European Review for Medical and Pharmacological Sciences 2020; 24: 1-10

MicroRNA-1286 inhibits osteogenic differentiation of mesenchymal stem cells to promote the progression of osteoporosis *via* regulating FZD4 expression

J.-G. ZHOU[1], Y. HUA[2], S.-W. LIU[1], W.-Q. HU[1], R. QIAN[1], L. XIONG[3]

[1]Department of Joint Surgery, The Affiliated Ganzhou Hospital of Nanchang University, Ganzhou, China
[2]Department of Emergency Trauma Surgery, The Affiliated Ganzhou Hospital of Nanchang University, Ganzhou, China
[3]Department of Orthopedics, The People's Hospital of Jiangxi Province, Nanchang, China

Jianguo Zhou and Yi Hua contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to investigate whether microRNA-1286 could inhibit the osteogenic differentiation of human marrow mesenchymal stem cells (hMSCs) by regulating FZD4 expression and promoting the progression of osteoporosis.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expression of microRNA-1286 in the serum of patients with osteoporosis. Meanwhile, microRNA-1286 expression in different stages of osteogenic differentiation of hMSCs was measured by qRT-PCR as well. After overexpression of microRNA-1286 and FZD4 in hMSCs, the mRNA expression levels of microRNA-1286, alkaline phosphatase (ALP), RUNX2 and osteocalcin (OCN) were detected by qRT-PCR. The protein expression levels of RUNX2 and OCN were detected by Western blot. Meanwhile, alkaline phosphatase (ALP) activity and expression in cells were examined using ALP assay kit and ALP staining method, respectively. Cell mineralized nodules were detected through the alizarin red staining test. Bioinformatics method was used to predict the binding site of microRNA-1286 to FZD4. Subsequent luciferase reporter gene assay was performed to verify whether microRNA-1286 could combine with FZD4. After overexpression or knockdown of microRNA-1286, the mRNA and protein expressions of FZD4 were analyzed using qRT-PCR and Western blot assay, respectively. After the simultaneous overexpression of microRNA-1286 and FZD4 in hMSCs, the mRNA expression levels of ALP, RUNX2 and OCN, ALP activity and content, and cell mineralization ability were successively examined.

RESULTS: The expression of microRNA-1286 in the serum of patients with osteoporosis was significantly higher than that of the normal population. Meanwhile, microRNA-1286 expression decreased with the increase of osteogenic differentiation days of hAMSCs. After the overexpression of microRNA-1286, ALP, RUNX2, and OCN levels, ALP activity, RUNX2, and OCN protein levels, as well as mineralized nodule formation were significantly reduced. However, results were reversed when FZD4 was simultaneously up-regulated. Luciferase reporter gene assay results verified that microRNA-1286 could bind to FZD4. After the overexpression of microRNA-1286, the mRNA and protein expressions of FZD4 were found significantly down-regulated. However, results were reversed after knocking down microRNA-1286. Furthermore, the simultaneous overexpression of microRNA-1286 and FZD4 could counteract the inhibitory effect of over-expression of microRNA-1286 on osteogenic differentiation of hMSCs.

CONCLUSIONS: MicroRNA-1286 can regulate FZD4 expression and inhibit osteogenic differentiation of hMSCs, thereby promoting the development of osteoporosis.

Key Words:
Osteoporosis, MicroRNA-1286, FZD4, HMSCs, Osteogenic differentiation.

Introduction

Osteoporosis is a systemic metabolic bone disease characterized by decreased bone mass, bone microstructural destruction, increased bone fragility, and easy fracture[1]. The prevention and treatment of osteoporosis are serious problems which need to be solved in the field of orthopedics. Mesenchymal stem cells (MSCs) are a type of pluripotent stem cells located in the mesoderm. Under different conditions, MSCs can differentiate into a variety

Corresponding Author: Long Xiong, MD; e-mail: ncxionglong2@126.com

of cells, such as adipocytes, osteoblasts, chondrocytes, and myocytes[2,3]. Osteogenic differentiation is a highly ordered regulatory process that begins with the activation of Runt-related transcription factor 2 (RUNX2). It has been found that RUNX2 is the key transcription factor in the process of osteogenesis. This results in an increase in the expression and activity of alkaline phosphatase (ALP). Eventually, this leads to changes in the morphology of terminal osteoblasts, characterized by the presence of calcium deposits in the extracellular matrix. Genes associated with mineralization include osteocalcin (OCN), osteopontin, and type I collagen (Col-I)[4,5].

MiRNAs are a class of non-coding RNA molecules with approximately 22 nucleotides in length. They can combine with target genes by base pairing, leading to the degradation or translational repression of target genes[6]. Scholars[7,8] have shown that miRNAs are abnormally expressed in patients with osteoporosis. An increasing number of studies[9-12] has indicated that miRNAs can regulate the differentiation of MSCs into osteoblasts, such as microRNA-346, microRNA-20a, microRNA-29b, microRNA-433, etc. At present, researchers[13,14] have found that microRNA-1286 is associated with lung cancer and cervical cancer. However, no study has investigated its role in the progression of osteoporosis.

FZD4 belongs to the Frizzled family and is closely related to malignant tumors. Through different signaling pathways, FZD4 participates in a series of biological behaviors of malignant tumors, including cell proliferation, apoptosis, invasion, and metastasis[15]. Ma et al[16] have shown that microRNA-505 affects the development of cervical cancer through the regulation of FZD4. At present, no research has explored the relationship between FZD4 and osteoporosis. Meanwhile, whether microRNA-1286 is involved in the regulation of FZD4 remains elusive. Therefore, in this work, we used hMSCs as a research object to investigate whether microRNA-1286 and its regulation on FZD4 could affect osteogenic differentiation of hMSCs. Our findings might help to provide new clues for the treatment of osteoporosis.

