastasis in patients with liver cancer (14). The knockout of FRZB in gastric cancer cells increases cell growth and migration/invasion, which is also accompanied by activation of Wnt/β-catenin and the downstream targets (11). In a mouse model, deletion of the FRZB not only increases articular cartilage loss during arthritis arising from enzyme damage or inflammation, but also leads to cortical bone thickening, increased stiffness after loading and cortical apical bone formation (15). A study on patients with early RA reports that the basic serum level of FRZB was high, but was reduced following treatment with anti-rheumatic drugs (16). As for the genetic study of this protein, a study conducted in patients with arthritis reported that FRZB was negatively correlated with this disease (12). Therefore, the mechanism of FRZB in bone related diseases needs further exploration. It is important to highlight that the intrinsic mechanism of the FRZB gene associated with the osteomyelitis remains to be elucidated.

In the present study, the expression profiles of the FRZB gene were primarily confirmed in human bone marrow derived stem cells (hBMSCs) and patients with osteomyelitis, then the influences of FRZB on cell activity, apoptosis and differentiation of hBMSCs treated with or without *Staphylococcus aureus (S. aureus)* were detected in different expression states. Further, it was verified that whether silencing of FRZB restrained *S. aureus*-induced osteomyelitis by regulating Wnt/β-catenin signal pathway.

Materials and methods

Cell culture. hBMSCs (cat. no. SCSP-405) were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Basic medium ingredients were low-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS, 100 U/ml streptomycin and 100 U/ml penicillin. Culture conditions were 37˚C, 5% CO_2, 95% air humidity with half changed every 48 h. The cells of the 3rd to 5th generation were used for the experiments (17). A previous study has shown that activation of Wnt/β-catenin pathway contributes to the BMSC osteogenic differentiation and osteogenesis (18). The present study used inhibitor of β-catenin responsive transcription [ICRT-3; 2-[[[2-(4-ethylphenyl)-5-methyl-4-oxazolyl]methyl] thio]-N-(2-phenylethyl)acetamide] as an inhibitor of Wnt signaling pathway that could inhibit the occurrence of osteogenic differentiation and osteogenesis in control and infected BMSCs. Hence, ICRT3 was used to investigate the function of Wnt/β-catenin signaling pathway in the FRZB-mediated osteomyelitis inhibition. hBMSCs were seeded in 96 well plates at a density of $2x10^5$ cells per ml. At 24 h after transfection, cells were pre-treated with 10 μM ICRT-3 (MilliporeSigma) or vehicle for 50 min at 37˚C and then infected with *S. aureus* or vehicle.

Bacterial culture and infection. The *S. aureus* (ATCC; cat. no. 53657) strain was cultured overnight in brain heart infusion broth medium (OXOID Ltd.) at 37˚C under 160 rpm rotation on a shaker (Shanghai Fuma Laboratory Instrument Co., Ltd.). Then the cultures were centrifuged (10,000 x g, 4˚C, 10 min) and washed before re-suspended in PBS to a final concentration of $0.5x10^6$ colony forming units (CFU) per μl. For bacterial infection process, hBMSCs were infected with a 100 MOI of *S. aureus* and incubated for 72 h under 37˚C. Extracellular *S. aureus* was removed with 20 mg/ml lysostaphin. Fresh medium was added to cells every 2-3 days to remove the bacteria in the supernatant.

FRZB overexpression and knockdown vectors. FRZB overexpression construct was obtained by sub-cloning PCR. FRZB was amplified from the cDNA ORF clone (Sino Biological) into the pcDNA 3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). Empty pcDNA 3.1 vector (vector) was used as the overexpression vector negative control. To complete short hairpin (sh)RNA knockdown, synthesized shRNA oligonucleotide sequences of FRZB (Shanghai Shenggong Biology Engineering Technology Service, Ltd.) were subcloned into the lentiviral pSilencer 4.1 vector backbone (Thermo Fisher Scientific, Inc.). The shRNA sequences for FRZB were as follows: 3'-GGAGATTCTAAAGTCCTCTTTCAAGAGAAG AGGACTTTAGAATCTCC-5'. A scramble shRNA was used as negative control: forward, 5'-AGGCGATTAAGTTGG GTA-3'; reverse, 5'-CGGTAGGCGTGTACGGTG-3'. Plasmids were transfected into hBMSCs for 48 h at 37˚C by using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocols. At 48 h post-transfection, the subsequent experiments were performed.

The 3rd generation lentiviral system was used for lentivirus packaging of sh-FRZB, including pSilencer 4.1-FRZB-shRNA, pCMV Delta R8.2 plasmid (Addgene, Inc.) and pCMV VSVG plasmid (Addgene, Inc.). Transfection mix was prepared as follows: Opti-MEM reduced serum medium (Thermo Fisher Scientific, Inc.) 1 ml; pCMV delta R8.2, 2 μg; pCMV-VSV-G, 0.5 μg; pSilencer 4.1-FRZB-shRNA, 1.5 μg; Xtreme gene 9 transfection reagent (MilliporeSigma), 12 μl. $2x10^6$ HEK293T cells (ATCC) were seeded in a 10 cm^2 plate and cultured for 24 h at 37˚C and 5% CO_2. The medium was replaced with fresh DMEM. After incubation for 20 min at 37˚C, the transfection mix was added to the HEK293T cells and the cells were incubated for 48 h at 37˚C and 5% CO_2. Subsequently, the viral supernatant was collected and filtered with a 0.45 μm PVDF filter (MilliporeSigma) and then centrifuged for 15 min at 4,000 x g and 4˚C with Plus-20 centrifugal ultrafiltration (MilliporeSigma) to obtain a high-titer lentivirus stock. The lentivirus without the transgene was used as the negative control and was produced in the same manner. hBMSCs were seeded at $1.0x10^5$ cells per well in 24-well plates in DMEM containing 10% FBS. After 24 h incubation at 37˚C, the hBMSCs were transduced with or without lentivirus (MOI=20) and then incubated for an additional 48 h at 37˚C. Following that, puromycin (2 μg/ml) was added to hBMSCs and fresh media with puromycin was added every two days until all hBMSCs not treated with any lentivirus were dead. Successfully screened hBMSCs were used for subsequent experiments. Successful transduction was demonstrated through reverse transcription-quantitative (RT-q) PCR and western blotting.

RT-qPCR. Expression of FRZB in $1x10^4$ hBMSCs was examined by RT-qPCR. Total RNA was extracted by the TRIzol® reagent (Thermo Fisher Scientific, Inc.) from the harvested hBMSCs. cDNA synthesis was accomplished using a PrimeScript™ RT Master Mix (Takara Bio, Inc.) according to the manufacturer's

protocols. qPCR was performed using SYBR® Premix EX Taq™ (Takara Bio, Inc.) according to the manufacturer's protocols. The qPCR conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. The set of primers for FRZB were as follows: Forward, 5'-GAGGAGCTGCCAGTG TACGAC-3' and Reverse, 5'-GAAAATCAGCTCCGTCCG C-3'; GAPDH: Forward, 5'-GGACCTGACCTGCCGTCTAG-3' and Reverse, 5'-GTAGCCCAGGATGCCCTTGA-3' respectively. The $2^{-\Delta\Delta Cq}$ (19) method was used to calculate the relative mRNA expression level and GAPDH was used as an internal parameter. The experiments were replicated three times.

