Dikkopf-1 promotes matrix mineralization of osteoblasts by regulating Ca+-CAMK2A-CREB

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Research Article

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Abstract

Dickkopf-1 (DKK1) is a secreted protein that acts as an antagonist of the canonical WNT/β-catenin pathway, which regulates osteoblast differentiation. However, the role of DKK1 on osteoblast differentiation has not yet been fully clarified. Here, we investigate the functional role of DKK1 on osteoblast differentiation. Primary osteoprogenitor cells were isolated from human spinal bone tissues. To examine the role of DKK1 in osteoblast differentiation, we manipulated the expression of DKK1, and the cells were differentiated into mature osteoblasts. DKK1 overexpression in osteoprogenitor cells promoted matrix mineralization of osteoblast differentiation but did not promote matrix maturation. DKK1 increased Ca\textsuperscript{2+} influx and activation of the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II Alpha (CAMK2A)-cAMP response element-binding protein (CREB) and increased translocation of p-CREB into the nucleus. In contrast, stable DKK1 knockdown in SaOS2 cells exhibited reduced nuclear translocation of p-CREB and matrix mineralization. Overall, we suggest that manipulating DKK1 regulates the matrix mineralization of osteoblasts by Ca\textsuperscript{2+}-CAMK2A-CREB, and DKK1 is a crucial gene for bone mineralization of osteoblasts.

Introduction

Bone homeostasis is maintained by bone resorption of osteoclasts and bone formation of osteoblasts [1, 2]. In particular, osteoblast differentiation is a dynamic process in which cell proliferation, extracellular matrix maturation, and extracellular matrix mineralization occur sequentially [3]. Osteoblast differentiation is regulated by various transcription factors and signaling [4].

The WNT/β-catenin pathway regulates cellular functions including cell proliferation, migration, apoptosis, and differentiation [5, 6]. This signaling is also a key regulator of osteoblast differentiation and is mediated by canonical and non-canonical pathways. The canonical pathway depends on β-catenin; and the calcium (Ca\textsuperscript{2+}) pathway, a non-canonical pathway, responds to Ca\textsuperscript{2+} influx [7, 8]. Ca\textsuperscript{2+} signaling is significant for osteoblast differentiation, and Ca\textsuperscript{2+}/calmodulin-dependent protein kinases (CAMKs) are targets of Ca\textsuperscript{2+}, especially after activated CAMK2A triggers phosphorylation of serine and threonine residues on cAMP-response element binding protein (CREB) [9]. Phosphorylated CREB translocates into the nucleus and acts as a transcription factor. Translocated p-CREB binds to the cAMP response element (CRE) and regulates the expression of genes involved in survival, proliferation, and differentiation [10, 11].

Dickkopf-1 (DKK1) acts as an inhibitor of the canonical WNT/β-catenin pathway by binding to low-density lipoprotein receptor-related proteins (LRP) 5/6 and Kremen [12, 13]. DKKs are deemed to have a negative effect on osteoblast differentiation, but there are contradictory reports regarding the effect of DKKs on bone formation. A decrease of Dkk1 expression leads to a concomitant increase of bone mass in mice [14]. In contrast, DKK2 has a role in mineralized matrix formation \textit{in vivo} and \textit{in vitro} [15]. We also showed that DKK1 plays a positive role in osteoblast mineralization [16, 17]. Here, we aim to determine the functional role of DKK1 in osteoblast differentiation.
Materials And Methods