Patients and Methods

Cell Culture and Induction of Osteogenic Differentiation

This investigation was approved by the Ethics Committee of The Affiliated Ganzhou Hospital of Nanchang University. Signed written informed consents were obtained from all participants before the study. Bone marrow samples were collected from healthy volunteers and placed in heparin anticoagulation tubes. Collected bone marrow samples were mixed with an equal volume of serum-free α-modified eagle medium (α-MEM) medium (containing 100 UI/mL of penicillin sodium and 0.1 μg/mL streptomycin) (HyClone, South Logan, UT, USA). After centrifugation at 800 × g for 10 min at room temperature, the fat layer was removed. Subsequently, cells were resuspended in α-MEM medium. Meanwhile, an equal volume of 1.073 g/mL Percoll was slowly injected. Next, the mixture was centrifuged at 400 × g for 30 min and the middle mononuclear cell layer was aspirated. After washing twice with phosphate-buffered saline (PBS), cells were resuspended in α-MEM medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 100 UI/mL penicillin sodium and 0.1 mg/mL streptomycin. After the concentration of cells was adjusted to 1×10^9/L, they were seeded in the culture flask. When the cells grew to 60-70% fusion, the medium was replaced with osteogenic induction medium (containing 10% FBS, 0.1 μmol/L dexamethasone, 10 mmol/L β-glycerophosphate, and 50 mg/L vitamin C in α-MEM) to induce the cell osteogenic differentiation.

Cell Transfection

The total RNA in hMSCs was extracted according to the instructions of the TRIzol kit (Invitrogen, Carlsbad, CA, USA). Extracted RNA was then reverse transcribed into complementary deoxyribose nucleic acid (cDNA) according to the instructions of One StepPrimeScript® miRNA cDNA Synthesis Kit (TaKaRa, Otsu, Shiga, Japan). FZD4 cDNA was amplified by PCR to construct a pcDNA-FZD4 recombinant plasmid. HMSCs with good growth were selected and seeded into 6-well plates at a density of 2×10^5/well, followed by culture in an incubator at 37°C with 5% CO_2 and saturated humidity. When the cells grew to about 50% confluence, cell transfection was performed according to the instructions of the Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA, USA). MicroRNA-1286-mimic and microRNA-1286-inhibitor, pcDNA-FZD4, microRNA-1286-mimic, and pcDNA-FZD4 were transfected into hMSCs. The fresh medium was replaced in 24 h after transfection.

ALP Staining

Cells in 24-well plates were first washed twice with PBS and the ALP fixative was added. After 3 min, the fixative was aspirated and the cells were

washed with PBS. After adding ALP incubation solution, the plate was put into a wet box and incubated at room temperature for 15-20 min in the dark. Then, cells were counterstained with methyl green staining solution for 3-5 min and washed with PBS. Finally, cells were observed and photographed under a microscope.

ALP Activity Detection

Cells in the collection dish were washed with 1 mL of PBS and centrifuged to obtain the precipitate. 0.1 mL PBS was added to grind the precipitate with a manual homogenizer in an ice water bath for 5 min. The ALP activity of cells was determined in strict accordance with the ALP kit (Sigma-Aldrich, St. Louis, MO, USA).

Alizarin Red Staining

Cells were washed twice with balanced salt solution and fixed with 75% ethanol solution for 10 min. After discarding the fixative, cells were rinsed with double distilled water for 3 times. After that, 1% alizarin red-Tris-HCl (pH 8.3) was added in cells, followed by incubation for 30 min at 37°C. Cells were then dried in an oven at 65°C. Finally, the pictures were taken under an inverted fluorescence microscope at 200×.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Detection

Total RNA in human serum and cells was extracted with TRIzol. The concentration and purity of RNA were determined by ultraviolet spectrophotometer. The expression levels of microRNA-1286, FZD4, ALP, RUNX2, and osteocalcin (OCN) were detected using a PCR detection kit. Primer sequences used in this study were as follows: microRNA-1286: F: 5'-GGGGTGCAGGACCAAGATG-3', R: 5'-CAGTGCGTGTCGTGGAGT-3'; FZD4: F 5'-AAACTAGCGGCCGCTAGTTCAGTTACCAGTGACCTTCAT-3'; R 5'-CTAGATGAAGGTCACTGGTAACTGAACTAGCGGCCGCTAGTTT-3'; U6 F 5'-TCCGATCGTGAAGCGTTC-3', R: 5'-GTGCAGGGTCCGAGGT-3'. ALP: F: 5'-AACATCAGGGACATTGACGTG-3', R: 5'-GTATCTCGGTTTGAAGCTCT-3'; RUNX2: F: 5'-GGAGCGGACGAGGCAAGAGT-3', R 5'-AGGAATGCGCCCTAAATCAC-3'; OCN, F: 5'- CAGCGAGGTAGTGAAGAGAC-3' R: 5'-TGAAAGCCGATGTCGTCAG-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F: 5'-TTCTTTTGCGTCGCCAGCCGA-3' R: 5'-GTCACCACCCGCCCAATACGA-3'.

Western Blot Assay

Cells were first lysed with protein lysis buffer, shaken on ice for 30 min and centrifuged at 14,000 g for 15 min at 4°C. Total protein concentration was calculated by the bicinchoninic acid (BCA) Protein Assay Kit (Abcam, Cambridge, MA, USA). Extracted proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Western blot analysis was performed according to standard procedures. Finally, the membranes were compressed in a dark room, followed by enhanced chemiluminescence (ECL) and development.

