Dishevelled-2 modulates osteogenic differentiation of human synovial fibroblasts in osteoarthritis

LIHUA ZHANG, LUAN LUAN and YINGYING MA

Department of Rheumatology, Jining No. 1 People's Hospital, Jining, Shandong 272000, P.R. China

Abstract. Dishevelled (Dvl)-2 represents one of the cytoplasmic proteins, which serves as a pivotal hub in signaling intermediates through a number of different signaling pathways associated with the Wnt family. The aim of the present study was to investigate the roles and mechanisms of Dvl-2 on synovial fibroblasts (SFBs) in osteoarthritis (OA). A Cell Counting kit-8 (CCK-8) assay was used to determine cell viability. An alkaline phosphatase (ALP) test kit was used to measure the activity of ALP. Western blot and reverse transcription-quantitative polymerase chain reaction analysis were used to evaluate the protein and mRNA expression, respectively. The results suggest that depletion of Dvl-2 significantly decreased the expression of osteoprotegerin (OPG) and ALP ($P<0.05$) and significantly increased the expression of receptor activator of nuclear factor-κB ligand (RANKL), ALP, osteonectin (ON), osteocalcin (OCN) and osterix ($P<0.05$). In addition, the depletion of Dvl-2 also significantly inhibited the expression of runt-related transcription factor 2 (Runx-2) and β-catenin in SFBs ($P<0.05$). The effect of Dvl-2 over-expression was opposite to the effect of Dvl-2 silencing. The inactivation of Wnt3a reversed the effect of Dvl-2 silencing. In conclusion, the results indicate that Dvl-2 regulated osteogenic differentiation of SFBs in OA.

Introduction

Osteoarthritis (OA) also known as degenerative arthritis or proliferative arthritis is a joint disease characterized by the cartilage progressive destruction, which results from the excessive degradation of cartilage extracellular matrix components (1). The risk factors of OA is various, which can be classified as person-level factors, such as sex, age, race, genetics, and diet,

Correspondence to: Dr Yingying Ma, Department of Rheumatology, Jining No. 1 People's Hospital, 6 Jiankang Road, Fuqiao Street, Jining, Shandong 272000, P.R. China
E-mail: yingyingma25my@163.com

and joint-level factors involving malalignment, injury, and abnormal loading of the joints (2). Though OA leads to great suffering to patients, seriously affects their life qualities, and further raises a serious burden on the entire social economy. However, there is no drug that is able to effectively delay or prevent the progression of OA. Joint replacement is the only medical treatment during the middle and late stages of OA diease. However, studies have shown that synovial inflammation plays a crucial role in the development and progression of OA. Due to the high expression of inflammatory mediators in early OA synovial tissues, acute synovitis may be the origin of OA (3). Synovial membrane is a special type of cementitious tissue that consists of a lining layer, a lining under-layer, and an outer edge of the lining fuses with the joint capsule (4). Synovial cells can be divided into macrophages, fibroblasts, and dendritic cells. In addition, synovial fibroblasts (SFBs) can secrete collagen, fibronectin, osteonectin (ON), and hyaluronic acid (5). It has also been reported that SFBs can generate multiple cytokines, involving osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) (6,7). Hence, the SFBs from joints may become the key in OA joint treatment.

As a prevalent membrane-bound glycoprotein, alkaline phosphatase (ALP) promotes the hydrolysis of phosphate monoesters at basic pH values (8). ALP is expressed in several osteocytes, including osteoblasts, osteoclasts, and bone marrow stromal cells (9-11). Studies have found that the activity of ALP was closely associated with the bone formation (12), mineral bone disorder (13), and osteogenic differentiation (14). Although ALP is expressed in many mammalian tissues and has been studied for several years, it is still little known. Moreover, regulatory mechanisms of ALP in the ossification of SFBs in OA are still little known to us.