Isolation of human osteoprogenitor cells and osteogenic differentiation

Human bones obtained from surgery were cut into small bone chips using a sterilized rongeur and operating scissors, and attached tissues around the bone chips were removed. The bone chips were washed with phosphate-buffered saline (PBS, Hyclone, UT, USA) containing 1% penicillin-streptomycin (P/S, Gibco, MA, USA) to remove non-adherent bone marrow cells. After washing twice, the bone chips were placed in cell culture plates to isolate osteoprogenitor cells and incubated in Dulbecco's modified eagle medium (DMEM, Hyclone) containing 10% fetal bovine serum (FBS, Gibco) and 1% P/S, followed by outgrowth culture methods [18, 19]. Isolated osteoprogenitor cells cultured to passage 2–4 were used in the experiments. For osteogenic differentiation, these cells were stimulated using osteogenic media containing supplements of 50 µM ascorbic acid (Sigma-Aldrich, MO, USA), 10 mM β-glycerophosphate (Santa Cruz, TX, USA), and 100 nM dexamethasone (Sigma-Aldrich), as described in our previous studies [20, 21]. Osteogenic media were changed every 3 days.

Microarray and RNA sequencing

Microarray data with human osteoprogenitor cells were analyzed with genes changed by osteogenic differentiation and were screened by canonical and non-canonical WNT/β-catenin signaling-related molecules. RNA sequencing was analyzed with changed genes by DKK1 and screened by DKKs and WNT/β-catenin signaling related molecules. All data visualization was conducted using MeV.

DKK1 overexpression and knockdown

For DKK1 overexpression, human osteoprogenitor cells were transfected with DKK1 (HG10170-CY) and an empty plasmid (CV013) using Lipo3000 (Thermo Fisher, MA, USA) for 48 h. These plasmids were purchased from Sino Biological (Wayne, Beijing, china).

To construct the DKK1 knockdown cells, SaOS2 cells were cultured in RPMI 1640 (Hyclone) medium containing 10% Tet-System Approved FBS (Gibco) and 1% P/S. Cells were seeded in a 6 cm culture dish and transfected with shRNA vectors using Lipo3000 (Thermo Fisher) for 48 h. Transfected SaOS2 cells were selected with 1 µg/mL of puromycin (Sigma-Aldrich) and treated with doxycycline (Sigma-Aldrich) to induce knockdown of DKK1.

The vector sequences for knockdown of DKK1 expression were as follows: Empty: tet-pLKO-puro (Addgene), shDKK1: tet-pLKO-puro with the targeting sequence 5’-CCGG-AATGGTCTGGTACTTATTCCC-CTCGAG-GGGAATAAAGTACCAGACCATT- TTTTTG-3’. Vectors were cloned by Cosmogenetech (Seoul).

Assessment of osteogenic differentiation

Maturation of the extracellular matrix (early stage of differentiation) was evaluated by alkaline phosphatase (ALP) activity (Biovision, CA, USA) and staining (Sigma-Aldrich) and Sirius Red (Abcam,
Cambridge, UK) staining. Matrix mineralization (late stage of differentiation) was evaluated by Alizarin Red (ARS, Sigma-Aldrich, MO, USA), hydroxyapatite (HA, Lonza, Basel-stadt, Swiss), and Von Kossa (VON, Sigma-Aldrich) staining, as described in our previous study [22, 23]. For quantification by ARS staining, stained wells were eluted with 200 µL of acetic acid at 37 °C for 2 h. The eluate solution was loaded in each well of a 96-well plate and measured at a wavelength of 450 nm with an ELISA plate reader. For VON quantification, captured images were analyzed with Image J. For HA quantification, stained wells were detected at an excitation wavelength of 492 nm and an emission wavelength of 550 nm using an ELISA plate reader.

**Western blotting and mRNA analysis**

Harvested cells were washed with PBS and lysed with 1X RIPA buffer containing phosphatase inhibitors and proteinase as supplements. The lysates underwent lysis in ice for 15 min and were centrifuged at 12,000 g for 15 min at 4 °C. Then the proteins in the lysates were quantified by a Bradford assay (Bio-Rad Laboratories, CA, USA), separated by SDS-PAGE, and transferred onto nitrocellulose membranes (Cytiva, MA, USA). The membranes were blocked with 5% skim milk, immunoblotted with primary and secondary antibodies, and detected with ECL detection kits (Thermo Fisher). The total RNA was extracted with Nucleozol (Macherey-Nagel, PA, USA) reagent. At least 0.2 µg of the total RNA was used for reverse transcription. RT-qPCR was performed on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) with SYBR Green Supermix (170-8882AP, Bio-Rad). The expression of each target gene was normalized to that of GAPDH. Normalized expression values were averaged, and then average fold changes were calculated. Antibodies used for western blotting and primers used for RT-qPCR are presented in Tables 1 and 2, respectively.
Table 1
Antibodies used for western blotting

