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Original Article

Targeting DKK1 prevents development of alcohol-induced osteonecrosis of the femoral head in rats

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Abstract: Osteonecrosis of the femoral head (ONFH) is a devastating bone disease characterized by avascular or aseptic necrosis of the femoral head, and alcohol consumption is reported one of the leading risks to this disease. Previous studies have linked Dickkopf-1 (DKK1) to the occurrence of ONFH, but the role of DKK1 in alcohol-induced ONFH (AONFH) has not been fully discussed. In this study, we found that the expression level of DKK1 was dramatically increased in serum and bone samples from AONFH patients, experimental AONFH rats, and cultured bone marrow mesenchymal stem cells (BMMSCs) with ethanol stimulation. Elevated DKK1 inhibited Wnt/β-catenin signaling *in vivo* and *in vitro*, while knockdown of DKK1 enhanced the nuclear translocation of β-catenin and promoted osteogenesis and inhibited adipogenesis in the BMMSC cell line C3H10T1/2. Local injection of DKK1 knockout lentivirus into the femoral head of rats alleviated the progression of AONFH, with activated Wnt/β-catenin signaling, increased bone formation, reduced number of empty adipose lacunae and restored blood supply. In conclusion, our findings confirmed the important role of DKK1 and canonical Wnt/β-catenin pathway in AONFH. We propose that DKK1 may be a prognostic marker of AONFH and targeting DKK1 to activate the canonical Wnt/β-catenin pathway and restore osteogenic potential could be a promising therapeutic strategy to prevent AONFH.

Keywords: Alcohol-induced ONFH, Dickkopf-1, BMMSCs, Wnt/β-catenin signaling, rats

Introduction

Osteonecrosis of the femoral head (ONFH) is a common cause of pain and disability that often affects young adults aged 30 to 50 [1]. The main features of ONFH are microarchitectural deterioration and bone tissue ischemia, usually advancing to femoral head collapse, and eventually resulting in total hip arthroplasty [2, 3]. Long-time steroid medicine intake, abused alcohol consumption, genetic mutation and thrombotic/fibrinolytic disorders are regarded as possible risk factors of ONFH [4, 5]. However, an epidemiological study in the Guangxi province of China showed that the incidence of alcohol-induced osteonecrosis of the femoral head (AONFH) is significantly higher than any other causes [6]. This is due to the chronic consumption of home-brewed low-alcohol wine among residents of Guangxi province. The current study aimed to investigate a potential strategy to halt the progression of AONFH.

Previous studies have revealed that impaired bone synthesis and reduced bone mineral density, and moreover, excessive fat accumulation and empty lacunae are often observed in damaged bone marrow cavity in bone samples from AONFH patients [7-10]. Bone marrow mesenchymal stem cells (BMMSCs) are characterized by their multipotent differentiation potential which enables them to differentiate into cell lineages including osteoblasts, adipocytes, endotheliocytes, and chondrocytes [11, 12]. Studies have shown that ethanol impairs osteogenic differentiation and promotes adipogenesis of BMMSCs, and restoration of osteogenic potential alleviates the progression of AONFH in animal experiments [13, 14]. Thus, there is a strong possibility that AONFH is a stem cell disease resulting from abnormal differentiation between osteogenesis and adipogenesis of BMMSCs. Determining how to promote osteogenesis and inhibit adipogenesis of BMMSCs is

therefore a crucial direction of treatment for ONFH.

The canonical Wnt/β-catenin signaling pathway has been demonstrated to play a vital role in the regulation of BMMSCs differentiation towards osteoblasts [15-18]. Stabilized β-catenin accumulates and translocates into the nucleus, and then binds to transcriptional activators and regulates the expression of some key osteogenic factors, including Runx2 and osterix [19]. Dickkopf-1 (DKK1), acts as a natural inhibitor of Wnt/β-catenin pathway, directly binds to the LRP5/6 receptor and Kremen-1/2, leading to internalization of the receptor complex and dampening of Wnt/β-catenin signaling [20]. Previous studies have demonstrated that glucocorticoids upregulate antagonists to Wnt/β-catenin signaling in steroid-induced ONFH [21, 22], and knockdown of DKK1 inhibited glucocorticoid-induced bone loss and reduced apoptosis of osteoblasts *in vivo* [23, 24]. However, the character of DKK1 and Wnt/β-catenin signaling in AONFH has not yet been fully clarified.

In this study, we noticed an accumulated expression of DKK1 in serum and bone samples from AONFH patients. We successfully established an AONFH model in rats with ethanol-containing liquid diet [25, 26]. Under alcohol stimulation, expression of DKK1 was confirmed to be upregulated, and elevated DKK1 inhibited the Wnt/β-catenin signaling *in vivo* and *in vitro.* Knock down of DKK1 promoted osteogenesis and inhibited adipogenesis in cultured BMMSCs. Moreover, injection of DKK1 knockout lentivirus into the femoral head of rats effectively alleviated the pathological change of AONFH. Thus, we demonstrated that ethanol-triggered accumulation of DKK1 expression leaded to the suppression of osteogenesis and enhancement of adipogenesis via inhibiting the canonical Wnt/β-catenin pathway, and then contributed to the progression of AONFH. Targeting DKK1 could be a potential therapeutic approach to prevent AONFH.