A large number of co-receptors, receptors, ligands, and regulatory components are involved in the complex Wnt pathway (15) that proved to participate in several evident signaling events, such as β-catenin signaling activation (16). Researchers found that Wnt pathway modulates the maturation, differentiation as well as the apoptosis of osteoblasts and osteoclasts, therefore maintaining the balance of organism's bone metabolism (17). There are usually two types of Wnt pathways, which are the classical β-catenin-dependent pathway and non-classical β-catenin-independent pathway (18). In the classical β-catenin-dependent Wnt pathway, dishevelled acts as a key molecule that upregulates the Wnt pathway (19). A recent

research indicates that the Wnt pathway may serve as a target for OA therapy (20). SFBs, as one of the bone progenitor cells, have a strong ability of reproduction division *in vitro* in organizational engineering. Moreover, FB could be directly turned into bone cells without OB cells, and which was recognized as a most effective way in bone formation (21). Therefore, it was important to further explore the exact mechanisms of Wnt pathway in osteogenic differentiation of SFBs.

Dishevelled (Dvl) is one of the cytoplasmic proteins, and it servea as a pivotal hub in signaling intermediates through a number of different signaling pathways of Wnt family (22). Three dishevelled homologs-Dvl-1/2/3 are expressed in human and mice. Dvl-2 has effective impacts on the progressions of gliomas, prostate tumor and esophageal squamous cell carcinoma (23-25). Due to the osculating relationship between Dvl and Wnt pathway (19), we thereby set out to investigate the definite roles and mechanisms of Dvl-2 in the ossification of SFBs in OA via regulating the Wnt pathway.

In the current study, we analyzed the correlation between Dvl-2 and the ossification of SFBs in OA. Furthermore, it was also fascinating to investigate the exact role and mechanisms of Dvl-2 together with Wnt pathway in the ossification of SFBs in OA.

Materials and methods

Cell culture, genes, plasmids, and inhibitor. Human SFBs were obtained as previously described (26). Synovial membranes were obtained from 16 OA patients (mean age, 65±4.5 years) during arthroplastic surgery with the informed consent from patient and the approval of Ethics Committee of Jining No. 1 People's Hospital (Jining, China). The synovial membranes collected from end-stage joint narrowed space of hip and knee joints. The dissected tissues were incubated in DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 1 mg/ml collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) with shaking for 90 min at 37°C. The cells were centrifuged at 400 x g at 20°C for 30 min. Then, released SFBs were maintained in tissue culture flasks at 37°C for 1 h. Then, SFBs were incubated in DMEM supplemented with 10% heat inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.), 10% penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C. Dvl-2 RNA and Dvl-2 siRNA were respectively cloned into 2 pcDNA3.1(+) empty vectors-Dvl-2 (Invitrogen; Thermo Fisher Scientific, Inc.) and si-Dvl-2. IWR-1-endo (Beyotime, Shanghai, China) was used as a Wnt inhibitor.

Grouping. Control group (SFBs), NC group (SFBs transfected with empty vector), Dvl-2 group (SFBs transfected with Dvl-2), and si-Dvl-2 group (SFBs transfected with si-Dvl-2) were prepared as four treatment groups in this study. At least three independent experiments were performed.

Cell viability analysis. Cell Counting Kit-8 (CCK-8; Beyotime) was used to determine SFBs' cell viability. About 6x10^4 cells/ml of SFBs in the logarithmic phase were sowed into the wells of 96-well plates, and then maintained in a 5% CO_2 atmosphere at 37°C for 12 h. Afterwards, SFBs were handled as described above. Cells were then maintained for 12, 24, and 48 h respectively. 10 μl of CCK reagent was

then added into the wells. After that, cells were maintained for 3 h. The absorbance at 450 nm was read by a Microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was evaluated by the percentage of cell survival.

Western blot analysis. A total of 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to segregate proteins lysates of cultured SFBs, which were then transferred to a PVDF membrane (EMD Millipore, Billerica, MA, USA). Blotting was carried out by using specific antibodies (anti-Dvl-2, dilution, 1:1,000, cat. no. ab22616, rabbit anti-human; anti-OPG, dilution, 1:1,000, cat. no. ab73400, rabbit anti-human; anti-RANKL, dilution, 1:1,000, cat. no. ab9957, rabbit anti-human; anti-ALP, dilution, 1:1,000, cat. no. ab83259, rabbit anti-human; anti-ON, dilution, 1:1,000, cat. no. ab8448, rabbit anti-human; anti-osteocalcin (OCN), dilution, 1:500, cat. no. ab93876, rabbit anti-human; anti-osterix, dilution, 1:1,000, cat. no. ab22552, rabbit anti-human; anti-Wnt3a, dilution, 1:1,000, cat. no. ab28472, rabbit anti-human; anti-β-catenin, dilution, 1:5,000, cat. no. ab32572, rabbit anti-human; anti-Runx-2, dilution, 1:1,000, cat. no. ab23981, rabbit anti-human; anti-β-actin, dilution, 1:2,000, cat. no. ab8227, rabbit anti-human; all from Abcam, Cambridge, UK). Horseradish peroxidase-conjugated secondary antibodies (dilution, 1:5,000, cat. no. ab205718, goat anti-rabbit; Abcam) were supplemented and incubated for 1 h at room temperature. Enhanced chemiluminescent reagents (EMD Millipore) using an ECL system (Amersham; GE Healthcare, Chicago, IL, USA) were performed on the evaluation of results.