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<tr>
<th>Antibody</th>
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<td>sc-374573</td>
</tr>
<tr>
<td>OCN</td>
<td>Santa-cruz</td>
<td>sc-365797</td>
</tr>
<tr>
<td>Active β-catenin</td>
<td>Cell signaling</td>
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Table 2
Primers for qRT-PCR

<table>
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</tr>
<tr>
<td></td>
<td>R - CAAGACAGACCTTCTCCACA</td>
</tr>
<tr>
<td>OCN</td>
<td>F - AGCCACCGAGACACCAGAGAGA</td>
</tr>
<tr>
<td></td>
<td>R - CTCCTGAAAGCCGATGTGGTC</td>
</tr>
<tr>
<td>Runx2</td>
<td>F - GTGCGCTTCAAGGTGTTAG</td>
</tr>
<tr>
<td></td>
<td>R - ACTCTTGCCTCGTCCACTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F - CAAGATCATCAGCAATGCC</td>
</tr>
<tr>
<td></td>
<td>R - CTGTGGTCATGAGTCCCTTCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F - GTCAGTGGTGGACCTGACCT</td>
</tr>
<tr>
<td></td>
<td>R - AGGGGTCTACATGGCACTG</td>
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</table>

Immunofluorescence (IF)

The cells were washed with PBS and fixed using 10% formalin for 15 min. Then, the cells underwent permeabilization with PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and 1% BSA (Rocky Mountain
Biologicals, Inc, MT, USA) for 1 h, followed by incubation with primary antibody overnight at 4 °C. These cells were washed twice with PBS and incubated with Cy3 or Alexa 488-conjugated secondary antibody for 1 h at room temperature. The stained cells were washed with distilled water and mounted with DAPI (Vector CA, USA). Images were obtained using a confocal microscope (Leica Microsystems). The antibodies used for IF are presented in Table 3.

<table>
<thead>
<tr>
<th>Antibody</th>
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<tr>
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**Table 3**

Antibodies for IF

**Transfection and luciferase assay**

All transfection experiments were performed with Lipo3000 (Thermo Fisher) according to the manufacturer's protocol. For the luciferase assay, human osteosarcoma cell line SaOS2 was co-transfected with each osteoblast-specific element (OSE) and osteocalcin (OCN) promoter (3 µg/well) and Renilla (0.3 µg/well). After transfection, the cells were reseeded and treated with DKK1 for 24 h in a dose-dependent manner. Cells were lysed and underwent a luciferase assay (Promega, WI, USA) according to the manufacturer's protocol. Luciferase activity was measured with a Luminometer (Berthold), and the data were normalized to that of Renilla luciferase. SaOS2 cells were kindly donated by Dr. Heekyoung Chung (Hanyang University, Korea). OSE and OCN promoters were received from Dr. Kwang Yeol Lee (College of Pharmacy, Chonnam National University, Korea) [24]. For laboratory-scale production, the plasmid was transformed by Dh5a (Dongin Company, Korea) and subjected to Exfection™ Plasmid LE midi kit (GeneAll, Korea).

**Ca⁺ influx assay**

For assessment of Ca⁺ influx, cells were treated with DKK1 (PeproTech, NJ, USA) and verapamil (Ca⁺ channel blocker, Sigma-Aldrich) in a dose-dependent manner. The cells were analyzed with a Ca⁺ influx assay kit (Abcam) according to the manufacturer's protocol.