Luciferase Reporter Gene Assay

First, hMSCs were seeded into 24-well culture plates. When the cells grew to 70-85% of confluence, they were co-transfected with luciferase reporter vector and miR-NC or microRNA-1286-mimic. Subsequently, the cells were cultured for 48 h. Transfected pRL-TK was used as the standard internal control. After that, cells were collected and lysed. Firefly luciferase activity and sea-renal luciferase activity were detected according to the method of dual luciferase reporter gene system. Relative luciferase activity = (firefly luciferase activity / *Renilla* luciferase activity) × 100% (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for all statistical analysis. Experimental results were expressed as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used to compare the difference between the two groups. One-way ANOVA test was performed to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant: $^*p<0.05$.

Results

Expression of MicroRNA-1286 in Serum of Osteoporosis Patients and During Osteogenic Differentiation of hMSCs

To determine whether there was a relation between microRNA-1286 and osteoporosis, serum samples were collected from both osteoporosis patients and normal controls. After RNA ex-

traction, the expression of microRNA-1286 in the serum of patients with osteoporosis was detected by qRT-PCR. The results showed that microRNA-1286 expression in the serum of patients with osteoporosis was significantly higher than that of normal controls (Figure 1A). The expression of microRNA-1286 gradually decreased after the induction of osteogenic differentiation of hMSCs for 1, 3, 7, and 14 days (Figure 1B). Above results indicated that microRNA-1286 might be associated with the progression of osteoporosis.

High Expression of MicroRNA-1286 Inhibited Osteogenic Differentiation of hMSCs

To further verify the relation between microRNA-1286 and osteogenic differentiation of hMSCs, we overexpressed microRNA-1286 in hMSCs. The following results found that osteogenic differentiation of hMSCs was significantly inhibited, showing a remarkable decrease in the mRNA expression levels of ALP, RUNX2, and OCN (Figure 2A). Meanwhile, a significant decrease in the ALP activity was also detected by the ALP activity assay (Figure 2B). The protein expressions of RUNX2 and OCN were significantly reduced (Figure 2C). Meanwhile, the ALP staining showed a significant decrease in the ALP content (Figure 2D). While the Alizarin red staining revealed a significant reduction in the formation of mineralized nodules (Figure 2E). These results indicated that high expression of microRNA-1286 significantly inhibited the osteogenic differentiation of hMSCs.

MicroRNA-1286 Could Targeted Bind to FZD4 and Regulate its Expression

As shown in Figure 3A, a binding site for microRNA-1286 and FZD4 was predicted using the website Starbase 3.0. The luciferase reporter gene assay demonstrated that overexpression of microRNA-1286 significantly quenched wild-type FZD4 fluorescence (Figure 3B). This indicated that microRNA-1286 was able to target bind to FZD4. To verify the regulation between the two molecules, we overexpressed microRNA-1286 *in vitro*. Subsequently, the mRNA and protein expression levels of FZD4 were detected. Results demonstrated that the mRNA and protein expression levels of FZD4 were both significantly reduced. However, the situation could be reversed after knockdown of microRNA-1286 (Figures 3C, 3D). These findings suggested that microRNA-1286 could regulate the FZD4 expression through targeted binding.

High Expression of FZD4 Promoted Osteogenic Differentiation of hMSCs

To investigate the regulatory role of FZD4 in the osteogenic differentiation of hMSCs, we overexpressed FZD4 in hMSCs. Results found that the mRNA expression levels of ALP, RUNX2,

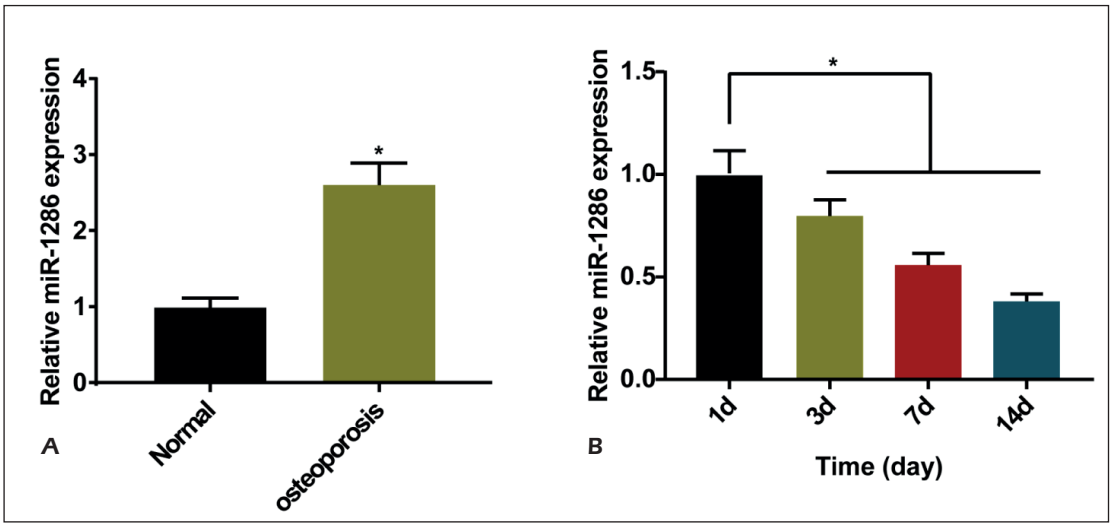

Figure 1. Expression of miR-1286 in the serum of osteoporosis patients and during osteogenic differentiation of hMSCs. **A**, QRT-PCR detection revealed that miR-1286 expression in the serum of patients with osteoporosis was significantly higher than normal controls. **B**, QRT-PCR detection showed that during the osteogenic differentiation of hMSCs, with the increase of induction days, miR-1286 expression gradually decreased.