Materials and methods

Human samples

Thirty-five patients with AONFH were included and osteonecrosis was confirmed in all cases using radiography and magnetic resonance images, all had a clear history of drinking alcohol (consumption of 400 mL per week or total alcohol consumption was more than 150 L). Patients with autoimmune, endocrine or metabolic diseases, or tumors that could affect bone metabolism were excluded. Thirty patients with sub cephalic femoral neck fracture who underwent hip arthroplasty were recruited as controls. The serum samples from all 65 patients were collected and bone samples were collected during surgery for total hip arthroplasty at the Affiliated Hospital of Youjiang Medical University for Nationalities (Baise, Guangxi, China). Patient consents were obtained and this study were approved by the Ethics Committee of the Affiliated Hospital of Youjiang Medical University for Nationalities.

Animals and treatments

All procedures were approved by the Animal Research Committee of Youjiang Medical University for Nationalities. Thirty Sprague-Dawley male rats aged 4-6 weeks were obtained from Hunan Changsha Tianqin Biotechnology Co., Ltd (Changsha, Hunan, China), and rats were divided into control (CON), model (AONFH) and AONFH with DKK-1 knockout group (n = 5 for each group). After a week of routine feeding in the Animal Center of Youjiang Medical University for Nationalities, they were switched to a nutritionally complete liquid diet containing 5.0% (weight/volume) ethanol (35% ethanol-derived calories) [25]. The duration of the study was 4 months, and rats in control group were fed with water for the same period. At 16th week of the experiment, animals were killed and the femoral head samples were harvested.

Micro-CT scanning

Micro-CT scanning of the rats' femoral heads from all groups were performed using a micro-CT scanner (Scanco Medical, Bassersdorf, Switzerland) at a voltage of 75 kV and a resolution of 12 μm per pixel. The BMD and BV/TV were calculated with the analysis system of the micro-CT. A total of 100 sections of the primary trabecular bone of the lower femoral metaphysis as areas of interest were quantified.

Histological, immunostaining and TUNEL analysis

Resected bone samples from each group were fixed in 4% paraformaldehyde and decalcifica-

tion with 10% EDTA for 2 months. After decalcification, bone samples were cut into 5-μm-thick sections and stained with hematoxylin & eosin (H&E) following a standard protocol. For immunostaining analysis, the slices were incubated in 10 mM citric acid buffer overnight at 60°C to unmask antigens. Then, we incubated the slices in diluted primary antibodies at 4°C overnight and appropriate secondary antibody for 1 h at RT. For IHC analysis, slices were visualized with diaminobenzidine solution (Zsbio, Beijing, China). For IF, nuclei were counterstained in 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies) and images were obtained on a confocal laser-scanning microscope (Olympus, Tokyo, Japan). For TdT-mediated dUTP nick end labeling (TUNEL), slices were deparaffinized and antigens were unmasked. The DeadEnd Fluorometric TUNEL System (Promega) procedure was performed following the manufacturer's instructions.

Cell culture

BMMSCs were isolated from rats aged 12 weeks. Both ends of femurs and tibias were cut off and the shafts were flushed with BMMSC culture medium, supplemented with 10% FBS (Gibco Biocult, Eggenheim, Germany), αMEM (Gibco, Grand Island, NY, USA), and penicillin/streptomycin (100 U/mL/100 μg/mL, respectively). C3H10T1/2 cells were also cultured with αMEM contained 10% FBS. Cells were cultured at 37°C in a humidified 5% CO_2 incubator. For osteogenic induction, 0.1 mM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 10 mM β-glycerol phosphate (Sigma-Aldrich) and 100 μg/mL ascorbic acid (Sigma-Aldrich) were added to cultured cells. For adipogenic induction, 1 μmol/L dexamethasone, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 100 μmol/L indomethacin and 5 μmol/L insulin (all from Sigma-Aldrich) were added to cultured cells. Alizarin red and Oil red O staining were performed following standard techniques.

siRNA knockdown

C3H10T1/2 cells were transfected with DKK1 small interfering RNA (siDKK1) with Lipofectamine iMAX (Invitrogen, Carlsbad, CA, USA) in Opti-MEM medium (Invitrogen), according to the manufacturer's instructions. The sequence of DKK1 siRNAs were as below: DKK1-1, forward, 5'-GCU CUC AUG GAC UAG AAA UDT DT-3'; reverse, 5'-AUU UCU AGU CCA UGA GAG CDT DT-3'. DKK1-2, forward, 5'-GGU UCU CAA AUU CCA ACG CUD TDT-3'; reverse, 5'-AGC GUU GGA AUU GAG AAC CDT DT-3'. Negative control siRNA was used as the control and western blot analysis was used to detected the efficiency of siRNA knockdown.

Western blotting

Cells and tissues lysed in 2X SDS buffer on ice and boiled for 10 min. Total proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membrane was blocked with 5% non-fat milk and then incubated with primary antibodies at 4°C overnight. Next, membranes were treated with HRP-coupled secondary antibody for 1 h and the signals were detected with an enhanced chemiluminescence reagent Kit (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

All experiments were repeated at least three times. Data are presented as the mean ± standard error (SD), using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). Student's t-tests assessed statistical significance of differences between groups. $P < 0.05$ was considered statistically significant.

Results

DKK-1 expression increased in serum and bone samples of patients with AONFH

To analyze the changing level of DKK-1 in AONFH, Dkk-1 concentrations were first detected by ELISA in 35 patients with AONFH (female 0, male 35, with age 51.19 ± 8.80), and 30 healthy male controls (aged 51.19 ± 1.42). As shown in **Figure 1A**, average levels of DKK-1 expression in the serum of AONFH patients were significantly higher than in normal controls. Next, the femoral head samples from AONFH and normal patients were embedded and cut into slices, and the expression of DKK-1 in the slices was measured by immunohistochemical analysis. The DKK-1-positive cells in the AONFH group were clearly more numerous than in the normal controls (**Figure 1B**). These data suggest that DKK-1 expression was elevated in serum and bone samples from patients with AONFH.