**Cytosolic and nucleus fractionation**

Osteoprogenitor and SaOS2 cells were differentiated into mineralized osteoblasts. Cells were washed with PBS and centrifuged at 700g at 4°C to obtain a cell pellet. For the cytosolic fraction, cell pellets were lysed with cytoplasmic lysis buffer (0.2% Triton X-100, 200 mM Tris-HCl pH 8.0, supplemented with proteinase and phosphatase inhibitors) by gently pipetting. The lysates were placed on ice for 10 min and
centrifuged at 700\(g\) for 10 min at 4 °C, and then the supernatants were collected as cytosol proteins. The pellets were washed twice with cytoplasmic lysis buffer, and the supernatant was discarded. The pellets were lysed with nucleus lysis buffer (1% Triton X-100, 200 mM Tris-HCl pH 8.0, 400 mM NaCl, supplemented with proteinase and phosphatase inhibitors), placed on ice for 10 min, and centrifuged at 12,000\(g\) for 10 min at 4 °C. The supernatant was collected as nucleus proteins. The proteins were analyzed with western blotting.

**Trichloroacetic acid (TCA) precipitation**

The method for TCA precipitation was previously reported [25]. Briefly, cells were seeded in a culture dish. The next day, the cells were transfected with DKK1 cDNA plasmid and empty plasmid using Lipo3000 for 2 days. After transfection, growth media was replaced with serum-free DMEM media for 1 day. To obtain the cell supernatants, the cultured media was collected and centrifuged at 700\(g\) for 10 min at 4 °C, and then the supernatants were subjected to TCA precipitation.

**Statistical analysis**

All experiments in this study were performed more than 3 times. Graph Pad Prism version 7 (GraphPad, CA, USA) was used to analyze and visualize the data. All data were analyzed by analysis of variance, followed by an unpaired or paired t-test. Values are given as mean ± standard deviation.

**Results**

**Expression of DKK1 increased during osteoblast differentiation.**

We performed the microarray analysis with differentiated human osteoblasts and analyzed the WNT pathway and bone formation-related genes. During osteoblast differentiation, there were no changes in WNT pathway-related genes or DKK2, 3, and 4 genes, but DKK1 expression increased significantly (Fig. 1a). We also showed that the mRNA and protein levels of DKK1 and OCN, a marker of osteoblast mineralization, increased gradually throughout differentiation of the osteoblasts (Fig. 1b, c). Thus, the expression of DKK1 increased during osteoblast differentiation.

**DKK1 promotes matrix mineralization of osteoblasts.**

To investigate the role of DKK1 in osteoblast differentiation, we overexpressed the DKK1 gene in human osteoprogenitor cells. DKK1 overexpression was successfully performed at both the RNA and protein levels (Fig. 2a). During osteogenic differentiation, DKK1 overexpression in osteoprogenitor cells showed enhanced matrix mineralization of osteoblasts but not in matrix maturation (Fig. 2b, c). The bone mineralization status of the osteoblasts was supported by quantified data (Fig. 2d). As shown in Fig. 2e, the mRNA levels of ALP showed no significant change, while the mRNA levels of Runx-related transcription factor 2 (Runx2) and OCN, osteoblast differentiation-related genes, were increased by DKK1 overexpression. In particular, increase of OCN was confirmed by IF (Fig. 2f). As shown in Supplementary Figure S1a, overexpression of DKK1 increased the secretion of DKK1, and exogenous soluble DKK1
enhanced only matrix mineralization of osteoblast differentiation (Supplementary Fig. 1b-e). We observed that overexpression and treatment with DKK1 had a similar effect on osteoblast differentiation. Treatment with DKK1 showed that it was not an effective human ALP promoter but did significantly promote human OSE and OCN activities (Fig. 2g). Based on these results, we suggest that DKK1 plays a positive role in matrix mineralization during osteoblast differentiation.