Western blotting. Total protein was extracted using RIPA reagent and then measured by a BCA Protein Assay kit (Beyotime Institute of Biotechnology). The proteins (40 μg/lane) were separated by a 10% SDS-PAGE gel before being blotted onto a PVDF membrane. Then, the membranes were blocked with 5% skimmed milk powder in phosphate-buffered saline solution with 0.05% Tween (PBST) for 1 h at room temperature and incubated with the specific primary antibody at 4˚C overnight. The primary antibodies used were anti-FRZB (1:1,000; Abcam; ab273582), anti-β catenin (1:1,000; Abcam; ab32572), anti-runt-related transcription factor 2 (RUNX2; 1:1,000; Abcam; ab236639), anti-alkaline phosphatase (ALP; 1:1,000; Abcam; ab224335), anti-COL1A1 (1:1,000; Abcam; ab138492), anti-osterix (Osx; 1:2500, Abcam; ab209484), anti-osteocalcin (OCN; 1:1,000; Abcam; ab133612) and anti-GAPDH (1:1,000; Abcam; ab8245). The membranes were washed with PBST and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies [Goat Anti-Rabbit IgG H&L (1:5,000; ab96899; Abcam) or Goat Anti-Mouse IgG H&L (1:5,000; ab96879, Abcam)] for 30 min at 37˚C. Protein signals were visualized using enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology). Densitometric analysis was performed using ImageJ software V1.52a (National Institutes of Health) and values were normalized to GAPDH.

Caspase-3 activity detection. As in a previous study (20), caspase-3 activity was assessed by Caspase-3 Assay kit (MilliporeSigma) according to the manufacturer's instructions. Briefly, cells in each group were lysed on ice for 10 min with lysate ($2x10^4$ cells/μl), then the supernatant was collected at 4˚C at 12,000 x g for 1 min. Protein quantification was carried out through a BCA detection kit (Beyotime Institute of Biotechnology). Supernatant (45 μl) was mixed with 50 μl of 2X reaction buffer (provided in the kit) and 5 μl reaction substrate (Ac-DEVD-pNA; provided in the kit) before incubating at 37˚C for 2 h. The free pNA was assessed at 450 nm with microplate reader (Bio-Rad Laboratories, Inc.) and the concentration of pNA was calculated through a standard curve obtained from the detection of a series of known concentrations of pNA. The number of nanomoles of pNA per 1 mg of total protein per minute was used to represent caspase-3 activity. The experiment was repeated three times in each group.

Alizarin red sulfate (ARS) staining. ARS staining was conducted to analyze the differentiation of hBMSCs. Briefly, after washing twice in PBS, the cells were fixed with 95% ethanol for 10 min at room temperature. The cells were then incubated with ARS staining buffer solution at 37˚C for 30 min. The mineralization nodules were imaged using an optical light microscope (magnification, x200) in five randomly selected fields of view. The darker the intensity of the red dots, the higher the number of calcium nodules and therefore the higher degree of differentiation. For semi-quantitative assessment of the formation of mineralization nodules, 10% cetylpyridinium chloride in 10 mM Na_2HPO_4 was added to the wells and incubated for 10 min at room temperature. Then absorbance at 562 nm was measured with a microplate reader.

Alkaline phosphatase (ALP) staining and ALP activity. ALP, as a marker of osteoblast differentiation (21), was assessed by an ALP staining kit (Beijing Solarbio Science & Technology Co., Ltd.). The cells were fixed for 10 min with 4% paraformaldehyde at room temperature and then washed with distilled water. ALP staining solution was added with and incubated for 20 min at 37˚C. After washing with distilled water, the results were examined using an optical light microscope (magnification, x200) in five randomly selected fields of view. The darker intensity of the red dots was associated with higher ALP activity.

ALP activity was evaluated using a commercial ALP activity colorimetric kit (BioVision, Inc.). The cell cultures were rinsed with pre-cooled PBS and treated with 1% Triton X-100 (MilliporeSigma), then transferred to distilled water. The absorbance at 405 nm was determined with microplate reader (Bio-Rad Laboratories, Inc.). Total protein concentration was detected by BCA protein Assay kit (Beyotime Institute of Biotechnology). ALP activity level was quantified by dividing the absorbance to the protein concentration.

Cells activity. Activity of the hBMSCs was detected by Cell Counting Kit-8 (MedChemExpress). hBMSCs were centrifuged at 1,000 x g for 5 min at 4˚C before being suspended in DMEM low-glucose medium containing 10% FBS and counted. The cells were inoculated at $5x10^4$ cells/well in 24-well plates; the volume of each well was 1,000 μl. A total of five multiple wells were made for each group and medium only wells were used as blank controls. PBS was added to the surrounding wells to slow liquid evaporation before adding 10 μl CCK-8 solution to each well 5 days post cell inoculation. Cells were cultured at 37˚C, 5% CO_2 for 2 h, followed by measuring the absorbance at 450 nm with a microplate reader.

Statistical analysis. The data was analyzed using SPSS 18.0 software (SPSS, Inc.) and the results were presented as mean ± standard deviation. Unpaired *t*-test was used to compare two groups. One-way ANOVA (followed by Tukey post hoc test) was used for multiple comparisons. SPSS 18.0 software was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FRZB is highly expressed in S. aureus infected hBMSCs. As FRZB is an essential factor contributing to the osteoarthritis pathogenesis (22), the role of FRZB in osteomyelitis was explored in the present study. *S. aureus* infected hBMSCs were used as a model of *S. aureus*-induced osteomyelitis *in vitro*

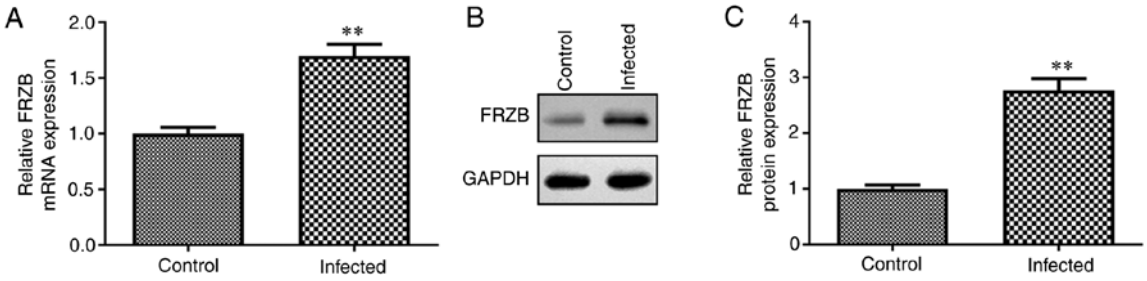

Figure 1. FRZB is overexpressed in *Staphylococcus aureus*-infected hBMSCs. (A) The mRNA expression of FRZB was detected by reverse transcription-quantitative PCR. (B and C) The protein expression was assessed by western blotting. **P<0.01 vs. control group. FRZB, frizzled related protein; hBMSCs, human bone marrow derived stem cells.

