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Lung cancer-derived Dickkopf1 is associated with bone metastasis and the mechanism involves the inhibition of osteoblast differentiation

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ABSTRACT

Wnt/β-catenin signaling and Dickkopf1 (DKK1) play important roles in the progression of lung cancer, which preferably metastasizes to skeleton. But the role of them in bone dissemination is poorly understood. This study aims to define the role of DKK1 in lung cancer bone metastases and investigate the underlying mechanism. Our results demonstrated that DKK1 over-expression was a frequent event in non-small-cell lung cancer (NSCLC) blood samples, and serous DKK1 level was much higher in bone metastatic NSCLC compared to non-bone metastatic NSCLC. We also found that conditioned medium from DKK1 over-expressing lung cancer cells inhibited the differentiation of osteoblast, determined by alkaline phosphatase activity and osteocalcin secretion, whereas the conditioned medium from DKK1 silencing lung cancer cells exhibited the opposite effects. Mechanistically, DKK1 reduced the level of β-catenin and RUNX2, as well as inhibiting the nuclear translocation of β-catenin. Taken together, these results suggested that lung cancer-produced DKK1 may be an important mechanistic link between NSCLC and bone metastases, and targeting DKK1 may be an effective method to treat bone metastase of NSCLC.

1. Introduction

Lung cancer is the most common cancer in terms of both incidence and mortality worldwide. It was reported that there were 1.61 million new cases, and 1.38 million deaths due to lung cancer in 2008 [1]. In the USA, the lifetime risk of developing lung cancer is 8% in men and 6% in women. Non-small-cell lung carcinoma (NSCLC), accounts for at least 85% of all lung cancers, and often spread beyond the initial tumor at the time of diagnosis [2].

Skeleton is one of the most frequently preferred metastatic sites for lung cancers. Once the osseous metastasis occurs, the local bone homeostasis is disturbed, and bone formation is decreased or/and bone resorption increased, causing patients' constant pain and leading to poor quality of life [3–5]. Osteoblasts are responsible for bone formation, which could synthesize and secrete proteins of the extracellular matrix (ECM) and express proteins necessary for inducing mineralization of specialized ECM [6]. RUNX2 is the key transcription factor in osteoblast function and plays essential roles in osteoblast differentiation and maturation. During the period of osteoblast formation, the marker genes, such as alkaline phosphatase (ALP) and osteocalcin are expressed sequentially to facilitate the function of osteoblast [7,8].

Other import signal pathways, such as Wnt/β-catenin signaling axis, also participates the regulation of osteoblast differentiation and function [9]. Wnt factors bind to the Frizzled receptor and co-receptor low density lipoprotein receptor related protein 5/6 (LRP5/6), leading to the activation of Dishevelled (Dvl) and the inactivation of degradation complex of AXIN/adenomatous polyposis coli/glycogen synthase kinase 3β (AXIN/APC/GSK-3β). The accumulated β-catenin translocates to nucleus and cooperates with TCF/LEF transcription co-factor to transcriptional activate downstream target genes [10,11].

Besides the bone-promoting role, Wnt/β-catenin signaling axis has also been reported to play important roles in several types of carcinoma including NSCLC [12,13]. Wnt1, Wnt2 and Dvl 3 were found to be over-expressed in several NSCLC cell lines and primary tumors. Inhibition of Wnt1 or Wnt2 induces apoptosis of NSCLC cell lines, and targeting Dvl resulted in retardation of cell growth [14–16]. However, some other groups got contrary results, demonstrating that higher expression level of β-catenin is associated with favorable prognosis, whereas lower levels with poor overall survival [17–19].

Wnt signaling is delicately regulated by extracellular agonists and antagonists. Dickkopf 1 (DKK1) is one kind of Wnt antagonists by binding and sequestering the co-receptors LRP5/6 to inhibit

Abbreviations: NSCLC, non-small-cell lung cancer; ECM, extracellular matrix; ALP, alkaline phosphatase; DKK1, Dickkopf1; Dvl, Dishevelled; GSK-3β, glycogen synthase kinase 3β; APC, adenomatous polyposis coli.

