PROSER2 is a poor prognostic biomarker for patients with osteosarcoma and promotes proliferation, migration and invasion of osteosarcoma cells

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Abstract. Proline- and serine-rich 2 (PROSER2) is encoded by the 47th open reading frame on human chromosome 10. Bioinformatic analysis has shown PROSER2 was significantly correlated with prognostic outcome of osteosarcoma patients. Its role in the progression and metastasis of human osteosarcoma has not been elucidated until now. Bioinformatics analysis was performed on 101 patients with osteosarcoma from The Cancer Genome Atlas database. High levels of PROSER2 were associated with a poor prognosis in patients with osteosarcoma. PROSER2 expression was significantly upregulated in clinical specimens from patients with osteosarcoma and osteosarcoma cell lines. MTT assay was performed to test the cell viability and Transwell assay was used to test the migration and invasion of MG63 cells. PROSER2 knockdown inhibited the viability, migration and invasion of MG63 cells. Gene Set Enrichment Analysis and Gene Ontology/Kyoto Encyclopedia of Genes and Genomes analysis showed that the differentially expressed genes were primarily involved in 'calcium signaling pathway' and 'Wnt signaling' in patients with osteosarcoma and high PROSER2 expression. Western blotting analysis revealed that PROSER2 regulated migration and invasion of osteosarcoma via the Wnt/nuclear factor of activated T-cells (NFAT)c1 signaling pathway. In conclusion, PROSER2 promoted the proliferation, migration and invasion of osteosarcoma cells via the Wnt/Ca^{2+}/NFATc1 signaling pathway by increasing nuclear localization of NFATc1.

Introduction

Osteosarcoma, the most common bone malignancy in children and adolescents (1), often manifests as serious pain in the bones and joints, as well as in the local mass. It is a highly malignant tumor with a poor prognosis (1). The treatment for high-grade osteosarcoma includes preoperative chemotherapy followed by surgical resection and postoperative chemotherapy (2,3). Although multimodal chemotherapy increases beneficial outcomes in patients with osteosarcoma, metastasis and recurrence contribute to poor prognosis in patients with advanced osteosarcoma (4,5). Thus, identifying effective prognostic markers in patients with osteosarcoma and clarifying the underlying molecular mechanisms is crucial to guide the clinical diagnosis of osteosarcoma.

Proline- and serine-rich 2 (PROSER2) is encoded by the 47th open reading frame on human chromosome 10 (6,7). A genome-wide associated study analysis involving 3,230 patients with pediatric fractures identified one locus on chromosome 10 (rs112635931), near PROSER2 antisense RNA 1 and PROSER2, which may be the candidate gene associated with pediatric fractures (8). PROSER2 accurately predicts survival in patients with breast cancer (9). Additionally, transcriptome analysis identified an optimal multivariable Cox regression model including five predictors (CD180, Myc, PROSER2, dynein axonemal intermediate chain 1 and fetal and adult testis expressed 1) in 82 osteosarcoma samples (10). However, the molecular mechanism of PROSER2 in regulating proliferation and metastasis of human osteosarcoma has remained unclear.

The Wnt signaling pathway is involved in cell proliferation, differentiation and embryonic development (11-13). Abnormal activation of this pathway has recently been reported to be involved in the pathogenesis, invasion and migration of various benign and malignant tumors (14,15). It includes two major pathways by activating distinct Wnt receptors: β-catenin-dependent and -independent (canonical and non-canonical, respectively) Wnt signaling pathways (16). The Wnt/Ca^{2+} cascade is a branch of non-canonical Wnt signaling activated in the presence of Wnt ligands, including Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a and Wnt11 (17). Wnt ligands attract and activate Dishevelled by binding to Frizzled family proteins and further activating phospholipase C. This catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate to 1,4,5-triphosphate inositol and diacylglycerol, leading to release of intracellular calcium (18). Increased intracellular Ca^{2+} activates calcineurin (CaN), Ca^{2+}/calmodulin-dependent kinase II and protein kinase C, which stimulate the activation of calcium-related pathway transcription factor and nuclear factor of activated T-cells

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(NFAT) and regulate transcription and expression of downstream target proteins (19). It has been reported that the NFATc family is a class of Ca^{2+}/CaN-dependent transcription factors, including NFATc1, NFATc2, NFATc3, NFATc4 and NFAT5, which are widely distributed in various human tissue (19). CaN is key for skeletal muscle differentiation and regeneration and conversion of type II skeletal muscle fibers to type I. The NFAT family regulates expression of type I and II skeletal muscle fiber genes. Specifically, NFATc1 activates the promoter of type I skeletal muscle fiber gene and upregulates its expression, but inhibits the promoter of the type II skeletal muscle fiber gene (20). CaN/NFATc1 pathway is involved in bone resorption and reconstruction, as well as changes in bone microenvironment, which is associated with occurrence of bone cancer (20). Thus, the present study investigated changes in the NFATc1-regulated pathway in osteosarcoma cells.

To the best of our knowledge, the mediation of the canonical Wnt/Ca^{2+} pathway by PROSER2 to regulate progression and metastasis of osteosarcoma has not been reported. Thus, in the present study, the prognostic value of PROSER2 in patients with osteosarcoma and the molecular mechanism of PROSER2 in the progression and metastasis of osteosarcoma was investigated. It is key to find new prognostic biomarkers, which is helpful to evaluate the clinical diagnosis and treatment effects in patients with osteosarcoma.

Materials and methods

Cell lines, reagents and clinical specimens. Human fetal osteoblasts (hFOB1.19, cat. no. CL-0353), MG63 (Cat. No. CL-0157) and U2OS (cat. no. CL-0236, all Procell Life Science &Technology Co., Ltd.) cells were cultured in Dulbecco's modified Eagle's medium (cat. no. 11965092; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (cat. no. SH30071.03; HyClone; Cytiva) and 1X penicillin-streptomycin solution (cat. no. P1400, Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C in a humidified atmosphere containing 5% CO_2. The complete medium for hFOB1.19 cells was supplemented with 0.3 mg/ml geneticin G418 (cat. no. A1720; Sigma-Aldrich; Merck KGaA). Lipofectamine™ 2000 (cat. no. 11668-027) was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). MTT was obtained from Sigma-Aldrich (Merck KGaA). The NFAT inhibitor VIVIT (cat. no. HY-P1026) was obtained from MedChemExpress. Clinical specimens collected from the Orthopedics Department at Chengdu Fifth People's Hospital (Chengdu, China) between February 2021 and January 2022 comprised four pairs of osteosarcoma specimens and paired paratumor (distance, <3 cm) tissue. The total number of patients was 4 including 1 male and 3 females. The age range of patients was 12.5-17.6 years old. The patients did not receive chemotherapy, radiotherapy or other clinical therapy. The specimens were obtained following routine surgery. The present study was conducted in accordance with the Declaration of Helsinki (2013) and approved by the Ethics Committee of Chengdu Fifth People's Hospital (approval no. 2020A-00173; Chengdu, China). All patients or their parent/legal guardian provided written informed consent for participation.

Analysis of PROSER2 expression and clinicopathological indicators. Patients with osteosarcoma in TCGA database (ocg.cancer.gov/programs/target) were divided into two groups according to the median value of PROSER2 expression, including 50 cases in the low and 51 cases in the high PROSER2 expression group. R software (R version 4.2.1) was used to analyze the correlation between PROSER2 expression and clinicopathological parameters of patients with osteosarcoma using the Wilcoxon rank-sum (continuous variables) or Pearson's χ^2 test (rank variables). Hazard ratio (HR) was calculated using univariate and multivariate analyses.

Survival analysis of patients with osteosarcoma. The survival probability of patients with osteosarcoma in low and high PROSER2 groups was analyzed using the Xiantao platform (https://xiantao.love). The survival probability of subgroup analysis was also determined by sex, metastasis, tumor region and primary site progression. HR and its corresponding log-rank P-value ($P<0.05$) were calculated for overall survival (OS).

