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Original Article

Dok5 regulates proliferation and differentiation of osteoblast via canonical Wnt/β-catenin signaling

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Abstract

Objective: In bone tissue engineering, the use of osteoblastic seed cells has been widely adopted to mediate the osteogenic differentiation so as to prompt bone regeneration and repair. It is hypothesized that Dok5 can regulate the proliferation and differentiation of osteoblasts. In this study, the role of Dok5 in osteoblast proliferation and differentiation was investigated. **Methods:** A lentiviral vector to silence Dok5 was transferred to C3H10, 293T and C2C12 cells. CCK-8 assay was used to detect the cell proliferation. Cells were stained by ALP and AR-S staining. Western blot and RT-PCR were used to detect the expression levels of related factors. **Results:** Dok5 expression level was gradually up-regulated during the osteoblast differentiation. Dok5 silencing down-regulated the expression levels of osteogenic biosignatures OPN, OCN, and Runx2 and suppressed the osteogenesis. Additionally, the osteoblast proliferation and canonical Wnt/β-catenin signaling were suppressed upon Dok5 knockdown, β-catenin expression level was significantly down-regulated in the knockdown group, while the expression levels of GSK3-β and Axin, negative regulators in the Wnt signaling pathway, were up-regulated. Furthermore, overexpression of Dok5 promoted the proliferation and osteogenesis and activated the canonical Wnt/β-catenin signaling pathway. **Conclusion:** Dok5 may regulate the osteogenic proliferation and differentiation via the canonical Wnt/β-catenin signaling pathway.

Keywords: Dok5, Osteogenesis, Proliferation, Wnt/β-catenin

Introduction

Bone is involved in specific metabolic activities, and is one of the few human organs with regenerative potential[1]. Bone regeneration is a critical factor in skeletal system diseases[2]. However, bone regeneration and repair are extremely poor, especially in patients with major traumatic defects, bone tumor resection or infection[3]. The gene expression is significantly regulated during the growth, development, regeneration and repair of bone defects[4-6]. Thus, understanding the mechanisms of bone development, regeneration and repair may improve the management efficiency for bone disorders.

Bone tissue engineering is a nascent approach to treat bone defects[7]. Bone marrow mesenchymal stem cells possess the capacity to differentiate into osteoblasts, chondroblasts, myoblasts or lipids[8]. Due to their easy accessibility and low immune response, bone marrow mesenchymal stem cells have captivated widespread attention in the repair of bone defects[9]. The seed cells have multidirectional differentiation potential, such as osteoblasts, bone marrow mesenchymal cells, mesenchymal stem cells, genetically-modified cells, embryonic stem cells. However, the efficiency of directional osteogenic differentiation is relatively low, which can be resolved by genetic modification of seed cells to enhance osteogenic differentiation[10]. Consequently, deeper understanding of osteogenic regulatory genes and underlying molecular mechanisms may improve the regeneration and repair outcomes of bone defects.

Doks are recently discovered cytosolic signaling molecules related to tyrosine kinases[11]. Previous studies have shown that Dok5, a member of the Doks family, can affect the proliferation and differentiation of neuronal cells,

The authors have no conflict of interest.

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Table 1. Sequences of Dok5-shRNA.

Group	Sense strand	Antisense strand
Dok5-shRNA1	**5'** -AGATTACATATGAGTACAT-**3'**	**5'**-ATGTACTCATATGTAATCTGC-**3'**
Dok5-shRNA2	**5'**-AGACGAATGGTGCAAAGTT-**3'**	**5'**-AACTTTGCACCATTCGTCTGC-**3'**
Dok5-shRNA3	**5'**-ACAAGGTTACAGAACTCAA-**3'**	**5'**-TTGAGTTCTGTAACCTTGTGG-**3'**
Control-shRNA	**5'**-TTCTCCGAACGTGTCACGT-**3'**	

Table 2. Primer sequences.

Gene	Forward	Reverse
Dok5	5'-CGAGCAAGGGTCCAAAGAGAC-3'	5'-TTGGCAATCGGGCTACATTTT-3'
OPN	5'-CTTTACAGCCTGCACCCAGA-3'	5'-TTCTGTGGCGCAAGGAGATT-3'
OCN	5'-CACTCCTCGCCCTATTGGC-3'	5'-CCCTCCTGCTTGGACACAAAG-3'
Runx2	5'-TTCAACGATCTGAGATTTGTGGG-3'	5'-GGATGAGGAATGCGCCCTA-3'
β-actin	5'-GGGACCTGACTGACTACCTC-3'	5'-TCATACTCCTGCTTGCTGAT-3'
GSK-3β	5'-AAGCGATTTAAGAACCGAGAGC-3'	5'-AGAAATACCGCAGTCGGACTAT-3'
Axin	5'-CATTGTGTCCAGACAAACCAAGC-3'	5'-GGAAGGGTAGGTATTCTCCTCCA-3'
β-catenin	5'-ATGGAGCCGGACAGAAAAGC-3'	5'-TGGGAGGTGTCAACATCTTCTT-3'

cardiomyocytes and tumor cells[12-14]. However, its effect on osteoblasts has not been studied. Therefore, the purpose of this study was to evaluate the function of Dok5 in osteoblast proliferation and differentiation and the possible mechanism.