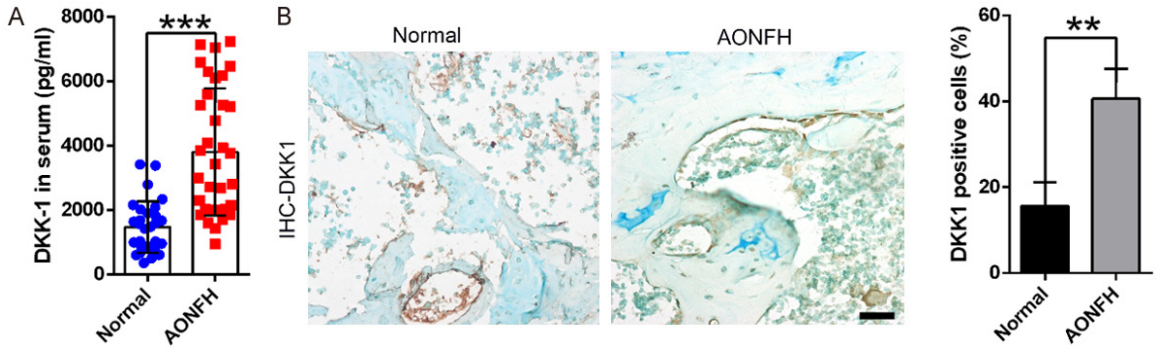

Figure 1. DKK-1 expression is increased in serum and bone samples of patients with AONFH. A. ELISA results of DKK-1 expression in serum of patients with AONFH (n = 35) and normal controls (n = 30). B. Representative photographs and quantification of DKK1 expression in bone specimens from patients with AONFH and normal controls; n = 5 for each group, scale bar = 50 μm, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's t test. All data are shown as means ± SD.

DKK-1 accumulates in osteoblasts of bone samples from the AONFH rat model

Next, we established an AONFH rat model by feeding an ethanol-containing liquid diet. After four months on this ethanol-containing liquid diet, rats were sacrificed and bone tissues of femoral heads from the AONFH and control groups were analyzed. Eight of the 10 rats in the AONFH group displayed obvious signs of AONFH, while none were observed in control group. Micro-CT scanning confirmed the presence of significantly fewer subchondral trabeculae (**Figure 2A**), and markedly decreased BMD and BV/TV in AONFH rats compared to the control group (**Figure 2B**). After scanning, the femoral heads from both groups were embedded and sectioned. H&E staining showed significantly increased numbers of bone marrow cell necrosis and empty lacunae, which were adipocytes in the subchondral trabecular area of AONFH rats compared to control rats (**Figure 2C**). And with TUNEL staining, we noticed a dramatically increment of apoptosis cells in the femur heads of AONFH rats compared to control rats (**Figure 2D**). Thus, we successfully constructed a rat model of AONFH. We next measured the expression of DKK-1 in the femur heads of AONFH rats. Immunohistochemical staining confirmed the up-regulation of DKK-1 in the AONFH rats versus control rats (**Figure 2E**). Double staining of osteocalcin (OCN) and DKK-1 by IF revealed that DKK-1 expression was mostly in the osteoblasts (**Figure 2F**). These data indicate that DKK-1 accumulates in osteoblasts of the femoral heads of AONFH rats.

Ethanol treatment increases DKK-1 expression and promotes adipogenesis in BMMSCs

To assess the impact of ethanol on DKK-1 expression, we cultured the rat BMMSCs and stimulated them with different concentrations of ethanol. As the ethanol concentration increased, the cultured BMMSCs showed enhanced adipocytic differentiation, demonstrated by increased areas of oil red O-positive staining (**Figure 3A**). Moreover, immunoblotting analysis revealed that the expression of DKK-1 and the adipogenic markers PPARγ and CEBPα gradually accumulated, while the expression of β-catenin dropped progressively as the ethanol stimulation period increased (**Figure 3B**). Based on these results, we conclude that ethanol treatment increases DKK-1 expression, leading to downregulation of Wnt/β-catenin signaling and promotion of adipogenesis in bone marrow mesenchymal stem cells.

Ethanol treatment increases DKK-1 expression and inhibits Wnt/β-catenin signaling in vitro and in vivo

DKK-1 is known as an inhibitor of β-catenin-dependent Wnt signaling. We next investigated changes in DKK-1 and Wnt/β-catenin signaling with alcohol treatment *in vitro* and *in vivo*. In the cultured BMMSCs, double staining of DKK-1 and β-catenin by IF confirmed the accumulation of DKK-1, while both the expression level and nuclear transcription of β-catenin were diminished under alcohol stimulation (**Figure 4A**). Similarly, in the femoral heads of AONFH rats, expression of DKK-1 increased and