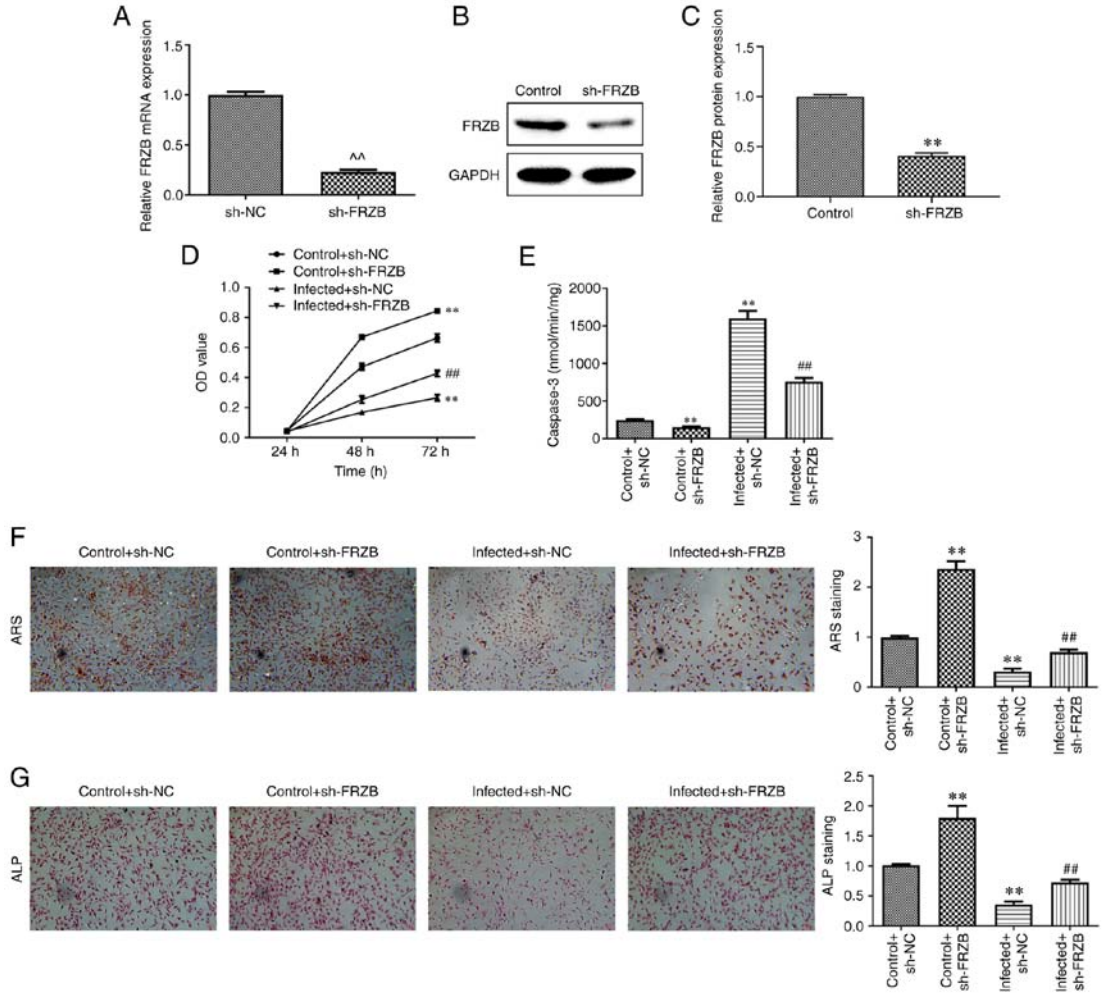

Figure 2. Downregulation of FRZB promotes cell viability and osteogenic differentiation and repressed apoptosis in hBMSCs. (A) Following transfection with sh-FRZB or sh-NC, the mRNA expression of FRZB was measured by reverse transcription-quantitative PCR. (B and C) the protein expression of FRZB was measured by western blotting. (D) The cell viability was assessed by CCK8 assay. (E) The apoptosis was detected by caspase-3 activity assay. The osteogenic differentiation was estimated by (F) ARS and (G) ALP staining. ^^P<0.01 vs. sh-NC group, **P<0.01 vs. control + sh-NC group, ##P<0.01 vs. infected + sh-NC. FRZB, frizzled related protein; hBMSCs, human bone marrow derived stem cells; sh, short hairpin; NC, negative control; ARS, Alizarin red sulfate; ALP, alkaline phosphatase.

and the mRNA and protein expression of FRZB in *S. aureus* infected hBMSCs was detected by RT-qPCR and western blotting, respectively. It was found that transcription level and protein expression level of FRZB in infected hBMSCs were all significantly higher compared with those in control group (Fig. 1A-C). The results suggested that *S. aureus* induced the upregulation of FRZB mRNA and protein in hBMSCs.

FRZB downregulation represses osteomyelitis by reducing apoptosis and promoting differentiation of hBMSCs. To study the FRZB-mediated effects in osteomyelitis, shFRZB or sh-NC was transfected into hBMSCs. The results showed that FRZB mRNA and protein expression was repressed in hBMSCs transfected with shFRZB (Fig. 2A-C). Meanwhile, apart from the significant inhibition effects caused by