Gene Ontology (GO)/Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA). Xiantao database (xiantao.love/products) is primarily used for gene expression, co-expression, enrichment and interaction network analysis and contains the RNA sequencing and microarray data from TCGA database and part of the Gene Expression Omnibus database. Numerous researchers have published articles using the Xiantao database and similar procedures to the present study (21-24). GO, KEGG and GSEA analyses were performed on PROSER2 differentially expressed genes (DEGs) using Xiantao platform. Firstly, single gene difference analysis was performed using the DESeq2 package (version 1.26.0) (25). GO and KEGG analysis criteria were absolute value $\log_2$ fold change >1 and adjusted P-value (p.adj)<0.01. GSEA was conducted according to ID and $\log_2$ fold change values of the DEGs. Gene set permutations were performed 1,000 times for each analysis and the screening criteria were false discovery rate (FDR)<0.25 and p.adj<0.05.

PROSER2 short hairpin (sh)RNA lentiviral particle transduction. PROSER2 human shRNA (locus ID 254427; cat. no. TL306312V) and control lentiviral particles (cat. no. TR30021V) were purchased from OriGene Technologies, Inc. Human shRNA lentiviral particles contained four unique 29-mer target-specific shRNA with lentiviral titer >$1x10^7$ TU/ml. Multiplicity of infection used to infect osteosarcoma cells was 30. The transduction of PROSER2 and control shRNA lentiviral particles (generation system used was 3rd) was performed according to the manufacturer's protocol (cat. no. TL306312V, OriGene Technologies). Briefly, $5x10^4$ MG63 or U2OS cells were plated in 24-well plates and incubated for 18 h at 37˚C with 5% CO_2. Appropriate amounts of lentiviral particles were supplemented with 8 μg/ml Polybrene® (cat. no. sc-134220, Santa Cruz Biotechnology, Inc.) to a total volume of 500 μl. After 24 h at 37˚C, the culture medium was removed and 1 ml fresh complete DMEM without Polybrene was added. Stable clones expressing PROSER2 or control shRNA were screened by splitting cells at a 1:10 ratio in complete DMEM containing 10 μg/ml puromycin (Santa Cruz

Biotechnology, Inc.). Finally, fresh puromycin-containing medium was replaced every 5 days until resistant colonies were identified for stable PROSER2 or control shRNA expression. The concentration of puromycin used for selection and maintenance is 10 and 2 μg/ml. The screening time continued for 1 month.

Wound healing assay. The stable cells infected with PROSER2 and control shRNA lentivirus were cultured on 6-well plates without serum ($3x10^5$ cells/well). Following adherence, cells at 100% confluence were scratched horizontally across the wall with a disposable pipette tip. The dislodged cells were washed with PBS buffer three times and discarded. Then, cells were cultured with serum-free DMEM for 24 and 48 h. The cells were photographed using a microscope(Olympus CX43, 40X magnification).

Migration and invasion assay. Migration and invasion assays were performed using Boyden chambers with Transwell membrane filter inserts (cat. no. 3422; Corning Costar, Inc.). Briefly, $3x10^4$ cells/well in serum-free DMEM were seeded into the upper chambers of a 24-well. DMEM containing 10% FBS were added into the lower chambers. Transwell chamber (pore size, 8 μm) for the migration assay and incubated at 37˚C for 24 and 48 h. MG63 cells on the lower surface of the filter were fixed with 10% formalin solution for 30 min and stained with 0.1% crystal violet for 30 min at room temperature. The number of migratory cells from five randomly selected fields of view in a single chamber of three samples was counted under a light microscope (mean ± SEM). For the invasion assay, Matrigel (cat. no. 354248; BD Biosciences) was applied at 37˚C for 5 h to coat the upper chamber, and the other steps were the same as that of the migration assay.

Nuclear and cytoplasmic protein extraction. PROSER2 and control shRNA lentiviruses infected MG63 cells were treated with 10 μM VIVIT at 37˚C for 24 h. Then, cells were collected and nuclear and cytoplasmic proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction kit (cat. no. P0027; Beyotime Institute of Biotechnology). This kit uses cytoplasmic protein extraction reagents A and B to fully expand cells under low osmotic pressure conditions, then destroy the cell membrane, release cytoplasmic proteins and then obtain cell nucleus pellets by centrifugation at 12,000 g 10 min, at 4˚C. Finally, nuclear protein was extracted using high-salt nuclear protein extraction reagent (cat. no. P0027; Beyotime Institute of Biotechnology).

Western blot analysis. The primary antibodies were as follows: Anti-Wnt5a (cat. no. ab235966; Abcam), NFATC1 polyclonal (A01; cat. no. H00004772-A01; Abnova), anti-cyclooxygenase-2 (COX2; cat. no. ab198646; Abcam), anti-MMP2 (EPR17003-25; cat. no. ab181286; Abcam), monoclonal anti-MMP9 (cat. no. SAB5300247; Sigma-Aldrich; Merck KGaA) and β-actin (SP124; cat. no. ab115777; Abcam). Cell lysate was prepared using Cell lysis buffer for Western and IP (cat. no. P0013; Beyotime Institute of Biotechnology). Protein determination method is BCA assay and 20 μg of protein loaded per lane. Total protein was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes at 300 mA for 2 h. The membranes were blocked with 5% bovine serum albumin (Thermo Fisher Scientific, Inc.) for 1 h at room temperature and incubated with primary antibody at 1:1,000 dilution at 4˚C overnight. The membranes were washed three times for 5 min with 1X Tris-buffered saline + 0.05% Tween 20. The membrane was incubated with secondary antibody at a dilution of 1:10,000 for 1 h and washed three times as aforementioned. Goat anti-rabbit IgG H&L (cat. No. ab6721; Abcam) HRP, 1:10,000 dilution; incubated at room temperature for 1 h. Bands were visualized using enhanced chemiluminescent kit (Pierce, Thermo Fisher Scientific, Inc.). ImageJ software (version 1.8.0; National Institutes of Health) was used to analyze the densitometry of the bands.

Apoptosis assay. MG63 cells were infected with PROSER2 and control shRNA lentiviruses and stable clones with PROSER2 shRNA and control shRNA were selected ($2x10^5$ cells/plate). Each group was treated with 3 μg/ml cyclosporine A (CsA, MedChemExpress LLC) for 24 h at 37˚C. The cells were centrifuged twice at 400 g for 10 min at 4˚C. The cell apoptosis rate (early + late apoptosis) was determined by FACS using BD LSRFortessa X-20 flow cytometer and BD FACSDiva™ software version 6.0 (BD Biosciences) with Annexin V-FITC dual staining kit (cat. no. C1062S-1; Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Confocal microscopy imaging. Intracellular Ca^{2+} was measured by incubation with Ca^{2+}-selective fluorescent indicator Fura-2 AM (cat. no. ab120873; Abcam) with a purity of >99%. Briefly, human osteosarcoma MG63 cells were infected with PROSER2 shRNA and control shRNA lentiviral particles for 48 h. The cells in each group were loaded with 2 μM Fura-2 AM in DMSO/HBSS for 30 min at room temperature in the dark. The cells were washed with prewarmed HBSS and incubated for 30 min at 37˚C in the dark. Finally, the cells were washed again with pre-warmed HBSS and live cells were imaged using a confocal microscope (excitation laser, 405 nm; emission gate center, 519 nm; magnification, x200).