Materials and methods

Reagents and antibodies

Alizarin red S (AR-S), ascorbic acid (AA), β-glycerophosphate (β-GP), and dexamethasone (DXMS) were obtained from Sigma Aldrich to prepare osteogenic induction fluid. Anti-Dok5 antibody was purchased from Abcam (Cat No. ab168343). Anti-β-catenin antibody was obtained from Santa Cruz Biotechnology (Cat No.sc-7963), and Anti-Axin (Cat No.20540-1-AP), Anti-GSK-3β (Cat No.22104-1-AP), and anti-β-actin (Cat No.60008-1-Ig) antibodies were purchased from Proteintech.

Cell culture and osteogenesis induction

The C3H10, 293T and C2C12 cell lines were obtained from the cell bank of Chinese Academy of Sciences, Shanghai. These cell lines were cultured in high-glucose DMEM media enriched with 10% FBS (Gibco, Cat No.16000-044), at 37°C, 5% CO_2, in a humified incubator. Osteogenic induction was performed using an osteogenic induction medium comprised of 10mM β-GP, 50μM AA, 100mM DXMS, in complete medium. The induction medium was replaced daily.

Plasmids and lentivirus production

Plasmids including pMD2.G, pLKO.1-EGFP-puromycin, and psPAX2 were designed by GeneChem, CHN. To silence Dok5, 3 shRNAs were designed and subsequently inserted into a lentiviral vector pLKO.1-EGFP-puromycin (Table 1). For lentivirus generation, Lipofectamine 2000 was utilized to facilitate the transfection of lentiviral vectors (pLKO.1-puro or pCDH-CMV-MCS-EF1-Puro), psPAX2, and pMD2.G into the HEK293T cells. The cells were then cultured in FBS-free high-glucose DMEM. Three lentiviruses were packaged and infected cells plated into 6-cm cell culture dishes at a cell density of 80%. At 48 h after transfection, stably-transfected cells were selected and treated with 5 μg/L puromycin for 2 weeks.

ALP and AR-S staining

When the confluency of C3H10 and C2C12 cells reached 80-90%, (cell density of 1×106/ml), the cells were trypsinized, adjusted and seeded into 24-well plates. Next, 1 ml of osteogenic induction solution was added into each well for 0, 3, and 7 days. The cells were then rinsed with PBS twice, and fixed in 4% PFA for 30 min. Subsequently, the cells were stained with ALP and AR-S reagents strictly according to the manufacturers' protocols. After staining, the cells were rinsed with PBS, air-dried and photographed under microscope.