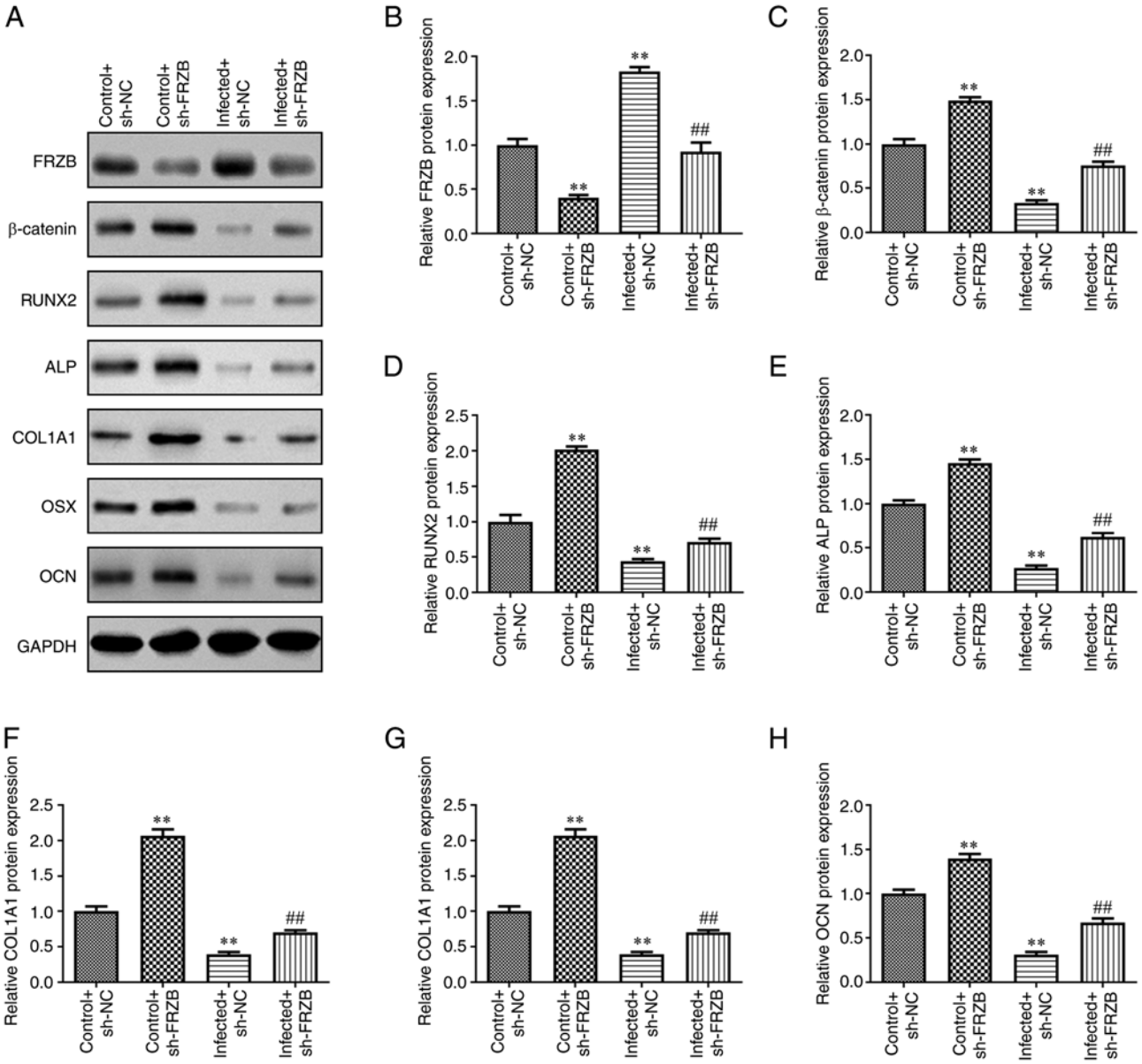

Figure 3. Downregulation of FRZB amplifies the protein expression of osteoblast-specific markers. (A) The protein expression of (B) FRZB, (C) β-catenin, (D) RUNX2, (E) ALP, (F) COL1A1, (G) Osx and (H) OCN were measured by western blotting. **P<0.01 vs. control + sh-NC group, $^{\#\#}$P<0.01 vs. infected + sh-NC. FRZB, frizzled related protein; RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; COL1A1, type I collagen; Osx, osterix; OCN, osteocalcin; sh, short hairpin; NC, negative control.

S. aureus, cell viability of either the sh-FRZB group or the *S. aureus* + sh-FRZB group showed an increase due to downregulation of FRZB (Fig. 2D). The results revealed a significant decrease of apoptosis in sh-FRZB group compared with control group and in *S. aureus* + sh-FRZB group compared with *S. aureus* group (Fig. 2E). ARS and ALP staining results showed that the sh-FRZB group and infected + sh-FRZB group exhibited different promoting effects compared with the sh-NC group and *S. aureus* infected sh-NC group, respectively (Fig. 2F-G). Further investigation into the pathways regulating cell differentiation using western blotting indicated that β-catenin, RUNX2, ALP, COL1A1, Osx and OCN allied to the Wnt/β-catenin signaling pathway all exhibited a significant elevating in sh-FRZB group and *S. aureus* + sh-FRZB group compared with their control groups, severally (Fig. 3A-H). These results indicated that silencing of FRZB promoted cell viability and osteogenic differentiation, while inhibiting apoptosis in *S. aureus* infected hBMSCs.

FRZB upregulation promotes osteomyelitis by increasing hBMSCs apoptosis and reducing differentiation. To identify the mechanism responsible for FRZB mediation in osteomyelitis, the overexpression of FRZB in hBMSCs was conducted through transfection of pcDNA-FRZB. The expression of FRZB was upregulated at the mRNA and protein level as determined by RT-qPCR (Fig. 4A-C). The effect of transient overexpression of FRZB on cell viability was measured using the CCK8 assay and the results showed that hBMSC viability of both the OE-FRZB group and the *S. aureus* + OE-FRZB group were reduced compared with their controls separately (Fig. 4D). From cell apoptosis assay, a significant enhancement of caspase-3 activity could be observed corresponding to the