Reverse transcription-quantitative reverse transcription PCR (RT-qPCR) analysis. Total RNA was extracted from cultured SFBs by TRIzol reagent (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). RNA was reverse transcribed to cDNA by Reverse Transcription kit (Beijing Solarbio Science & Technology Co., Ltd.) according to the direction. RT-qPCR analysis was performed on ABI 7500 Thermocycler (Applied Biosystems Thermo Fisher Scientific, Inc.). PCR cycling conditions were as follows: One pretreatment at 95°C for 10 min, 94°C for 15 sec, 62°C for 45 sec (45 cycles), 94°C for 15 sec, 62°C for 1 min, 95°C for 15 sec, a final extension at 75°C for 10 min and was held at 4°C. Relative expressions of target genes were calculated by $2^{-\Delta\Delta CT}$ method (27). The primers were purchased from Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China): Dvl-2 forward, 5'-CATCCAGCCAAT TGACCCTG-3' and reverse, 5'-AGGGATGGTGATCTTGA GCC-3' (product, 241 bp); OPG forward, 5'-GGCACCAAA GTAAACGCAGA-3' and reverse, 5'-TCCCGGTAAGCTTTC CATCA-3' (product, 228 bp); RANKL forward, 5'-CGCTCG TGTTTCTGGACATC-3' and reverse, 5'-GGGGCTGCAGTA TAGACACT-3' (product, 233 bp); ALP forward, 5'-CCTCTT CCCCTTCCTGGTG-3' and reverse, 5'-GATGCCACAAGT GTCAGGAC-3' (product, 196 bp); ON forward, 5'-CAACGA AAGCCATGACCACA-3' and reverse, 5'-ACCTCGGCCATC ATATGTGT-3' (product, 247 bp); OCN forward, 5'-GCAGAG TCCAGCAAAGGTG-3' and reverse, 5'-TCACAGTCCGGA TTGAGCTC-3' (product, 161 bp); osterix forward, 5'-TCT CTGGACATGACACACCC-3' and reverse, 5'-AGGGGA GCAAAGTCAGATGG-3' (product, 233 bp); Wnt3a forward, 5'-ATCGAGTTTGGTGGGATGGT-3' and reverse, 5'-CGC
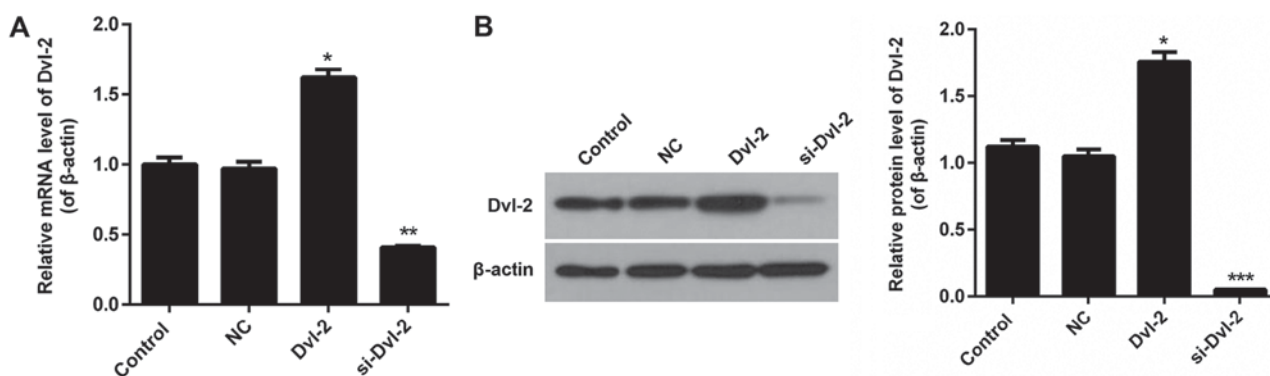
Figure 1. Over-expression and interference of Dvl-2 in SFBs. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis were performed to detect the mRNA and protein expression levels of Dvl-2 in SFBs, SFBs transfected with empty vector, SFBs transfected with Dvl-2 and SFBs transfected with si-Dvl-2. *P<0.05, **P<0.01 and ***P<0.001 vs. NC. Dvl-2, dishevelled-2; SFB, synovial fibroblasts; si, short interfering; NC, negative control.

