Dkk1 is a negative regulator for the pathogenesis of ossification of the posterior longitudinal ligament

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Research article

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Abstract

Purpose

Ossification of the posterior longitudinal ligament (OPLL) is an osteogenic disorder characterized by ectopic bone formation in the posterior longitudinal ligament of the spine. dinkkopf 1 (Dkk1) is a secreted inhibitor of the Wnt pathway, which negatively regulates bone formation during skeletal development. However, whether Dkk1 impacts the pathogenesis of OPLL has not been reported. This study is to investigate the implication of Dkk1 in the pathogenesis of OPLL.

Methods

The serum level of Dkk1 in OPLL patients was determined using ELISA. The expression of Dkk1 and activation of the Wnt/β-catenin signaling were examined in OPLL ligament-derived fibroblasts. The modulation of Dkk1 on OPLL cell proliferation, apoptosis, as well as BMP2-induced osteogenic differentiation was also investigated.

Results

The serum level of Dkk1 is decreased in OPLL patients as compared to non-OPLL patients. The expression of Dkk1 is also reduced in OPLL fibroblasts. Downregulation of Dkk1 in OPLL cells is associated with stabilized β-catenin and increased TCF-dependent transcriptional activity, indicating an activation of the Wnt/β-catenin signaling pathway. Functionally, Dkk1 exerts a growth-inhibitory effect by repressing proliferation but promoting apoptosis of OPLL fibroblasts. Dkk1 also suppresses BMP2-induced entire osteogenic differentiation of OPLL cells and this suppression is mediated via its inhibition of the Wnt pathway.

Conclusion

Dkk1 acts as an important negative regulator in the pathogenesis of OPLL. Targeting the Wnt pathway using Dkk1 or small molecule inhibitors may represent a potential therapeutic strategy for the treatment of OPLL.

Introduction

Ossification of the posterior longitudinal ligament (OPLL) is a spinal disorder characterized by progressive ectopic bone formation in the posterior longitudinal ligament (PLL). OPLL mainly occurs in the cervical spine (70%) and can result in various degrees of neurological symptoms, ranging from discomfort to severe myelopathy due to compression of the spinal cord and nerve roots by increasingly calcified PLL tissues [1]. So far, there is a lack of effective drug therapy against OPLL. Patients suffering from significant myelopathy usually require surgical treatment, yet the outcomes are not often satisfactory, and the surgery itself may cause adverse complications. As such, there is a need to identify

the signaling pathway that regulates the pathogenesis of OPLL in order to develop novel molecular therapy for this disease.

Wnt pathway plays a central role in skeletal development and bone regenerative process such as fracture healing [2–5]. In the canonical Wnt signaling, Wnt ligands bind to the transmembrane receptor Frizzled (Fz) and the co-receptor low-density lipoprotein receptor-related protein 5 or 6 (LRP-5/6), which leads to the activation of the Dishevelled (Dvl) and subsequently inhibits the action of glycogen synthase kinase 3β (GSK-3β). Inhibition of GSK-3β reduces phosphorylation of β-catenin, preventing its degradation by the ubiquitin-mediated pathway. The stabilized β-catenin translocates to the nucleus and activates T cell factor/lymphoid enhancer factor (TCF/LEF)-mediated transcription of target genes (e.g., *c-myc*, *cyclin D1*) that regulate proliferation, apoptosis, differentiation, and other processes [6]. Hence, the Wnt/β-catenin signaling pathway is a critical regulator of cellular activities.

Wnt signaling can be negatively regulated by its inhibitors, such as Dickkopf-1 (Dkk1), sclerostin (SOST), secreted frizzled-related protein-1 (SFRP1), and Wnt inhibitory factor 1(WIF1). Among these inhibitors, Dkk1 forms a ternary complex with LRP-5/6 and another receptor, Kremen, followed by endocytosis of this complex and removal of LRP-5/6 from the cell surface, thus blocking the signaling transduction of Wnt. In the musculoskeletal system, although inhibition of the Wnt pathway by Dkk1 is associated with reduced osteoblast differentiation and low bone mass [7, 8], whether Dkk1 impacts the development and progression of OPLL remains unknown and this warrants investigation.