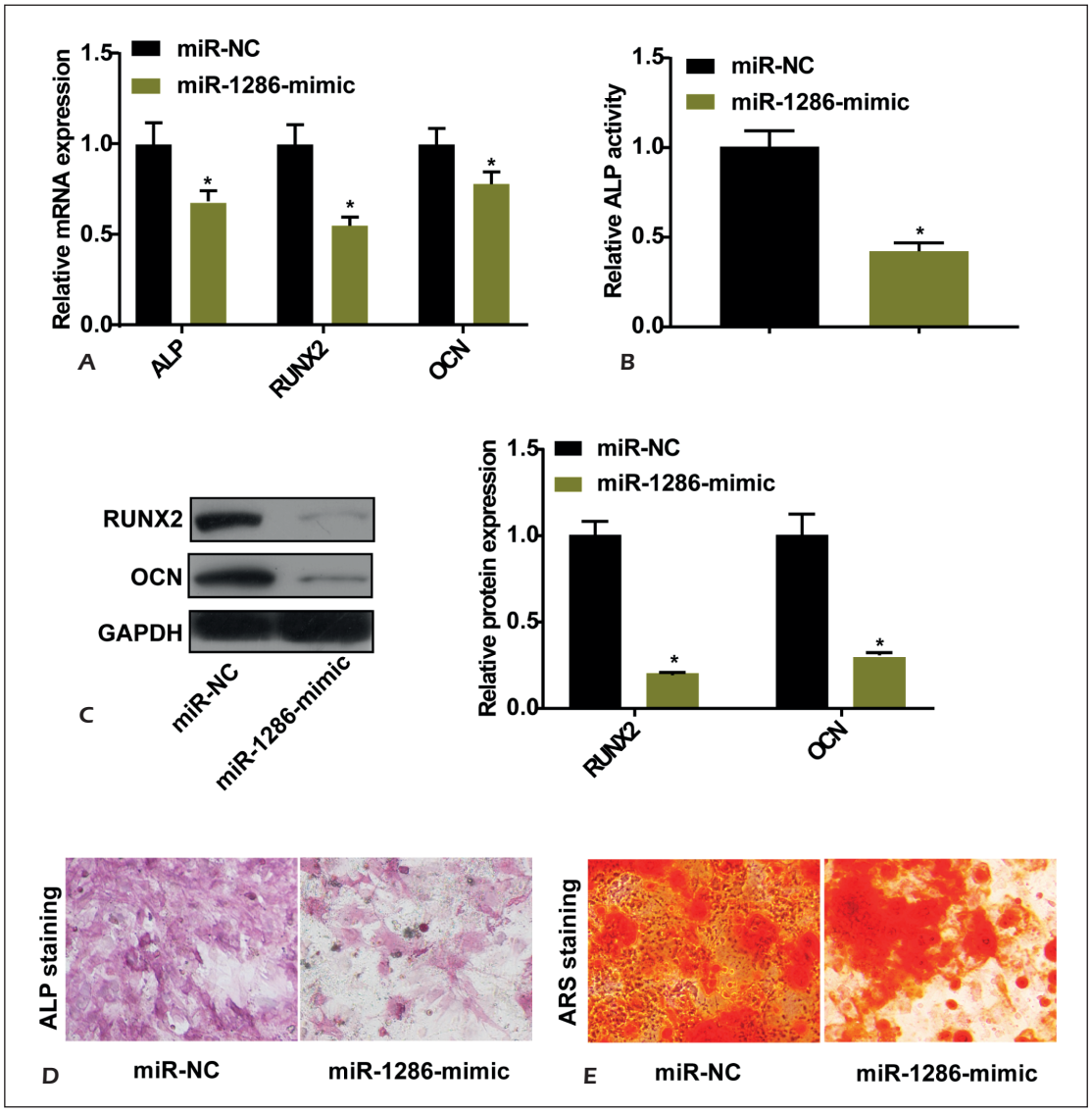

Figure 2. Overexpression of miR-1286 inhibited osteogenic differentiation of hMSCs. **A**, QRT-PCR detection indicated that after overexpression of miR-1286 in hMSCs cells, ALP, RUNX2, and OCN expression levels decreased significantly. **B**, ALP viability test indicated that ALP activity decreased significantly. C, Western blot found that the protein levels of RUNX2 and OCN were significantly reduced. **D**, ALP staining showed that ALP content was significantly reduced. **E**, Alizarin red staining indicated that the formation of mineralized nodules was significantly reduced. (200X).

and OCN, as well as the ALP activity increased significantly (Figures 4A, 4B). Furthermore, Western blot assay revealed the protein levels of RUNX2 and OCN were remarkably up-regulated (Figure 4C). The ALP staining showed a significant increase in the ALP content (Figure 4D). Meanwhile, the alizarin red staining indicated an evident increase in the mineralized nodule formation (Figure 4E). Above results indicated that FZD4 could promote osteogenic differentiation of hMSCs.

