BMB
Reports

BMB Rep. 2022; 55(12): 627-632
www.bmbreports.org

Dikkopf-1 promotes matrix mineralization of osteoblasts by regulating Ca^{+}-CAMK2A- CREB1 pathway

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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^{+} influx and activation of the Ca^{+}/calmodulin-dependent protein kinase II Alpha (CAMK2A)-cAMP response element-binding protein 1 (CREB1) and increased translocation of p-CREB1 into the nucleus. In contrast, stable DKK1 knockdown in human osteosarcoma cell line SaOS2 exhibited reduced nuclear translocation of p-CREB1 and matrix mineralization. Overall, we suggest that manipulating DKK1 regulates the matrix mineralization of osteoblasts by Ca^{+}-CAMK2A-CREB1, and DKK1 is a crucial gene for bone mineralization of osteoblasts. [BMB Reports 2022; 55(12): 627-632]

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 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 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^{+}) pathway, a non-canonical pathway, responds to Ca^{+} influx (7, 8). Ca^{+} signaling is significant for osteoblast differentiation, and Ca^{+}/calmodulin-dependent protein kinases (CAMKs) are targets of Ca^{+}, especially after activated CAMK2A triggers phosphorylation of serine and threonine residues on cAMP-response element binding protein 1 (CREB1) (9, 10). Phosphorylated CREB1 translocate into the nucleus and acts as a transcription factor. Translocated p-CREB1 binds to the cAMP response element (CRE) and regulates the expression of genes involved in survival, proliferation, and differentiation (11, 12).

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 (13, 14). DKKs are deemed to have a negative effect on osteoblast differentiation, but there are contradictory reports regarding the effect of DKKs on bone formation (15, 16). A decrease of Dkk1 expression leads to a concomitant increase of bone mass in mice (17). In contrast, DKK2 has a role in mineralized matrix formation *in vivo* and *in vitro* (18). We also showed that DKK1 plays a positive role in osteoblast mineralization (16, 19). Here, we aim to determine the functional role of DKK1 in osteoblast differentiation.

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 statistical differences in WNTs 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,

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https://doi.org/10.5483/BMBRep.2022.55.12.103

Received 23 June 2022, Revised 2 August 2022, Accepted 7 October 2022

Keywords: Ca^{+} influx, CAMK2A-CREB1, DKK1, Matrix mineralization, Osteoblast differentiation

ISSN: 1976-670X (electronic edition)

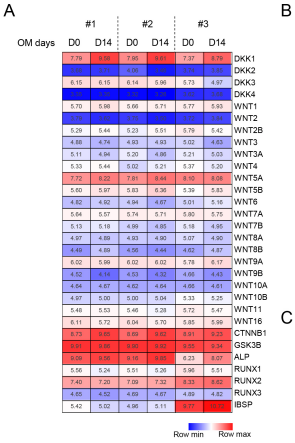

Fig. 1. DKK1 expression was increased during osteogenic differentiation. Osteoprogenitor cells were stimulated with osteogenic differentiation as indicated days. Differentiated cells were subjected to (A) Microarray analysis, (B) RT-qPCR for DKK1 and OCN mRNA levels, and (C) immunoblotting for DKK1 and OCN protein levels. OM, osteogenic media; D, days; DKK1, dickkopf-1; OCN, osteocalcin. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

a mineralization marker of osteoblast differentiation, 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 mRNA 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 Runt-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 Fig. 1A, 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.

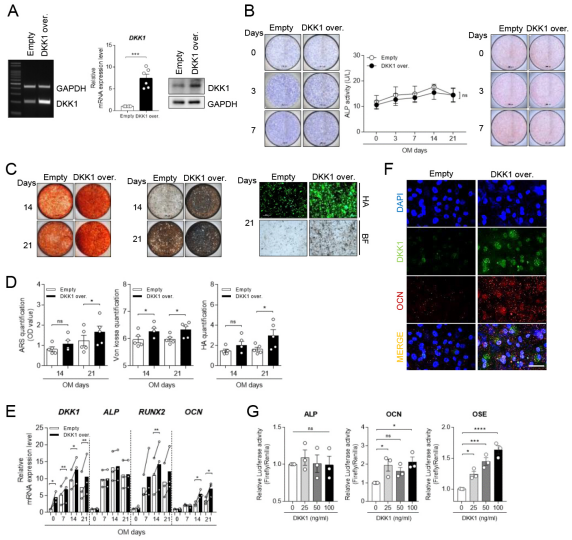

Fig. 2. DKK1 overexpression promotes matrix mineralization of osteoblast differentiation. (A-F) Osteoprogenitor cells were transfected with Empty and DKK1 Plasmid DNA. (A) After transfection for 2 days, overexpression of DKK1 was confirmed by RT-PCR (left), RT-qPCR (center) and immunoblotting (right). (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 (right); scale bar is 200 μm. (D) Quantification of (C). (E) At the indicated days, cells were analyzed by RT-qPCR with DKK1, RUNX2, and 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, or OSE promoter plasmids for 24 h, treated with DKK1 dose-dependent manner for 24 h, and analyzed with a luciferase assay. over., overexpression; OM days, osteogenic days; ALP, alkaline phosphatase; ARS, alizarin red s; HA, hydroxyapatite; BF, bright field. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