In this study, we explored the role of Dkk1 in the pathogenesis of OPLL, focusing on the regulating effect of this Wnt inhibitor in ligament-derived cellular behaviors. We report that the serum level of Dkk1 is reduced in OPLL patients as compared to non-OPLL patients. Dkk1 is also downregulated in OPLL fibroblasts, which is associated with increased β-catenin-mediated transcriptional activity. Functionally, Dkk1 acts as a negative regulator for the cellular growth and osteogenic differentiation in association with its inhibition of the Wnt pathway.

Materials And Methods

Acquisition of human biomaterials

This study was approved by the Human Ethics Committee of the Shandong Provincial Hospital and written informed consent was obtained from each patient. The peripheral blood samples (10 ml) were collected from 12 patients (48.3 ± 3.5 years) with cervical OPLL and another 10 non-OPLL patients (50.2 ± 4.2 years) that suffered cervical vertebral fractures or cervical intervertebral disc herniation. The diagnosis of cervical OPLL was made based on radiographic examinations, including lateral X-ray radiography and CT scanning. After coagulation, the serum samples were centrifuged and stored at -80^{o}C. The ligament tissues from both OPLL and non-OPLL patients were harvested during cervical anterior decompression surgery.

Cell culture

For ligament-derived cell culture, the non-OPLL ligament tissues were minced into 1-mm^3 pieces, washed twice with PBS, and plated into 90-mm culture dishes containing growth medium: Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen). Primary cells were cultured in an incubator at 37 °C in a humidified atmosphere with 95% air and 5% CO_2. When cells derived from the explants reached 90% confluence, they were trypsinized and passaged for further study. For cell culture from OPLL tissues, the ligaments closely adjacent to the ossified site were carefully extracted to avoid any possible contamination with osteogenic cells. Cells were then isolated and cultured using the same procedure as described above.

qPCR & western blot

For quantitative real-time RT-PCR (qPCR), total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using qScript cDNA SuperMix (QuantaBio). qPCR was performed using the SYBR green master mix. The primers designed for qPCR analysis were listed in **Table 1**. PCR products were analyzed with the ABI PRISM 7900HT Sequence Detections System (Applied Biosystems). The relative mRNA level of the target genes was determined with the ABI software (RQ Manager Version 1.2) using the ΔΔCt method with normalization to *GAPDH*. For western blot, proteins from ligament tissues or cell lysates were prepared using RIPA Lysis Buffer (Sigma-Aldrich). The samples (25 μg) were electrophoresed on an SDS polyacrylamide gel (Invitrogen) and were transferred onto a polyvinylidene difluoride membrane (PVDF, Millipore). Membranes were washed with TBS/0.1% Tween 20 (TBST) and blocked with 1% BSA in TBST for 30 minutes. The primary rabbit anti-human β-catenin antibody (Cell Signaling) was added to the membranes overnight at 4 °C. Blots were washed 3 times with TBST and followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (Cell Signaling) for 1 hour. After washing with TBST, the membrane was detected with ECL Detection Reagents (Thermo Scientific) and exposed to X-ray film. Protein bands were analyzed for densitometry using ImageJ software (NIH).

ELISA assay

For enzyme-linked immunosorbent assay (ELISA), the serum level of Dkk1 in patients was examined using a highly sensitive Human Dkk-1 Quantikine ELISA Kit (R&D Systems) according to the manufacturer's protocol. The absorbance at 450 nm was measured using a microplate reader.

Proliferation & apoptosis assay

The proliferation of ligament cells was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay kit (Roche) according to the manufacturer's protocol. In brief, cells plated at a density of 5×10^3/well in 0.1 ml medium in 96-well plates. After 24 hours, 10 μl of MTT labeling reagent was added to each well and incubated for 2 hours. A volume of 0.1 ml of the isopropanol/HCl solution was added to each well to dissolve the formazan product. The absorbance at 570 nm was measured using a microplate reader. For apoptosis assay, ligament cells were cultured under serum starvation conditions (0.5% FCS) for 24 hours. The proapoptotic activity was evaluated with the colorimetric

Caspase-3 Assay Kit (Novus Biologicals) using the manufacturer's protocol. The absorbencies at 405 nm were recorded.

Osteoblast differentiation

When cells reached 80% confluence, the growth medium was replaced with osteogenic medium containing DMEM-10% FCS, 5 mM sodium β-glycerophosphate, 50 μg/ml L-ascorbic acid, as well as 200 ng/ml recombinant human BMP2 (Sigma-Aldrich). Alkaline phosphatase (ALP) activity in cell lysates was examined using a colorimetric ALP Assay Kit (Abcam) using the manufacturer's protocol. For mineralization, cells were cultured in osteogenic medium for 2 weeks and were stained with von Kossa reagent (Sigma-Aldrich).