MicroRNA- 1286 Regulated Osteogenic Differentiation of hMSCs Via FZD4

To clarify the regulatory relation between microRNA-1286 and FZD4 in the osteogenic differentiation of hMSCs, we divided the cells into three groups, including control group, microRNA-1286 overexpression group, and simultaneous overexpression of microRNA-1286 and FZD4 group. It was found that the overexpression of microRNA-1286 and FZD4 reversed the effect of microRNA-1286 overexpression alone on cell

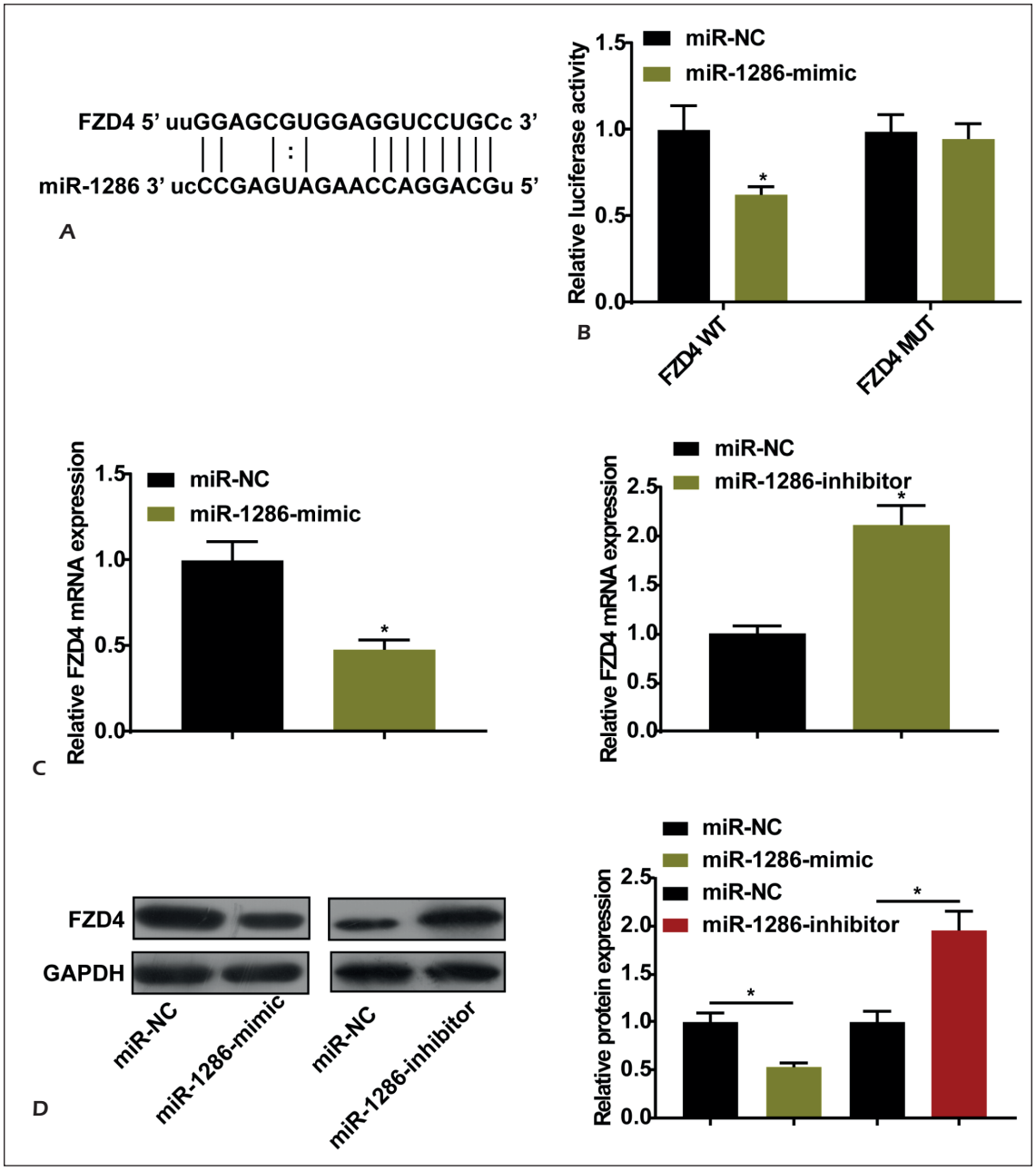

Figure 3. MiR-1286 could targeted regulate FZD4. **A**, Binding site of miR-1286 to FZD4 was predicted. **B**, Luciferase reporter gene assay revealed that overexpression of miR-1286 significantly quenched wild-type FZD4 fluorescence. **C**, QRT-PCR detection indicated that overexpression of miR-1286 reduced the mRNA expression level of FZD4, while knocking down miR-1286 significantly increased FZD4 expression. **D**, Western blot indicated that overexpression of miR-1286 reduced the protein expression level of FZD4, while knocking down miR-1286 significantly increased the protein expression level of FZD4.

function changes, including reduced mRNA expression levels of ALP, RUNX2, and OCN, decreased ALP activity and content, and weakened mineralization forming ability (Figures 5A, 5B, 5C, and 5D). These results suggested that microRNA-1286 could inhibit the osteogenic differentiation of hMSCs by interacting with FZD4.

Discussion

With the advent of an aging society, the incidence of osteoporosis has increased year by year, seriously affecting the health of the elderly. Therefore, its prevention and research have already received an increasing attention. During the process