TGTCGTACTTGTCCTTG-3' (product, 238 bp); β-catenin forward, 5'-AGTTCCTTACCGTCCCCAAG-3' and reverse, 5'-CAGACACGCCTGTTTCGAAT-3' (product, 249 bp); Runx-2 forward, 5'-ATTCTGCTGAGCTCCGGAAT-3' and reverse, 5'-AGCTTCTGTCTGTGCCTTCT-3' (product, 211 bp); and β-actin forward, 5'-GTTACAGGAAGTCCCTCA CCC-3' and reverse, 5'-CAGACCTGGGCCATTCAGAAA-3' (product, 194 bp). β-actin was used as the control of the input RNA level.

Statistical analysis. Results are shown as the mean ± SD. Following Dunnet's test, all experimental data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparisons post hoc test. GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform the statistical analysis. The statistical significance was defined as P<0.05.

Results

Over-expression and interference of Dvl-2 in SFBs. RNA and siRNA vectors targeting Dvl-2 gene named Dvl-2 and si-Dvl-2 were constructed in our study. After being transfecting with Dvl-2, the expression level of Dvl-2 in SFBs was clearly upregulated, and the knockdown efficiency was about 60% in SFBs after being stably transfected with si-Dvl-2 (P<0.05; Fig. 1A and B).

Dvl-2 silence reduced the cell viability of SFBs. CCK-8 assay was carried out to determine the cell viability of SFBs coped with the treatment groups as previously described. The results shown that both the over-expression of Dvl-2 over-expression and depletion of Dvl-2 slightly reduced the cell viability of SFBs (Fig. 2). No significant difference was found among these groups.

Dvl-2 affected the activity of ALP in SFBs. We also evaluated the activity of ALP in SFBs coped with the treatment groups as described above. An obvious increase of ALP activity was observed in Dvl-2 group compared with NC (P<0.05; Fig. 3A). However, the ALP activity in SFBs was markedly reduced by si-Dvl-2 (P<0.01; Fig. 3A). Therefore, it was determined that Dvl-2 silence lessened the activity of ALP in SFBs.
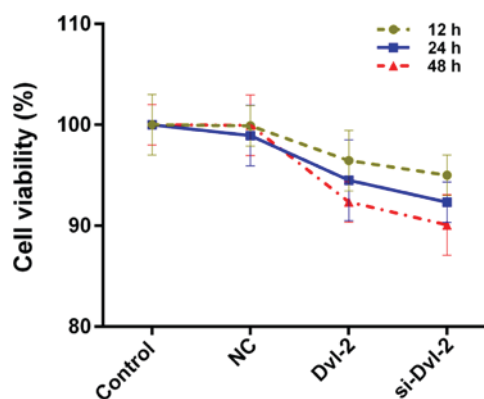

Figure 2. Effect of Dvl-2 on the cell viability of SFBs. A Cell Counting kit-8 assay was performed to measure the cell viability of SFBs, SFBs transfected with empty vector, SFBs transfected with Dvl-2 and SFBs transfected with si-Dvl-2 at 12, 24 and 48 h. Dvl-2, dishevelled-2; SFB, synovial fibroblasts; si, short interfering; NC, negative control.