TCF reporter assay

For TCF reporter assay, cells were transfected with the β-catenin-responsive firefly luciferase reporter plasmid TOPflash or FOPflash using Lipofectamine 2000 reagent (Invitrogen). After 24 hours following transfection, cells were harvested and washed with PBS. Cell lysates were isolated using Passive Lysis Buffer (Promega). The reporter activity was measured by the Dual-Luciferase Assay kit (Promega) using a BD Monolight 2010 luminometer (BD Biosciences).

Statistical analyses

Data are expressed as mean ± SD. All calculations were performed using GraphPad Prism (V5.0) software. Two-tailed Student's *t-test* was used for statistical analysis between two groups. One-way ANOVA was used for statistical analysis among multiple groups. The variance is similar between the groups in the same experiment. $P < 0.05$ was considered significant.

Results

Dkk1 is downregulated in OPLL patients

To investigate the role of Dkk1 in the pathogenesis of OPLL, we first determined the serum level of Dkk1 in patients. We collected serum samples from 12 patients with OPLL and another 10 non-OPLL patients (age-matched) suffering from cervical fracture or cervical intervertebral disc herniation. Using ELISA analysis, we found that the serum level of Dkk1 was significantly ($P < 0.05$) lower in OPLL patients (345.65 ± 89.34 pg/ml) than in non-OPLL patients (658.54 ± 112.78 pg/ml) (Fig. 1A). To explore if the expression of Dkk1 was also downregulated in OPLL cells, we harvested tissues from OPLL and non-OPLL patients and established primary culture for ligament cells. Under microscopy, the ligament-derived cells from OPLL or non-OPLL patients exhibit a fibroblast-like phenotype (Fig. 1B). qPCR analysis showed that the expression of *Dkk1* mRNA in OPLL fibroblasts was reduced as compared to that of non-OPLL fibroblasts (Fig. 1B). These results suggest that Dkk1 is downregulated in OPLL patients and ligament cells.

β-catenin signaling activity is increased in OPLL fibroblasts

Since Dkk1 acts as an inhibitor of the Wnt/β-catenin signaling, downregulation of Dkk1 in OPLL implies that activation of the Wnt pathway may be associated with the development of OPLL. To test this hypothesis, we first evaluated the expression level of β-catenin. Using qPCR analysis, we found that the β-catenin gene (*Ctnnb1*) was expressed at a higher level in OPLL fibroblasts as compared to non-OPLL cells (Fig. 2A). Western blot analysis indicated that the protein level of β-catenin was also upregulated in OPLL cells (Fig. 2B), indicating a stabilization of β-catenin. Importantly, TCF reporter assay indicated that both basal level and LiCl-stimulated endogenous luciferase activity was higher in OPLL fibroblasts than in non-OPLL cells (Fig. 2C). Besides, the expression of several β-catenin-mediated transcription target genes, such as *c-myc*, *cyclin D1*, and *Axin2* was also increased in OPLL cells (Fig. 2D). These results suggest that β-catenin-mediated TCF-dependent signaling is enhanced in OPLL ligament cells.

Dkk1 exerts a growth-inhibitory effect in OPLL fibroblasts

Since Dkk1 is downregulated in OPLL patients and ligament fibroblasts, it seems plausible that this Wnt inhibitor may function as a negative regulator in the development of OPLL. To functionally investigate the role of Dkk1 at the cellular level, we treated ligament fibroblasts harvested from OPLL patients with recombinant human Dkk1 (R&D Systems) and explored the modulating effect of Dkk1 in cell behaviors. As shown in Fig. 3A, the MTT assay showed that treatment with Dkk1 decreased the proliferation rate of OPLL fibroblasts. We also determined the apoptotic level using a colorimetric approach and observed a 1.48-fold increase of caspase 3 activity in Dkk1-treated cells as compared to control cells (Fig. 3B). These results suggest that Dkk1 exerts a growth-inhibitory effect in OPLL fibroblasts.