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Wnt signaling. Several studies showed that DKK1 plays essential role in NSCLC. For instance, high serologic level of DKK1 expression was associated with poor prognosis [20], and DKK1 hypermethylation was correlated with better overall survival [21,22]. Other studies also suggested a role of DKK1 in bone metastases of cancers with bone tropism, such as prostate cancer [23,24] and breast cancer [25]. However, there was no evidence indicating the role of DKK1 in NSCLC bone metastases.

In this study, aiming to explore the biology role of DKK1 in bone metastasis of NSCLC, we examined the effect of lung cancer cell derived DKK1 in osteoblast differentiation and tried to investigate the underlying molecular mechanism.

2. Materials and methods

2.1. Clinical samples and cell lines

Blood samples from 21 non-small-cell lung cancer patients and 7 healthy people were collected from Department of Pulmonary Medicine, Shanghai Chest Hospital, Shanghai Jiaotong University, with prior informed consent of the patients. All patients were pathologically confirmed by two certified pathologists and 7 patients with bone metastatic lesions confirmed by certified radiologists. Study protocol was approved by the ethics committee of Shanghai Chest Hospital, Shanghai Jiaotong University. Four human lung cancer cell lines including A549, H1299, SPC-A-1, Calu-1 and mouse mesenchymal cell C2C12 were obtained from American type culture collection (ATCC, MD, USA). All cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Semi-quantitative reverse transcription PCR and quantitative Real-time PCR

Cell total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), and blood RNA was extracted using QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA). Extracted RNA was treated with DNAse to eliminate genomic DNA. 2 μg of total RNA from each sample was reversely transcribed to double-stranded cDNA using Superscript II First Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Semi-quantitative reverse transcription-PCR (RT-PCR) experiments were optimized for the number of cycles to ensure product intensity to be within the linear phase of amplification. Quantitative Real-time PCR was carried out using SYBR Premix Ex Taq™ II (Takara, Japan) and the results were analyzed using comparative threshold cycle (Ct) method for relative quantification. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as internal control. The specific primers for indicated genes were as follows: DKK1: 5′-TAGAGTCTAGAACGCAAGGATCTC-3′, and 5′-CAAAAACTATCACAGCCTAAAGGG-3; GAPDH: 5′-GAGGTG ATAGCATTGCTTTCG-3′, and 5′-CAAGTCAGTGTACAGGTAAGC-3′.

2.3. DKK1 over-expression

Plasmid containing DKK1 full length cDNA was obtained from addgene (Cambridge, MA, USA) and DKK1 was subcloned into pcDNA3.1 vector to construct recombinant DKK1-pcDNA3.1 vector using *Kpn* I and *Xho* I. SPC-A-1 cells were cultured as described above and transfected with either the control empty pcDNA3.1 vector or *DKK1-pcDNA3.1*. After transfection with Superfect Transfection Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's procedure, cells were replenished with fresh culture medium and incubated for 48 h before sample collection.

2.4. DKK1 knockdown

A549 cells were cultured as described above and transfected with 200 nM of siRNA against human scramble sequence or siRNA against human DKK1 using Superfect Transfection Reagent (Qiagen, Valencia, CA, USA). siRNA against DKK1 was a combination of three duplexes as follows:

- 5′-GCCGGAUACAGAAAGAUCACCAUCA-3′, and
- 5′-UGAUGGUGAUCUUUCUGUAUCCGGCAA-3′;
- 5′-GUAUCACACCAAAGGACAAGAAGGT- 3′, and
- 5′-ACCUUCUUGUCCUUUGGUGUGAUACAU-3′;
- 5′-AGAACGGAAGUGUGAUAUGUUUAAA-3′, and
- 5′-UUUAAACAUAUCACACUUCCGUUCUUG-3′

(Invitrogen, Carlsbad, CA, USA).

2.5. Western blot

Protein was extracted using M-PER reagent (Pierce, Rockford, IL, USA) or the Nuclear and Cytoplasmic Protein Extraction Kit (Pierce, Rockford, IL, USA). After the concentrations were determined by the BCA protein assay (Pierce, Rockford, IL, USA), the protein were separated on 12% SDS–PAGE and then transferred to nitrocellulose membrane. Membranes were incubated with primary antibodies for DKK1 (1:1000 dilution, Abcam, Cambridge, MA, USA), Tubulin (1:1000 dilution, Santa Cruz, Dallas, TEX, USA), β-catenin (1:1000 dilution, Abcam, Cambridge, MA, USA), RUNX2 (1:500 dilution, Santa Cruz, Dallas, TEX, USA) and Histone H2A (1:500, Cell Signaling Technology, Inc. Danvers, MA, USA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000, Abcam, Cambridge, MA, USA) or goat anti-mouse antibody (1:2000, Abcam, Cambridge, MA, USA). Signal was detected by chemiluminescent ECL Plus™ Western blotting detection reagent (Pierce, Rockford, IL, USA).