DKK1 activates the Ca^{+}-CAMK2A-CREB1 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). 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 β-catenin at the early stage but increased p-CAMK2A and p-CREB1 at the late stage. Furthermore, CREB1 in cytosol was decreased, while p-CREB1 in the nucleus was increased by DKK1 overexpression (Fig. 3D, E). Collectively, we suggest that DKK1 overexpression stimulates CAMK2A-CREB1 activation during osteoblast differentiation.

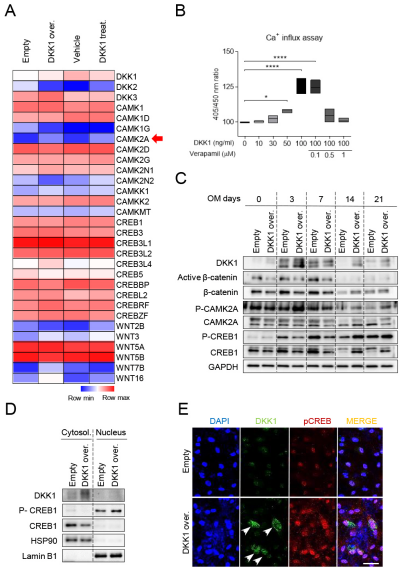

Fig. 3. DKK1 overexpression induces non-canonical WNT signaling during osteoblast differentiation. (A) Osteoprogenitor cells were treated DKK1 for 24 h and transfected with Empty and DKK1 plasmid DNA for 48 h. The cells were analyzed by RNA sequencing, and screened by DKKs, CAMKs, CREBs and WNTs. (B) 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^{+} influx. (C-E) Osteoprogenitor cells were transfected by Empty and DKK1 plasmid DNA, and the cells were stimulated by osteogenic differentiation. (C) At indicated days, the cells were analyzed by immunoblotting. (D, E) Osteogenic differentiation for 14 days. (D) The cells were harvested and fractionated into cytosol and nucleus proteins. The proteins were analyzed by immunoblotting. (E) The cells were subjected to immunofluorescence; DKK1 (green), p-CREB1 (red) and DAPI (blue); scale bar is 50 μm. over., overexpression; treat., treatment; Ca^{+}, calcium; OM days, osteogenic days. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

Knockdown of DKK1 inhibits mineralization of osteoblast differentiation

We established and generated stable DKK1 knockdown in the human osteosarcoma cell line SaOS2. 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. 2) 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-CREB1 by DKK1 knockdown at osteogenic differentiation day 7. DKK1 knockdown reduced p-CREB1 in the nucleus (Fig. 4D). As shown in Fig. 4E, DKK1 knockdown decreased mRNA expression of Runx2 and OCN. Taken together, these findings indicate that DKK1 knockdown reduces nuclear translocation of p-CREB1 and inhibits matrix mineralization of osteoblasts.

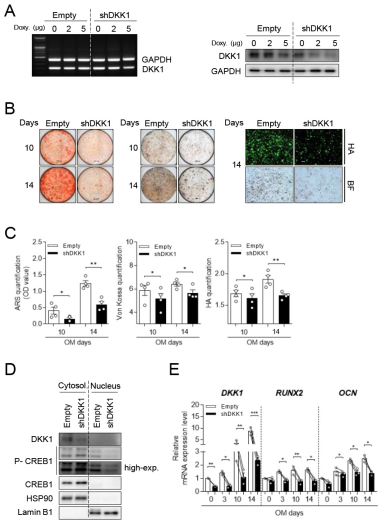

Fig. 4. DKK1 knockdown suppresses matrix mineralization of osteoblast differentiation. (A) For knockdown of DKK1, cells were transfected by shRNA and then treated with doxycycline 0, 2, 5 μg/ml for 48 h. The treated cells were analyzed by RT-PCR (left) and immunoblotting (right). (B-E) The DKK1 knockdown cells were stimulated with osteogenic media containing doxycycline 5 μg/ml during indicated days. Osteogenic differentiation was assessed by (B) ARS staining (left), Von kossa staining (center) and HA staining (right); scale bar is 200 μm. (C) Quantification of (B). (D, E) stable DKK1 knockdown cells were treated with osteogenic media containing doxycycline 5 μg/ml. (D) After 7 days, the cells were fractionated into cytosol and nucleus proteins and analyzed by immunoblotting. (E) At indicated days, cells were analyzed by RT-qPCR for DKK1, RUNX2, and OCN mRNA levels. Doxy, doxycycline; Days, osteogenic days; ARS, alizarin red s; HA, hydroxyapatite; BF, bright field. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