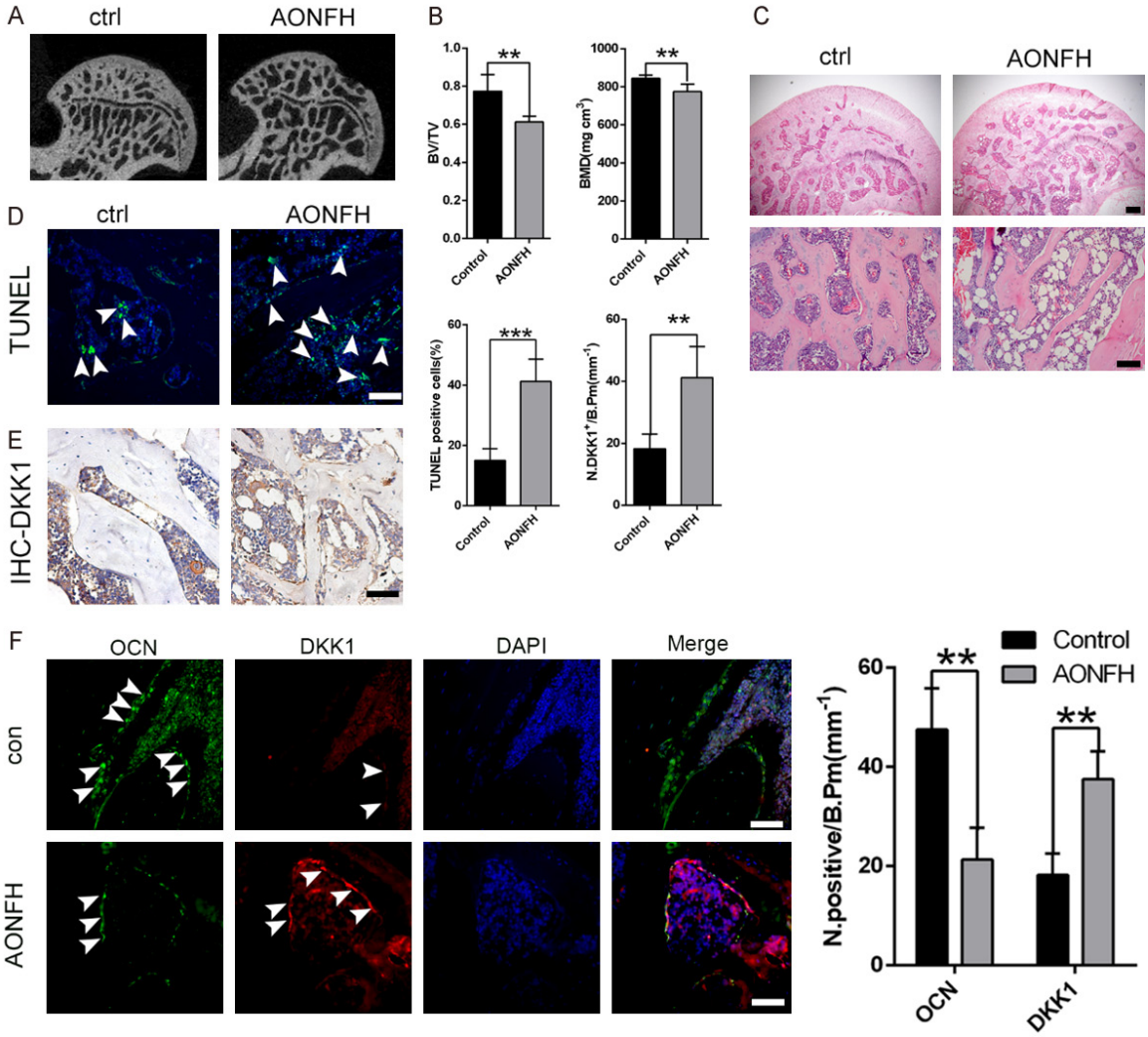

Figure 2. DKK-1 accumulates in osteoblasts of bone samples from an AONFH rat model. A. Micro-CT scanning images of femoral heads from AONFH and control rats; n = 5 for each group. B. Quantification of the value of BV/TV and BMD in both groups; n = 5 for each group. C. H&E staining of the femoral head in AONFH and control rats; White arrows indicate necrotic bone marrow cells and black arrows indicate empty lacunas. n = 5 for each group, scale bar = 50 μm. D. Images and quantification of TUNEL staining in the femoral heads from both groups. n = 5 for each group. E. IHC staining of DKK-1 and quantification of DKK-1-positive cells in the femoral heads from both groups. n = 5 for each group. F. Co-staining of OCN (green) and DKK-1 (red) by IF and quantification of positive cells in the femoral head from both groups; n = 5 for each group, scale bar = 50 μm. $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's t test. All data are shown as means ± SD.

β-catenin deceased compared to control rats (**Figure 4B**). In summary, our data suggest that ethanol treatment increases DKK-1 expression and inhibits Wnt/β-catenin signaling *in vitro* and *in vivo*.

Knockdown of DKK-1 promotes osteogenesis and inhibits adipogenesis in BMMSCs via activation of Wnt/β-catenin signaling

Further, we analyzed the influence of knocking down DKK-1 on the differentiation of BMMSCs. The BMMSC line C3H10T1/2 cell was transfected with two different DKK-1 siRNA and was cultured in osteogenic induction medium. Immunoblotting analysis ascertained the knockdown efficiency of DKK-1 protein, while expression of β-catenin and the osteogenic markers OCN, RUNX2 and Osterix were dramatically upregulated (**Figure 5A**). Enhanced mineralization of the extracellular matrix emerged in the DKK-1 knockdown cells (**Figure 5B**). C3H10T1/2 cells were also stimulated with