2.6. DKK1 and osteocalcin secretion

DKK1 concentrations in the blood samples and cell culture media were measured using DuoSet Human DKK1 ELISA Kit (R&D Systems, Minneapolis, MN, USA) as described previously [26]. Osteocalcin secretion was tested using Osteocalcin ELISA Kit (R&D Systems, Minneapolis, MN, USA).

2.7. Conditioned medium collection and use

DKK1 expression modified cells were treated as described above, 24 h later the medium was replaced with serum-free medium, and cells were cultured for additional 24 h. The conditioned medium (CM) was collected and stored at −80 °C until used. To normalize for differences in cell density due to proliferation, cell number was determined and CM was then normalized to cell number between samples. C2C12 cells were seeded at a density of 5×10^4 cells per cm^2 and cultured with or without 200 ng/ml BMP2. The CM was added to the culture media at a ratio of 25% or 50% while additional BMP2 was added to maintain the final concentration of 200 ng/ml. To test the specificity of DKK1, 10 μg/ml of anti-human DKK1-neutralizing antibody (R&D Systems, Minneapolis, MN, USA) or unimmunized goat IgG was added to the culture medium, in addition with 200 ng/ml BMP2 and 50% CM. The medium was changed every other day.

2.8. Alkaline phosphatase activity assay

C2C12 cells were treated with BMP2 and lung cancer cell CM as described above, 5 d later cells were harvested for assay of alkaline phosphatase (ALP) activity (Pierce, Rockford, IL, USA) by

determining the amount of *p*-nitrophenol synthesized from *p*-nitrophenylphosphate according to the manufacturer's instructions. Absorbance was determined at 405 nm and compared with a *p*-nitrophenol standard titration curve. ALP activity was normalized to total protein content (Bio-Rad Protein Assay, Hercules, CA, USA).

2.9. Statistics

Statistical analyses were performed by the Statistical Package for the Social Sciences software 15.0 (SPSS, Chicago, IL, USA). Continuous variables were expressed as mean and standard deviation. Paired Student's t test was used and $P < 0.05$ was considered statistically significant.

3. Results

3.1. DKK1 is associated with bone metastatic characteristic of NSCLC

To examine the role of DKK1 in NSCLC, we analyzed the serous DKK1 level in clinical samples from 21 NSCLC and 7 healthy people by quantitative real-time RT-PCR and ELISA. All the samples were from patients with ages ranging from 33 to 76, and of them 7 samples were from patients with bone metastatic lesions confirmed by certified radiologists. As seen in Fig. 1A and B, we found that DKK1 expression was exclusively low in all normal controls, whereas all NSCLC samples exhibited high levels of DKK1, especially in those with bone metastatic lesions. Then we tested the expression level of DKK1 in several lung cancer cell lines with various metastatic characteristics. Semi-quantitative RT-PCR results indicated that DKK1 were highly expressed in metastatic lung cancer cell lines (Fig. 1C), and Western blot confirmed the results (Fig. 1D). These findings indicated that DKK1 over-expression was a frequent event in human NSCLC tissue, and DKK1 was correlated with NSCLC metastatic status.

