Am J Cancer Res 2021;11(6):3354-3374
www.ajcr.us /ISSN:2156-6976/ajcr0134909

Original Article

DLX5 promotes osteosarcoma progression via activation of the NOTCH signaling pathway

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Received May 4, 2021; Accepted May 25, 2021; Epub June 15, 2021; Published June 30, 2021

Abstract: The distal-less (dlx) homeobox transcription factors have been implicated roles in bone development. DLX5, in particular, was shown to play essential roles in osteoblast differentiation by targeting RUNX2, a master transcription factor for bone development. Interestingly, DLX5 has also been shown to play an oncogenic role in lung and other cancers, possibly via regulation of MYC expression. Given its dual roles in bone and cancer, this study aimed to investigate the effect of DLX5 on progression of osteosarcoma (OS), the primary bone cancer that is characterized by abnormal bone formation and osteoblast activity. Expression of DLX5 in OS cell lines was detected by quantitative real-time PCR (qRT-PCR) and western blot (WB). In vitro and in vivo assays were performed to investigate the oncogenic function of DLX5 in OS cells and xenograft models. Luciferase reporter assay was performed to determine the underlying mechanism of DLX5-mediated OS aggressiveness. The results showed that DLX5 was differentially expressed in OS cell lines, with significantly upregulated levels in HOS and MG-63 and relatively low levels in U2OS and 143B cell lines, compared with the normal bone cell line. DLX5 knockdown in HOS and MG-63 cell lines by siRNA inhibited OS cell growth and progression, and induced cell apoptosis and cell cycle changes both in vitro and in vivo. Meanwhile, DLX5 overexpression had the opposite effect on U2OS and 143B cell lines. Notably, a positive correlation between the expression patterns of NOTCH1 and DLX5 was also observed. The expression levels of NICD (NOTCH1 intracellular domain) and HES1 (classical target of NOTCH) were closely associated with DLX5 expression. Whereas knockdown of DLX5 in OS cells resulted in decreased expression of NOTCH1 and reduced cell proliferation and migration, which were rescued by overexpression of NOTCH1. We further analyzed DLX5 and NOTCH1 genes using JASPAR software and found two potential DLX5 binding sites within the *NOTCH1* promoter. Dual-luciferase assay demonstrated that DLX5 specifically activates the NOTCH1 promoter and controls its expression. Taken together, our results support that DLX5 plays an oncogenic role in OS development, which can at least partially, be attributed to activation of the NOTCH signaling pathway.

Keywords: DLX5, osteosarcoma, NOTCH signaling

Introduction

Osteosarcoma (OS) is the most common and malignant bone cancer with high morbidity in children and adolescents. With the application of neoadjuvant chemotherapy, the survival rate in OS patients with localized disease has been largely increased [1]. The five-year survival rate of OS may reach approximately 60-70% after effective surgical resection and neo-adjuvant chemotherapy. However, OS tends to have a high potential of local recurrence and lung metastasis [2, 3]. The underlying mechanism is still unclear. Therefore, it remains a challenge to identify new OS molecular markers and novel therapeutic targets for more effective treatment strategies [4].

DLXs family of transcription factors are known to participate in multiple tissue and organ development, which mainly affects cell growth and differentiation of the appendages, nervous

system, branchial arches, as well as tissues of hematopoiesis [5]. In mammals, six *DLX* gene clusters are arranged in three pairs of closely linked transcription units: Dlx1/Dlx2, Dlx3/Dlx4, and Dlx5/Dlx6 [6]. All *Dlx* genes have been shown to play a role in chondrogenesis and/or osteogenesis [7, 8]. Specifically, Dlx5 is expressed at very early stages of bone development and may play an essential role in the control of osteogenesis [9]. Osteoblast differentiation is achieved by activating a transcriptional network in which Dlx5, Runx2 and Osx/SP7 have fundamental roles [10]. Dlx5 can interact with Runx2 enhancer and other bone-specific genes, such as BMP, Msx to form a signaling network and influence osteoblast differentiation [11-13]. Multiple previous studies have also demonstrated a role of Dlx5 in craniofacial skeletal development and linked to expressions of Runx2, Osterix, OCN, and the Shh signaling pathway [14-18].