Statistical analysis. The data from MTT, migration and FACS assays were analyzed using SPSS software version 24 (IBM Corp.). The results are presented as the mean ± standard deviation. One-way ANOVA followed by Tukey's post hoc test or two-way ANOVA, followed by Bonferroni's post hoc test was used for comparisons between >2 groups. Matched data, such as tumor and adjacent normal tissue, were compared using a paired t test (parametric data). All experiments were repeated twice with technical duplicates. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Correlation of PROSER2 expression and clinicopathological parameters. The data of patients with osteosarcoma was analyzed (Table I). Briefly, according to the PROSER2 expression level, 101 patients with osteosarcoma were divided into high and low PROSER2 groups using Wilcoxon rank-sum test (continuous variables). The overall survival rate was significantly different between the two groups. Univariate and multivariate analyses were performed, and HRs were

Table I. Baseline data in patients with osteosarcoma.

Characteristic	Low expression of PROSER2 (n=50.00)	High expression of PROSER2 (n=51.00)	P-value
Age, years (%)			0.597
<18	37.00 (36.60)	41.00 (40.60)	
≥18	13.00 (12.90)	10.00 (9.90)	
Sex, n (%)			0.626
Female	22.00 (21.80)	19.00 (18.80)	
Male	28.00 (27.70)	32.00 (31.70)	
Race, n (%)			Not analyzed
Native American or Indigenous Alaskan	0.00 (0.00)	1.00 (1.30)	
Asian	4.00 (5.30)	3.00 (3.90)	
Black or African American	3.00 (3.90)	7.00 (9.20)	
Native Hawaiian or Pacific Islander	0.00 (0.00)	0.00 (0.00)	
White	35.00 (46.10)	23.00 (30.30)	
Metastasis, n (%)			0.074
No	42.00 (41.60)	34.00 (33.70)	
Yes	8.00 (7.90)	17.00 (16.80)	
Primary site progression, n (%)			>0.999
No	14.00 (27.50)	18.00 (35.30)	
Yes	8.00 (15.70)	11.00 (21.60)	
Overall survival event, n (%)			<0.001
Alive	38.00 (38.40)	20.00 (20.20)	
Dead	12.00 (12.10)	29.00 (29.30)	
Median age, years (IQR)	15.35 (12.78, 18.06)	15.06 (12.23, 17.56)	0.632

PROSER2, proline- and serine-rich 2.

calculated (Table II). The results showed that expression of PROSER2 and metastasis were independent prognostic factors for patients with osteosarcoma.

Prognostic value of PROSER2 in patients with osteosarcoma. The association between PROSER2 expression and survival in patients with osteosarcoma was investigated using Kaplan-Meier plotter in the Xiantao database. High expression of PROSER2 was significantly associated with poorer OS (HR=3.66; 95% CI, 1.86-7.21; Fig. 1). Significant effects in subgroup analysis were exhibited only in patients with osteosarcoma without metastasis, with distal tumor regions, no primary site progression and in males with osteosarcoma. The HR and 95% CIs were 2.73 (1.13-6.60), 2.66 (1.04-6.82), 4.66 (1.54-14.10) and 4.30 (1.78-10.36). All data demonstrated that high PROSER2 expression was associated with poor survival probability in patients with osteosarcoma. Collectively, these results suggest that PROSER2 was a valuable prognostic predictor in patients with osteosarcoma.

Expression of PROSER2 is upregulated in clinical specimens and osteosarcoma cell lines. Expression of PROSER2 was detected in four pairs of osteosarcoma clinical specimens, including tumor and paired paratumor tissue. Expression of PROSER2 was significantly higher in osteosarcoma than in paratumor tissue (Fig. 2A). Expression of PROSER2 in human osteoblast and osteosarcoma cell lines was detected by western blot analysis. Expression of PROSER2 was significantly increased in MG63 and U2OS cells compared with that in hFOB1.19 cells (Fig. 2B). These data suggested that PROSER2 expression was higher in osteosarcoma cancer cells.

PROSER2 knockdown inhibits viability of MG63 cells and U2OS cells. Next, PROSER2 and control shRNA lentiviruses were used to infect osteosarcoma cells and western blotting was performed to test expression of PROSER2 in MG63 and U2OS cells. Expression of PROSER2 was significantly suppressed in PROSER2 shRNA lentivirus-infected MG63 and U2OS cells compared with control shRNA lentivirus-infected MG63 and U2OS cells (Fig. 2C).

To investigate whether interference with PROSER2 affects osteosarcoma cell proliferation, MTT assay was performed to test the viability of PROSER2 and control shRNA lentivirus-infected MG63 and U2OS cells. PROSER2 knockdown inhibited the viability of MG63 and U2OS cells compared with control shRNA lentivirus at 48 and 72 h (Fig. 2D).

PROSER2 knockdown inhibits cell proliferation, migration and invasion of MG63 cells. To identify whether PROSER2 regulates proliferation, migration and invasion of osteosarcoma cells, wound closure, cell migration and invasion assays were

Table II. Univariate and multivariate analysis of patient characteristics.

		Univariate analysis		Multivariate analysis	
Characteristic	Total, n	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Age, years	99				
<18	76	Reference			
≥18	23	0.732 (0.325-1.653)	0.454		
Metastasis	99				
No	75	Reference			
Yes	24	3.679 (1.964-6.892)	<0.001[a]	4.102 (2.162-7.783)	<0.001[a]
Race	74				
White	57	Reference			
Black or African American and Asian	17	1.246 (0.494-3.142)	0.641		
Primary site progression	50				
No	31	Reference			
Yes	19	1.769 (0.864-3.626)	0.119		
PROSER2 expression	99				
Low	50	Reference			
High	49	3.662 (1.860-7.208)	<0.001[a]	4.016 (2.020-7.984)	<0.001[a]

[a]Statistically significant. PROSPER2, proline- and serine-rich 2.

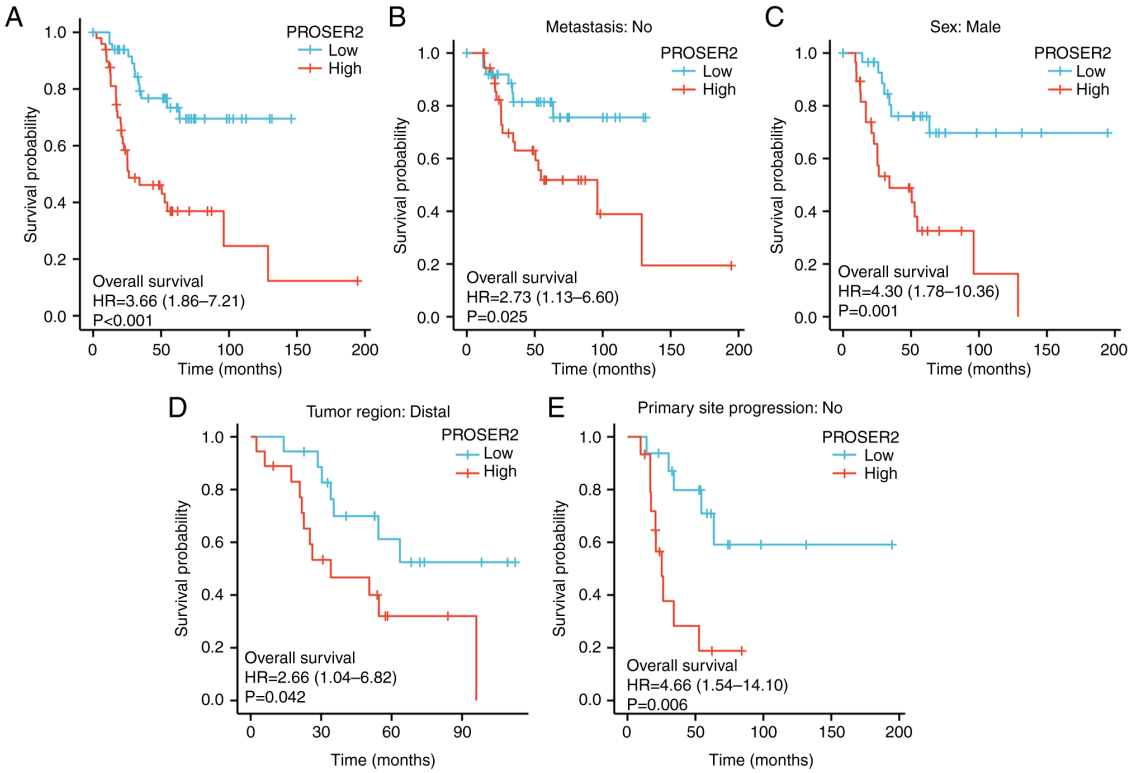

Figure 1. Prognostic value of PROSER2 in patients with osteosarcoma. (A) Survival probability shown by Kaplan-Meier plotter in patients with osteosarcoma. Subgroup analysis of overall survival in osteosarcoma for (B) non-metastasis, (C) males, (D) distal tumor region and (E) no primary site progression. PROSPER2, proline- and serine-rich 2; HR, hazard ratio.