3.2. Over-expression of DKK1 in lung cancer cells results in retardation of osteoblast differentiation

To further verify the correlation between bone metastasis and high DKK1 level, we examined the effect of lung cancer cell-produced

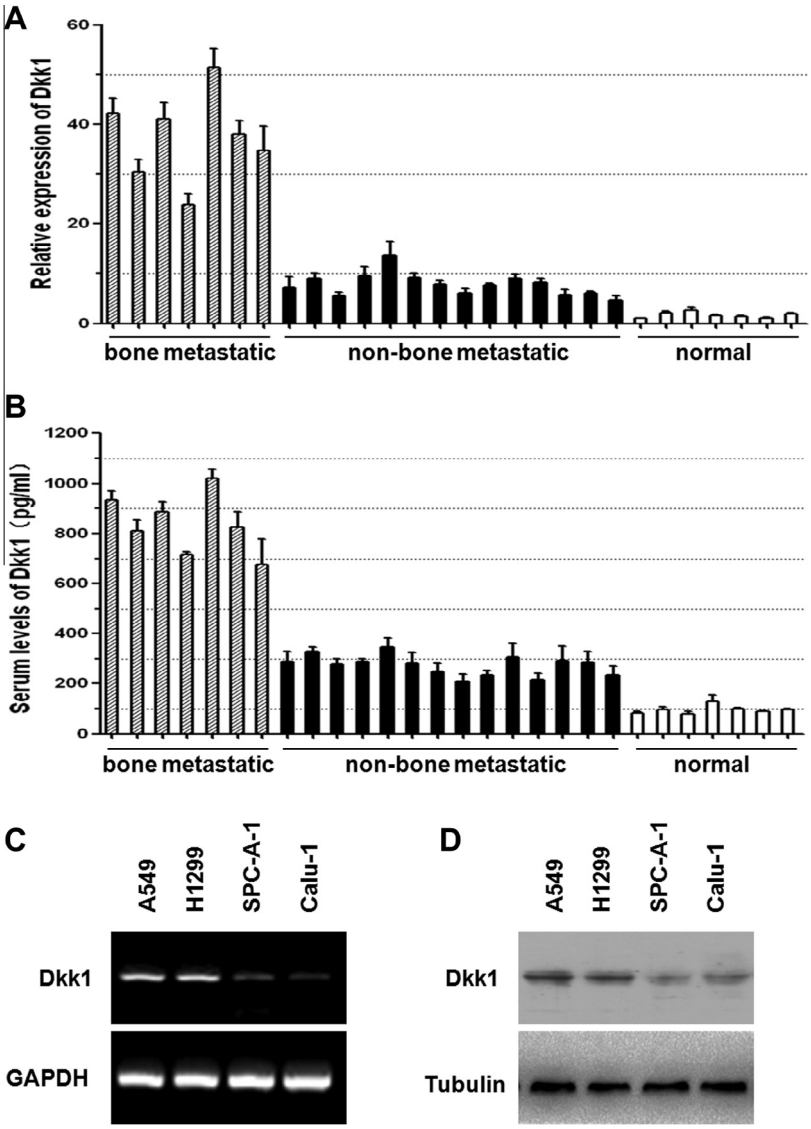

Fig. 1. DKK1 is associated with bone metastatic characteristic of NSCLC. (A) mRNA levels of DKK1 in the blood were up-regulated in NSCLC samples compared to normal controls, especially in samples with bone metastatic characteristics. (B) Serous levels of DKK1 determined by ELISA. (C) Different mRNA levels of DKK1 in human lung cancer cell lines. (D) Different protein levels of DKK1 in human lung cancer cell lines.

DKK1 on bone microenvironment. We collected the conditioned medium (CM) of DKK1 over-expressing SPC-A-1 cells or control cells, and added to the culture media of C2C12 cells, which could differentiate to osteoblastic cells in the presence of BMP2.

The efficiency of over-expression was confirmed by analysis of mRNA and protein level of SPC-A-1 cells (Fig. 2A), in addition with results from ELISA determining the secretion level of DKK1 (Fig. 2B). The differentiation of C2C12 cells was assessed by detection of specific marker of osteoblast differentiation, including ALP activity and osteocalcin secretion. It was found that only low activity of ALP was detectable in C2C12 cells without BMP2 stimulation, but was significantly elevated after BMP2 treatment. CM of DKK1 over-expressing SPC-A-1 cell effectively blocked the up-regulation of ALP activity induced by BMP2, in a dose-dependent manner. However, CM of control SPC-A-1 cell had no obvious effects on BMP2-induced elevation of ALP activity. Moreover, when DKK1 neutralizing antibody was introduced to the co-cultures in the presence of 50% DKK1-CM, the blockade was disappeared, suggesting the specific role of DKK1 again (Fig. 2C).