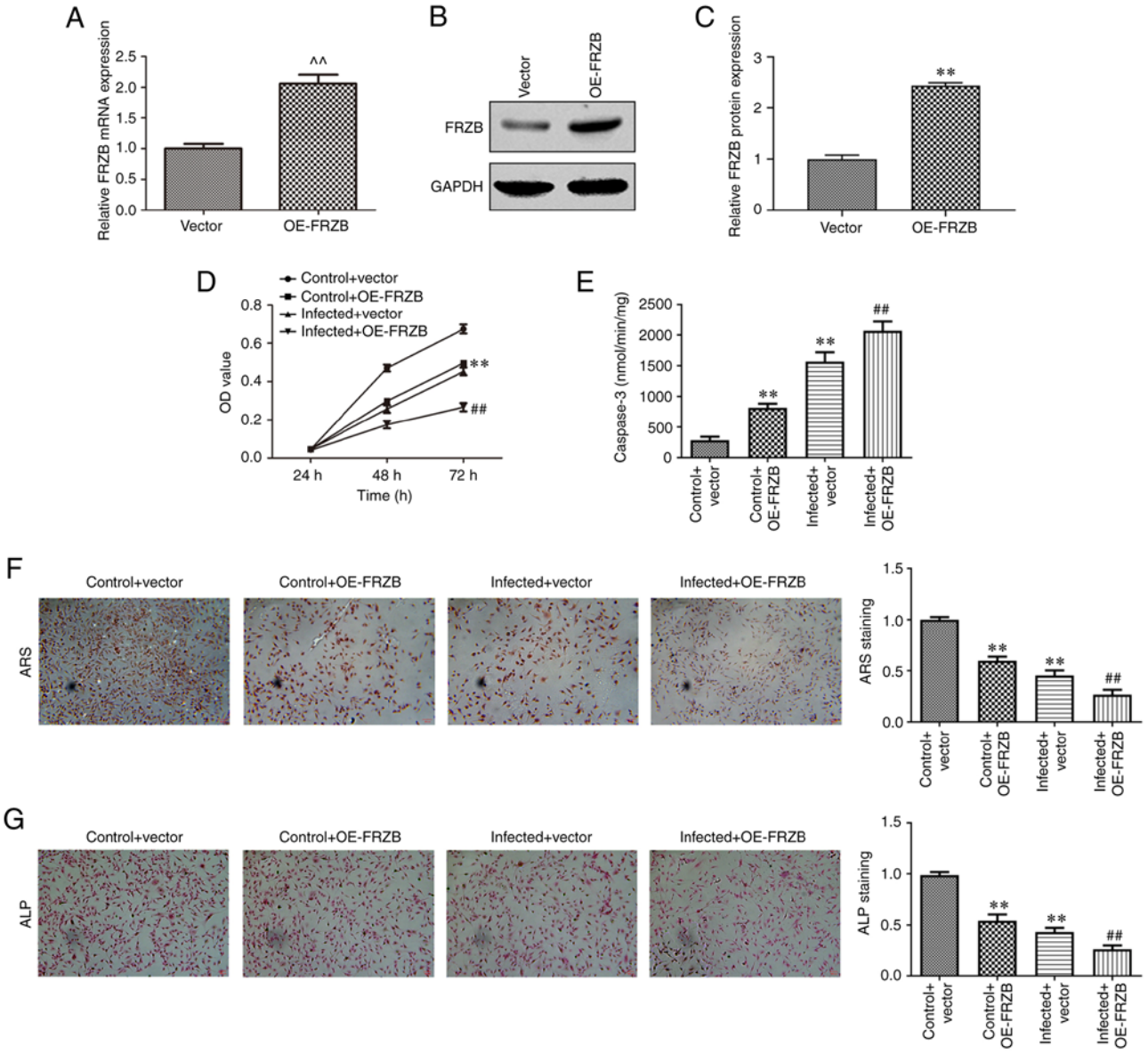

Figure 4. Upregulation of FRZB limits cell viability and osteogenic differentiation and enhances apoptosis in hBMSCs. (A) After transfection with pcDNA-FRZB or vector, the mRNA expression of FRZB was measured by reverse transcription-quantitative PCR. (B and C) the protein expression of FRZB was measured by western blotting. (D) Cell viability was assessed by CCK8 assay. (E) The apoptosis was detected by caspase-3 activity assay. Osteogenic differentiation was estimated by (F) ARS (magnification, x200) and (G) ALP staining (magnification, x200). $^{\wedge\wedge}$P<0.01 vs. vector group, **P<0.01 vs. control + vector group, $^{\#\#}$P<0.01 vs. infected + vector. FRZB, frizzled related protein; hBMSCs, human bone marrow derived stem cells; OE, overexpression; ARS, Alizarin red sulfate; ALP, alkaline phosphatase.

FRZB upregulation (Fig. 4E). To further confirm the mode of action of FRZB on cell differentiation, ARS and ALP staining were conducted. Staining intensities of OE-FRZB group and the *S. aureus* + OE-FRZB group were weaker than their separate controls (Fig. 4F and G). The phenotypical quantification was also supported by a reduction in osteogenic markers such as β-catenin, RUNX2, ALP, COL1A1, Osx and OCN following FRZB overexpression (Fig. 5A-H). These results demonstrated that upregulation of FRZB repressed cell viability and osteogenic differentiation while enhancing apoptosis in *S. aureus* infected hBMSCs.

FRZB downregulation inhibits osteomyelitis by activating the Wnt/β-catenin signaling pathway. To further investigate the function of Wnt/β-catenin signaling pathway in the FRZB-mediated osteomyelitis inhibition, hBMSCs were treated with ICRT3 (23), an inhibitor of both Wnt and β-catenin responsive transcription. CCK8 assay was conducted to evaluate the effect of FRZB and the inhibitor to the activity of hBMSCs and the observable promotion due to FRZB downregulation was counteracted by the Wnt inhibitor in the normal hBMSCs group and the *S. aureus* infected group (Fig. 6A). In the cell apoptosis detection assay, activities of the caspase-3 for the sh-FRZB group and the *S. aureus* + sh-FRZB were both markedly increased while all raised to their comparative levels subsequently after joining of the Wnt inhibitor (Fig. 6B). As for hBMSCs differentiation, staining intensities of ARS and ALP staining all depicted a significant decrease attributed to the effect of the Wnt inhibitor when compared with their FRZB downregulation controls,

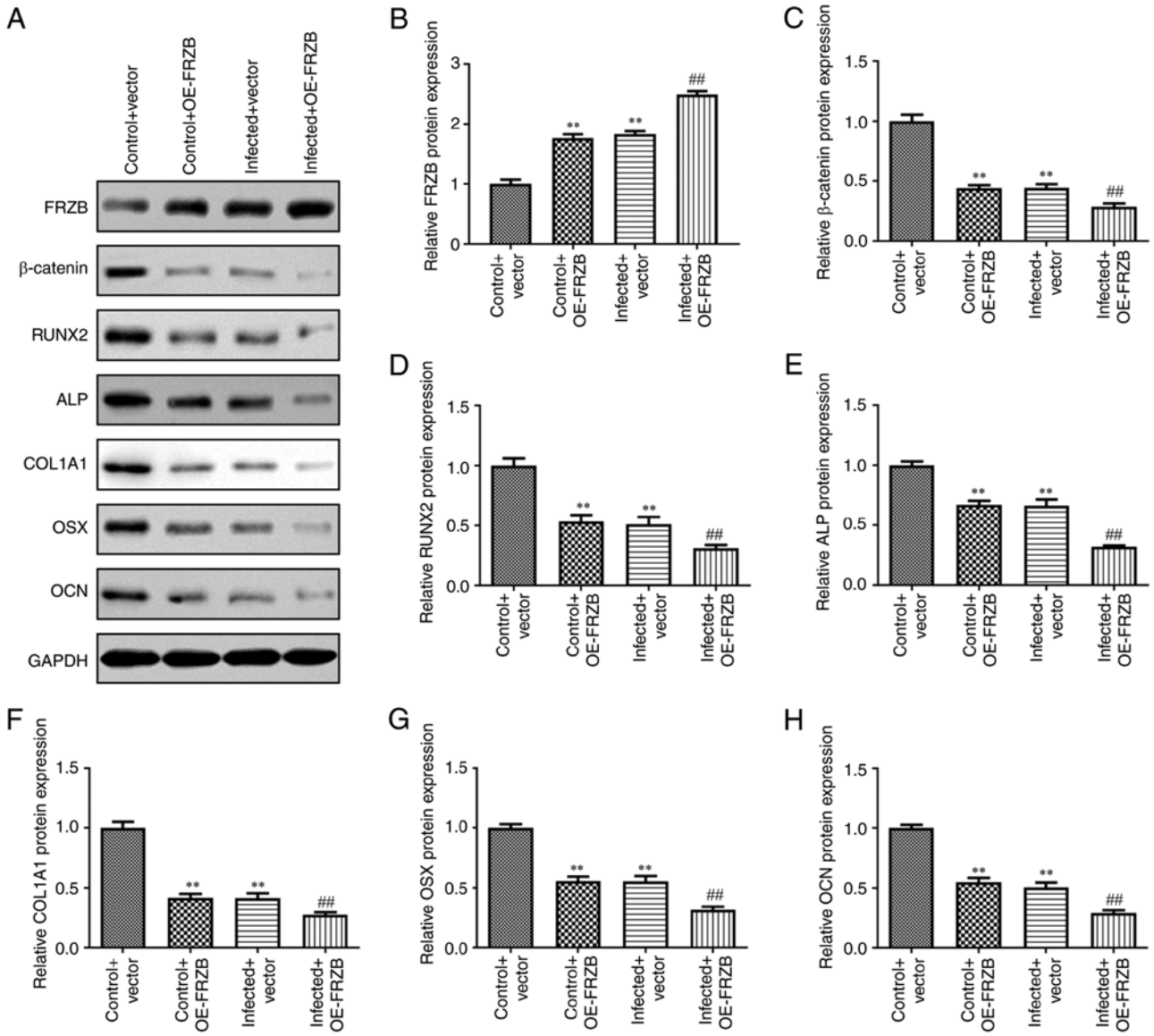

Figure 5. Upregulation of FRZB suppresses the protein expression of osteoblast-specific markers. (A) The protein expression of (B) FRZB, (C) β-catenin, (D) RUNX2, (E) ALP, (F) COL1A1, (G) Osx and (H) OCN were measured by western blotting. **P<0.01 vs. vector group, **P<0.01 vs. control + vector group, $^{\#\#}$P<0.01 vs. infected + vector. FRZB, frizzled related protein; RUNX2, runt-related transcription factor 2; ALP, alkaline phosphatase; COL1A1, type I collagen; Osx, osterix; OCN, osteocalcin; OE, overexpression.

respectively (Fig. 6C-E). These results showed that activation of Wnt/β-catenin signaling pathway participated in the effect of FRZB on hBMSCs induced by *S. aureus*.