performed. MG63 cells were infected with PROSER2 and control shRNA lentiviruses for 24 and 48 h (Fig. 3A and C). Wound closure was significantly lower in PROSER2 shRNA lentivirus-infected MG63 cells than in control shRNA-infected MG63 cells at both timepoints (Fig. 3D). The migratory ability of PROSER2 shRNA lentivirus-infected MG63 cells

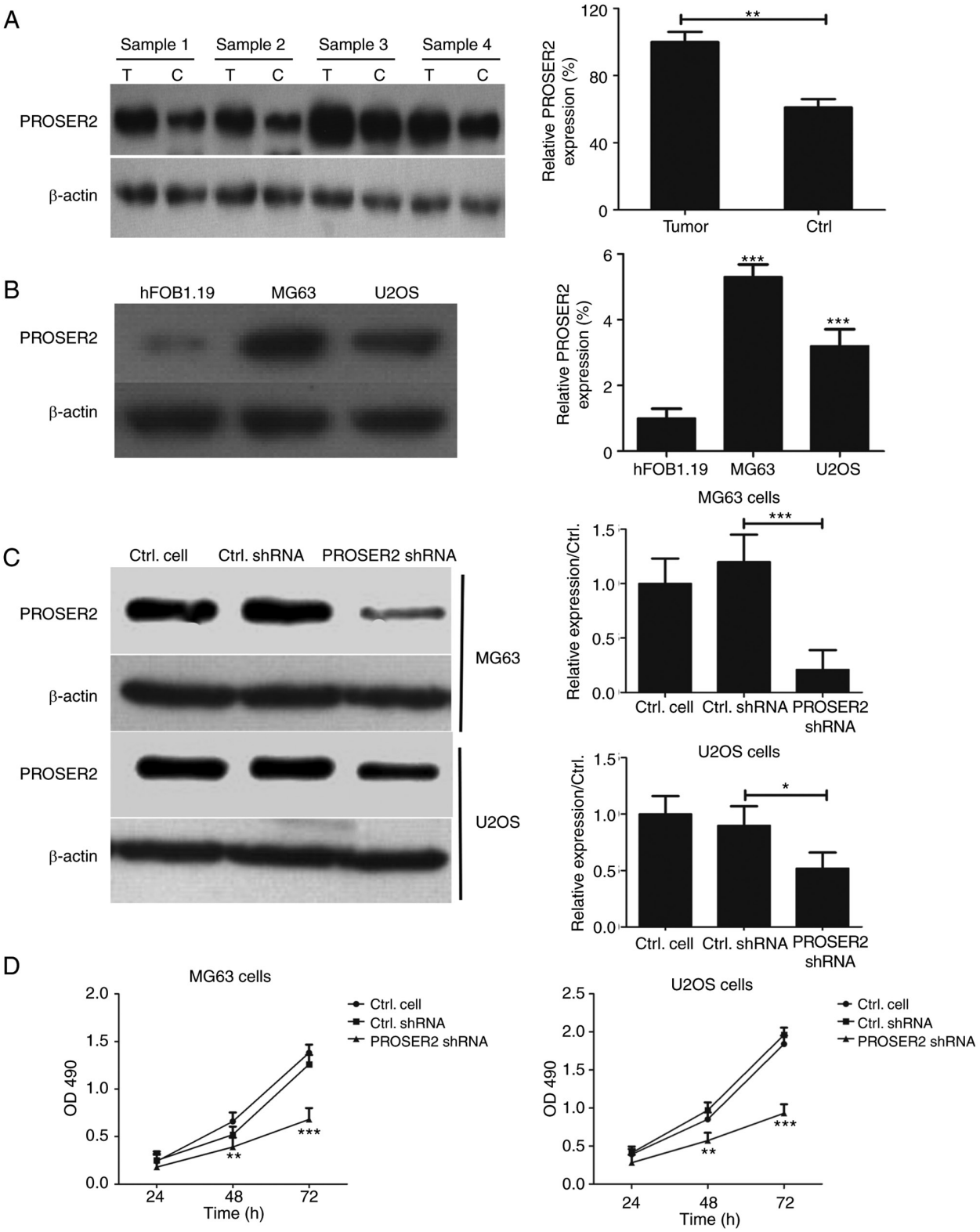

Figure 2. PROSER2 knockdown inhibits viability of osteosarcoma cells. (A) Expression of PROSER2 was determined by western blot analysis in four pairs of clinical specimens. **P<0.01. (B) PROSER2 levels were determined by western blot analysis in hFOB1.19, MG63 and U2OS cells. ***P<0.001 vs. hFOB1.19. (C) MG63 and U2OS cells were infected with PROSER2 and control shRNA lentivirus for 24 h and PROSER2 expression was determined by western blotting analysis. *P<0.05, ***P<0.001. (D) Viability of MG63 and U2OS cells infected with PROSER2 or Ctrl shRNA lentivirus and cultured for 24, 48 and 72 h was assessed by MTT assay. **P<0.01, ***P<0.001 vs. Ctrl shRNA. PROSPER2, proline- and serine-rich 2; Ctrl, control; T, tumor tissue; C, paratumor tissue; OD, optical density; sh, short hairpin.

was assessed using Transwell assay. The migratory ability of PROSER2 shRNA lentivirus-infected MG63 cells was significantly decreased compared with that of control shRNA lentivirus-infected cells at 24 and 48 h (Fig. 3B and D) suggesting the invasive ability of MG63 cells were inhibited after PROSER2 knockdown. These data revealed that