**DKK1 activates the Ca\(^{+}\)-CAMK2A-CREB signal during osteoblast differentiation.**

We analyzed RNA sequencing to obtain candidates of genes regulated by DKK1. CAMK2A, a Ca\(^{+}\) pathway molecule, was increased by DKK1 (Fig. 3a; red arrow). Ca\(^{+}\) influx was gradually increased by DKK1 treatment, while treatment with verapamil decreased Ca\(^{+}\) influx in a dose-dependent manner (Fig. 3b). Next, we identified molecules related to the canonical and non-canonical (Ca\(^{+}\) pathway) WNT pathways (Fig. 3c). During osteoblast differentiation, DKK1 overexpression reduced active \(\beta\)-catenin at the early stage but increased p-CAMK2A and p-CREB at the late stage. Furthermore, CREB in cytosol was decreased, while p-CREB in the nucleus was increased by DKK1 overexpression. (Fig. 3d, e). Collectively, we suggest that DKK1 overexpression stimulates CAMK2A-CREB activation during osteoblast differentiation.

**Knockdown of DKK1 inhibits mineralization of osteoblast differentiation.**

We established and generated stable DKK1 knockdown in the SaOS2 cell line. Doxycycline was applied dose-dependently, and then the mRNA and protein expression of DKK1 was confirmed. The DKK1 knockdown effect had a high efficiency in 5 µg/mL of doxycycline (Fig. 4a). DKK1 knockdown had no effect on matrix maturation (Supplementary Fig. S2) but inhibited matrix mineralization of osteoblast differentiation (Fig. 4b). The matrix mineralization of osteoblasts was supported by quantification data (Fig. 4c). We confirmed the nuclear translocation of p-CREB by DKK1 knockdown at osteogenic differentiation day 7. DKK1 knockdown reduced p-CREB in the nucleus (Fig. 4d). As shown in Fig. 4e, DKK1 knockdown decreased mRNA expression of Runx2 and OCN, osteoblast differentiation-related genes. Taken together, these findings indicate that DKK1 knockdown reduces nuclear translocation of p-CREB and inhibits matrix mineralization of osteoblasts.

**Discussion**

In this study, we showed the functional role of DKK1 in osteoblast differentiation. An increase of DKK1 during osteoblast differentiation promoted only matrix mineralization but not matrix maturation of the osteoblasts. Moreover, we found that regulated matrix mineralization of osteoblast by DKK1 was related to the non-canonical WNT pathway (Ca\(^{+}\) signaling). DKK1 increased the intracellular Ca\(^{+}\) influx significantly as well as activating CAMK2A-CREB. Conversely, DKK1 knockdown inhibited matrix mineralization of osteoblast differentiation and nuclear translocation of p-CREB. Taken together, these results suggest that DKK1 regulates matrix mineralization of osteoblasts through the Ca\(^{+}\)-CAMK2-CREB axis.
In the RNA sequencing data, DKK1 did not markedly change the WNT molecules and did not have an effect in the early stage of differentiation (Fig. 3a, c). Active β-catenin increased in the early stage and then decreased in the late stage of differentiation. However, p-CREB gradually increased in the late stage of osteoblast differentiation. Thus, we suggest that WNT/β-catenin and DKK1 play crucial roles in the matrix maturation and matrix mineralization of osteoblasts, respectively. The WNT/β-catenin pathway acts as a beneficial signal on osteoblast differentiation and activity in mice and humans [26, 27]. Canonical WNT/β-catenin pathway antagonists, such as DKKs, are considered to have a negative role in osteoblast differentiation. However, there are opposing reports suggesting a positive role for DKKs in osteoblast differentiation and bone formation [15]. One study found that DKK2 deficiency led to osteopenia and suppressed mineralization, and that DKK2 overexpression showed a mineralization and increased expression of OCN and osteopontin. These findings support the idea that DKKs not only function as WNT antagonists, but also perform other roles. In our previous report, we showed that 1,25D3-induced DKK1 expression was required for osteoblast differentiation [16]. Next, transforming growth factor β1 (TGFβ1) inhibited mineralization by reducing the expression of DKK1 [17]. Here, we show that DKK1 promotes only matrix mineralization during osteoblast differentiation.