Dkk1 inhibits osteogenic differentiation of OPLL fibroblasts

Given that OPLL is characterized by progressive ectopic bone formation in the PLL tissues, we then explored whether Dkk1 impacts osteogenic differentiation of ligament cells. For this purpose, we cultured OPLL ligament fibroblasts from OPLL patients in osteogenic medium containing recombinant BMP2. First, we evaluated the activity of ALP, an early osteoblastic differentiation marker. We found that the ALP activity was decreased in cells treated with recombinant Dkk1 as compared to control cells (Fig. 4A). qPCR assay showed that the expression of Runx2 and osterix, two transcription factors for osteoblasts, was reduced in Dkk1-treated cells. The expression of osteoblast differentiation markers including ALP and collagen type I (COL1A1) was also inhibited by Dkk1 (Fig. 4B). Besides, von Kossa staining showed that treatment with Dkk1 impaired the mineralization of osteoblasts (Fig. 4C). These results suggest that Dkk1 inhibits the entire osteogenic differentiation process of OPLL cells.

Dkk1 inhibits osteogenic differentiation by targeting the Wnt pathway

We have shown that Dkk1 is downregulated in OPLL cells, and this is associated with activation of β-catenin-mediated TCF-dependent transcription. To further confirm that Dkk1 inhibits osteogenic differentiation of ligament fibroblasts by targeting the Wnt/ β-catenin pathway, we cultured OPLL cells in BMP2-containing osteogenic medium and treated with recombinant Wnt3a (R&D Systems) or Dkk1. As shown in Fig. 5A, treatment with Wnt3a increased ALP activity, whereas this induction was attenuated by the addition of Dkk1. Wnt3a also increased the expression of osteoblast differentiation markers including ALP, collagen type I, and osteocalcin. However, this upregulation was reversed in the presence of Dkk1 (Fig. 5B). These results suggest that Dkk1 suppresses osteogenic differentiation via its negative regulation of the Wnt pathway.

Discussion

OPLL is an osteogenic disorder characterized by the ectopic bone formation of the PLL in the spine. Although the histopathology of this disease has been well studied, the knowledge of the signaling pathway that regulates the development and progression of OPLL is still at its infancy. Being a secreted antagonist of the Wnt/β-catenin pathway, Dkk1 inhibits osteoblast differentiation and bone formation during skeletal development and may function as an important serological molecular marker for osteoporosis and malignant bone diseases, such as bone metastasis and osteosarcoma [9–11]. However, it remains unknown whether Dkk1 impacts the development and progression of OPLL. In this study, we examine the serum level of Dkk1 and show that this Wnt inhibitor is downregulated in OPLL patients as compared to non-OPLL patients. The expression of Dkk1 is also reduced in OPLL ligament-derived fibroblasts. These findings imply that Dkk1 may act as a negative regulator in the pathogenesis of OPLL. Notably, our results are supported by a previous study, in which a decreased serum level of Dkk1 has been documented in patients suffering from spondyloarthropathies, including ankylosing spondylitis (AS), diffuse idiopathic skeletal hyperostosis (DISH), ossification of the yellow ligament (OLY), as well as OPLL [12].

The central role of the Wnt/β-catenin signaling in bone development and fracture repair has been extensively studied. However, the implication of this critical pathway in the pathogenesis of OPLL has not been reported. DISH (also called ankylosing hyperostosis) is closely associated with OPLL, in that this disease is most commonly characterized by ossification of the anterior longitudinal ligament (ALL) on the anterolateral surface of vertebrae, eventually leading to fusion of the spine [13]. As such, DISH and OPLL have some common histopathological features such as ossification of spinal longitudinal ligament [14]. Overexpression of β-catenin in endplate cartilage cells has been reported to induce extensive osteophyte formation and fusion of adjacent vertebra in the entire spine, which is similar to the defects observed in DISH disease [15]. Interestingly, activation of the Wnt pathway also plays an important role in the process of excessive bone formation in AS, an inflammatory spine disorder that causes abnormal bony outgrowth [16]. In our study, we find that the expression of β-catenin is increased in ligament fibroblasts of OPLL, indicating the accumulation of this protein. Importantly, we report a higher TCF reporter activity and gene expression of Wnt target genes including c-myc, cyclin D1, and Axin2 in OPLL

fibroblasts than in non-OPLL cells. These results support the notion that downregulation of Dkk1 in OPLL cells is associated with stabilized β-catenin, which activates TCF-dependent transcriptional activity.