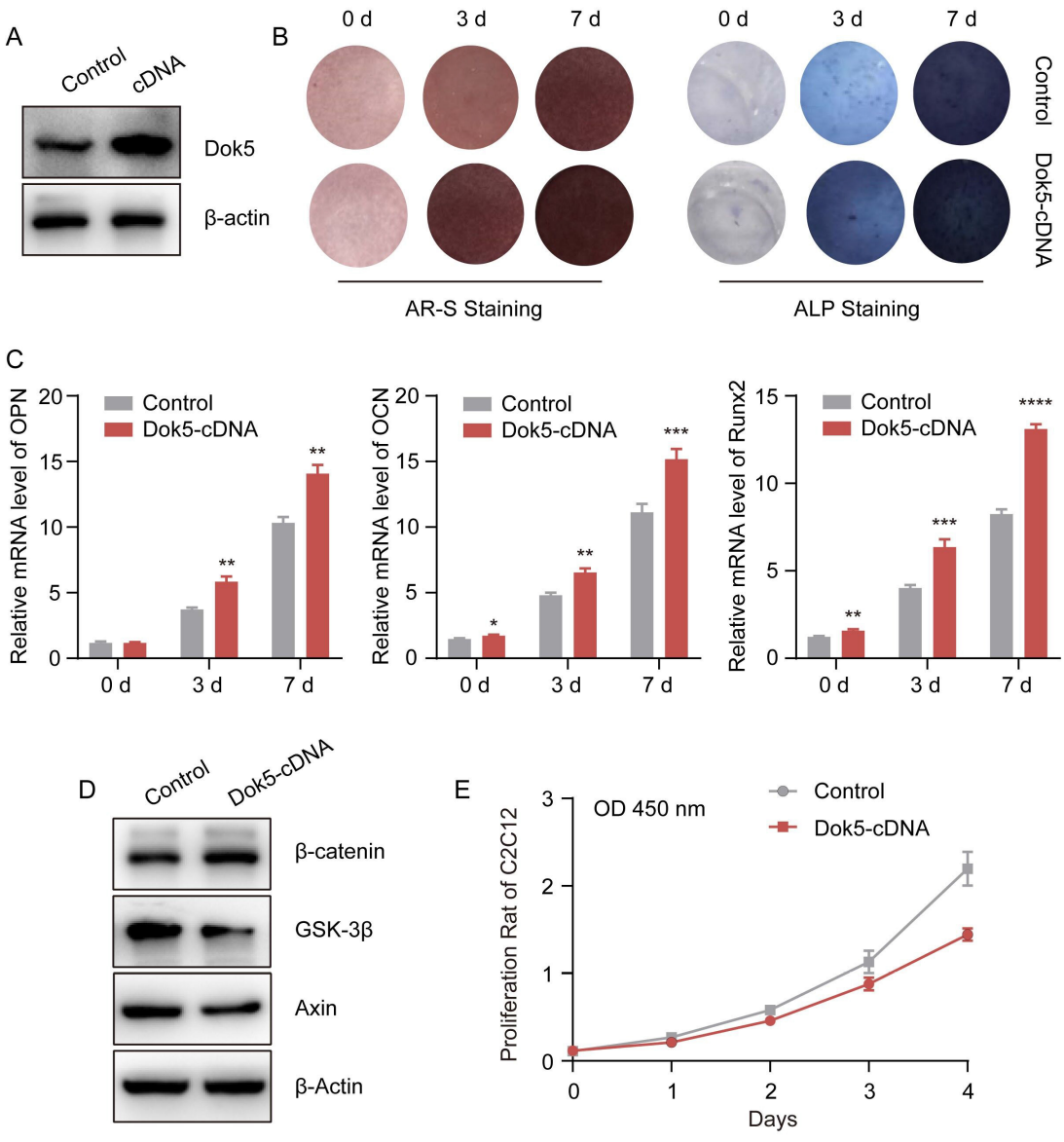

Figure 1. Osteogenesis induces Dok5 expression. A) The protein expression of Dok5 in C2C12 and C3H10 cell lines. B-C) The expression pattern during osteogenesis of Dok5 in C2C12 and C3H10 cell lines. D)The relative mRNA levels of OPN, OCN and Runx2 during osteogenesis. E) ALP staining and AR-S staining in C2C12 and C3H10 cells. F. Data are indicated as the mean ± SD. (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001).

CCK-8 assay

C2C12 and C3H10 cells were cultured into 96-well plates at 1500 cells/well. Each experimental group was assayed in 6 replicates. Once the cells adhered to the plate, 100 μL of serum-free medium containing CCK-8 reagent was supplemented to each well and cultured for 2 h under normal culture conditions. The absorbance value was detected at a wavelength of 450 nm on a Tecan Infinity 200 Pro, multi-well plate reader. The experiment was repeated for triple times.

Western blotting

Osteogenic induction and differentiation were performed in the C2C12 and C3H10 cell lines. Protein lysates were harvested on 0, 3, and 7 days, and Dok5 expression was detected. When the cell confluence reached 80-90%, they were lysed with RIPA buffer supplemented with 1% volume protease inhibitor, PMSF, on ice for 30 min. Cell debris were eliminated by centrifugation for 10 min at 12000 *rpm* at 4°C, and the protein lysate was collected. The concentration of the obtained protein samples was determined using BCA assay.