Interestingly, previous studies have reported DLX5 as an oncogene in lymphomas and lung cancers possibly via controlling expression of *MYC* oncogene [19]. It was shown that Knockdown of DLX5 in lung cancer cells resulted in decreased expression of MYC and reduced cell proliferation, while overexpression of MYC rescued the phenotypic consequence [19]. In an integrated analysis using microarray technologies, a genome-wide genetic and epigenetic profile was obtained from a dataset containing 19 human OS cell lines, and upregulated expression of DLX5 and RUNX2 was detected in some of the OS cell lines and clinical OS samples [20]. These research findings demonstrated that DLX5 plays dual functions in bone and cancer and directly interacts with Runx2, a master transcription factor essential for bone and cancer formation [10]. However, the effects and the underlying mechanisms of DLX5 on progression of OS, the primary bone cancer, remain to be determined.

The Notch signaling pathway, which includes multiple Notch receptors and ligands, is known to regulate multiple fundamental cellular processes essential for development [21]. Notch signaling molecules have also been associated with multiple malignancies, including T-ALL, gastric and esophageal cancer, colorectal cancer, uterine corpus endometrial cancer, lung adenocarcinoma, renal cell carcinoma, neuroblastoma and other cancers [22-29]. Mechanistically, binding of Notch ligands induces proteolytic cleavage of Notch receptor and releases the Notch intracellular domain (NICD), which enters into the nucleus to act on its target genes, including the hairy enhancer of split 1 (HES1) [30-32]. Interestingly, DLX5 was shown to regulate *NOTCH1* expression and may participate in cell differentiation of squamous cell carcinoma [33]. It is also well known that Notch signaling plays an important role in skeletal development and bone remodeling [34]. However, it is not clear whether DLX5 and NOTCH signaling work together to control OS development. Here, we surmise that DLX5 and NOTCH1 might both be involved in OS pathogenesis. In the present study, we analyzed the expression of DLX5 in OS cell lines, its correlation with NOTCH1 and the effect and potential mechanism of DLX5 and NOTCH1 signaling pathway on OS progression.

Material and methods

Cell culture

Human osteoblast cell line (hFOB1.19) and OS cell lines (HOS, MG-63, U2OS) were purchased from the Shanghai Cell Collection (Shanghai, China). 143B cells were purchased from the FuHeng Biology (Shanghai, China). hFOB1.19 cells were maintained in F12 medium. HOS and MG-63 cells were maintained in DMEM medium, 143B and U2OS cells were maintained in RPMI-1640 and MoCoy's 5A medium respectively. All medium (Biological Industries, USA) contained high glucose, 10% fetal bovine serum (Biological Industries, USA). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Cell transfection and establishment of stable cell lines

To overexpress DLX5, the full-length *DLX5* coding sequence was cloned into the LV18 lentiviral vector (GenePharma, Shanghai, China), and LV18 was used as a negative/vector control. According to the manufacturer's instructions, 143B and U2OS cells were infected with LV18-DLX5 or LV18. The infection efficiency can be evaluated by qRT-PCR and western blot. Puromycin was then used to select and establish stable expression cell lines. For DLX5 knockdown experiment, the siRNAs for DLX5

Table 1. Primers of transcription factors designed for qRT-PCR

	Sense Primers (5'-3')	Antisense Primers (5'-3')
GAPDH	CAGGAGGCATTGCTGATGAT	GAAGGCTGGGGCTCATTT
DLX5	CAACTTTGCCCGAGTCTTCA	GTTGAGAGCTTTGCCATAGGAA
NOTCH1	GGCACTTTCTGTGAGGAGGA	GCAGTCAGGCGTGTTGTTCT
COL10A1	ATGCTGCCACAAATACCCTTT	GGTAGTGGGCCTTTTATGCCT
RUNX2	TGGACGAGGCAAGAGTTT	CTTCTGGGTTCCCGAGGT
SOX2	GCTACAGCATGATGCAGGACCA	TCTGCGAGCTGGTCATGGAGTT
SOX9	TCAACGGCTCCAGCAAGAACAAG	ACTTGTAATCCGGGTGGTCCTTCT

(5-GUGCAGCCAGCUCAAUCAA-3) and the negative control siRNA (5-UUCUCCGAACGUGUCACGU-3) were synthesized by GenePharma (Shanghai, China). si-DLX5 or negative control (NC) were transfected into HOS and MG-63 cells using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific). Transfection of NICD (GenePharma, Shanghai, China) and luciferase reporter plasmids (GenePharma, Shanghai, China) was performed by using a Lipofectamine 3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. At 48 h after transfection, cells were processed for further experiments.

Real-time PCR (RT-PCR)

Total RNAs from OS cell lines were extracted using TRIzol Reagent (Invitrogen). RNA was reversely transcribed into cDNA using PrimerScript regent Kit with DNA Eraser (TAKARA). We performed real-time PCR to quantitate mRNA expression as described in the protocol supplied with UltraSYBR Mixture (CWBIO, China). *GAPDH* was chosen as an internal loading control. The relative expression of relevant genes was calculated by comparative Ct method. Specific primers were synthesized by Sangon Biotech for the amplification of the target genes. The primer sequences used for relevant genes were as listed (**Table 1**).