Notably, our results seem different from Shi et al., who have reported that OPLL is mediated by osterix (a transcription factor for osteoblasts) via the inhibition of the β-catenin signaling, in that during dexamethasone (Dex)-induced osteogenic differentiation of PLL cells, osterix was increased whereas β-catenin was reduced in response to Dex treatment [17]. This discrepancy seems highly plausible due to the different experimental conditions used. In Shi's study, Dex was used to initiate the osteogenic differentiation of PLL cells [17]. Although Dex can induce osteoblast differentiation in a variety of cell types, this glucocorticoid has been shown to inhibit the Wnt pathway. For instance, Dex promotes adipogenic differentiation of mesenchymal progenitor cells by inducing Wnt antagonists (including Dkk1 and WIF1) and activating GSK-3β, thus leading to the inhibition of the Wnt/β-catenin pathway [18]. Dex also inhibits chondrogenic differentiation by repressing β-catenin and TCF/LEF-mediated transcription [19]. Therefore, it is not surprising that treatment with Dex in PLL cells could lead to the downregulation of β-catenin.

Besides the observations that Dkk1 is downregulated whereas β-catenin signaling is upregulated in OPLL ligament fibroblasts, we further provide evidence that Dkk1 functionally regulates OPLL at the cellular level. Dkk1 has been shown to inhibit proliferation in diverse cell types [20–22]. This Wnt inhibitor is also known to promote apoptosis [23, 24]. In agreement with these studies, we show here that treatment with recombinant Dkk1 also inhibits proliferation but promotes the apoptotic activity of OPLL fibroblasts. As such, Dkk1 possesses a growth-inhibitory effect in ligament cells.

As a Wnt inhibitor, Dkk1 has been critically implicated in the skeletal system. Heterozygous knockout of the *Dkk1* gene increases bone formation and bone mass [8]. In contrast, overexpression of Dkk1 in osteoblasts leads to severe osteopenia [7]. In our study, we provide evidence that Dkk1 inhibits BMP2-induced osteogenic differentiation of ligament cells, supported by the following observations that 1) treatment with Dkk1 reduces ALP activity; 2) Dkk1 inhibits gene expression of osteoblast markers in OPLL cells; 3) Dkk1 also represses osteoblast mineralization. Moreover, we show that Dkk1 attenuates Wnt3a-upregulated ALP activity and expression of osteoblast markers in OPLL cells, suggesting that Dkk1 inhibits osteogenic differentiation via its suppression of the Wnt pathway. All these findings support that Dkk1 functions as a negative regulator in the pathogenesis of OPLL, and downregulation of Dkk1 favors the ossification of the ligament by promoting cell growth and survival, as well as accelerating osteogenic differentiation. In addition, our results also suggest that targeting the Wnt/β-catenin pathway using Dkk1 or small molecule inhibitors may represent a potential treatment against the development and progression of OPLL.

In general, we report that Dkk1 is downregulated in OPLL patients and ligament-derived cells, which is associated with activation of the β-catenin-mediated signaling pathway. Functionally, we show that Dkk1 exerts a growth-inhibitory effect and inhibits osteogenic differentiation of ligament cells by targeting the Wnt pathway. Our results demonstrate for the first time that Dkk1 acts as a negative regulator in the

pathogenesis of OPLL. Targeting the Wnt pathway using Dkk1 may serve as a therapeutic strategy against OPLL.

Abbreviations

ALP, alkaline phosphatase; AS, ankylosing spondylitis; BMP2, bone morphogenetic protein 2; Dex, dexamethasone; DISH, diffuse idiopathic skeletal hyperostosis; Dkk1, dickkopf 1; Fz, Frizzled; GSK3β, glycogen synthase kinase 3β; LEF, lymphoid enhancer factor; LRP5/6, low-density lipoprotein receptor-related protein 5 or 6; OPLL, ossification of the posterior longitudinal ligament; PLL, posterior longitudinal ligament; qPCR, quantitative real-time RT-PCR; SFRP1, secreted frizzled-related protein-1; SOST, sclerostin, TCF, T cell factor

Declarations

Acknowledgements

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Availability of data and materials

Not applicable

Author contributions

J.D. and B.T. designed the study and wrote the manuscript. J.D., X.X., Q.Z., N.W., and Z.N. collected data and performed experiments. J.D., X.X., Q.Z., N.W., and B.T. analyzed the data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not Applicable.

Patient consent for publication

This study was approved by the Human Ethics Committee of the Shandong Provincial Hospital and written informed consent was obtained from each patient.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

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Table

Due to technical limitations, table 1 is only available as a download in the supplemental files section.