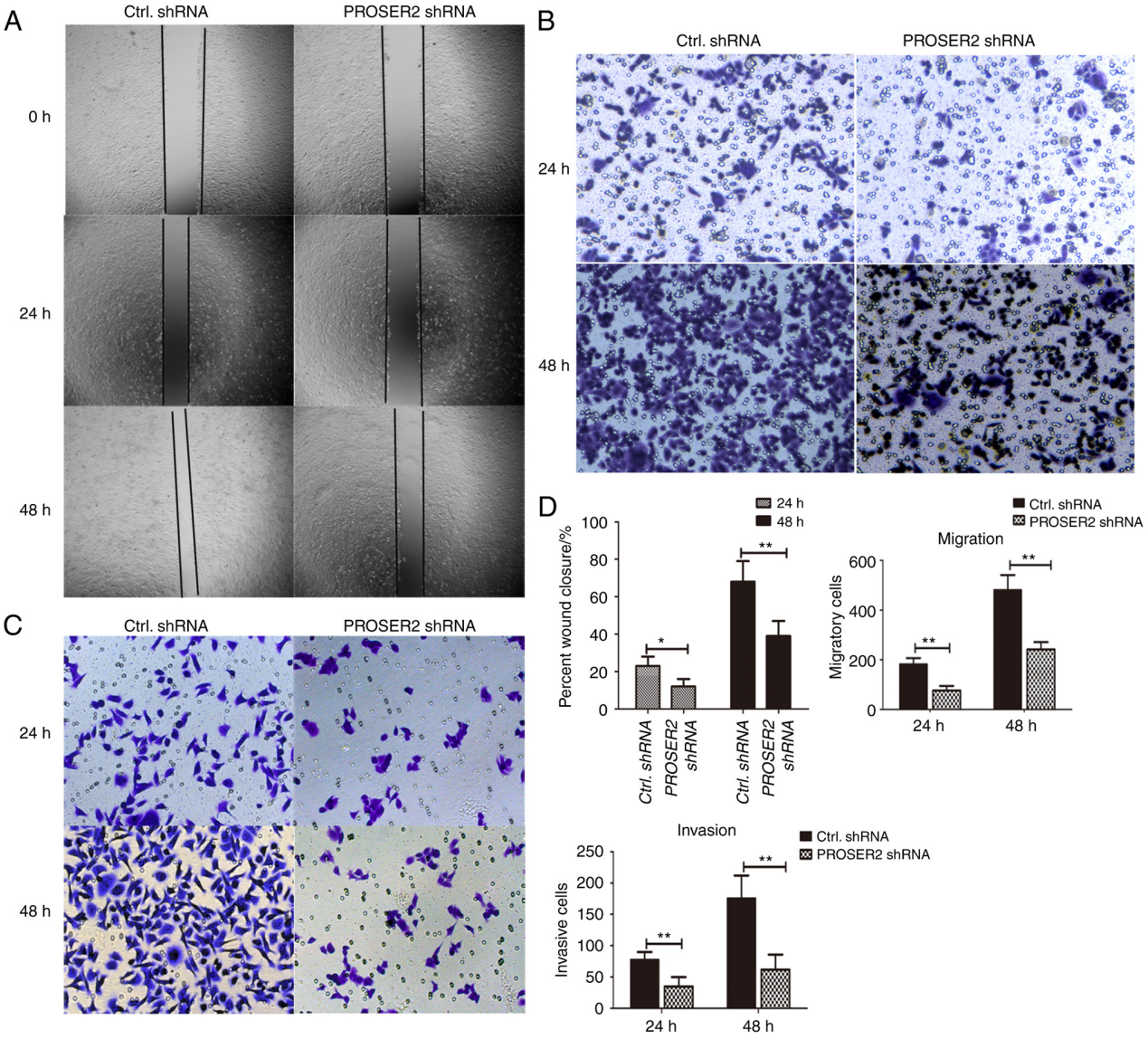

Figure 3. PROSER2 knockdown inhibits proliferation and migration of MG63 cells. MG63 cells were infected with PROSER2 and Ctrl shRNA lentiviruses for 24 and 48 h. (A) Wound healing assay. Magnification, x40. (B) Migration and (C) invasion assay were performed to investigate the role of PROSER2 on proliferation and migratory ability of MG63 cells. Magnification, x100. (D) Wound closure, migratory and invasive cells. *P<0.05 and **P<0.01 vs. Ctrl shRNA. PROSPER2, proline- and serine-rich 2; Ctrl, control; sh, short hairpin.

PROSER2 knockdown inhibited proliferation, migration and invasion of MG63 cells.

GSEA and GO/KEGG analysis. GSEA and GO/KEGG analyses were performed using the Xiantao platform. Firstly, patients with osteosarcoma were divided into high and low PROSER2 expression groups. DEGs were screened with the threshold values of $|\log_2(FC)|>1$ and p.adj<0.01, from which 2,348 coding genes were selected for GSEA. A total of 105 signaling pathways were significantly enriched at FDR<0.05 and p.adj<0.05. The differentially enriched terms included 'KEGG pathways in cancer', 'GPCR ligand binding', 'calcium signaling pathway' and 'Wnt signaling pathway' (Fig. 4A; Table SI). KEGG analysis results (Fig. 4B; Table SII) revealed that PROSER2 DEGs were primarily involved in pathways such as 'neuroactive ligand-receptor interactions', 'gastric cancer' and 'retinol metabolism'. GO analysis revealed that PROSER2 DEGs were primarily involved in 'muscle tissue development', 'regulation of ion transmembrane transport', 'cellular calcium ion homeostasis' and 'calcium ion transport' in the biological process group (Table SII). In the molecular function group, the DEGs were primarily enriched in 'receptor-ligand activity', 'DNA-binding transcription activator activity', 'ion channel activity' and 'G protein-coupled receptor binding'. In the cellular component group, DEGs were primarily enriched in 'collagen-containing extracellular matrix', 'transmembrane transporter complex', 'ion channel complex' and 'transporter complex'.

To determine the molecular mechanisms of PROSER2-regulated proliferation, migration and invasion of MG63 cells, a Venn diagram was constructed based on 'Wnt signaling pathway' and 'pathways in cancer' terms and ten overlapping genes were screened: Frizzled 10(FZD10), WNT10B, WNT1, WNT4, WNT3A, WNT11, WNT2B, FZD9, WNT2 and WNT6 (Fig. 4C). Among these, WNT4, WNT11