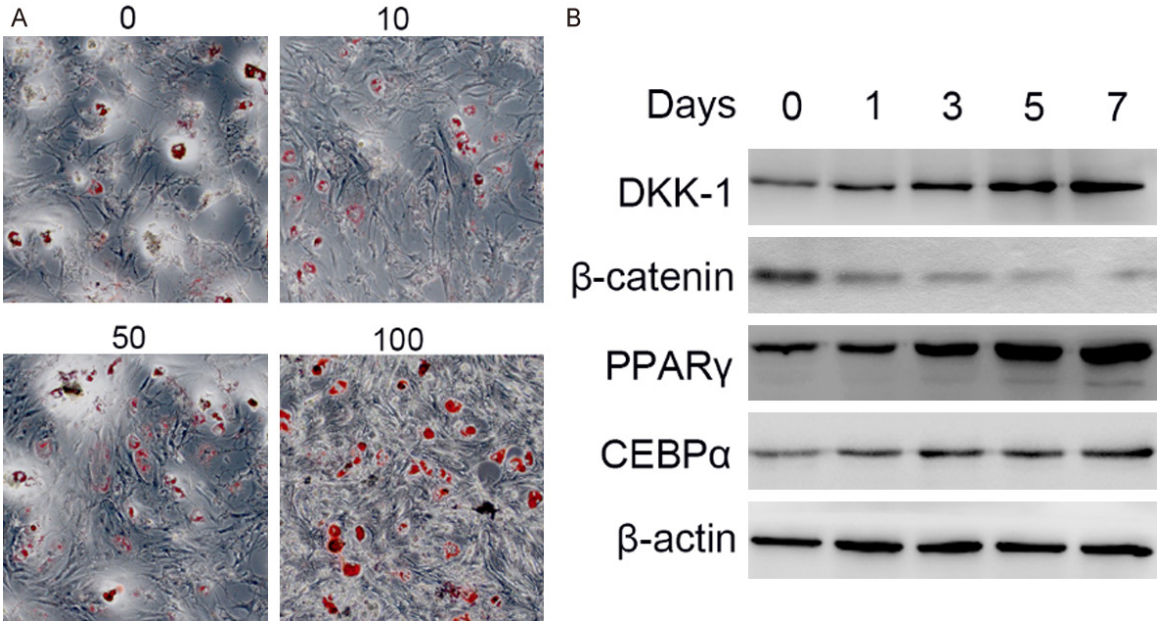

Figure 3. Ethanol treatment elevates DKK-1 expression and promotes adipogenesis in bone marrow mesenchymal stem cells. A. Images of Oil red O staining of cultured BMMSCs stimulated with different concentrations of ethanol (0, 10, 50, 100 mmol/L) for 3 days. B. Western blot results of DKK-1, β-catenin, PPARγ and CEBPα in BMMSCs stimulated with 50 mmol/L ethanol for different time periods (0, 1, 3, 5, and 7 days). β-actin was used as an internal control. The experiment was repeated three different times.

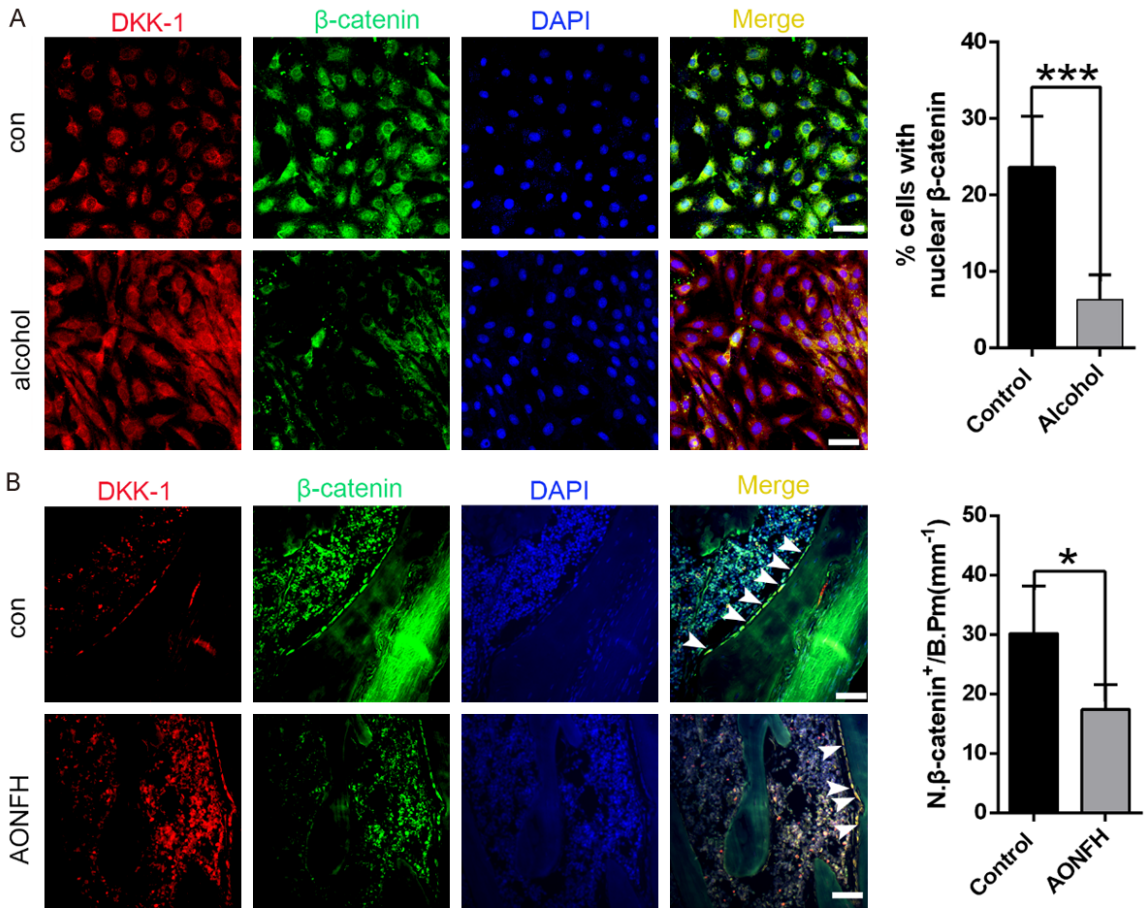

Figure 4. Ethanol treatment increases DKK-1 expression and inhibits Wnt/β-catenin signaling *in vitro* and *in vivo*. A. Co-staining of DKK-1 (red) and β-catenin (green) by IF in BMMSCs stimulated with 50 mmol/L ethanol or control (saline). Expression of β-catenin in the nuclei was quantified. The experiment was repeated three different times. Scale bar = 20 μm. B. Co-staining of DKK-1 (red) and β-catenin (green) by IF in the femoral head from AONFH and control rats; n = 5 for each group, scale bar = 50 μm. **P* <0.05, ****P* < 0.001 by Student's t test. All data are shown as means ± SD.