Figures

Figure 1

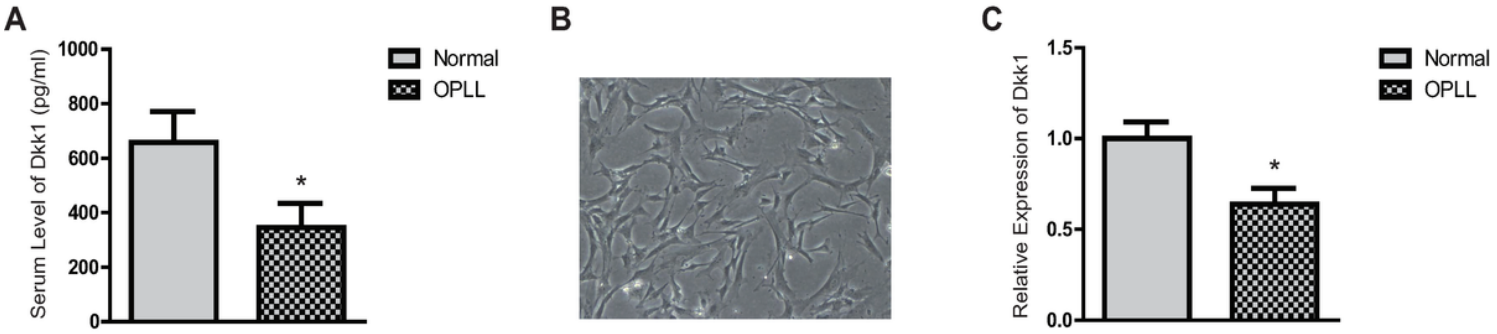

Figure 1

Dkk1 is downregulated in OPLL. (A) ELISA for the serum level of Dkk1 in OPLL and non-OPLL patients (N=8). (B) Ligament-derived fibroblast-like cells. (C) qPCR analysis for Dkk1 mRNA in ligament fibroblasts harvested from OPLL and non-OPLL patients (N=3). *P<0.05.