Similarly, as shown in Fig. 2D, treatment of C2C12 cells with media containing BMP2 greatly induced osteocalcin secretion, which was blocked by CM from DKK1 over-expressing SPC-A-1 cell but not CM from control cell. DKK1 neutralizing antibody eliminated the down-regulation of osteocalcin secretion induced by CM from DKK1 over-expressing cells. These findings demonstrated that over-expression of DKK1 in SPC-A-1 cells caused blockade of osteoblast differentiation.

3.3. Knockdown of DKK1 in lung cancer cells leads to acceleration of osteoblast differentiation

Then we tested the effect of CM of DKK1-silencing A549 cells on C2C12 cell differentiation. The efficiency of knockdown using specific siRNA against DKK1 was confirmed by RT-PCR, Western Blot, and ELISA (Fig. 3A and B). As shown in Fig. 3C, CM of DKK1 silencing A549 cells caused dramatic enhancement of BMP2-induced ALP activity, whereas CM of control cells had no similar impact. As for the amount of osteocalcin released to the culture medium, lower DKK1 level caused dramatic increase of osteocalcin secretion (Fig. 3D). The introduction of DKK1 neutralizing antibody abolished the rise of osteoblastic markers expression induced by CM from DKK1-silencing cells (Fig. 3C and D). Taken together, these results showed that DKK1 silencing in A549 cells stimulated osteoblastic differentiation of C2C12 cells.

3.4. Tumor cell derived DKK1 increase the level of β-catenin and RUNX2, as well as changing the distribution of β-catenin

DKK1 is an antagonist of Wnt/β-catenin signaling, which positively regulates osteoblast differentiation. So we tested the effect of tumor cell derived DKK1 on expression of β-catenin in C2C12 cells, and examined whether the change of Wnt/β-catenin signaling is the underlying molecular mechanism for osteoblast differentiation regulation. As shown in Fig. 4A and B, the expression level of β-catenin was significantly up-regulated by