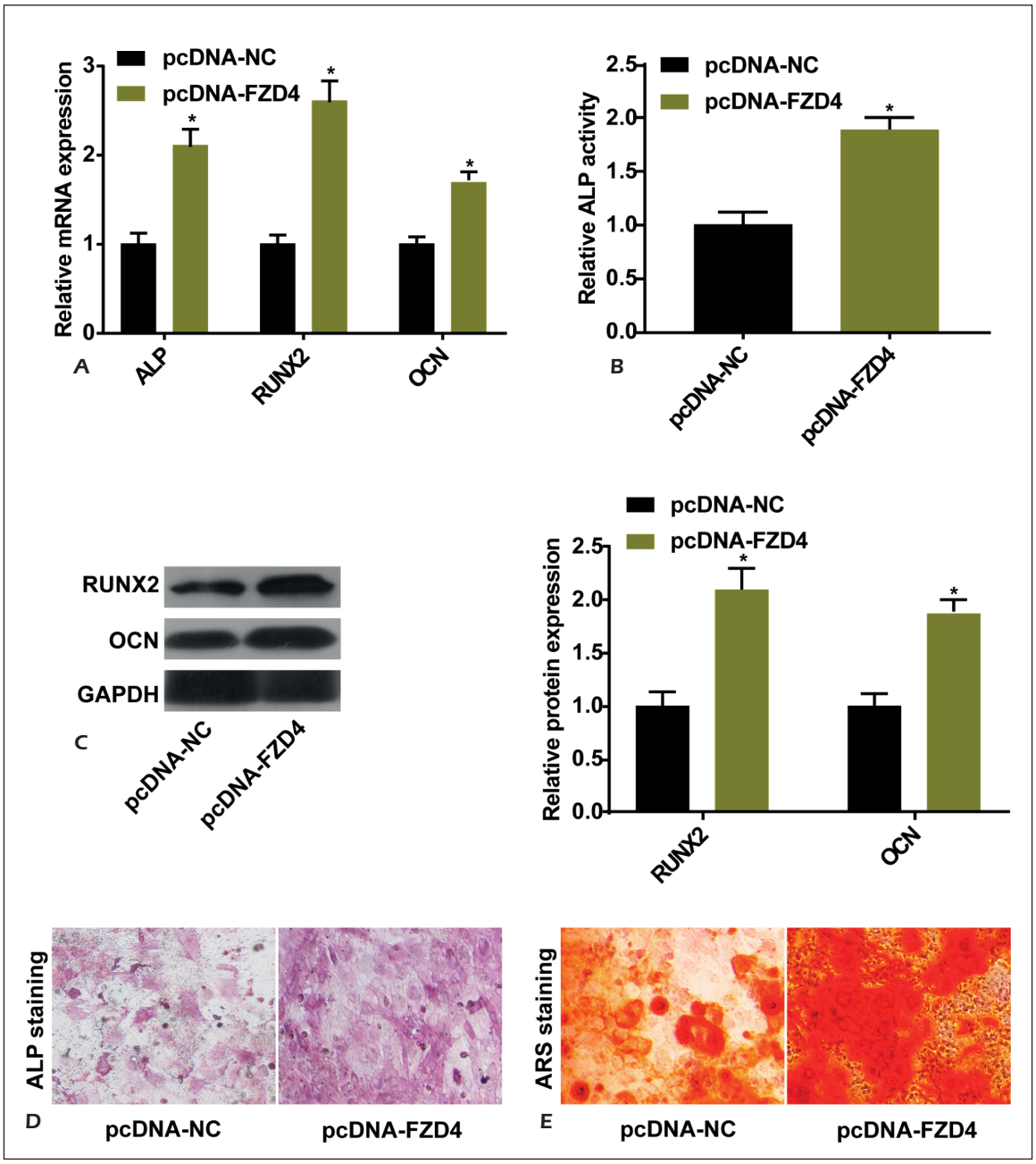

Figure 4. High expression of FZD4 promoted osteogenic differentiation of hMSCs. **A**, QRT-PCR detection indicated that after overexpression of FZD4 in hMSCs cells, the expression levels of ALP, RUNX2, and OCN were significantly up-regulated. **B**, ALP viability test showed that ALP activity increased significantly. **C**, Western blot detected that the protein levels of RUNX2, and OCN were significantly elevated. **D**, ALP staining result showed that ALP content was significantly enhanced. E, Alizarin red staining indicated that the formation of mineralized nodules increased significantly. (200X).

of bone remodeling, there is a dynamic balance between the osteogenic capacity of osteoblasts (derived from MSC) and bone resorption capacity of osteoclasts. If the balance is broken, bone resorption increases, easily leading to osteoporosis[17]. Therefore, researches of osteoporosis have a far-reaching significance.

As a kind of pluripotent stem cells, MSCs are widely found in bone marrow and cancellous bone. MSCs are common precursor cells of osteoblasts and fat cells, which play a vital role in bone metabolism. Treatment of osteoporosis is a novel idea to regulate the differentiation of MSCs, promote the differentiation of MSCs into osteo-