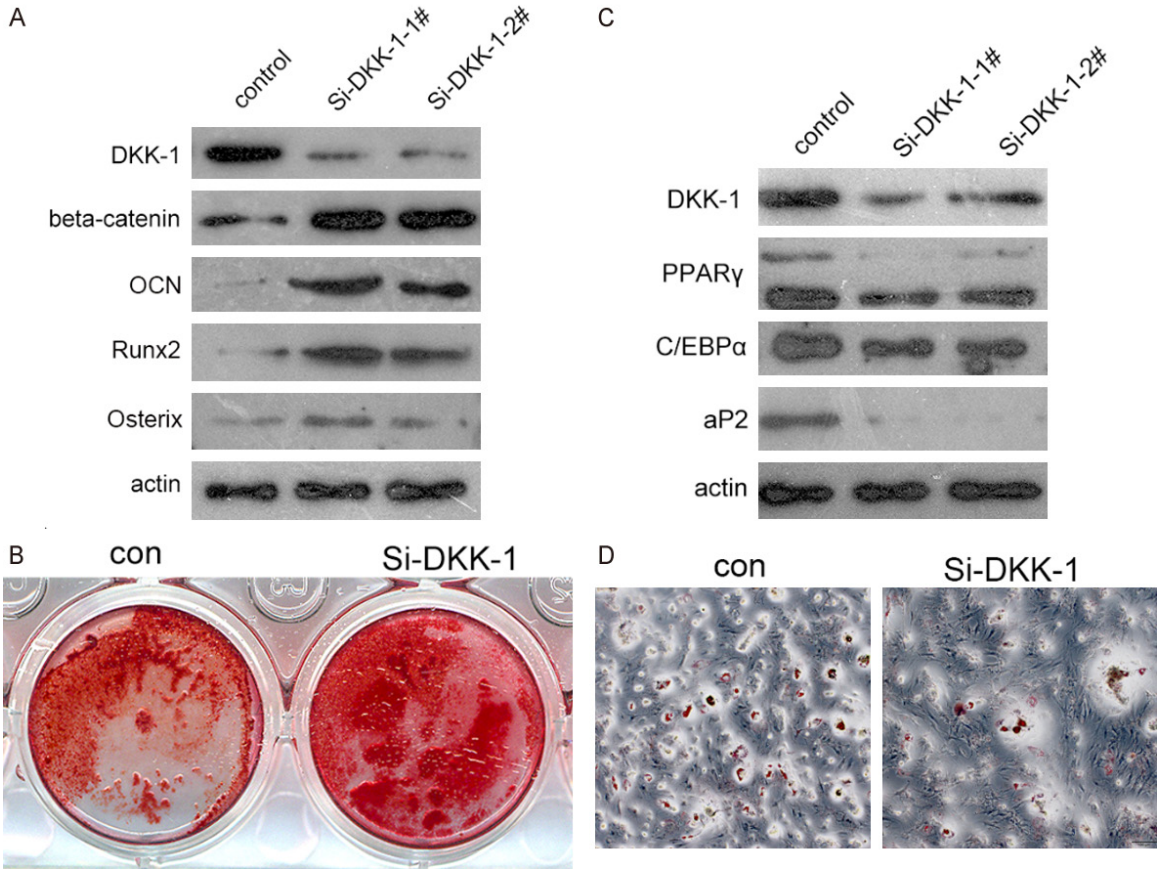

Figure 5. Knockdown of DKK-1 promotes osteogenesis and inhibits adipogenesis in bone marrow mesenchymal stem cells via activation of Wnt/β-catenin signaling. A. Western blot results of DKK-1, β-catenin, OCN, Runx2 and Osterix in C3H10T1/2 cells transfected with negative control or DKK-1 siRNAs, and cultured in osteogenic induction medium for 3 days. B. Alizarin red staining of C3H10T1/2 cells transfected with negative control or DKK-1 siRNA, and cultured in osteogenic induction medium for 14 days. C. Western blot results of DKK-1, PPARγ, CEBPα and aP2 in C3H10T1/2 cells transfected with negative control or DKK-1 siRNAs, and cultured in adipogenic induction medium for 3 days. D. Oil red O staining of C3H10T1/2 cells transfected with negative control or DKK-1 siRNAs, and cultured in adipogenic induction medium for 14 days. All experiments were repeated three different times.

adipogenic induction medium after transfection with DKK-1 SiRNAs. Expression of adipogenic markers, including PPARγ, C/EBPα and aP2, were notably reduced (**Figure 5C**), while Oil red O staining showed smaller areas of red staining, indicating diminished lipid accumulation in the DKK-1 knockdown cells (**Figure 5D**). Collectively, these data demonstrate that knocking down DKK-1 promotes osteogenesis and inhibits adipogenesis in BMMSCs by activating Wnt/β-catenin signaling.