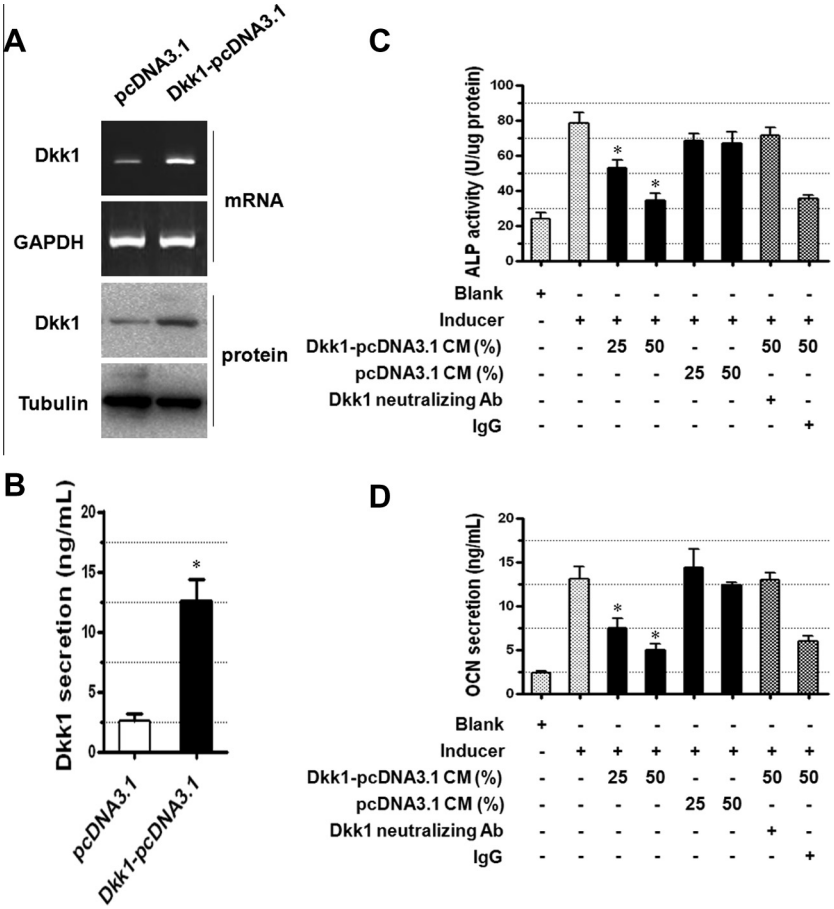

Fig. 2. Over-expression of DKK1 in SPC-A-1 cells results in retardation of osteoblast differentiation. (A) Verifying the efficiency of over-expression by Western Blot and semi-quantitative RT-PCR. (B) The secretion of DKK1 was increased after DKK1 expression was enhanced.*$P < 0.05$ indicated a significant difference compared to control. (C) Conditioned medium from DKK1-overexpressing SPC-A-1 cells inhibited the elevation of ALP activity induced by BMP2. *$P < 0.05$ indicated a significant difference compared to cells induced by BMP2. (D) Conditioned medium from DKK1-overexpressing SPC-A-1 cells inhibited the release of osteocalcin induced by BMP2. *$P < 0.05$ indicated a significant difference compared to cells induced by BMP2.

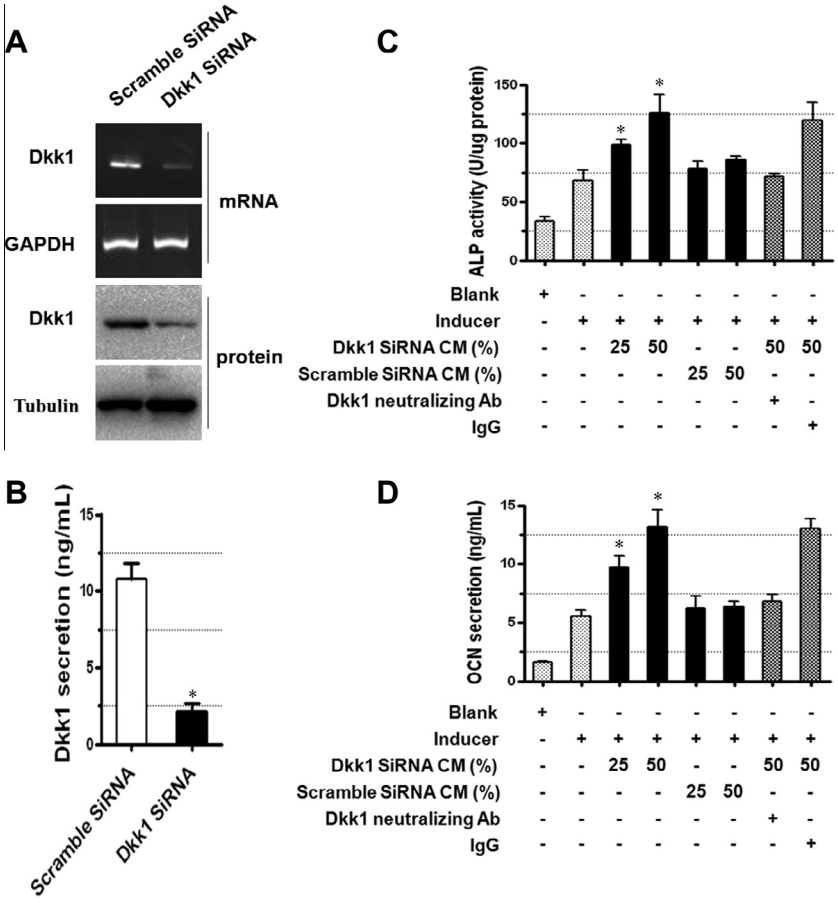

Fig. 3. Knockdown of DKK1 in A549 cancer cells leads to acceleration of osteoblast differentiation. (A) Verifying the efficiency of knockdown by Western Blot and semi-quantitative RT-PCR. (B) The secretion of DKK1 was decreased after DKK1 was silenced. $^{*}P < 0.05$ indicated a significant difference compared to control. (C) Conditioned medium from DKK1-silencing A549 cells boosted the elevation of ALP activity induced by BMP2.$^{*}P < 0.05$ indicated a significant difference compared to cells induced by BMP2. (D) Conditioned medium from DKK1-silencing A549 cells stimulated the release of osteocalcin induced by BMP2. $^{*}P < 0.05$ indicated a significant difference compared to cells induced by BMP2.