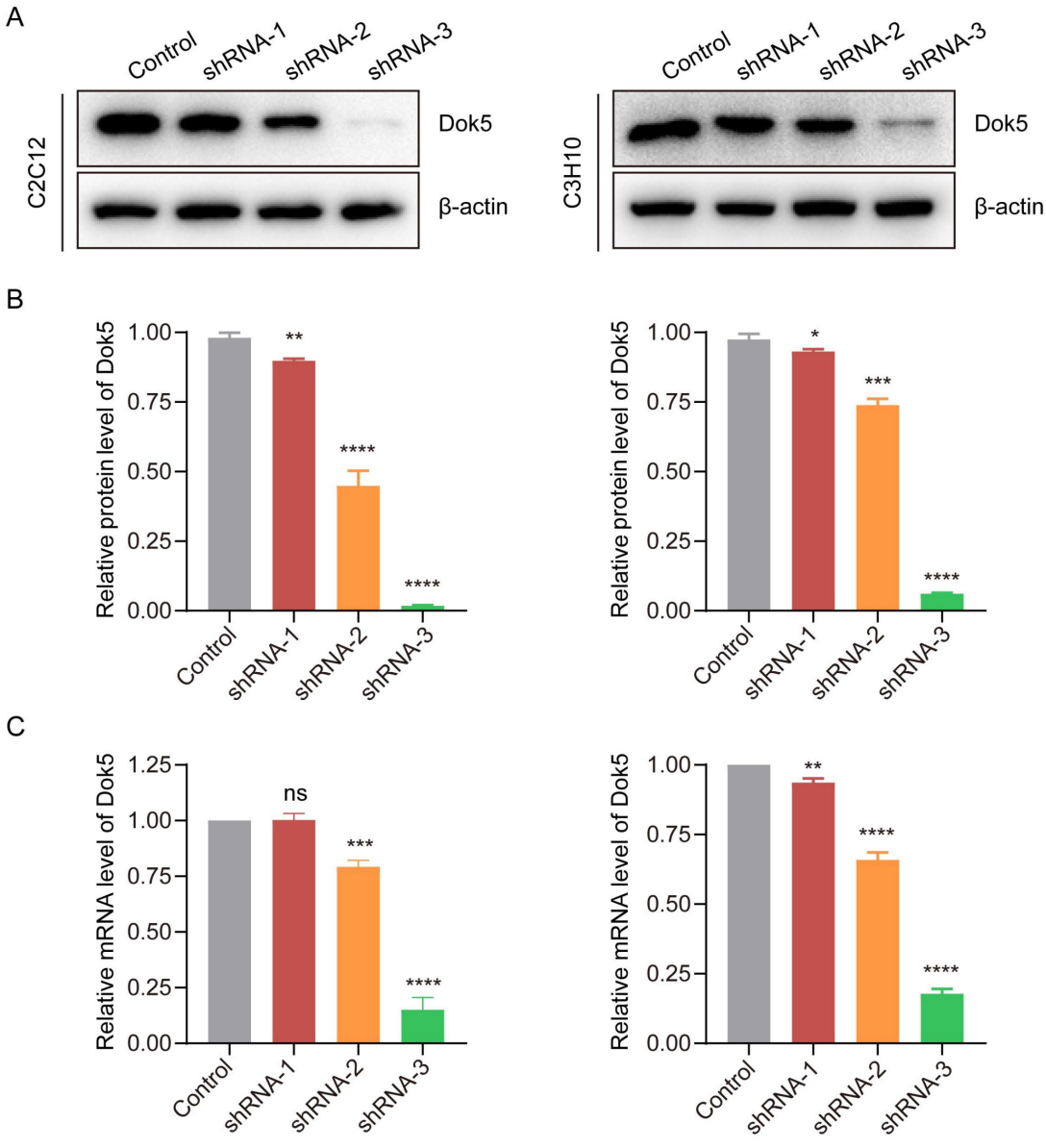

Figure 2. Determination of Dok5 knockdown cell lines.A) C2C12 and C3H10 cell lines were infected with 3 anti-Dok5 lentiviral shRNA or control, and Dok5 protein level measured by western blotting. B) Measurement of Dok5 silencing efficiency by protein level analysis in the C2C12 and C3H10 cell lines. C) RT-qPCR confirmed Dok5 knockdown. Data are indicated as the mean ± SD of 3 independent assays (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001).

Next, the samples (25 g of total proteins) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% skimmed milk at room temperature for 2 h and then treated with primary antibodies overnight 4°C while shaking. We then rinsed the membranes thrice using TBST, 15 min for each time, followed by conjugation with the appropriate HRP-conjugated secondary antibodies at RT for 1 h. ECL was used to develop the chemiluminescence signal.

RT-q PCR

On 0, 3, and 7 days, the Trizol reagent was used to extract total RNA from the C3H10 and C2C12 cells following the protocol provided by the manufacturer. The quality, as well as the concentration of the purified RNA was read on a Tecan Infinity 200 Pro porous plate reader. Reverse transcription (cDNA synthesis) was performed using PrimeScript™ RT Master Mix (Takara, Cat No. RR036B). RT-qPCR was done