Dvl-2 regulated the osteogenic differentiation in SFBs. To further investigate the funtion of Dvl-2 in the osteogenic differentiation of SFBs, we assessed the expression of osteogenic factors in SFBs that were transfected with Dvl-2 or si-Dvl-2, using OPG, RANKL, ALP, ON, OCN, and osterix. We found that a rise in the OPG/RANKL ratio in Dvl-2 group, which is in contrast to a decline in the si-Dvl-2 group (P<0.05; Fig. 3B and C). Moreover, compared to control group, the expression levels of ON, OCN, and osterix in SFBs transfected with si-Dvl-2 were significantly upregulated However, a sharp decrease of ALP expression was observed in si-Dvl-2 group; and an increase in Dvl-2 group (P<0.05; Fig. 4A and B).

Dvl-2 affected the activity of Runx-2. For the purpose of exploring the mechanisms of Dvl-2 in the osteogenic differentiation of SFBs, we therefore measured the expression levels of Runx-2 in SFBs from all treatment groups. The results indicate that compared to control group, the expression levels of Runx-2 was upregulated by Dvl-2 and downregulated by si-Dvl2. Moreover, though the expression of Wnt3a and β-catenin increased at the presence of Dvl-2, the depletion of Dvl-2 decreased the levels of Wnt3a and β-catenin (P<0.05; Fig. 5A and B). Furthermore, Wnt3a was inhibited to estimate the effect of Dvl-2. The expression of Wnt3a was shown in
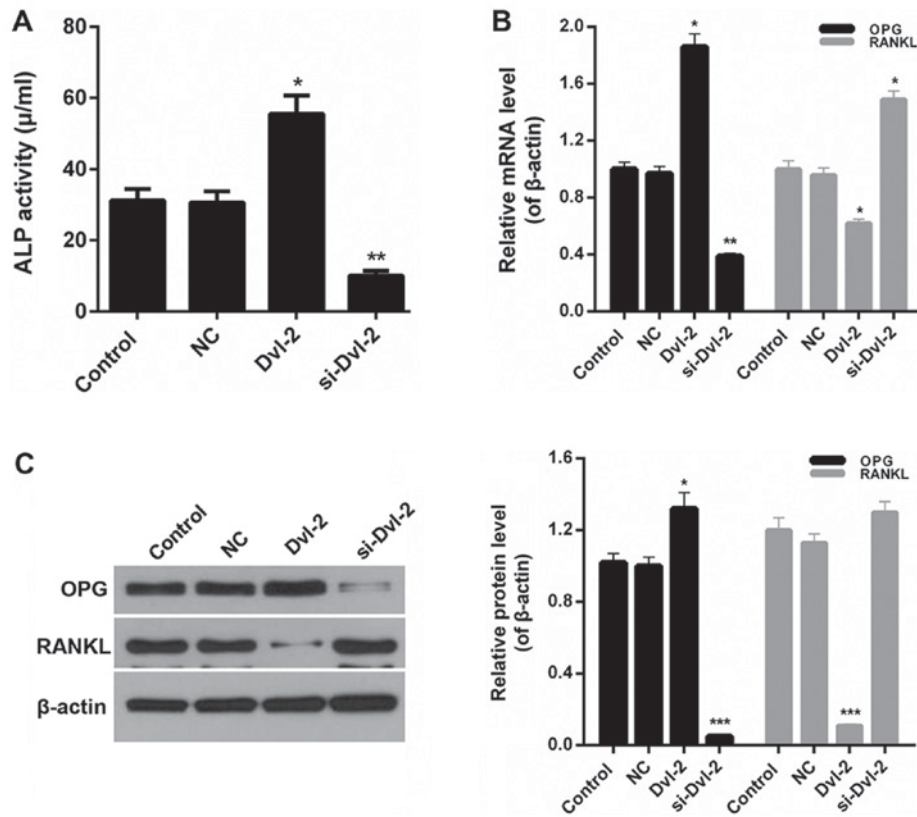
Figure 3. Effect of Dvl-2 on ALP activity and OPG and RANKL expression in SFBs. SFBs were transfected with empty vector, Dvl-2 and si-Dvl-2. (A) An ALP test kit was used to measure the activity of ALP in SFBs. (B) Reverse transcription-quantitative polymerase chain reaction (C) and western blot analysis were performed to evaluate the mRNA and protein expression levels of OPG and RANKL in SFBs. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. NC. Dvl-2, dishevelled-2; SFB, synovial fibroblasts; si, short interfering; ALP, alkaline phosphatase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; NC, negative control.
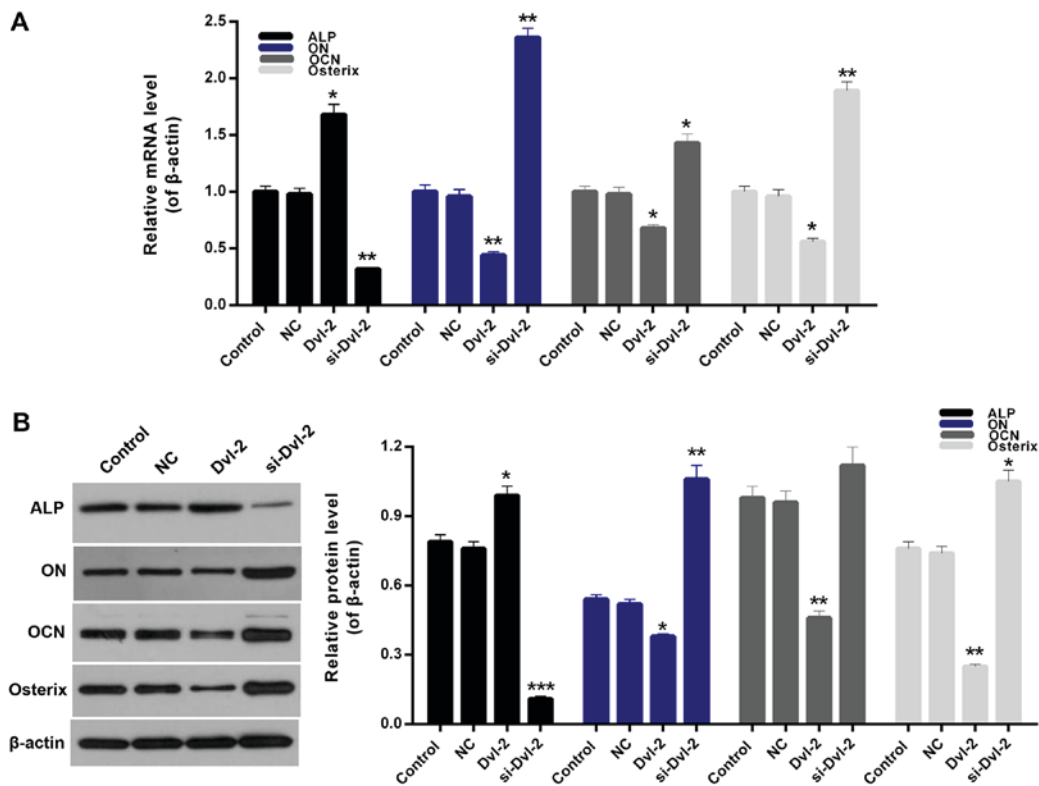