DKK1 deficiency, when recombinant vector containing DKK1 was introduced after DDK1 knockdown, the expression of β-catenin was reduced to the previous level.

RUNX2, a critical transcriptional factor in osteoblast differentiation, is a target of Wnt/β-catenin signaling. To find out whether the transcriptional regulation of RUNX2 was effected by Wnt/β-catenin signaling, we detected the protein expression of RUNX2 by Western Blot. As expected, DKK1 silencing caused dramatic increase in RUNX2 protein level, and when DKK1 expression was rescued RUNX2 expression returned to the basal level (Fig. 4A and B).

Both β-catenin and RUNX2 play roles in nucleus by binding with other co-factors to regulate target genes expression. Thus it was necessary to investigate the subcellular distribution of β-catenin and RUNX2. As seen in Fig. 4C, the amount of β-catenin in both cytoplasm and nucleus were remarkably elevated by DKK1 knockdown, and almost returned to the normal level after DKK1 expression was rescued. Grayscale scanning analysis showed that more β-catenin translocated to nucleus when DKK1 was depleted (Fig. 4D). As for RUNX2, nulear and cytolasmic RUNX2 levels were remarkably increased by DKK1 silencing, but the distribution to nucleus or cytoplasm remained unchanged (Fig. 4E and F).

4. Discussion

Bone metastases are common in NSCLC and are associated with considerable morbidity. The mechanism for the development of NSCLC bone metastasis is not yet fully understood, however, it is believed that lung cancer cells invade to the bone surface by secreting some kinds of cytokines and growth factors, which result in the disturbance of bone homeostasis [27]. Therefore, the osteoblast function and bone formation are inhibited and bone resorption is activated relatively, causing growth and proliferation of malignant cells and development of bone metastases. Thus, it is of great value to find out a key factor involved in NSCLC bone metastases and make preparation for translation into improvement in treatment for NSCLC bone metastases.

In this study, we found a correlation between DDK1 expression and NSCLC bone metastasis. Then we modified the expression level of DKK1 in lung cancer cells and collected the conditioned medium to treat the C2C12 cells. Our objective is to investigate the role of DKK1 produced by NSCLC cells in osteoblast differentiation and bone metastases. We found that lung tumor cell derived DKK1 negatively regulated osteoblast differentiation, and β-catenin and RUNX2 were involved in this regulation. Our results supplied an important component to the current knowledge of NSCLC bone metastases.

DKK1 is an antagonist of Wnt and negatively modulates the Wnt/β-catenin pathway. Contrary to other antagonists, DKK1 levels are elevated in many malignant tissues and associated with poor prognosis, including multiple myeloma [28], hepatoblastomas, Wilms' tumors [29] and breast cancer [30]. In the case of lung cancer, it was reported that DKK1 was over-expressed and hypermethylation of DKK1 was correlated with better overall