Discussion

Bone marrow mesenchymal stem cells (BMSCs) have osteogenic differentiation ability and serve an important role in bone formation (18,24). Osteomyelitis usually occurs during bone healing and is known to inhibit osteogenesis and bone formation (25,26). However, the regulatory mechanism of osteomyelitis on osteogenic differentiation of BMSCs remains to be elucidated. *In vivo*, the inflammatory environment of osteomyelitis is complex, so it is difficult to replicate the inflammatory environment of osteomyelitis. *S. aureus* is the most common bacterial species causing bone infection, causing 80% of osteomyelitis secondary to bone infection (27). Previous studies have shown that *S. aureus* can induce inflammation *in vitro* (28,29). In the present study, *S. aureus* was selected to trigger the inflammatory environment in hBMSCs to build a cell model that could partially replicate the inflammatory environment of osteomyelitis *in vitro*. The present study then investigated the role of FRZB in hBMSCs infected by *S. aureus* and confirmed that FRZB participated in the regulation of bone differentiation of hBMSCs infected by *S. aureus* by affecting Wnt/β-catenin signaling pathway.

In previous studies, FRZB has been implicated in a range of developmental processes and diseases (30-32). FRZB serves as a secreted Wnt antagonist that can decrease growth and invasiveness of fibrosarcoma cells (33). In addition, FRZB expression level also shows a deep relationship with the Wnt/β-catenin pathway in gastric cancer (11,34). However, the correlation between FRZB and osteomyelitis, along with the possible relevant regulatory mechanisms

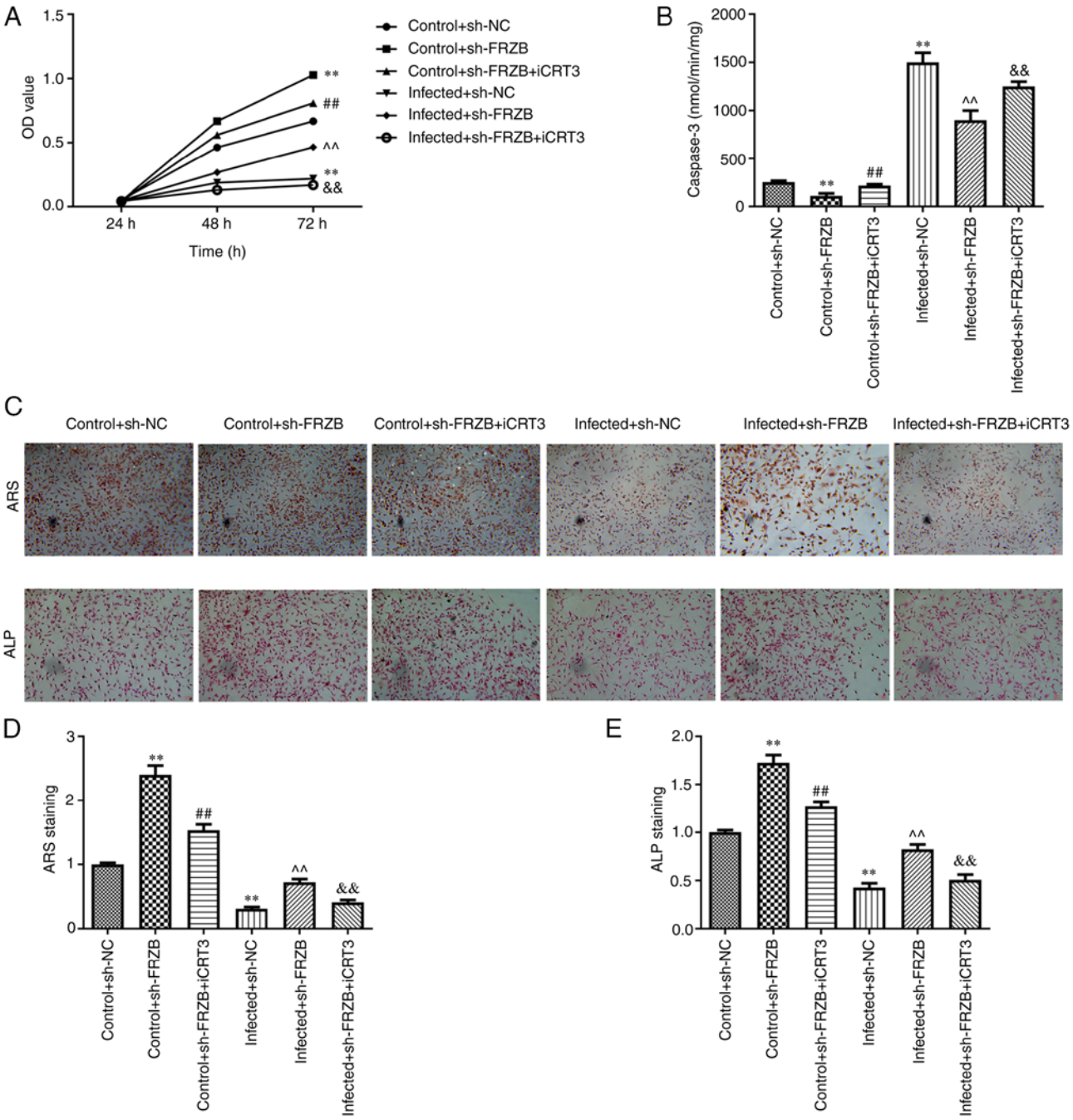

Figure 6. Silencing of FRZB represses *Staphylococcus aureus*-induced osteomyelitis in hBMSCs by regulating the Wnt/β-catenin pathway. (A) The cell viability was assessed by CCK8 assay. (B) The apoptosis was detected by caspase-3 activity assay. (C-E) The osteogenic differentiation was estimated by ARS staining (magnification, x200) and ALP staining (magnification, x200). **P<0.01 vs. control + sh-NC group, $^{\#\#}$P<0.01 vs. control + sh-FRZB group, $^{\wedge\wedge}$P<0.01 vs. infected + sh-NC, $^{\&\&}$P<0.01 vs. infected + sh-FRZB group. FRZB, frizzled related protein; hBMSCs, human bone marrow derived stem cells; ARS, Alizarin red sulfate; ALP, alkaline phosphatase; sh, short hairpin; NC, negative control; ICRT-3, 2-[[[2-(4-ethylphenyl)-5-methyl-4-oxazolyl]methyl]thio]-N-(2-phenylethyl)acetamide.

therein, remain to be elucidated. The present study found that FRZB was upregulated in hBMSCs with *S. aureus* infection. To investigate the function of FRZB, FRZB expression was firstly downregulated using shRNA. The role of FRZB on osteogenic differentiation of hBMSCs treated with *S. aureus* was then assessed. Based on these results, FRZB silencing ameliorated *S. aureus*-inhibited proliferation and osteogenic differentiation in hBMSCs. It has been reported that FRZB antagonizes Wnt signaling by binding to the Wnt ligands and induces signal transduction (10). This initiates the transcription of osteogenic canonical transducers, such as Runx2 and β-catenin, which further regulate cell proliferation and differentiation (35,36). As the results of the present study showed, downregulation of FRZB in *S. aureus*-infected BMSCs could amplify the expression of β-catenin, RUNX2, ALP, COL1A1, OSC and OCN, considered as markers of osteogenesis and bone formation (37). Hence, a role of FRZB in either direct or indirect regulation of the osteomyelitis through inhibiting apoptosis and facilitating differentiation can be presumed in hBMSCs.