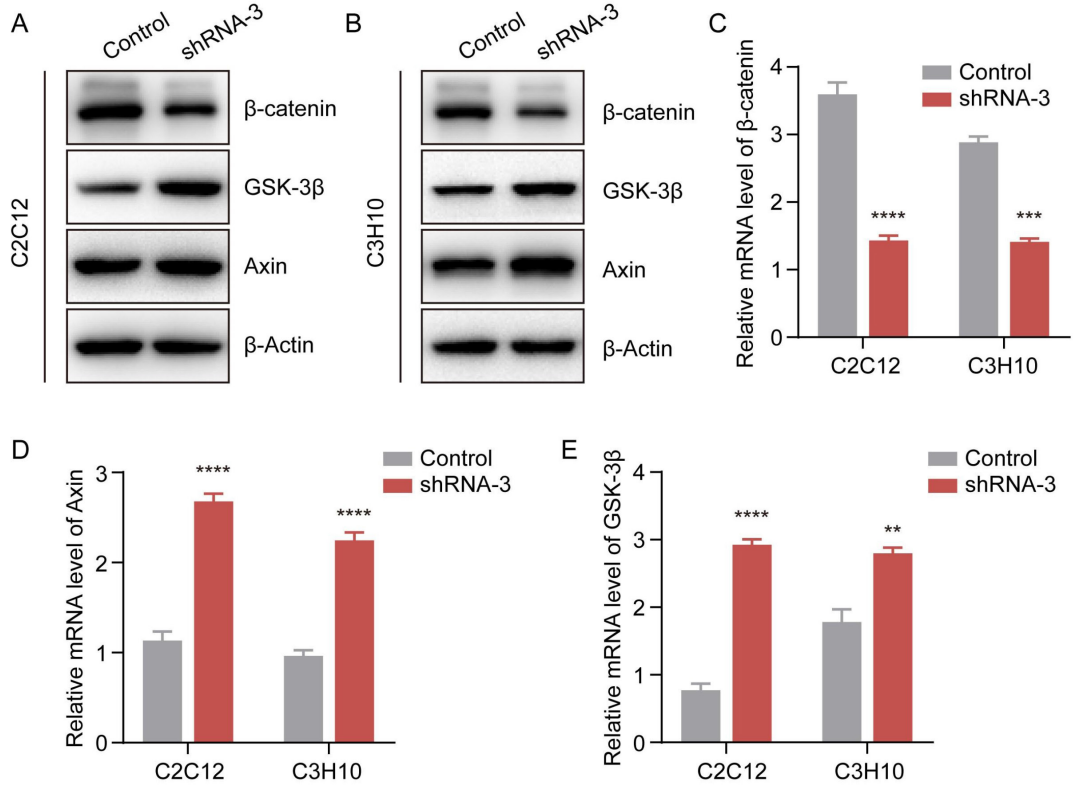

Figure 3. Dok5 silencing suppresses osteoblast proliferation and differentiation. A) Dok5 knockdown inhibited osteogenesis of C2C12 and C3H10 cells, detected by ALP staining and AR-S staining. B) Expression of OPN, OCN and Runx2 during osteogenesis when Dok5 knockdown. C) Cell proliferation was measured using CCK8 analysis when Dok5 silencing. Data are indicated as the mean ± SD of 3 independent assays (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001).

using the SYBR Green Premix Ex Taq™ (Takara, Cat No. RR820B) reagent in 3 wells per sample. Real-time PCR was conducted on an ABI7500 real-time PCR system under the following cycling conditions: 1) 95°C 10 min, 2) 95°C 15 s, 57°C 15s, 72°C 15s, for 40 cycles. The $2^{-\Delta\Delta}$ Ct method was used to estimate the relative gene expression, with the β-actin gene as the internal reference gene. The sequences of the primers used herein are indicated in Table 2.

Statistical analysis

All experiments were performed as independent triplicates. Data were analyzed using GraphPad Prism 7 and expressed as mean ± standard deviation (SD). The data between two groups were statistically compared by unpaired two-tailed student *t*-test. The data among multiple groups were statistically compared by using one-way ANOVA. A *P* value of less than 0.05 was considered as statistical significance.

Results

Osteogenesis induces Dok5 expression

Our results showed that Dok5 was stably expressed in both C2C12 and C3H10 cell lines (Figure 1A). On 0, 3, and 7 days, the expression levels of Dok5, OPN, OCN, and Runx2 in the C2C12 and C3H10 cell lines was detected by western blot and RT-qPCR. This analysis revealed a gradual rise in Dok5 expression levels along with a longer duration of osteogenic induction (Figure 1B-C). Moreover, the osteogenic differentiation markers, OPN, OCN, and Runx2, were elevated expression levels on 0, 3, and 7 days (Figure 1D). ALP and AR-S staining analyses revealed that the staining intensity of ALP and AR-S was increased along with a longer duration of osteogenic induction (Figure 1E), indicating a positive correlation between Dok5 expression and osteogenic differentiation. The staining intensity of ALP and AR-S in the C2C12 and C3H10 cell lines is shown in Figure 1F.

Determination of Dok5 knockdown cell lines

Western blot and RT-qPCR assays revealed Dok5-shRNA3 had the most potent knockdown (Figure 2A-C). Dok5-shRNA3 was therefore employed in all downstream experiments.

Dok5 silencing suppresses osteoblast proliferation and differentiation

ALP and AR-S staining analyses detected an increased staining intensity in both Dok5 knockdown and control cells

along with a longer duration of osteogenesis induction. However, the staining intensity was significantly weaker in Dok5 knockdown cells compared with that in control cells at all time points (Figure 3A), indicating that osteogenic potential was reduced upon Dok5 silencing. The expression levels of OPN, OCN, and Runx2 were down-regulated in Dok5 knockdown cells compared with that in the control group on 0, 3, and 7 days (Figure 3B). These data found that DOK5 silencing inhibits osteogenic differentiation. CCK-8 assay revealed a significantly slower proliferation of Dok5 knockdown cells at0, 24, 48, and 96 h compared with that of the control cells (Figure 3C), indicating that Dok5 is required for osteoblast proliferation.