Knockout of DKK-1 with lentivirus injection alleviates AONFH in rats

To investigate whether DKK-1 inhibition could alleviate AONFH *in vivo*, we injected DKK-1 knockout lentivirus into the femoral heads of rats once every one month from the start of the liquid diet, and AONFH rats in the control group were injected with saline. Micro-CT analyses showed a considerable increase in subchondral trabeculae, BMD, and BV/TV in the DKK-1

knockout AONFH rats compared to the control group (**Figure 6A**, **6B**). With H&E staining, we observed an increase in subchondral trabeculae and decreased numbers of empty lacunae in the DKK-1 knockout AONFH rats (**Figure 6C**). TUNEL staining showed decreased apoptosis cells in the femoral heads DKK-1 knockout AONFH rats (**Figure 6D**). IF analysis confirmed the inhibition of DKK-1 and enhancement of β-catenin expression (**Figure 6E**), and notably, expression of the osteogenic marker OCN and angiogenic marker CD31 were elevated in the femoral heads of DKK-1 knockout AONFH rats (**Figure 6F**).

In conclusion, these data indicate that knockout of DKK-1 by lentivirus injection activates Wnt/β-catenin signaling, promotes osteogenesis and angiogenesis in the femoral heads, and thus alleviates AONFH in rats.

Discussion

DKK1 is a natural inhibitor of Wnt/β-catenin signaling, which has been reported involved in the development of skeletal tissue, as well as in rheumatoid arthritis joint disorders, osteoporosis and glucocorticoid-induced ONFH [20, 27]. In our study, we identified a significantly increased expression of DKK1 in serum and bone samples from AONFH patients in Guangxi Province of China. Meanwhile in an alcohol-induced rat AONFH model, we confirmed that knockdown of DKK-1 in the femoral head prevented the development of AONFH. *In vitro*, alcohol stimulation elevated DKK1 expression and resulted in increased adipogenesis in BMMSCs. Further, knockdown of DKK1 promoted osteogenesis and inhibited adipogenesis in cultured BMMSCs, and the underlying mechanism may be through promote the nuclear translocation of β-catenin and activate the canonical Wnt/β-catenin pathway. In conclusion, we claim that DKK1 is a biomarker of AONFH, and targeting DKK1 could be a potential therapeutic strategy for the treatment of AONFH.

The main characteristics of ONFH are bone ischemia and destruction of the microstructure, accompanied by bone loss and increased adipose vacuoles in the femoral head. The exact mechanism of ONFH is still nebulous, but abnormal osteogenic and adipogenic differentiation of BMMSCs relates to its pathological change. It is well known that BMMSCs are able to differentiate into several cell types, including osteoblasts and adipocytes. In our study, after treating rat BMMSCs with ethanol, adipogenic differentiation was dramatically enhanced along with increased adipogenic markers. Moreover, in the femoral head of AONFH rats, the number of adipose vacuoles was apparently greater, and micro CT scanning revealed that bone formation was significantly retarded. In the development of therapies for ONFH, BMMSCs are a promising candidate cell source in tissue engineering and regenerative medicine [28]. Thus, we speculate that the occurrence of AONFH may be largely due to damage to the differentiation balance of BMMSCs towards osteoblasts and adipocytes caused by sustained alcohol stimulation. Restoration of osteogenic differentiation ability could be a potential therapeutic strategy to alleviate AONFH.

Numerous studies have studied the role of canonical Wnt/β-catenin signaling in regulating the osteogenic differentiation of BMMSCs [29-31]. Activation of canonical Wnt/β-catenin signaling, in which β-catenin accumulates and translocates to the nucleus, results in enhanced osteogenic differentiation and bone formation [15, 32]. Previous studies have shown that ethanol inhibits osteogenic differentiation and upregulates adipogenic differentiation of BMMSCs partly through suppressing Wnt/β-catenin signaling [33]. In the present study, we demonstrated that the Wnt/β-catenin pathway was inhibited by ethanol treatment in cultured BMMSCs and in AONFH rats. Further, we observed that the expression of DKK1, an inhibitor of the Wnt/β-catenin pathway, was elevated in serum and femoral head samples from AONFH patients, as well as in cultured BMMSCs with ethanol stimulation and in the femoral heads of AONFH rats. The consistent expression of DKK1 in all these tissues indicates its important role in AONFH, and the elevated DKK1 expression in AONFH tissue reveals a new spectrum of molecular pathology that is associated with the incidence of AONFH. We inferred that DKK1 may be a prognostic marker of AONFH.

Previous studies have reported that DKK1 upregulation accelerates the deterioration of bone microstructure and is associated with the occurrence of ONFH, but little research has been carried out to analyze the effect of DKK1 deletion in ONFH, especially in AONFH. In our