Western blot

Total protein was extracted from OS cell lines using RIPA buffer with proteinase inhibitor (Beyotime Biotechnology). Nuclear proteins were extracted from OS cells using Nuclear Protein Extraction Kit (Beijing Solarbio Science & Technology). Proteins of different molecular weights were separated by 10% SDS-PAGE (sodium dodecyl sulfate/polyacrylamide gel) electrophoresis and transferred onto nitrocellulose membranes. The membrane was blocked with Blocking Buffer (Beyotime Biotechnology) for 10 min and then incubated with a specific primary antibody overnight at 4°C. Then the membrane was incubated with the horseradish peroxidase enzyme (HRP) conjugated second antibody (Beyotime Biotechnology) at room temperature for 1 hour. The protein bands were detected and visualized by using the chemiluminescence Kit (Beyotime Biotechnology). Anti-GAPDH antibody was used to monitor the loading amount. The antibodies used were as the following: GAPDH (Sangon Biotech, D110016), DLX5 (Abcam ab109737), NOTCH1 (Santa Cruz Biotechnology, sc-376403), Histone H3 (Abcam, ab32356), NICD (Cell Signaling Technology, Val1744), HES1 (Cell Signaling Technology, D6P2U).

Cell counting kit-8 assay

Cells were seeded into a 96-well plate with a density of 5,000 cells per well after different treatments. At 24 h intervals, the medium was removed and then the cells were incubated with 10% CCK8 (Dojindo) medium for 2 h and the absorbance was detected at the wavelength of 450 nm using a microplate reader (Geneomaga).

Transwell migration and invasion assays

Migration and invasion assays of OS cells in vitro were performed using transwell chambers with 8 μm pores (BD Bioscience). For the migration assay, cells were harvested after the specific treatment, followed with trypsin digestion, washed twice with PBS, and resuspended in 100 μl serum-free medium at a concentration of 5×10^5 cells/ml. 100 μl cell suspension of each group was transferred into the upper transwell chamber, 600 μl complete medium was transferred into the 24 well-plate. After incubation for 24 h in an atmosphere containing 5% CO_2 at 37°C, the cells were fixed in 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 40 min. The number of migrated cells was counted in five randomly selected fields at 100× magnification using a microscope (OLYMPUS DP73). For the invasion assay, the transwell chambers were coated with Matrigel (BD Bioscience), and same proce-

dures as those for the migration assay above were followed.

Colony formation assay

For colony formation assays, approximately 500 cells were plated into 6-well plate and incubated for 15 days. Cells were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 40 min. The number of colonies was counted under a microscope (Nikon ECLIPSE).

Cell apoptosis and cell cycle analysis

Cell apoptosis analysis was performed using an FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA). Briefly, cells were harvested after the specific treatment, then digested with Trypsin Solutions without EDTA (Beijing Solarbio Science & Technology), washed twice with ice-cold PBS, and resuspended in 1× Binding buffer at a concentration of 1×10^6 cells/ml. Then, 100 μl cell suspension of each group was transferred into 1.5 ml EP tube, and 5 μl each of FITC Annexin V and propidium iodide (PI) staining solution were added. The single staining tubes of FITC and PI were prepared simultaneously. After incubation for 15 min at room temperature in the dark, 400 μl 1× Binding buffer was added to each EP tube, and the stained cells were analyzed using a FACSC alibur Flow Cytometer (Beckman CytoFLEX FCM).

Cell cycle analysis was performed using a BD Cycletest Plus DNA reagent kit (BD Biosciences). Briefly, cells were harvested after the specific treatment, followed with trypsin digestion, washed twice with PBS, and fixed in ice-cold 70% ethanol overnight at 4°C. The cells were centrifuged and washed twice with PBS to remove ethanol and were immediately added with 0.5 ml propidium iodide staining solution using a BD Cycletest Plus DNA reagent kit. After incubation for 15 min at room temperature in the dark, stained cells were further analyzed using FACS and at least 10000 cells were counted for each sample. Percentage of cells in each phase of cell cycle was measured using ModFitLT software.

Ethynyldeoxyuridine assay

OS cell lines were investigated using a Cell-Light EDU Apollp567 In Vitro Kit (RiboBio) according to the manufacturer's protocol. Briefly, cells were subjected to incubation with 10 μM EDU for 2 h. Then, the cells were fixed with 4% paraformaldehyde for 30 min, allowed for permeabilization with 0.5% TritonX-100 for 10 min, and washed with PBS for 5 min. For staining, cells were incubated with the prepared EDU staining reaction solution for 30 min and nuclei were counterstained with 5 μg/ml Hoechst33342 for 30 min. The cell proliferative ability was evaluated in five randomly selected fields under a microscope (Nikon ECLIPSE).