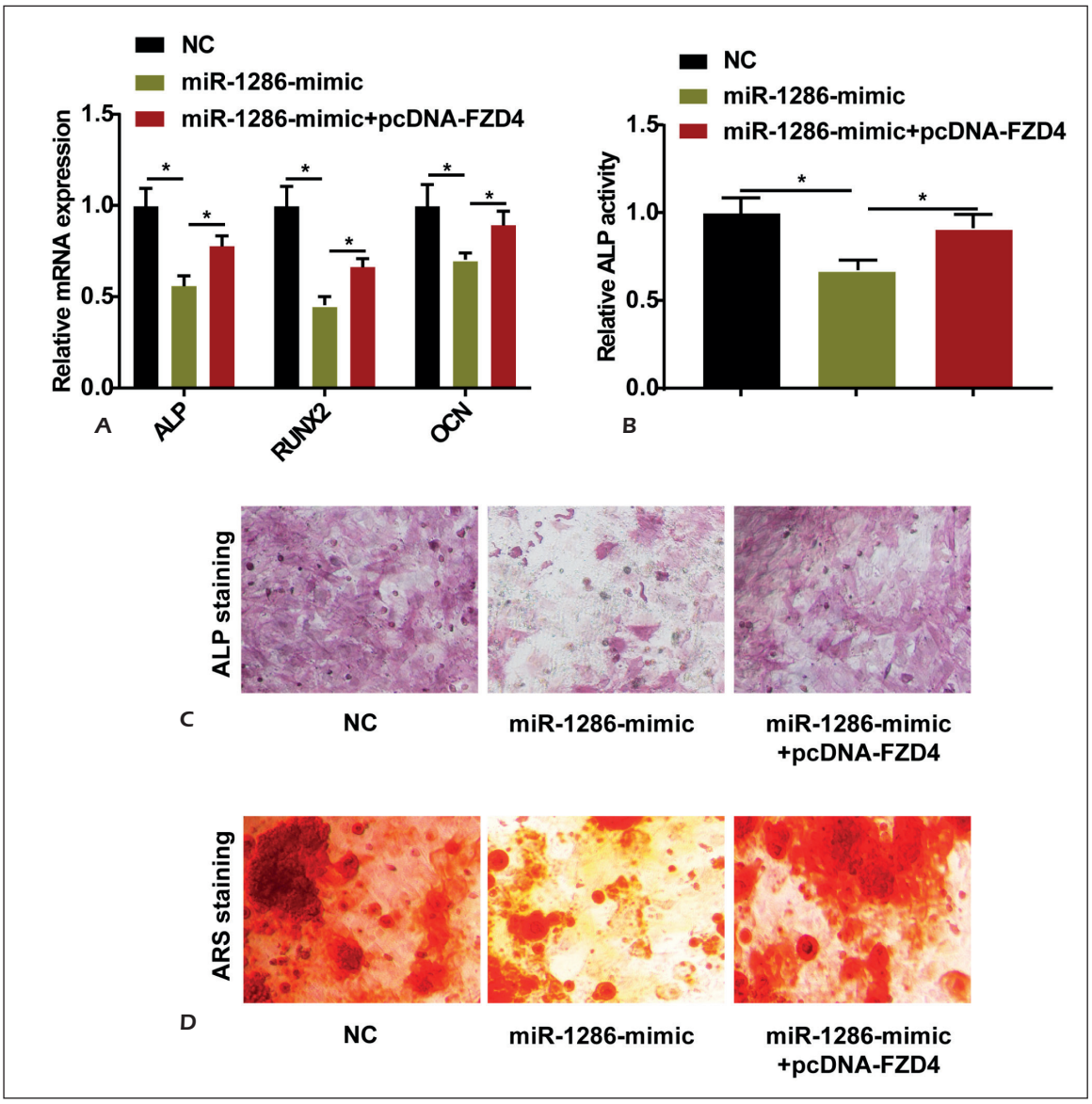

Figure 5. MiR-1286 regulated osteogenic differentiation of hMSCs via regulating FZD4 expression. **A**, Simultaneous overexpression of miR-1286 and FZD4 in hMSCs reversed the decrease in the mRNA expression levels of ALP, RUNX2, and OCN induced by overexpression of miR-1286. **B**, ALP activity decreased. **C**, ALP content decreased. **D**, Cell mineralization capacity decreased. (200X).

blasts, and inhibit the differentiation of BMSCs into adipocytes. This may increase the number and activity of osteoblasts, promote bone formation, and increase bone mass[18]. In this work, human-derived MSCs were used as a research model to explore the occurrence and development of osteoporosis.

MiRNA therapy has become one of the most attractive research areas in tissue engineering[19]. Several researches have reported that miRNAs are important regulators of stem cell therapy or differentiation. Meanwhile, dozens of miRNAs have been identified as important negative or positive regulators of transcriptional gene expression, playing a key role in osteogenesis[20-22]. Authors[12,23] have shown that miR-145 inhibits osteogenic differentiation by targeted regulating Sp7. MiR-433 inhibits osteogenic differentiation by combining with Runx2 to regulate its expression. Similar to these conclusions, in this study, we found that microRNA-1286 exerted a significant inhibitory effect on osteogenic differentiation of hMSCs.

FZD4 is an important downstream gene in the Wnt signaling pathway. It is involved in many biological processes, such as cell proliferation, invasion, and apoptosis[24]. In this research, our results indicated that FZD4 was involved in the regulation of osteogenic differentiation of hMSCs and showed a promoting effect. There is a close relation between miRNA and various signaling pathways of osteoporosis. Several reports have shown that miR-20a can promote the osteogenic differentiation of human bone marrow MSCs by targeting PPARγ, Bambi and Crim1 and regulating BMP/Runx2 signaling pathway[10]. Wang et al[9] have reported that the overexpression of miR-346 promotes differentiation of hMSCs into osteoblasts by regulating the Wnt signaling pathway by binding to GSK3b. The results of this study found that microRNA-1286 could target FZD4 and play a key role in the osteogenic differentiation of hMSCs. An in-depth study on the regulation mechanism of microRNA-1286 and FZD4 on the osteogenic differentiation process of hMSCs can help to better understand the mechanism of osteoporosis and provide new ideas for its clinical treatment.

Conclusions

We detected that microRNA-1286 was highly expressed in the serum of patients with osteoporosis. Furthermore, it could decrease the expression of FZD4 and inhibit the osteogenic differentiation of hMSCs.

Acknowledgments

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of Interests

The Authors declared that they have no conflict of interests.