As well as the potential salutary effects of the FRZB downregulation, FRZB was overexpressed in hBMSCs to

further affirm its functional role. The results indicated that FRZB expression effectively activated caspase-3 and inhibited osteogenic differentiation in *S. aureus*-infected BMSCs. FRZB also acted as a major suppressor to the various transducers in Wnt signaling pathway. Thus, FRZB was confirmed to be a key factor affecting osteomyelitis and it can be hypothesized that the regulation center was located in Wnt signaling pathway. To verify this hypothesis, an inhibitor (ICRT-3) targeting the Wnt/β-catenin signaling pathway was used to block the crosstalk between Wnt and other transducers. FRZB cannot validly bind to the receptors and cause the inhibition of β-catenin nuclear transcription (38,39). With Wnt inhibition, the elevation in cell activity, drive to differentiation and apoptosis resistance caused by downregulation of FRZB were all eliminated almost completely. Previous studies show that aberrant changes in FRZB expression are associated with pathophysiological states including osteoarthritis and cancer (attenuated expression) (40,41) and limb-girdle muscular dystrophy (increased expression) (42). Combined with the findings of the present study, it could be concluded that silencing of FRZB may inhibit osteomyelitis under the mediation of Wnt/β-catenin signaling pathway.

However, *S. aureus*-induced osteomyelitis is associated with multiple signaling pathways besides the Wnt/β-catenin, such as the NF-κB (43), SMAD (44), MyD88 and IL-1R signaling pathways (45). Whether FRZB participates in the regulation of osteomyelitis by mediating other signaling pathways needs further research. Meanwhile, lack of clinical samples and *in vivo* experimental data are two limitations to the present study. In addition, the effects of ICRT-3 alone on untransfected control and infected cells were not assessed in the present study, which was a limitation in experimental grouping. Those limitations will be studied in future work.

The present study thereby proposed a novel controlling gene involving FRZB in the pathogenesis of osteomyelitis, whereby during *S. aureus* infection, FRZB inhibited the Wnt/β-catenin signaling pathway, which in turn reduced osteogenic differentiation of hBMSCs, contributing to osteomyelitis. FRZB may also serve as therapeutic targets for treatment against osteomyelitis.

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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

LZ and XL conceived and designed the present study and wrote the main manuscript text. XL and WP performed the experiments and data acquisition. HF and HW analyzed and interpreted the data and performed literature searches. HF and HW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Cui Y, Lu S, Tan H, Li J, Zhu M and Xu Y: Silencing of long non-coding RNA NONHSAT009968 ameliorates the staphylococcal protein A-Inhibited osteogenic differentiation in human bone mesenchymal stem cells. Cell Physiol Biochem 39: 1347-1359, 2016.
2. Josse J, Velard F and Gangloff SC: Staphylococcus aureus vs. Osteoblast: Relationship and consequences in osteomyelitis. Front Cell Infect Microbiol 5: 85, 2015.
3. Fantoni M, Taccari F and Giovannenze F: Systemic antibiotic treatment of chronic osteomyelitis in adults. Eur Rev Med Pharmacol Sci 23 (2 Suppl): S258-S270, 2019.
4. Sanchez CJ Jr, Ward CL, Romano DR, Hurtgen BJ, Hardy SK, Woodbury RL, Trevino AV, Rathbone CR and Wenke JC: Staphylococcus aureus biofilms decrease osteoblast viability, inhibits osteogenic differentiation, and increases bone resorption in vitro. BMC Musculoskelet Disord 14: 187, 2013.
5. Liu W, Konermann A, Guo T, Jäger A, Zhang L and Jin Y: Canonical Wnt signaling differently modulates osteogenic differentiation of mesenchymal stem cells derived from bone marrow and from periodontal ligament under inflammatory conditions. Biochim Biophys Acta 1840: 1125-1134, 2014.
6. Wang Y, Li YP, Paulson C, Shao JZ, Zhang X, Wu M and Chen W: Wnt and the Wnt signaling pathway in bone development and disease. Front Biosci (Landmark Ed) 19: 379-407, 2014.
7. Qin X, Jiang Q, Komori H, Sakane C, Fukuyama R, Matsuo Y, Ito K, Miyazaki T and Komori T: Runt-related transcription factor-2 (Runx2) is required for bone matrix protein gene expression in committed osteoblasts in mice. J Bone Miner Res 36: 2081-2095, 2021.
8. Kawaguchi H: Regulation of osteoarthritis development by Wnt-beta-catenin signaling through the endochondral ossification process. J Bone Miner Res 24: 8-11, 2009.
9. Wang H, Sun W, Ma J, Pan Y, Wang L and Zhang WB: Biglycan mediates suture expansion osteogenesis via potentiation of Wnt/β-catenin signaling. J Biomech 48: 432-440, 2015.
10. Kwan T, Kazamel M, Thoenes K, Si Y, Jiang N and King PH: Wnt antagonist FRZB is a muscle biomarker of denervation atrophy in amyotrophic lateral sclerosis. Sci Rep 10: 16679, 2020.
11. Qin S, Zhang Z, Li J and Zang L: FRZB knockdown upregulates β-catenin activity and enhances cell aggressiveness in gastric cancer. Oncol Rep 31: 2351-2357, 2014.
12. Fernández-Torres J, Zamudio-Cuevas Y, López-Reyes A, Garrido-Rodríguez D, Martínez-Flores K, Lozada CA, Muñóz-Valle JF, Oregon-Romero E and Martínez-Nava GA: Gene-gene interactions of the Wnt/β-catenin signaling pathway in knee osteoarthritis. Mol Biol Rep 45: 1089-1098, 2018.