Figure 2

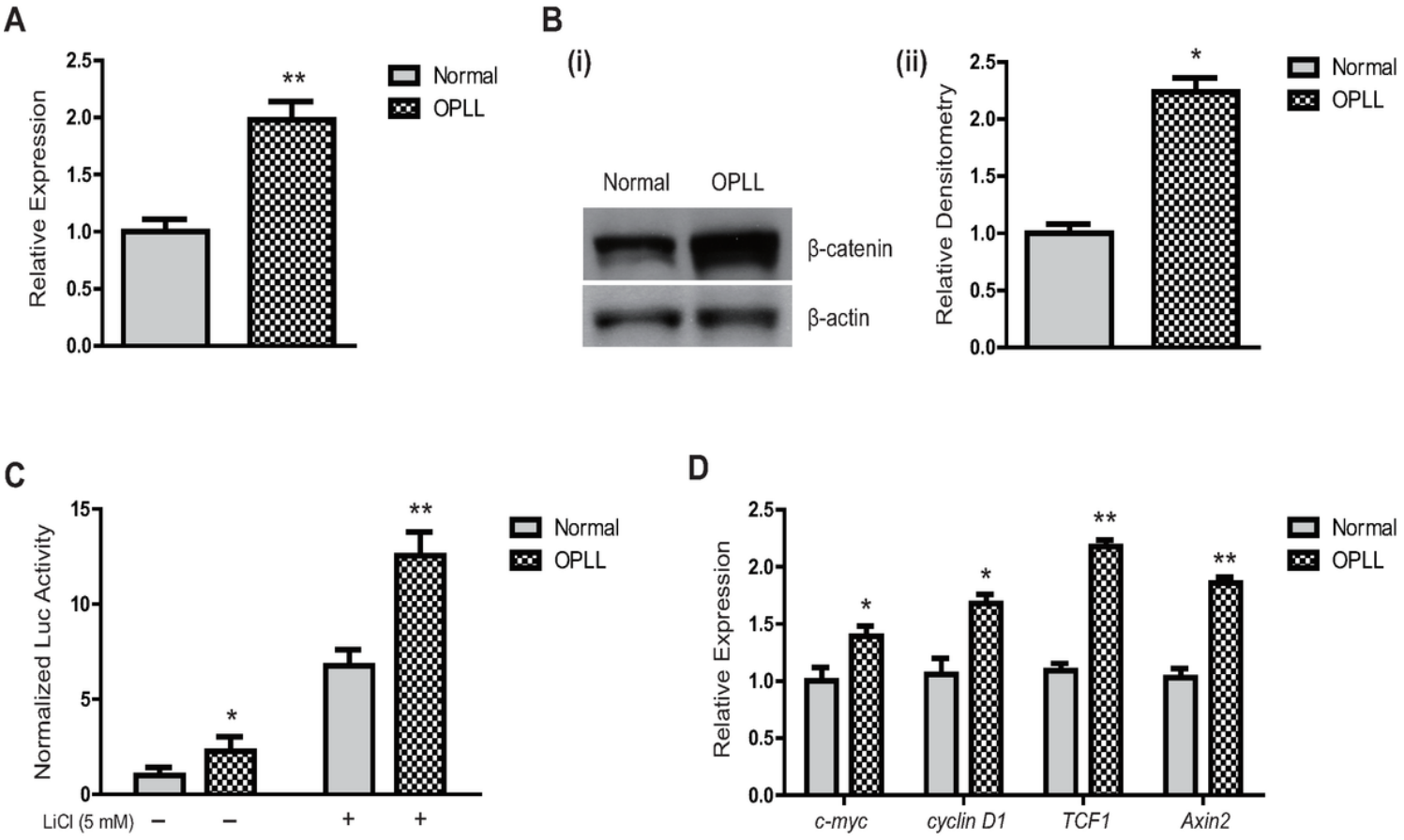

Figure 2

β-catenin signaling activity is increased in OPLL fibroblasts. (A) qPCR analysis for Ctnnb1 mRNA in ligament fibroblasts harvested from OPLL and non-OPLL patients. (B) Western blot for β-catenin in ligament cells. Densitometry for the protein band of β-catenin normalized to that of β-actin was analyzed using ImageJ. (C) TCF reporter assay in ligament fibroblasts, treated with LiCl (5 mM). (D) qPCR analysis for Wnt target genes in ligament fibroblasts. N=3. *P<0.05, ** P<0.01.

Figure 3

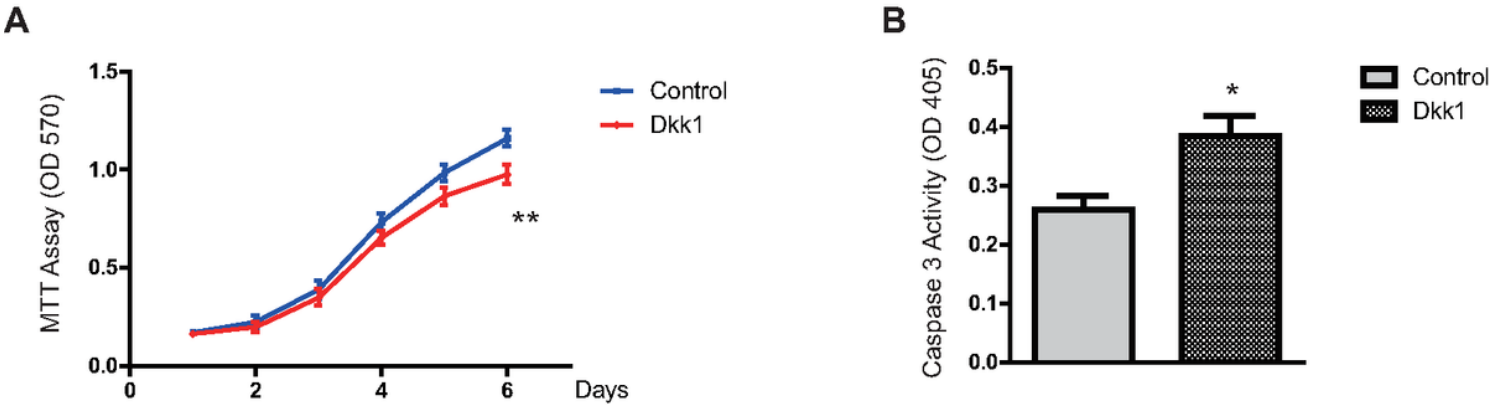

Figure 3

Dkk1 exerts a growth-inhibitory effect in OPLL fibroblasts. (A) MTT assay in OPLL fibroblasts treated with recombinant human Dkk1 (500 ng/ml) (N=5). (B) Caspase 3 activity assay in OPLL fibroblasts treated with Dkk1 (500 ng/ml) for 24 hours (N=3). *P<0.05, **P<0.01.