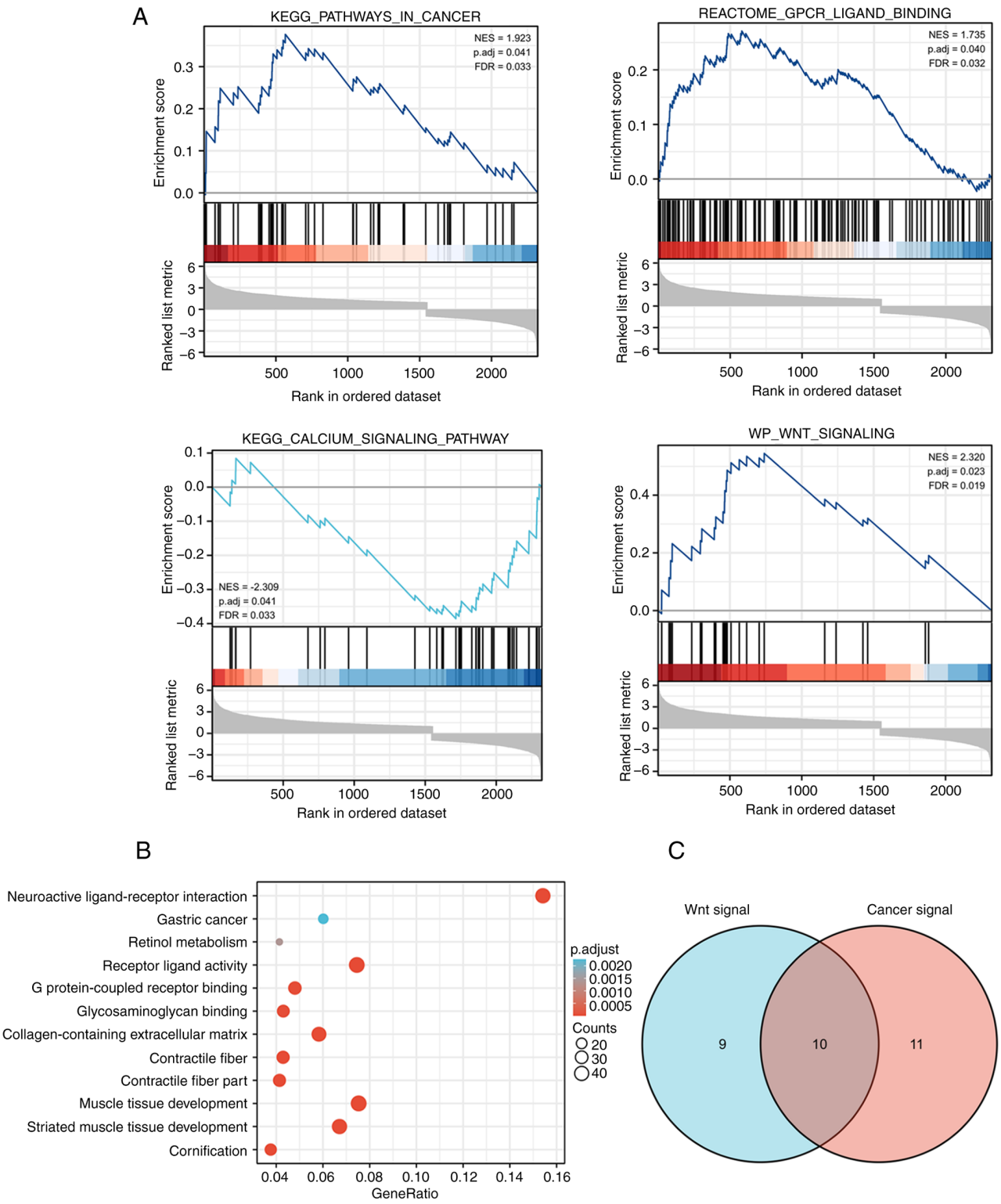

Figure 4. GSEA and GO/KEGG analysis. (A) GSEA and (B) GO/KEGG analyses were performed using the Xiantao platform. FDR<0.05 and p.adj<0.05. (C) Venn diagram was constructed based on Wnt signaling pathway and other pathways in cancer and the overlapping genes were screened. GSEA, Gene Set Enrichment Analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate; p.adj, adjusted P-value; GPCR, G protein-coupled receptor; NES, Normalized Enrichment Score; WP, Wnt pathway.

and WNT6 are key molecules regulating the Wnt/Ca^{2+} signaling pathway in cancer progression (17).

PROSER2 is involved in the Wnt/Ca^{2+} signaling pathway in MG63 cells. As aforementioned, calcium and Wnt signaling pathways were significantly enriched based on the GSEA. Therefore, whether PROSER2 regulated the proliferation, migration and invasion of osteosarcoma cells via the Wnt/Ca^{2+} signaling pathway was evaluated. Expression of Wnt5a, COX2, MMP-2 and MMP-9 was significantly decreased in PROSER2 shRNA lentivirus-infected MG63 compared with control shRNA lentivirus-infected MG63 cells (Fig. 5A and B).

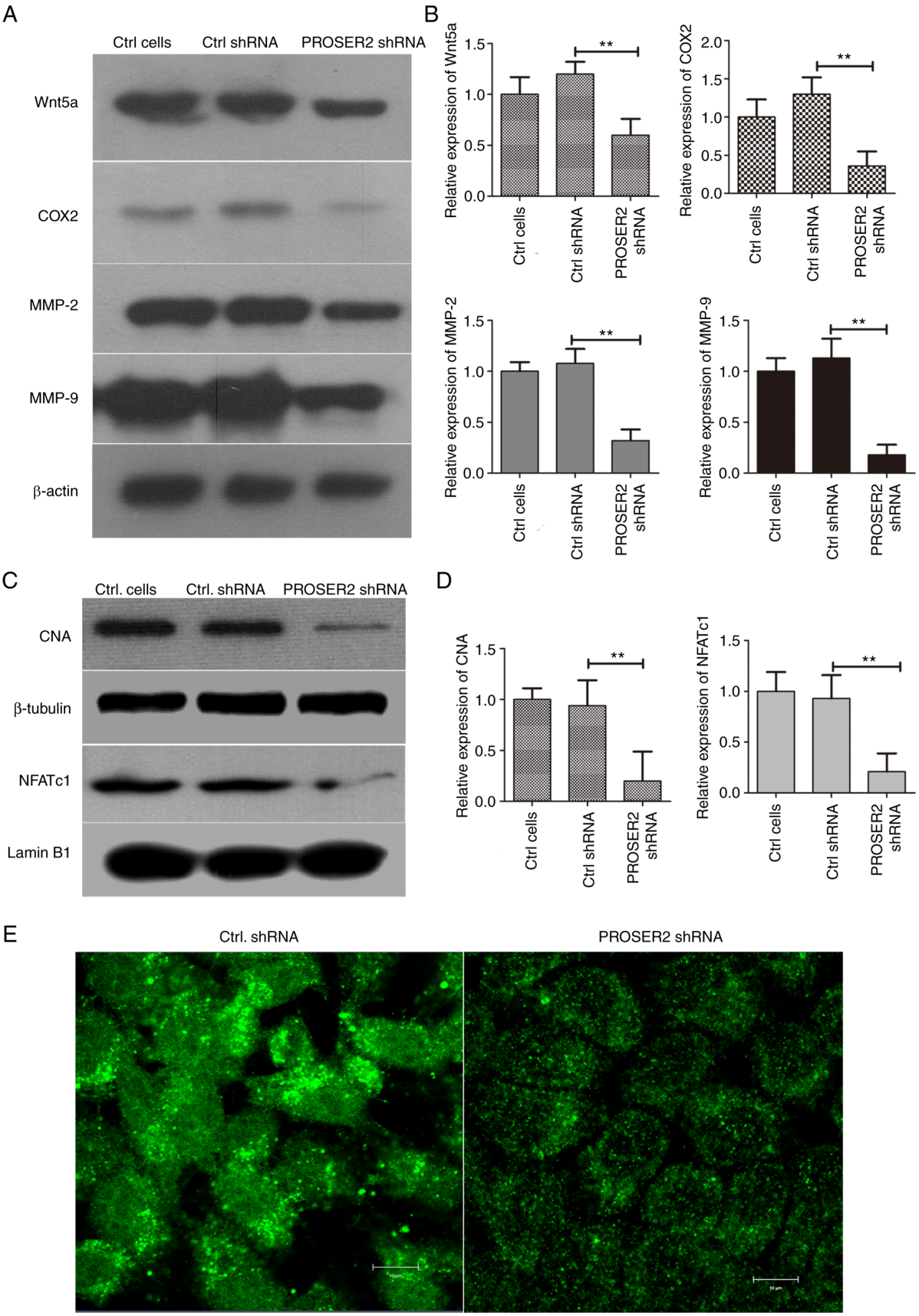

Figure 5. PROSER2 is involved in the Wnt/Ca^{2+} signaling pathway in MG63 cells. (A) MG63 cells were infected with PROSER2 and Ctrl shRNA lentivirus for 24 h. The expression of Wnt5a, COX2, MMP-2 and MMP-9 was detected by western blot analysis in PROSER2 and Ctrl shRNA lentivirus-infected MG63 cells. (B) Histograms of relative expression of Wnt5a, COX2, MMP-2 and MMP-9. (C) Nuclear expression of NFATc1 and cytoplasmic CNA were detected by western blotting analysis in PROSER2 and Ctrl shRNA lentivirus-infected MG63 cells. (D) Histograms of relative expression of NFATc1 and CNA. **P<0.01. (E) Confocal microscopy imaging. Intracellular Ca^{2+} was measured by incubation with the Ca^{2+} selective fluorescent indicator, Fura-2 AM. Scale bar, 10 μM. PROSPER2, proline- and serine-rich 2; NFATc1, nuclear factor of activated T-cells 1; CNA, catalytic subunit of calcineurin; Ctrl, control; sh, short hairpin; COX, cyclooxygenase; MMP, matrix metalloproteinase.