13. Ma Z, Wang X, Lv Q, Gong Y, Xia M, Zhuang L, Lu X, Yang Y, Zhang W, Fu G, *et al*: Identification of underlying hub genes associated with hypertrophic cardiomyopathy by integrated bioinformatics analysis. Pharmgenomics Pers Med 14: 823-837, 2021.
14. Huang J, Hu W, Lin X, Wang X and Jin K: FRZB up-regulated in hepatocellular carcinoma bone metastasis. Int J Clin Exp Pathol 8: 13353-13359. eCollection 2015, 2015.
15. Killock D: Osteoarthritis: Frzb knockout reveals the complexity of Wnt signaling in joint homeostasis. Nat Rev Rheumatol 8: 123, 2012.
16. Corallini F, Secchiero P, Castellino G, Montecucco M, Trotta F and Zauli G: Circulating levels of frizzled-related protein (FRZB) are increased in patients with early rheumatoid arthritis and decrease in response to disease-modifying antirheumatic drugs. Ann Rheum Dis 69: 1733-1734, 2010.
17. Li H, Zhang S, Nie B, Long T, Qu X and Yue B: KR-12-a5 reverses adverse effects of lipopolysaccharides on HBMSC osteogenic differentiation by influencing BMP/Smad and P38 MAPK signaling pathways. Front Pharmacol 10: 639, 2019.
18. Wang Y, Zhang X, Shao J, Liu H, Liu X and Luo E: Adiponectin regulates BMSC osteogenic differentiation and osteogenesis through the Wnt/β-catenin pathway. Sci Rep 7: 3652, 2017.
19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
20. Guo C, Wang SL, Xu ST, Wang JG and Song GH: SP600125 reduces lipopolysaccharide-induced apoptosis and restores the early-stage differentiation of osteoblasts inhibited by LPS through the MAPK pathway in MC3T3-E1 cells. Int J Mol Med 35: 1427-1434, 2015.
21. Jin Q, Yuan K, Lin W, Niu C, Ma R and Huang Z: Comparative characterization of mesenchymal stem cells from human dental pulp and adipose tissue for bone regeneration potential. Artif Cells Nanomed Biotechnol 47: 1577-1584, 2019.
22. Lories RJ, Peeters J, Bakker A, Tylzanowski P, Derese I, Schrooten J, Thomas JT and Luyten FP: Articular cartilage and biomechanical properties of the long bones in Frzb-knockout mice. Arthritis Rheum 56: 4095-4103, 2007.
23. Xiao H, Zeng Y, Wang Q, Wei S and Zhu X: A novel positive feedback loop between NTSR1 and Wnt/β-Catenin contributes to tumor growth of glioblastoma. Cell Physiol Biochem 43: 2133-2142, 2017.
24. Li F, Wang X and Niyibizi C: Bone marrow stromal cells contribute to bone formation following infusion into femoral cavities of a mouse model of osteogenesis imperfecta. Bone 47: 546-555, 2010.
25. Jin T, Zhu YL, Li J, Shi J, He XQ, Ding J and Xu YQ: Staphylococcal protein A, Panton-Valentine leukocidin and coagulase aggravate the bone loss and bone destruction in osteomyelitis. Cell Physiol Biochem 32: 322-333, 2013.
26. Beck-Broichsitter BE, Smeets R and Heiland M: Current concepts in pathogenesis of acute and chronic osteomyelitis. Curr Opin Infect Dis 28: 240-245, 2015.
27. Nasser A, Azimi T, Ostadmohammadi S and Ostadmohammadi S: A comprehensive review of bacterial osteomyelitis with emphasis on Staphylococcus aureus. Microb Pathog 148: 104431, 2020.
28. Dubus M, Varin J, Papa S, Chevrier J, Quilès F, Francius G, Audonnet S, Mauprivez C, Gangloff SC, Siboni R, *et al*: Bone marrow mesenchymal stem cells offer an immune-privileged niche to Cutibacterium acnes in case of implant-associated osteomyelitis. Acta Biomater 137: 305-315, 2022.
29. Ding R, Wei S and Huang M: Long non-coding RNA KCNQ1OT1 overexpression promotes osteogenic differentiation of staphylococcus aureus-infected human bone mesenchymal stem cells by sponging microRNA miR-29b-3p. Bioengineered 13: 5855-5867, 2022.
30. Oh CK, Ko Y, Park JJ, Heo HJ, Kang J, Kwon EJ, Kang JW, Lee Y, Myung K, Kang JM, *et al*: FRZB as a key molecule in abdominal aortic aneurysm progression affecting vascular integrity. Biosci Rep 41: BSR20203204, 2021.
31. Thysen S, Luyten FP and Lories RJ: Loss of Frzb and Sfrp1 differentially affects joint homeostasis in instability-induced osteoarthritis. Osteoarthritis Cartilage 23: 275-279, 2015.
32. Person AD, Garriock RJ, Krieg PA, Runyan RB and Klewer SE: Frzb modulates Wnt-9a-mediated beta-catenin signaling during avian atrioventricular cardiac cushion development. Dev Biol 278: 35-48, 2005.
33. Guo Y, Xie J, Rubin E, Tang YX, Lin F, Zi X and Hoang BH: Frzb, a secreted Wnt antagonist, decreases growth and invasiveness of fibrosarcoma cells associated with inhibition of Met signaling. Cancer Res 68: 3350-3360, 2008.
34. Shi Q, Zhou C, Xie R, Li M, Shen P, Lu Y and Ma S: CircCNIH4 inhibits gastric cancer progression via regulating DKK2 and FRZB expression and Wnt/β-catenin pathway. J Biol Res (Thessalon) 28: 19, 2021.
35. Kim HY, Choi S, Yoon JH, Lim HJ, Lee H, Choi J, Ro EJ, Heo JN, Lee W, No KT and Choi KY: Small molecule inhibitors of the Dishevelled-CXXC5 interaction are new drug candidates for bone anabolic osteoporosis therapy. EMBO Mol Med 8: 375-387, 2016.
36. Zuo GL, Zhang LF, Qi J, Kang H, Jia P, Chen H, Shen X, Guo L, Zhou HB, Wang JS, *et al*: Activation of HIFa pathway in mature osteoblasts disrupts the integrity of the osteocyte/canalicular network. PLoS One 10: e0121266, 2015.
37. Tang CY, Chen W, Luo Y, Wu J, Zhang Y, McVicar A, McConnell M, Liu Y, Zhou HD and Li YP: Runx1 up-regulates chondrocyte to osteoblast lineage commitment and promotes bone formation by enhancing both chondrogenesis and osteogenesis. Biochem J 477: 2421-2438, 2020.
38. Goldring SR: The osteocyte: Key player in regulating bone turnover. RMD Open 1(Suppl 1): e000049, 2015.
39. Matsumoto Y, La Rose J, Lim M, Adissu HA, Law N, Mao X, Cong F, Mera P, Karsenty G, Goltzman D, *et al*: Ubiquitin ligase RNF146 coordinates bone dynamics and energy metabolism. J Clin Invest 127: 2612-2625, 2017.
40. Bovolenta P, Esteve P, Ruiz JM, Cisneros E and Lopez-Rios J: Beyond Wnt inhibition: New functions of secreted Frizzled-related proteins in development and disease. J Cell Sci 121(Pt 6): 737-746, 2008.
41. Lories RJ, Corr M and Lane NE: To Wnt or not to Wnt: The bone and joint health dilemma. Nat Rev Rheumatol 9: 328-339, 2013.
42. Sáenz A, Azpitarte M, Armañanzas R, Leturcq F, Alzualde A, Inza I, García-Bragado F, De la Herran G, Corcuera J, Cabello A, *et al*: Gene expression profiling in limb-girdle muscular dystrophy 2A. PLoS One 3: e3750, 2008.
43. Ren LR, Wang H, He XQ, Song MG, Chen XQ and Xu YQ: Staphylococcus aureus Protein A induces osteoclastogenesis via the NF-κB signaling pathway. Mol Med Rep 16: 6020-6028, 2017.
44. Song B, Estrada KD and Lyons KM: Smad signaling in skeletal development and regeneration. Cytokine Growth Factor Rev 20: 379-388, 2009.
45. Putnam NE, Fulbright LE, Curry JM, Ford CA, Petronglo JR, Hendrix AS and Cassat JE: MyD88 and IL-1R signaling drive antibacterial immunity and osteoclast-driven bone loss during Staphylococcus aureus osteomyelitis. PLoS Pathog 15: e1007744, 2019.