Dok5 knockdown inhibits canonical Wnt/β-catenin signaling pathway

Compared with the control cells, the mRNA and the protein expression levels of β-catenin were significantly down-regulated, whereas the expression levels of GSK-3β and Axin, the negative modulators of canonical Wnt signaling pathway, were significantly up-regulated in the knockdown group (Figure 4A-B), suggesting that Dok5 can modulate the osteoblast differentiation via the canonical Wnt/β-catenin signaling pathway.

Overexpression of Dok5 promotes proliferation and osteogenesis and activates the canonical Wnt/β-catenin signaling pathway

To further verify the role of Dok5 in the proliferation and differentiation of osteoblasts, C2C12 (C2C12-cDNA) cells with Dok5 overexpression were established (Figure 5A). ALP and AR-S staining analyses showed that Dok5 overexpression significantly enhanced osteogenesis and extracellular mineralization (Figure 5B). Moreover, the expression levels of osteogenic biomarkers, OPN, OCN, and Runx2, in the C2C12-cDNA group were significantly up-regulated compared with those in the control group under osteogenesis induction (Figure 5C). In addition, overexpression of Dok5 increasingly activated the canonical Wnt/b-Catenin signaling pathway (Figure 5D). CCK-8 assay revealed a significantly faster proliferation in Dok5 overexpression cells compared with that in the control cells (Figure 5E).

Discussion

In this study, we demonstrated that Dok5 knockdown suppresses osteoblastic differentiation, as well as the expression of osteogenic biosignatures. Additionally, the canonical Wnt/β-catenin signaling cascade was suppressed by Dok5 knockdown. Besides, overexpression of Dok5 promoted the proliferation and osteogenesis and activated the canonical Wnt/β-catenin signaling pathway. Dok5 may regulate the osteogenic proliferation and differentiation through the canonical Wnt/β-catenin signaling pathway.

It is a challenging task to treat bone defects in clinical practice[15]. In previous studies, researchers have attempted to develop efficacious treatment options for bone defects by utilizing adult stem cells, and they proposed that bone marrow mesenchymal stem cells may offer novel clinical options for treating bone defects[16]. Osteogenic differentiation of bone marrow mesenchymal stem cells is a complex process influenced by physical, chemical and biological factors and governed by a wide variety of factors[17,18]. However, the efficiency of bone marrow mesenchymal stem cells into osteogenic differentiation is relatively low[19]. Consequently, profound comprehension of the underlying regulatory mechanisms is needed to develop certain strategies to promote the osteogenesis, thereby improving the efficacy in treating bone defects.

Dok5 is a member of the Doks family, and has been documented to modulate the proliferation and differentiation of various types of cells[20]. However, its role in osteoblasts has been poorly understood. In the present study, we found that Dok5 expression is up-regulated along with the longer duration of osteogenic induction, indicating that Dok5 may modulate the osteogenic differentiation. After silencing Dok5 in C2C12 and C3H1O cells, ALP and AR-S staining analyses revealed that osteogenic potential is reduced by DOK5 knockdown. Moreover, we observed that the expression levels of the osteogenic differentiation markers RUNX2, OCN and OPN are down-regulated, and the cell proliferation is reduced upon Dok5 knockdown, indicating that Dok5 knockdown inhibits the osteogenic proliferation and differentiation. To validate the above results, we established Dok overexpression cells and further confirmed that Dok5, as a positive regulator, can regulate the the proliferation and differentiation of osteoblasts.

In addition, our data show that Dok5 regulates the proliferation and differentiation of osteoblasts via the canonical Wnt/β-catenin signaling cascade. The Wnt/β-catenin signaling cascade is a pivotal regulator of stem cell proliferation, as well as differentiation, and is thought to be active in osteoblasts[21]. Wnt ligands are secreted proteins that bind to frizzled (FZD), a 7-pass transmembrane receptor, triggering the activation of downstream β-catenin pathway[22]. Wnt/β-catenin signaling pathway is thought to be involved in bone healing and influences cell proliferation, differentiation, self-renewal and cell fate decisions[23-26]. Importantly, Wnt signaling promotes the osteoblast production and osteogenic differentiation, thereby enhancing the quality of bone formation[27]. On the other hand, inactivating mutations affecting Wnt signaling antagonists, including Axin, GSK-3β, and APC, may enhance the bone mass[28]. In the present study, we observed that the β-catenin is decreased, and GSK-3β and APC are increased upon Dok5 silencing. In addition, after Dok5 overexpression, β-catenin is increased, whereas GSK-3 and APC are decreased. These findings are congruent with the transduction mechanism of the Wnt/β-catenin signaling cascade and highlight its importance in osteogenic differentiation.