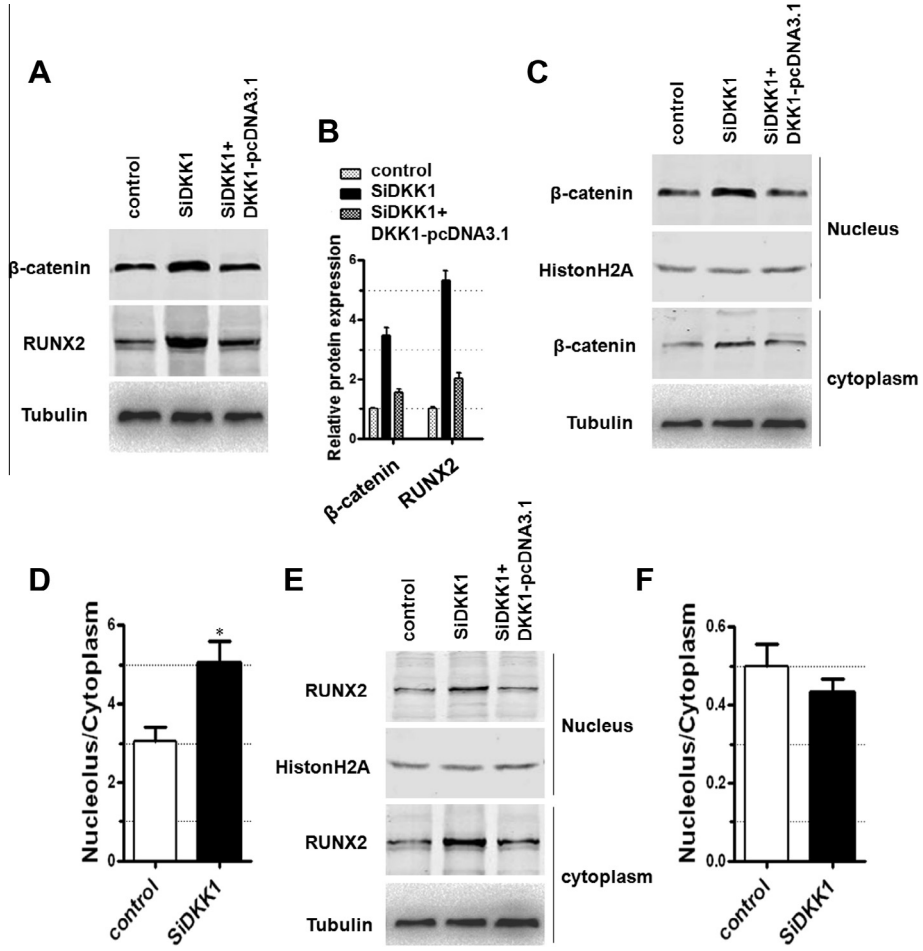

Fig. 4. Tumor cell derived DKK1 increase the level of β-catenin and RUNX2, as well as changing the distribution of β-catenin. (A) DKK1 silencing resulted in increase in the total cellular level of β-catenin and RUNX2, and this effect was abolished after DKK1 expression was rescued. (B) Quantification of β-catenin and RUNX2 levels showed in A. (C) The nuclear and cytoplasmic levels of β-catenin were increased after DKK1 was silenced, and was restored to basal levels when DKK1 expression was rescued. (D) Quantification of β-catenin level showed in *C*. DKK1 depletion caused nuclear translocation of β-catenin. $^{*}P < 0.05$ indicated a significant difference compared to control. (E). The nuclear and cytoplasmic levels of RUNX2 were increased after DKK1 was silenced, and was restored to basal level when DKK1 expression was rescued. F. Quantification of RUNX2 level showed in D. The distribution of RUNX2 in nucleus and cytoplasm remained unchanged.

survival [20–22]. DKK1 is a downstream target of Wnt/β-catenin signaling pathway, and activation of the canonical Wnt signaling leads to an increase in transcription and translation levels of DKK1 [31]. In addition, DKK1 was capable of mediating its affects independent of canonical Wnt signaling, such as Jun N-terminal kinase (JNK) pathway [32]. These evidences may explain the reason why DKK1 expression is up-regulated in NSCLC and other cancers. However, there were several contrary reports, demonstrating that DKK1 had tumor growth inhibitory effects in breast cancer [33], melanoma [34] and colon cancer [31]. It was speculated that DKK1 inhibit oncogenic Wnt/β-catenin signaling in these circumstances. In view of this, the role of DKK1 in cancer progression is much more complicated than we previously estimated. Be promoter or suppressor depends on the cellular and micro-environmental context of specific cancer type. Certainly, our results demonstrated that DKK1 function as a pro bone metastatic factor in NSLCL.

Our results proved that DKK1 knockdown resulted in the accumulation of β-catenin in nucleolus, being consistent with the fact that DKK1 silencing leads to the activation of Wnt/β-catenin signaling. Moreover, we also found that DKK1 depletion induced the elevation of DKK1 level in total cellular component. Whether it was due to the inhibition of β-catenin degradation or other reasons such as transcriptional activation cooperated with other factors or both remained to be further investigated. In addition, much more work will be required to define the function of DKK1 in the autocrine aspect, such as effect on tumor cell growth, apoptosis, migration and invasion.

We demonstrated that DKK1 affected RUNX2 expression but not its nucleus translocation. But we did not know this regulation was direct or indirect though Wnt/β-catenin signaling, since RUNX2 is one of target genes of β-catenin [35]. Further study is required to investigate the downstream of DKK1 expression and is warranted to better appreciate functions of DKK1. Role of lung tumor cell derived DKK1 in osteoclast differentiation and bone resorption is also need to be elucidated.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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