Figure 4. Effect of Dvl-2 on the osteogenic differentiation of SFBs. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis were performed to determine the mRNA and protein expression levels of ALP, ON, OCN and osterix in SFBs, SFBs transfected with empty vector, SFBs transfected with Dvl-2 and SFBs transfected with si-Dvl-2. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. NC. Dvl-2, dishevelled-2; SFB, synovial fibroblasts; si, short interfering; ALP, alkaline phosphatase; ON, osteonectin; OCN, osteocalcin; NC, negative control.
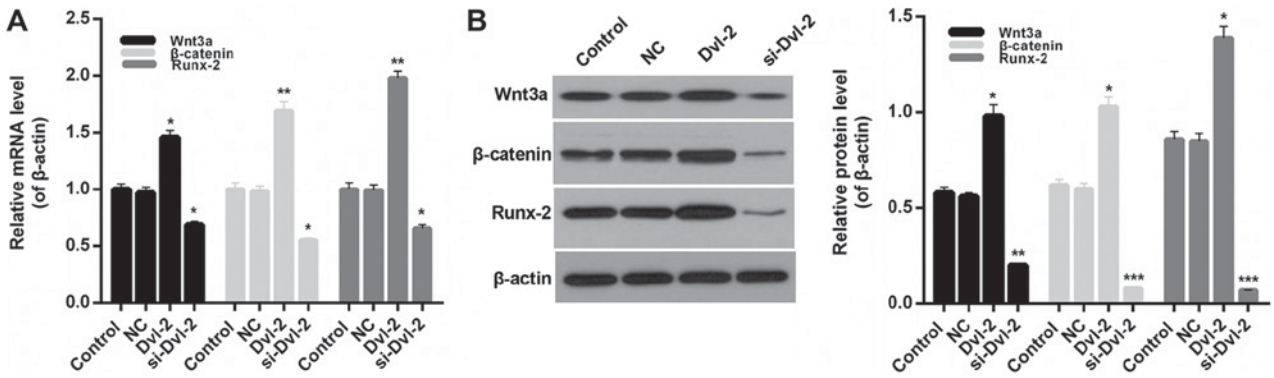
Figure 5. Effect of Dvl-2 on the expression of Runx-2, Wnt3a and β-catenin. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis were performed to determine the mRNA and protein expression levels of Wnt3a, β-catenin and Runx-2 in SFBs, SFBs transfected with empty vector, SFBs transfected with Dvl-2 and SFBs transfected with si-Dvl-2. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. NC. Dvl-2, dishevelled-2; SFB, synovial fibroblasts; si, short interfering; Runx-2, runt-related transcription factor 2; NC, negative control.
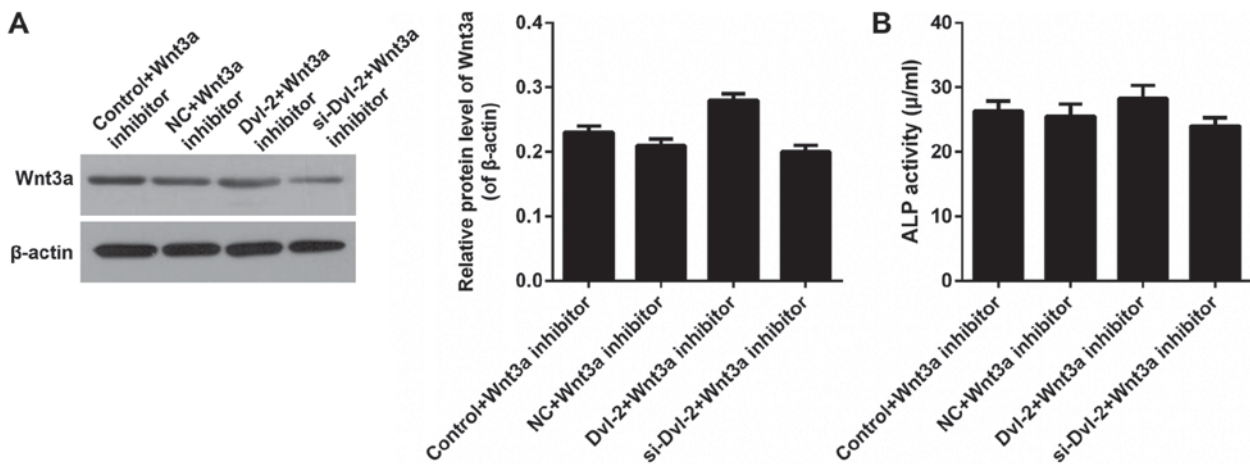