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-CREB1. Conversely, DKK1 knockdown inhibited matrix mineralization of osteoblast differentiation and nuclear translocation of p-CREB1. Taken together, these results suggest that DKK1 regulates matrix mineralization of osteoblasts through the Ca^{+}-CAMK2A-CREB1 axis.

We showed the positive correlation between p-CREB1 and matrix mineralization. To address this point, we treated CREB1 inhibitor (666-15; Sigma-Aldrich) to SaOS2 cells during osteoblast differentiation. Treatment with CREB1 inhibitor dramatically inhibited the matrix mineralization of SaOS2, but not in matrix maturation (ALP and COL) (Supplementary Fig. 3A). Furthermore, we treated CREB1 inhibitor to osteoprogenitor cells for 24 hours

to investigate the molecular mechanism. Treatment with CREB1 inhibitor reduced the expression of DLX5 (Supplementary Fig. 3B, C), which are known to be crucial transcriptional factors for osteoblast differentiation (20). Also, DKK1 overexpression increased the mRNA expression of DLX5 (Supplementary Fig. 3D). Taken together, we propose that DKK1 expression positively regulates matrix mineralization of osteoblasts via CAMK2A-CREB1-DLX5 axis (21).

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-CREB1 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 (22, 23). 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 (18). 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 (19). Here, we show that DKK1 promotes only matrix mineralization during osteoblast differentiation. DKK1 transgenic mice showed the reduction of new bone formation by inhibiting canonical WNT signaling (24). The common point of the above paper and our data is the reduction of active β-catenin protein by DKK1 overexpression *in vitro* and *in vivo*, while difference point is that active β-catenin protein is negatively regulated by different canonical or non-canonical pathway. In this study, we demonstrated that DKK1 overexpression inhibits active β-catenin protein via non-canonical pathways such as calcium signaling.

Normal bone differentiation progression is tightly regulated, and a sequential and dynamic process. Our research aims are to understand the normal bone differentiation progression and interpret it for bone diseases. Intriguingly, DKK1 is reported to therapeutic target for multiple myeloma and osteoporosis (25, 26), yet where DKK1 affects the bone and whether/how this is related to pathological bone remain unknown. In this perspective, it is thought that aberrant high expression of DKK1 for osteoblast differentiation may lead to pathological bone because it passes through matrix maturation and accelerate matrix mineralization.

We found that the expression of Secreted Frizzled Related Protein 4 (SFRP4) decreased during differentiation (Supplementary Fig. 4). 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 (27). It has been reported that SFRP4 TG mice have bone loss phenotype, and SFRP4 deficiency decreases cortical thickness but increases bone volume (28, 29). Additionally, SFRP4 has been implicated in adipogenesis, and osteogenesis has an inverse correlation with adipogenesis (30, 31). However, there was no significant change in the expression of SFRP4 by DKK1 in our system (data not shown).

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. 5). 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.

MATERIALS AND METHODS

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.

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 (32, 33). 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 (34-36). Osteogenic media were changed every 3 days.

Microarray and RNA sequencing

Total RNA was analyzed Affymetrix Whole-transcript Expression array by Macrogen (Korea) and RNA sequencing by Ebiogen (Korea). 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. Microarray and RNA sequencing data visualization was conducted using MeV provided by Ebiogen.

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-AATGG TCTGGTACTTATTCCC-CTCGAG-GGGAATAAGTACCAGAC CATT-TTTTTG-3′. Vectors were cloned by Cosmogenetech (Seoul).

Assessment of osteogenic differentiation, immunoblotting, RT-qPCR, immunofluorescence, transfection, luciferase assay, Ca^{+} influx assay, cytosolic and nucleus fractionation, and trichloroacetic acid (TCA) precipitation analysis

See the Supplementary materials and methods for details.

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.

ACKNOWLEDGEMENTS

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (2019 R1A2C2004214, 2020R1A2C1102386, and 2021R1A6A1A0303 8899).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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