References

1) Popp AW, Isenegger J, Buergi EM, Buergi U, Lippuner K. Glucocorticosteroid-induced spinal osteoporosis: scientific update on pathophysiology and treatment. Eur Spine J 2006; 15: 1035-1049.
2) Jackson WM, Nesti LJ, Tuan RS. Concise review: clinical translation of wound healing therapies based on mesenchymal stem cells. Stem Cells Transl Med 2012; 1: 44-50.
3) Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966; 16: 381-390.
4) Rodriguez JP, Astudillo P, Rios S, Pino AM. Involvement of adipogenic potential of human bone marrow mesenchymal stem cells (MSCs) in osteoporosis. Curr Stem Cell Res Ther 2008; 3: 208-218.
5) Hess R, Pino AM, Rios S, Fernández M, Rodriguez JP. High affinity leptin receptors are present in human mesenchymal stem cells (MSCs) derived from control and osteoporotic donors. J Cell Biochem 2005; 94: 50-57.
6) Eskildsen T, Taipaleenmäki H, Stenvang J, Abdallah BM, Ditzel N, Nossent AY, Bak M, Kauppinen S, Kassem M. MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo. Proc Natl Acad Sci U S A 2011; 108: 6139-6144.
7) Qiao L, Liu D, Li CG, Wang YJ. MiR-203 is essential for the shift from osteogenic differentiation to adipogenic differentiation of mesenchymal stem cells in postmenopausal osteoporosis. Eur Rev Med Pharmacol Sci 2018; 22: 5804-5814.
8) Li Y, Fan L, Hu J, Zhang L, Liao L, Liu S, Wu D, Yang P, Shen L, Chen J, Jin Y. MiR-26a rescues bone regeneration deficiency of mesenchymal stem cells derived from osteoporotic mice. Mol Ther 2015; 23: 1349-1357.
9) Wang Q, Cai J, Cai XH, Chen L. MiR-346 regulates osteogenic differentiation of human bone marrow-derived mesenchymal stem cells by targeting the Wnt/beta-catenin pathway. PLoS One 2013; 8: e72266.
10) Zhang JF, Fu WM, He ML, Xie WD, Lv Q, Wan G, Li G, Wang H, Lu G, Hu X, Jiang S, Li JN, Lin MC, Zhang YO, Kung HF. MiRNA-20a promotes osteogenic differentiation of human mesenchymal stem cells by co-regulating BMP signaling. RNA Biol 2011; 8: 829-838.
11) Li Z, Hassan MQ, Jafferji M, Aqeilan RI, Garzon R, Croce CM, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem 2009; 284: 15676-15684.
12) Kim EJ, Kang IH, Lee JW, Jang WG, Koh JT. MiR-433 mediates ERRγ-suppressed osteoblast differentiation via direct targeting to Runx2 mRNA in C3H10T1/2 cells. Life Sci 2013; 92: 562-568.
13) Yao T, Rao Q, Liu L, Zheng C, Xie Q, Liang J, Lin Z. Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in cervical cancer. Virol J 2013; 10: 175.
14) Mairinger FD, Ting S, Werner R, Walter RF, Hager T, Vollbrecht C, Christoph D, Worm K, Mairinger T, Sheu-Grabellus SY, Theegarten D, Schmid KW, Wohlschlaeger J. Different micro-RNA expression profiles distinguish subtypes of neuroendocrine tumors of the lung: results of a profiling study. Mod Pathol 2014; 27: 1632-1640.
15) Ueno K, Hirata H, Majid S, Yamamura S, Shahryari V, Tabatabai ZL, Hinoda Y, Dahiya R. Tumor suppressor microRNA-493 decreases cell motility and migration ability in human bladder cancer cells by downregulating RhoC and FZD4. Mol Cancer Ther 2012; 11: 244-253.
16) Ma C, Xu B, Husaiyin S, Wang L, Wusainahong K, Ma J, Zhu K, Niyazi M. MicroRNA-505 predicts prognosis and acts as tumor inhibitor in cervical carcinoma with inverse association with FZD4. Biomed Pharmacother 2017; 92: 586-594.

17) Makras P, Delaroudis S, Anastasilakis AD. Novel therapies for osteoporosis. Metabolism 2015; 64: 1199-1214.
18) Deng P, Chen QM, Hong C, Wang CY. Histone methyltransferases and demethylases: regulators in balancing osteogenic and adipogenic differentiation of mesenchymal stem cells. Int J Oral Sci 2015; 7: 197-204.
19) Lian JB, Stein GS, van Wijnen AJ, Stein JL, Hassan MQ, Gaur T, Zhang Y. MicroRNA control of bone formation and homeostasis. Nat Rev Endocrinol 2012; 8: 212-227.
20) Li J, Hu C, Han L, Liu L, Jing W, Tang W, Tian W, Long J. MiR-154-5p regulates osteogenic differentiation of adipose-derived mesenchymal stem cells under tensile stress through the Wnt/PCP pathway by targeting Wnt11. Bone 2015; 78: 130-141.
21) Qadir AS, Um S, Lee H, Baek K, Seo BM, Lee G, Kim GS, Woo KM, Ryoo HM, Baek JH. MiR-124 negatively regulates osteogenic differentiation and in vivo bone formation of mesenchymal stem cells. J Cell Biochem 2015; 116: 730-742.
22) An X, Ma K, Zhang Z, Zhao T, Zhang X, Tang B, Li Z. MiR-17, miR-21, and miR-143 enhance adipogenic differentiation from porcine bone marrow-derived mesenchymal stem cells. DNA Cell Biol 2016; 35: 410-416.
23) Jia J, Tian Q, Ling S, Liu Y, Yang S, Shao Z. MiR-145 suppresses osteogenic differentiation by targeting Sp7. FEBS Lett 2013; 587: 3027-3031.
24) Gupta S, Iljin K, Sara H, Mpindi JP, Mirtti T, Vainio P, Rantala J, Alanen K, Nees M, Kallioniemi O. FZD4 as a mediator of ERG oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Res 2010; 70: 6735-6745.