Figure 6. Wnt3a impacted the activity of ALP in SFBs. SFBs, SFBs transfected with empty vector, SFBs transfected with Dvl-2 and SFBs transfected with si-Dvl-2 were treated with Wnt3a inhibitor. (A) A western blot assay was performed to determine the protein expression levels of Wnt3a in SFBs. (B) An ALP test kit was used to measure the activity of ALP in SFBs. Dvl-2, dishevelled-2; SFB, synovial fibroblasts; si, short interfering; NC, negative control.

Fig. 6A. After the inhibition of Wnt signaling by IWR-1-endo, there was no significant difference of ALP activity in SFBs from all of the treatment groups (Fig. 6B).

Discussion

OA is one of the articular degenerative disorders. It can lead to bone destruction around the joint, ossification in the surrounding tissues, subsequent loss of bony rigidity and even joint activity (28). Based on the pathological investigation of OA ossification sites, it was considered that non-osteocytes at the attachment sites can proliferate and gradually differentiates into chondrocytes. The fiber textures at attachment sites gradually grow thicker and the cartilage-based pathological nodules will occur as a consequence (29). Furthermore, these possessing secretion function cells releases a large number of ALP matrix vesicles, which will cause the partial formation of hydeoxyapatite crystal and then gradually calcification (30-32). Multiple cell types are involved in the progress of osteogenic differentiation. SFBs have been widely investigated in the previous research about OA (33-36). And SFB palyed important roles both in bone resorption and bone

formation (37,38). Thus, in this study, we selected SFBs as our research objects to further explore the the osteogenic differentiation in OA.

Previous studies showed that the close connections between Dvl-2 and Wnt pathway (39-41). However, to the best of our knowledge, the roles and mechanisms of Dvl-2 in the osteogenic differentiation of SFBs have not been studied yet. Thus, we selected Dvl-2 as the study object, and transfected the SFBs with Dvl-2 and si-Dvl-2. Over-expression and silencing of Dvl-2 in SFBs were observed, and the knock-down efficiency of Dvl-2 was about 60%. We first measured the cell viability of SFBs, which are transfected with Dvl-2 and si-Dvl-2. The results indicated that Dvl-2 silence could inhibit the cell viability of SFBs, especially for 48 h-treatment. Then, we measured the activity of ALP in SFBs, which were transfected with Dvl-2 and si-Dvl-2. The results showed that Dvl-2 significantly influenced the ALP activity in SFBs. In order to investigate the functions of Dvl-2 in the osteogenic differentiation of SFBs, the expressions of OPG and RANKL in SFBs were examined in reference to previous studies (6,7). Based on the experimental results, we found that the OPG/RANKL ratio was remarkably reducced by si-Dvl-2.

Moreover, over-expression of Dvl-2 significantly enhanced the OPG/RANKL ratio in SFBs. Additionally, Dvl-2 silence significantly reduced the ALP expression, while it upregulated the expression levels of ON, OCN, and osterix in SFBs. It was confirmed that Dvl-2 suppressed bone absorption of SFBs in OA by regulating the expression levels of OPG, RANKL, ALP, ON, OCN, and osterix.

In different stages of osteogenic differentiation, signaling pathways involved/participated are not the same. It has been proved that ossification was largely affected by BMP pathway at an early stage, while the Wnt pathway impacts ossification in the advanced ossification (42-44). Among them, Wnt/β-catenin pathway plays an important role in stem cell differentiation, bone formation, and the regulation of balance from the embryonic period (45). The abnormal regulation of Wnt pathway is closely associated with the bony ankylosis in OA, and was considered as one of the important factors in osteogenic differentiation. In the present study, the Wnt3a, β-catenin, and Runx-2 expressions in SFBs were studied. Our results showed that Dvl-2 silence significantly downregulated the expression levels of Wnt3a, β-catenin, and Runx-2 in SFBs. After downregulating the Wnt3a expression, we found that there was no significant difference in the activities of ALP in SFBs. Such results remind us of that Dvl-2 regulated the activity of Runx-2 by affecting the Wnt pathway in SFBs. Thus, it can concluded that Dvl-2 modulated the osteogenic differentiation of SFBs in OA via Wnt/β-catenin/Runx-2 pathway, to some extent.

Taken together, our research demonstrated that the Dvl-2 plays a critical role in osteogenic differentiation of SFBs in OA, which was related to the Wnt pathway. Also, the results provided a new thread for understanding the pathogenesis of OA and put forward a fascinating approach for the therapy of OA.

In conclusoin, our study highlights that Dvl-2 silence modulates the ossification of SFBs in OA by downregulating the Wnt pathway. The findings of our research are crucial to unfolding the mechanisms of Dvl-2 in the ossification of SFBs. The potential effects of Dvl-2 in the ossification of SFBs suggest that Dvl-2 might be an effective target for OA therapies.

Acknowledgements

Not applicable.

Availability of data and materials

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

Authors' contributions

LZ wrote the main manuscript. LL performed the experiments. YM designed the study. LZ and LL performed data analysis. LZ, LL and YM contributed to manuscript revisions and all authors reviewed the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Jining No. 1 People's Hospital. Written informed consent was obtained from all patients prior to their inclusion within the study.

Consent for publication

Written informed consent was obtained from all patients for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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