We found that the expression of Secreted Frizzled Related Protein 4 (SFRP4) decreased during differentiation (Supplementary Fig. S3). SFRP4 is a member of the SFRP family, which contains a cysteine-rich domain homologous to the WNT-binding site of Frizzled proteins and acts as a soluble antagonist of WNT signaling [28]. It has been reported that SFRP4 TG mice have bone loss phenotype, and SFRP4 deficiency decreases cortical thickness but increases bone volume [29, 30]. Additionally, SFRP4 has been implicated in adipogenesis, and osteogenesis has an inverse correlation with adipogenesis [31, 32]. However, there was no significant change in the expression of SFRP4 by DKK1 in our system (data not shown).

We investigated the role of DKK1 on osteoblasts. Considering bone homeostasis, DKK1 might have an effect on osteoclasts and in bone metabolism. We show the association between DKK1 and osteoprotegrin (OPG) (Supplementary Fig. S4). Receptor activator of nuclear factor kappa-B ligand (RANKL) and OPG act as important regulators in the interaction between osteoblasts and osteoclasts [33, 34]. DKK1 overexpression does not affect the mRNA level of RANKL but regulates OPG. The OPG protein was increased by DKK1 overexpression in whole lysate and cytosol. Furthermore, the transcriptional factor CREB has a potential effect on OPG expression by binding at the CRE site [35]. We suggest that OPG increased by DKK1 has the potential to inhibit RANKL-dependent osteoclast differentiation. Further experiments are needed on the direct effect of DKK1 on osteoclast differentiation.

This study has a few limitations. First, we did not show the effect of DKK1 knockdown in human osteoprogenitor cells. Because it was important that the DKK1 knockdown effect continued until mineralization, we used SaOS2 cells, which differentiate rapidly. DKK1 overexpression promoted matrix mineralization but not maturation of SaOS2 cells (Supplementary Fig. S5). Second, possibilities associated with receptors of DKK1 need to more study. we state that Dkk1 activates calcium signaling. However, it is not clear whether these effects are mediated via LRP5/6 and kremen signaling, which are
receptors for DKK1, or whether they are independent of this pathway or involve other receptors. Third, the mechanism of DKK1-induced Ca\(^{+}\) influx is unclear. We found that treatment with verapamil, an L-type calcium channel blocker, inhibited the increase in Ca\(^{+}\) influx by DKK1 (Fig. 3b). However, we did not reveal an association between DKK1 and Ca\(^{+}\) influx or calcium channels. Despite these limitations, our study proposes novel insights into the underlying mechanisms for the positive role of DKK1 in matrix mineralization of human osteoblasts.

In conclusion, DKK1 enhances the matrix mineralization of osteoblasts without matrix maturation. DKK1 regulation might promotes osteoblast differentiation by increasing nucleus translocation of p-CREB. DKK1 might be crucial gene for the matrix mineralization of osteoblasts.

**Declarations**

**Author contributions** Hyosun Park, Sungsin Jo, and Tae-Hwan Kim designed the experiment. Hyosun Park and Sungsin Jo performed the experiment. Mi-Ae Jang analyzed the RNA sequencing data. Sung Hoon Choi provided the clinical samples. Hyosun Park wrote the manuscript. Hyosun Park, Sungsin Jo, and Tae-Hwan Kim reviewed and edited the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

**Ethical approval** This study was approved by the Institutional Review Board of Hanyang University Hospital (2014-05-002) and was carried out in accordance with the Declaration of Helsinki. A group of 29 patients (16 males and 13 females, mean age 58 ± 11.5 years) who had non-inflammatory spinal diseases were enrolled. All patients provided written informed consent, and all data were de-identified and anonymous.