A limitation of this study is that it only proves that Dok5 regulates the proliferation and differentiation of osteoblasts

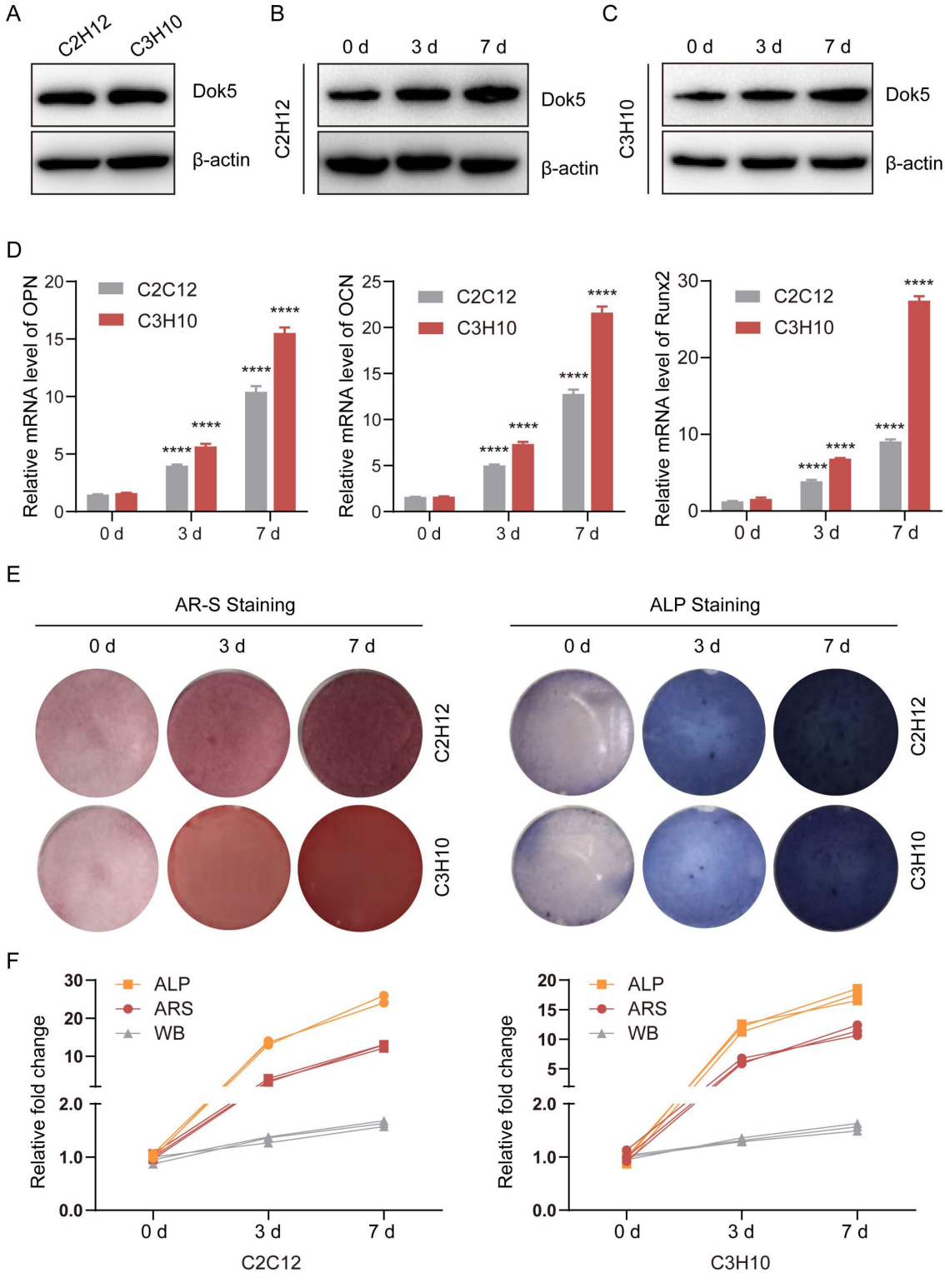

Figure 4. Dok5 knockdown inhibits canonical Wnt/ β-catenin signaling. A-B) Western blot analysis of β-catenin, GSK-3β and Axin, upon Dok5 knockdown vs control. C-E) Expression of β-catenin, GSK-3β and Axin when Dok5 downregulated. Data are shown as mean±SD of 3 independent experiments (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001).