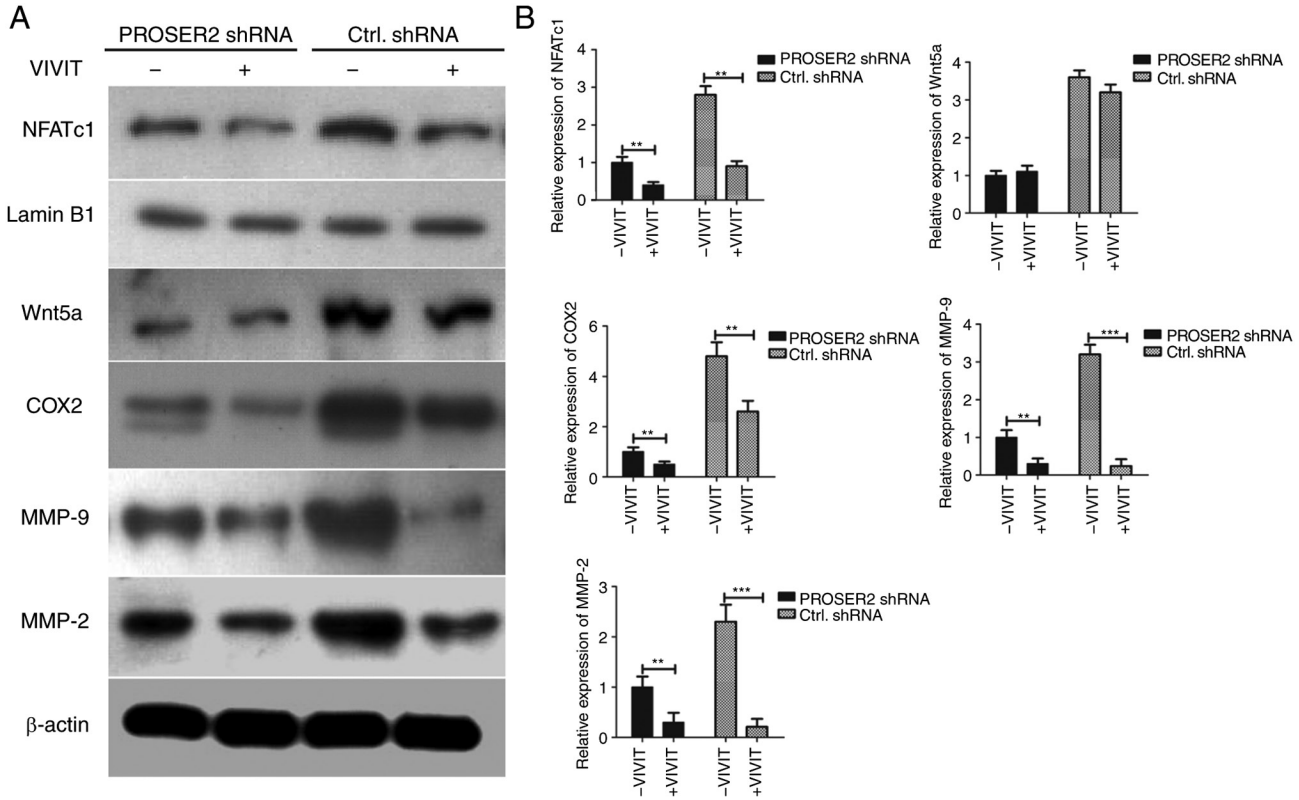

Figure 6. Interference with PROSER2 promotes VIVIT-induced inhibition of NFATc1 in MG63 cells via suppressing nuclear localization of NFATc1. (A) MG63 cells were transfected with PROSER2 or Ctrl shRNA lentivirus and treated with 10 μM VIVIT for 24 h. The nuclear expression of NFATc1 and lamin B1 was detected by western blotting. Lamin B1 was used as a nuclear internal reference protein. The expression of Wnt5a, COX2, MMP-2 and MMP-9 were determined by western blotting analysis. (B) Histograms of relative expression of NFATc1, Wnt5a, COX2, MMP-2 and MMP-9. **P<0.01 and ***P<0.001. PROSPER2, proline- and serine-rich 2; NFATc1, nuclear factor of activated T-cells 1; Ctrl, control; sh, short hairpin; COX, cyclooxygenase; MMP, matrix metalloproteinase.

Moreover, to test whether nuclear translocation of NFATc1 was changed in PROSER2 shRNA-transfected MG63 cells, nuclear NFATc1 expression was examined by western blot analysis. Expression of nuclear NFATc1 was significantly decreased in PROSER2 shRNA lentivirus-infected MG63 cells compared with that in control cells (Fig. 5C and D). CaN promotes nuclear translocation of NFATc1 via dephosphorylation and activates downstream target genes (19). Therefore, expression of the catalytic subunit of CaN (CNA) was measured in the cytoplasm of PROSER2 shRNA lentivirus-infected MG63 cells using western blotting. The expression of CNA was significantly decreased in PROSER2 shRNA lentivirus-infected MG63 cells compared with that in control cells (Fig. 5C and D). Moreover, the intracellular Ca^{2+} concentration in PROSER2 shRNA and control shRNA lentivirus-infected osteosarcoma cells was assessed using the Ca^{2+} sensing probe, Fura-2 AM. Ca^{2+} concentration was notably decreased in PROSER2 shRNA lentivirus-infected osteosarcoma cells compared with that in control shRNA lentivirus-infected osteosarcoma cells (Fig. 5E). These data revealed that PROSER2 regulated migration and invasion of osteosarcoma cells, potentially via the Wnt/Ca^{2+}/NFATc1 signaling pathway.

Interference with PROSER2 promotes apoptosis of MG63 cells via suppression of nuclear localization of NFATc1 mediated by VIVIT and CsA. Whether PROSER2 affected the nuclear translocation of NFATc1 was investigated. VIVIT is a specific inhibitor of NFAT and is a cell-permeable peptide that selectively inhibits CaN-mediated dephosphorylation of NFAT (19). PROSER2 and control shRNA lentivirus MG63 cells were treated with 10 μM VIVIT at 37˚C for 24 h. Nuclear expression of NFATc1, as well as total Wnt5a, COX2, MMP-9 and MMP-2 expression levels, were detected by western blot analysis. Nuclear NFATc1 expression, as well as total COX2, MMP-9 and MMP-2 expression were significantly decreased in VIVIT-treated PROSER2 shRNA lentivirus-infected MG63 cells compared with non-VIVIT-treated cells (Fig. 6). However, expression of Wnt5a was not significantly changed in VIVIT-treated and non-VIVIT-treated groups, suggesting that interference with PROSER2 inhibited the expression of Wnt5a but VIVIT treatment did not affect the expression of Wnt5a in MG63 cells. These results revealed that interference with PROSER2 promoted inhibition of NFATc1 by VIVIT in MG63 cells (Fig. 6).

PROSER2 knockdown with lentivirus significantly inhibited nuclear translocation of NFATc1 in MG63 cells (Fig. 7A). CsA was used to inhibit NFATc1 translocation to the nucleus of MG63 cells. Interference with PROSER2 promoted inhibition of NFATc1 by CsA in MG63 cells (Fig. 7A). The apoptosis rate of PROSER2 shRNA lentivirus-infected osteosarcoma cells was examined by FACS assay. Apoptosis in MG63 cells infected with PROSER2 shRNA lentivirus for 48 h was significantly upregulated compared with cells infected with control