**References**


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Figures

![Figure 1](image_url)
Expression of DKK1 increased during osteogenic differentiation. Osteoprogenitor cells were stimulated with osteogenic differentiation during indicated days. **a** analysis of Microarray data. Differentiated cells were analyzed expression of DKK1 and OCN by **b** qRT-PCR for mRNA level and **c** western blotting for protein level at indicated days. *p < 0.05; **p < 0.01; ***p < 0.001; N.S. Not significant; OM days, Osteogenic medium days.

**Figure 2**

Overexpression of DKK1 enhancing mineralization of osteoblast differentiation. **a-f** Osteoprogenitor cells were transfected with Empty and DKK1 Plasmid DNA. **a** After transfection for 2 days, overexpression of DKK1 was confirmed by RT-PCR (left), qRT-PCR (center) and western blotting (right). **b-f** Transfected cells were stimulated with osteogenic differentiation. At the indicated days, osteogenic differentiation was evaluated by **b** ALP staining (left), ALP activity (center) and Collagen staining (right); scale bar is 200 μm, **c** ARS staining (left), Von kossa staining (center) and HA staining;
scale bar is 200 μm. d Quantification of c, e At the indicated days, cells were analyzed by qRT-qPCR with DKK1, RUNX2, OCN and normalized to GAPDH. f Transfected cells were stimulated with osteogenic differentiation for 14 days and immunostaining with DKK1 (green), OCN (red), and DAPI (blue) were analyzed; scale bar is 50 μm. g SaOS2 cells were transfected with ALP, OCN, OSE promoter plasmid for 24 h, treated DKK1 dose-dependent manner for 24 h. Then analyzed with a luciferase assay. * p < 0.05; ** p < 0.01; *** p < 0.001; N.S. Not significant; Over, overexpression; OM days, Osteogenic medium days; ALP, Alkaline phosphatase; ARS, Alizarin Red S; HA, hydroxyapatite; BF, Bright field.

Figure 3

Overexpression of DKK1 inducing non-canonical WNT signaling during osteoblast differentiation.

a Osteoprogenitor cells were treated DKK1 for 24 h and transfected with Empty and DKK1 plasmid DNA for 48 h. The cells were analyzed by RNA sequencing, and screened by DKKs, CAMKs, CREBs and WNTs. b Cells were treated DKK1 and verapamil in dose-dependent manner. DKK1 was treated for 1 h, verapamil was pre-treated 10 min before DKK1 treatment. The cells were measured intracellular Ca\(^{2+}\) influx. c-e Osteoprogenitor cells were transfect by Empty and DKK1 plasmid DNA, and the cells were stimulated by osteogenic differentiation. c At indicated days, the cells were analyzed by western blotting. d, e Osteogenic differentiation for 14 days. d The cells harvested and fractionated into cytosol and nucleus proteins. The proteins were analyzed by western blotting. e The cells were performed
immunofluorescence; DKK1 (green), p-CREB (red) and DAPI (blue); scale bar is 50 μm; Over, overexpression; OM, Osteogenic medium days.

Figure 4

Knockdown of DKK1 suppresses mineralization in osteoblast differentiation.

a For knockdown of DKK1, cells were transfected by shRNA and then treated with doxycycline 0, 2, 5 μg/ml for 48 h. The cells were analyzed by RT-PCR (left) and western blotting (right).

b-f Knockdown cells were stimulated by osteogenic media with doxycycline 5μg/ml during indicated days. Osteogenic differentiation was assessed by b ARS staining (left), Von kossa staining (center) and HA staining; scale bar is 200 μm. c Quantification of b. d, e DKK1 knockdown stable cells were treated with osteogenic media containing doxycycline 5 μg/ml. d After 7 days, the cells fractionated into cytosol and nucleus proteins, analyzed by western blotting. e At indicated days, cells analyzed by qRT-PCR for mRNA level.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfigures.pdf