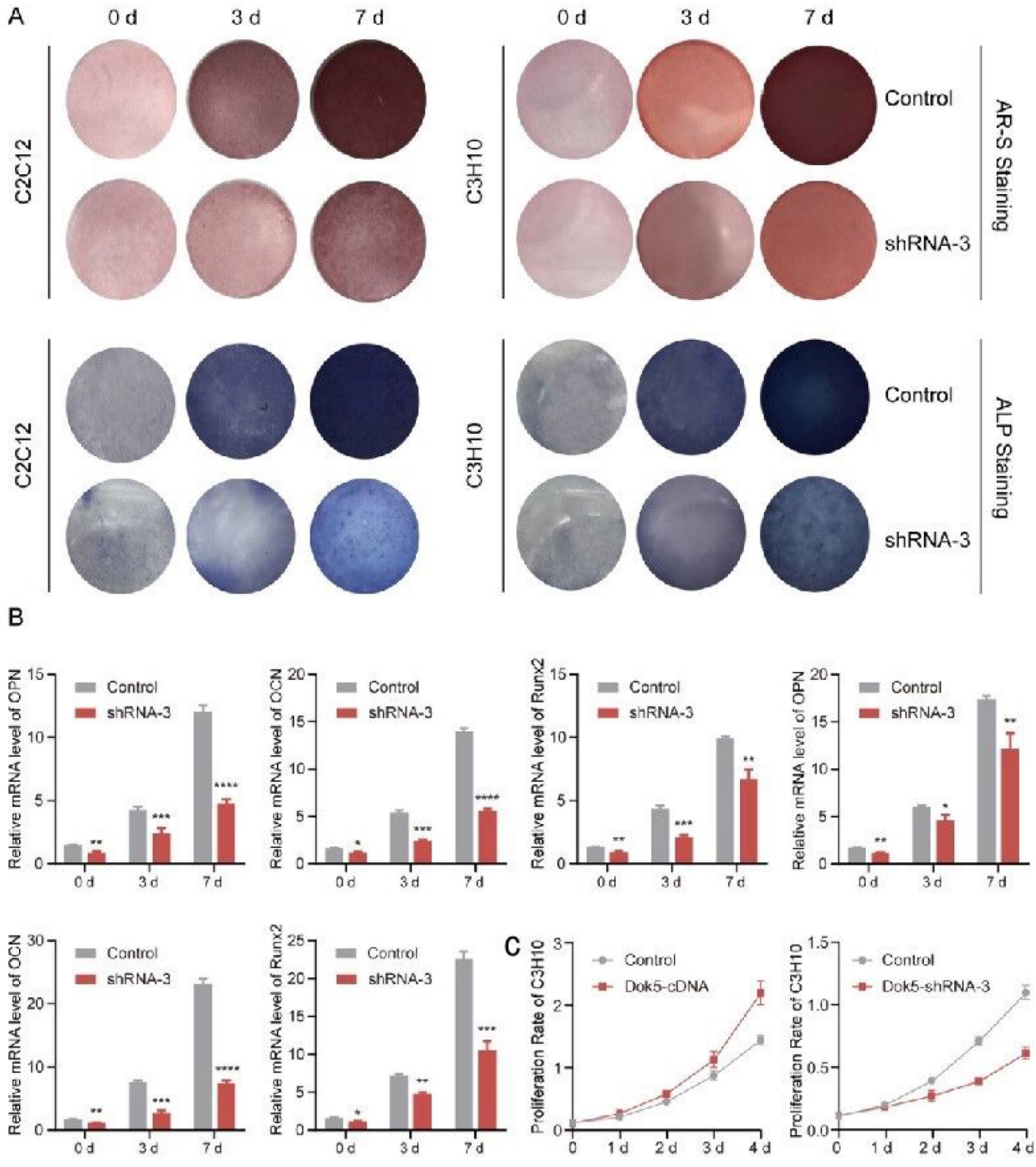

Figure 5. Overexpression of Dok5 promotes proliferation and osteogenesis, and activates the canonical Wnt/β-catenin signaling. A) Establishment of Dok5 overexpression C2C12 cells. B) Dok5 overexpression promoted osteogenesis of C2C12 cells, detected by ALP staining and AR-S staining. C) Expression levels of osteogenic biomarkers OPN, OCN, Runx2 were increased in Dok5-cDNA group. Compared with control group. D) Wnt/β-Catenin pathway was significantly activated when Dok5 upregulated. E) Cell proliferation was measured using CCK8 analysis when Dok5 upregulated. Data are indicated as the mean ± SD of 3 independent assays (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001).

in vitro, whereas *in vivo* experiment is lacking. Additionally, other signaling pathways, such as Notch, BMP/TGF-β, and PI3K/Akt/mTOR signaling pathways, may affect the bone development. The findings merely indicate that the Wnt/β-catenin signaling pathway is involved with the role of Dok5 in regulating the osteogenic differentiation. Whether other signaling pathways are also involved and the underlying regulatory mechanisms remain to be validated.

Conclusions

Taken together, Dok5 is an important negative modulator of osteoblast proliferation and differentiation *in vitro*. It can partially influence osteogenic differentiation via the canonical Wnt/β-catenin signaling pathway. Dok5 may be a promising target to develop a gene therapeutics to solve the problem of difficult regeneration of large bone defects. These findings provide experimental basis for promoting mesenchymal stem cell osteogenic differentiation, which may improve the clinical efficacy in treating bone defects.

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Authors' contributions

LX designed the study and drafted the manuscript. JW, HL and YY were responsible for the collection and analysis of the experimental data. SS and MW revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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