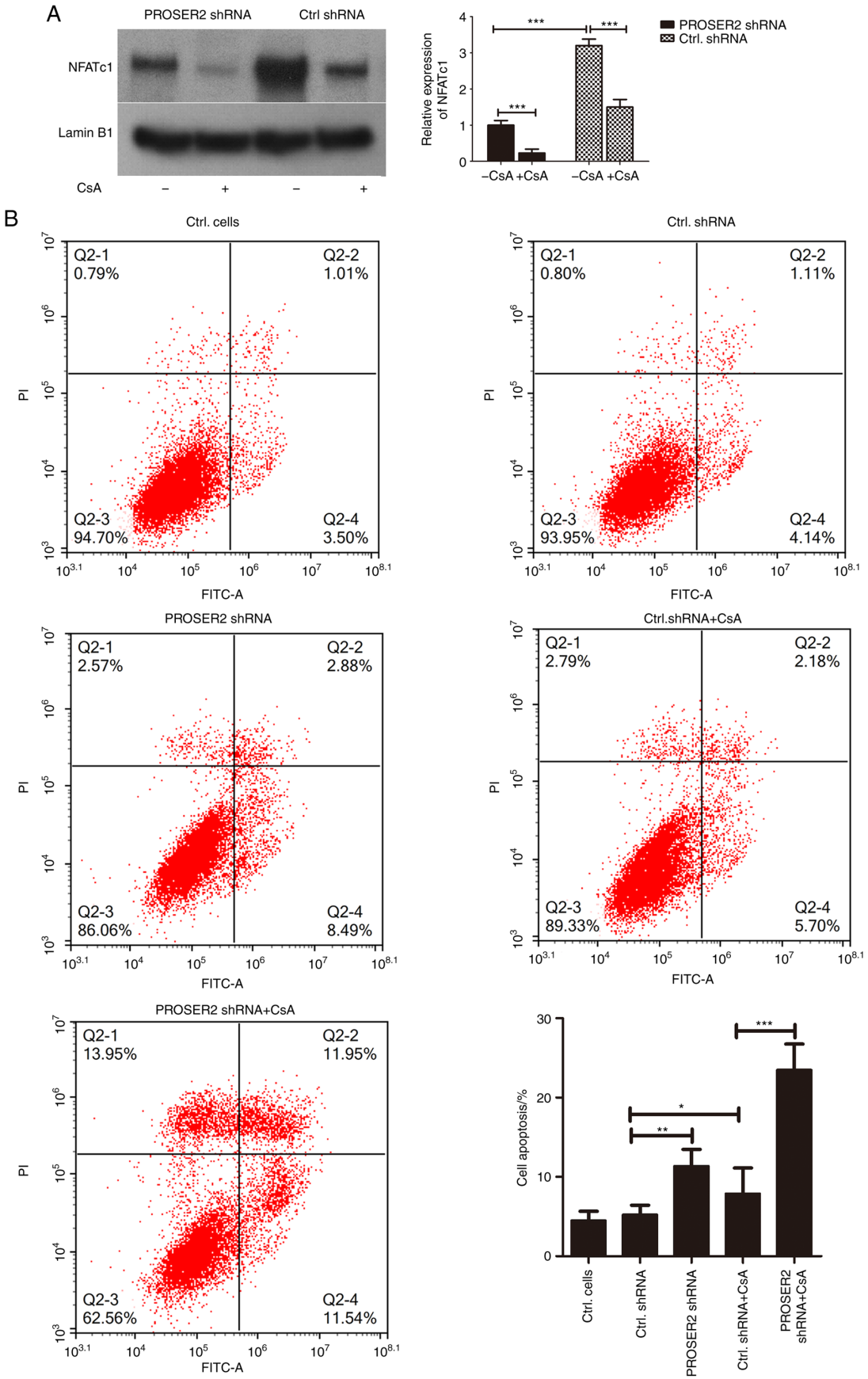

Figure 7. Interference with PROSER2 promotes apoptosis of MG63 cells via suppressing nuclear localization of NFATc1 mediated by CsA. (A) MG63 cells were infected with PROSER2 and Ctrl shRNA lentivirus and treated with 3 μg/ml CsA for 24 h. Expression of NFATc1 was detected by western blot analysis. Lamin B1 was used as a nuclear internal reference protein. ***P<0.001. (B) Apoptosis was determined by fluorescence-activated cell sorting assay in PROSER2 and Ctrl shRNA lentivirus-infected MG63 cells with or without CsA. *P<0.05, **P<0.01 and ***P<0.0001. PROSPER2, proline- and serine-rich 2; NFATc1, nuclear factor of activated T-cells 1; CsA, cyclosporine A; sh, short hairpin; Ctrl, control; PI, Propidium iodide; FITC-A, fluorescein isothiocyanate; Q, quadrant.

shRNA lentivirus (Fig. 7B). Moreover, CsA promoted apoptosis of MG63 cells and interference with PROSER2 by lentivirus promoted CsA-mediated apoptosis of MG63 cells (Fig. 7B).

Discussion

Osteosarcoma predominantly occurs in children and young adults prone to malignant transformation and metastasis (26). The present study of the pathogenesis of osteosarcoma may provide an effective reference for the clinical treatment of patients with osteosarcoma. Here, Therapeutically Applicable Research to Generate Effective Treatments (ocg.cancer.gov/programs/target) transcripts per million reads (TPM) format data in mRNA-sequencing data files from the Osteosarcoma project in TCGA database was analyzed and revealed that PROSER2 was an independent prognostic biomarker for patients with osteosarcoma. High expression of PROSER2 was associated with poor prognosis in patients. Next, expression of PROSER2 in four pairs of clinical specimens from patients with osteosarcoma was detected. Western blot analysis revealed that PROSER2 levels were significantly higher in osteosarcoma tissue than in paired normal control tissue. Moreover, PROSER2 levels were significantly higher in the osteosarcoma cell lines MG63 and U2OS than in hFOB1.19. These data revealed that PROSER2 served as an oncogene in the progression of osteosarcoma.

PROSER2 shRNA lentivirus was used to infect MG63 and U2OS cells. The role of PROSER2 in phenotypic effects was investigated; interference with PROSER2 significantly decreased viability, migration and invasion of MG63 and U2OS cells. GSEA was performed on the Xiantao and the 'GPCR ligand binding', 'calcium signaling pathway', and 'Wnt signaling pathway' were found to be enriched in the PROSER2 high-expression group of patients with osteosarcoma from TCGA database. Next, western blot analysis to was used to investigate whether PROSER2 regulates proliferation, migration and invasion of MG63 cells via the Wnt/Ca^{2+} signaling pathway. It was revealed that levels of Wnt5a, NFATc1, COX2, MMP-2 and MMP-9 were significantly decreased in PROSER2 shRNA lentivirus-infected MG63 cells. Wnt5a has previously been found to promote migration and invasion of osteosarcoma cells via the Wnt5a/receptor tyrosine kinase-like orphan receptor (ROR) 2 (27), Wnt5a/ROR1/disheveled-associated activator of morphogenesis 1 (28) and SRC/ERK/MMP-14 pathway (29), which was consistent with the results from the present study indicating that Wnt5a exerted a tumorigenic role in progression, migration and invasion of osteosarcoma. Overexpression of COX2 has been detected in various types of human malignancy and inhibition of COX2 has been found to suppress tumor cell proliferation and osteosarcoma metastasis (30,31). MMP-2 and MMP-9 participate in degrading the extracellular matrix and are involved in the invasion and migration of cancer cells (32,33). In the present study, PROSER2 knockdown significantly decreased expression of NFATc1, COX2, MMP-2 and MMP-9 in osteosarcoma cells. Notably, nuclear NFATc1 was detected in PROSER2 shRNA lentivirus-infected MG63 cells using western blot analysis. The results revealed that interference with PROSER2 significantly decreased translocation of NFATc1 in MG63 cells, which increased the inhibitory role of CsA in suppressing apoptosis, migration and invasion of MG63 cells.

However, the present study did not collect enough clinical specimens to prove the role of PROSER2 in the progression of osteosarcoma. Future studies should verify its role and test whether it could be used as the prognostic or diagnostic biomarker for clinical therapy of osteosarcoma.

In conclusion, PROSER2 was associated with poor prognosis in patients with osteosarcoma and served an oncogenic role in promoting proliferation, migration and invasion of osteosarcoma cells via the Wnt/Ca^{2+}/NFATc1 signaling pathway by increasing nuclear localization of NFATc1.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZB designed the experiments, performed statistical analysis and wrote the manuscript draft. ZL performed western blotting analysis and the flow cytometry assay. YZ and YL performed bioinformatics and the MTT assay. SX performed the lentivirus transfection assay and helped with the western blotting. SL and JL collected the clinical samples and performed cell culture. All authors have read and approved the final manuscript. ZB and ZL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of the Chengdu Fifth People's Hospital (approval no. 2020A-00173).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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