Dual-luciferase reporter assay

To elucidate the mechanism by which DLX5 regulates the expression of NOTCH1, we performed JASPAR (http://jaspar.genereg.net/) analysis to identify putative binding sites of DLX5 within the NOTCH1 promoter. The luciferase reporter plasmids containing wild-type or mutant NOTCH1 promoter sequences were constructed by GenePharma (Shanghai, China). The luciferase reporter plasmids and the Renilla luciferase reporter vector (pRL-TK) were co-transfected into the stable cell lines expressing DLX5 using Lipofectamine 3000 solution (Invitrogen). At 48 h after transfection, we performed luciferase assays using a luciferase reporter assay system (Promega, Madison, WI, USA). Luciferase/b-galactosidase activity was used to calculate luciferase activity.

Tumor xenograft

For in vivo assays, the HOS cells with DLX5 down-regulation were used. Nude mice (male BALB/c nu/nu, 4-5 weeks old) were purchased from Gem Pharmatech Co., Ltd. (Nanjing, China). The HOS cells with DLX5 down-regulation or negative control HOS cells were trypsinized, counted and resuspended in PBS. We established heterotopic subcutaneous and orthotopic tumor models in nude mice by injecting 5×10^6 HOS cells into the right abdominal back and the upper tibia of the right hind limb (n = 6 per group). Mice were weighed every 3 days for 30 days after injection. At the end of the experiment, the tumor tissue was removed and the tumor weight in each group was measured to evaluate the effect of DLX5 down-regulation on tumor progression. The tumor tissue was then collected for H&E and IHC assays to further analyze the expression of NOTCH1 and NICD. All procedures in this study were reviewed

in advance by the UJS IACUC committee and met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (UJS-IACUC-2020110401).

Immunohistochemistry

Fresh 5 μm sections were prepared from paraffin embedded OS and control tissue samples. The slides were baked at 65°C for 1 h, dewaxed in xylene and then hydrated. The slides were treated with 0.01 M (pH = 8.0) EDTA under high temperature and high pressure, and then incubated with 100 μl 3% hydrogen peroxide for 10 min at room temperature to block the activity of endogenous peroxidase. After cleaning, each slide was incubated with 50-100 μl primary antibody at 4°C overnight, and then incubated with 50-100 μl vision biosystems TM (rq7100) at room temperature for 20 minutes. The sections were incubated with DAB (3,3-diaminobenzidine), back stained, dehydrated, fixed, and then observed and photographed under the microscope. The antibodies were DLX5 (Abcam, ab109737), NOTCH1 (Signalway Antibody, #30991) and NICD (cell signaling technology, val1744).

H&E staining

Paraffin embedded tumor tissues were dewaxed with xylene and stained with hematoxylin for 5 min. After treatment with hydrochloric acid ethanol solution and ammonium hydroxide for 30 sec, the dye was stained with eosin for 1 min. The slides were dehydrated with 70%, 80%, 90% and 100% alcohol for 10 sec respectively. Then the slides were treated with xylene three times for 3 min each time. Finally, the film was sealed with neutral gum.

Statistical analysis

The data from in vitro experiments were expressed as mean ± SD from at least three independent experiments, and the data from in vivo experiments were expressed as mean ± SD from each group of independent experiments. The differences between two groups were analyzed using Student's t-test, and when more than two groups were compared, one-way ANOVA was used for analysis. $P<0.05$ was considered statistically significant. All the data were analyzed using GraphPad Prism software Version 6.0.

Results

DLX5 was differentially expressed in OS cell lines

To investigate whether DLX5 plays a role in OS development, DLX5 mRNA and protein expression in the normal bone cell line (hFOB1.19) and OS cell lines (HOS, MG-63, U2OS, and 143B) were detected by qRT-PCR and western blot analyses. The DLX5 expression level in OS cell lines HOS and MG-63 was markedly upregulated compared to normal bone cell line (hFOB1.19), but this difference was not significant in U2OS and 143B cells (**Figure 1A**). These results suggest a correlation between DLX5 expression and OS pathogenesis.