Figure 4

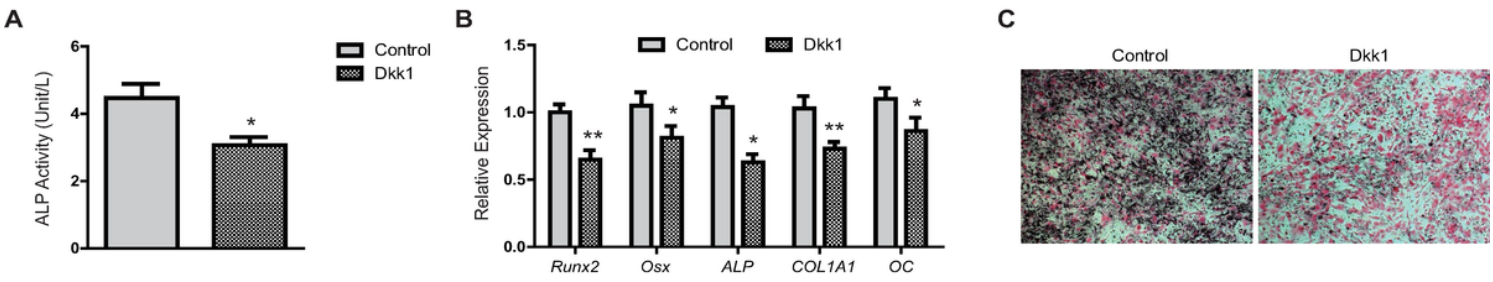

Figure 4

Dkk1 inhibits osteogenic differentiation of OPLL fibroblasts. OPLL fibroblasts were cultured in BMP2 (200 ng/ml)-containing osteogenic medium, treated with recombinant Dkk1 (500 ng/ml). (A) ALP activity assay at day 3. (B) qPCR analysis for Runx2, osterix (Osx), ALP, COL1A1, and osteocalcin (OC) at day 3. (C) von Kossa staining after 3 weeks. N=3. *P<0.05, ** P<0.01.

Figure 5

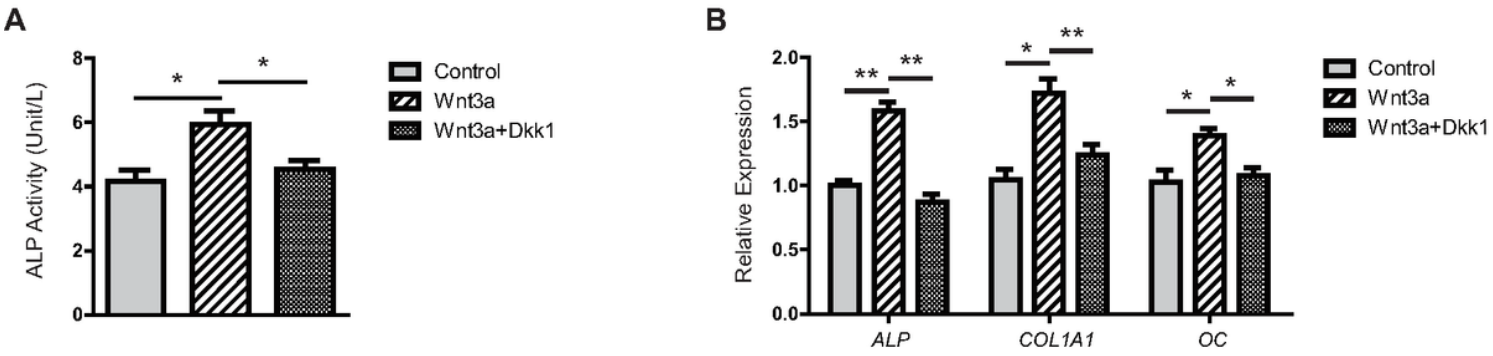

Figure 5

Dkk1 inhibits osteogenic differentiation by targeting the Wnt pathway. OPLL cells were cultured in BMP2 (200 ng/ml)-containing osteogenic medium, treated with recombinant Wnt3a (200 ng/ml) or Dkk1 (500 ng/ml) for 3 days. (A) ALP activity assay. (B) qPCR analysis for ALP, COL1A1, and osteocalcin. N=3. *P<0.05, ** P<0.01.

Supplementary Files

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- Table1PCRPrimers.xlsx