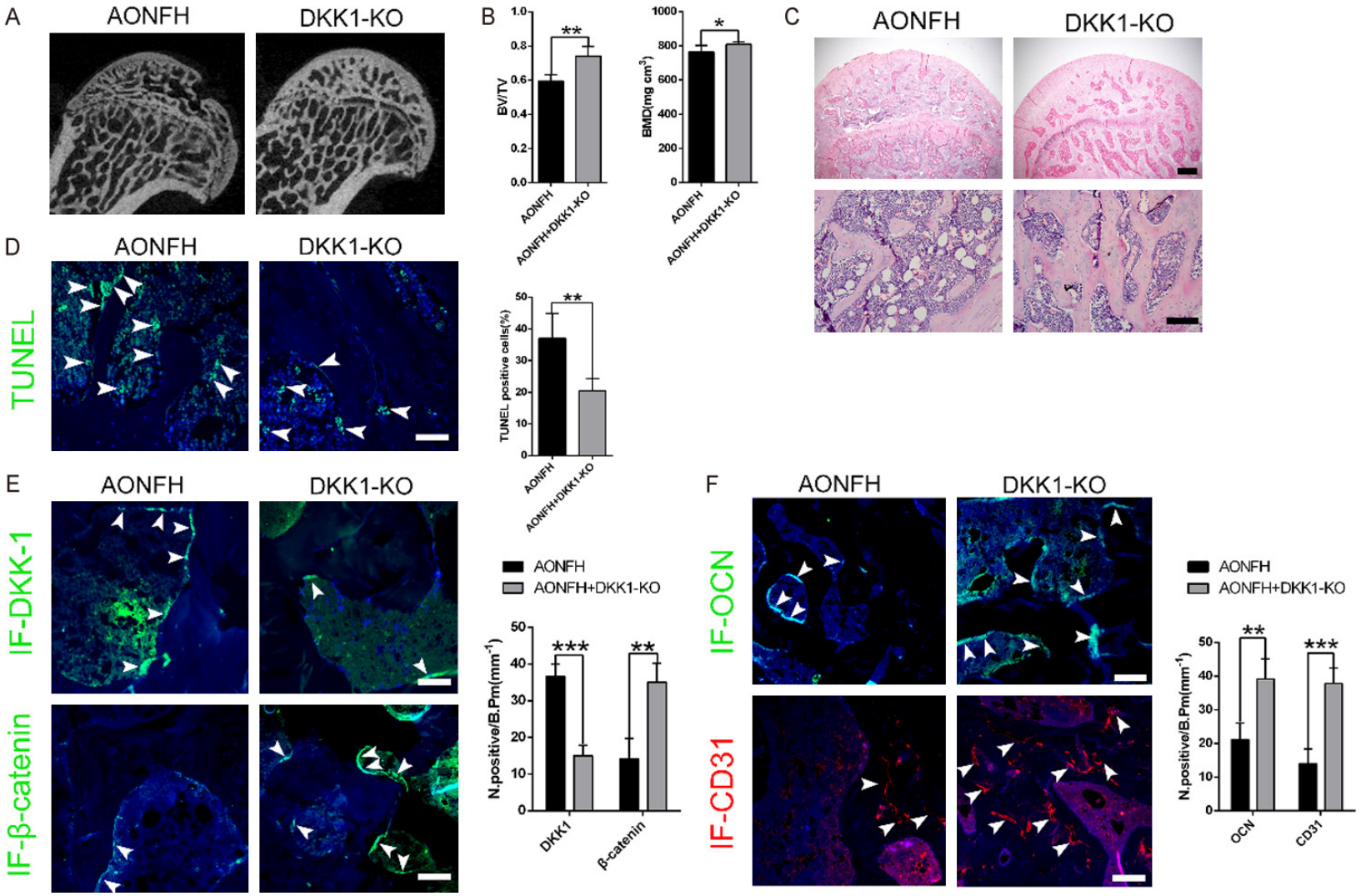

Figure 6. Knockout of DKK-1 with lentivirus injection alleviates AONFH in rats. A. Micro-CT scans of the femoral heads from AONFH and AONFH with DKK-1-KO rats; n = 5 for each group. B. Quantification of the value of bone volume/tissue volume (BV/TV) and bone mineral density (BMD) in both groups; n = 5 for each group. C. H&E staining of the femoral head in AONFH and AONFH with DKK-1-KO rats; n = 5 for each group, scale bar = 50 μm. D. Images and quantification of TUNEL staining in the femoral heads from both groups. n = 5 for each group. E. Representative IF staining photographs and quantification of DKK-1 and β-catenin in femoral heads from AONFH and AONFH with DKK-1-KO rats; n = 5 for each group. F. Representative IF staining photographs and quantification of OCN and CD31 in femoral heads from AONFH and AONFH with DKK-1-KO rats; n = 5 for each group, scale bar = 50 μm. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by Student's t test. All data are shown as means ± SD.

study, we used SiRNA to knock down DKK1 expression in BMMSCs, resulting in activation of Wnt/β-catenin signaling via enhancing nuclear translocation of β-catenin, decreased adipogenesis and, unsurprisingly, enhanced osteogenesis. *In vivo*, knocking down of DKK1 in the local site of femoral heads in AONFH rats could activate the Wnt/β-catenin pathway, resulting in enhanced bone formation and even restoring the blood supply of the femoral heads. Our findings indicate that reducing the expression of DKK1 restored osteogenic differentiation both *in vitro* and *in vivo*, and targeting DKK1 to activate Wnt/β-catenin signaling is a practicable way of alleviating the progression of AONFH. We speculate that developing drugs targeting deletion of DKK1 or activating the Wnt/β-catenin pathway, given by local injection or combined with BMMSCs for implanting, are promising strategies to treat AONFH.

In summary, this is the first report showing that the expression of DKK1 is increased in serum and bone samples from AONFH patients, as well as an experimental AONFH rat model and cultured BMMSCs with ethanol treatment. Knocking down of DKK1 promoted osteogenesis and inhibited adipogenesis in cultured BMMSCs, and injection of DKK1 knockout lentivirus to the femoral head of rats effectively alleviated the progression of AONFH. We conclude that DKK1 may be a prognostic marker of AONFH, and targeting DKK1 could be a potential therapeutic strategy to prevent AONFH via activation of the canonical Wnt/β-catenin pathway.

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Disclosure of conflict of interest

None.

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