Knockdown of DLX5 inhibits OS cell growth in vitro

To investigate the effects of DLX5 on OS cell growth, OS cell lines HOS and MG-63 were transfected with si-DLX5, which leads to decreased expression of DLX5 compared to cells treated with si-NC (**Figure 1B**). These cells were then subjected to CCK-8 assay for assessment of cell viability. The results showed that inhibition of DLX5 in OS cells significantly inhibited their viability in a time dependent manner (**Figure 2A**). The EDU staining assay was also carried out to validate the effects of DLX5 silencing on OS cell proliferation. Compared with the cells transfected with non-specific siRNA, the cell proliferation transfected with DLX5 siRNA was remarkably decreased (**Figure 2B**). We have performed colony formation assay to investigate the effect of DLX5 silencing on OS cell clonogenicity and found that the average number of colonies formed by DLX5-siRNA cells were significantly less than the number formed by NC cells, and the sizes of the colonies were also reduced (**Figure 2C**). We further investigated the effect of DLX5 inhibition on the migration and invasion of OS cells by Boyden chamber. The cell migration assay showed that inhibition of DLX5 in HOS and MG-63 cells caused a significantly decreased number of migrated cells (**Figure 2D**), and knockdown of DLX5 in MG-63 cells significantly reduced the number of invaded cells (**Figure 2E**). Collectively, these results indicated that knockdown of DLX5 inhibits OS cell viability, proliferation, migration and invasion in vitro.

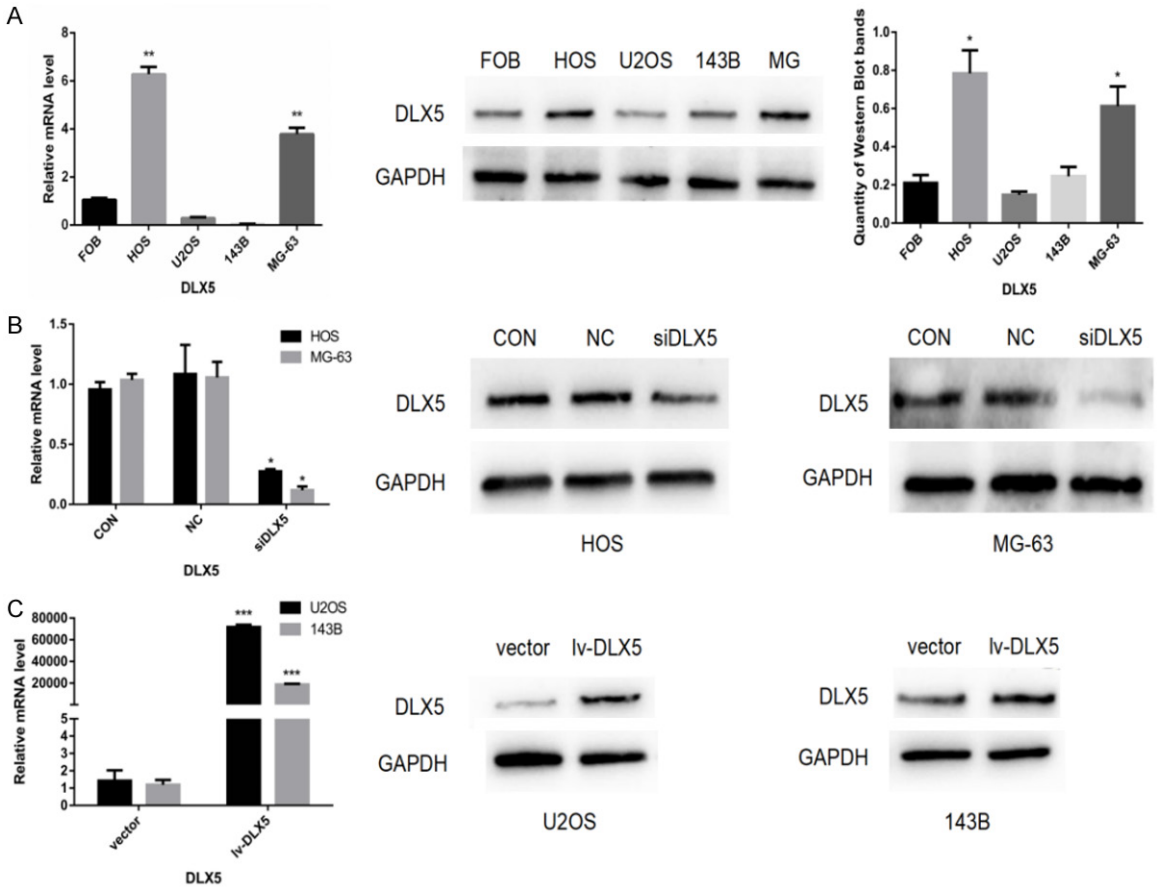


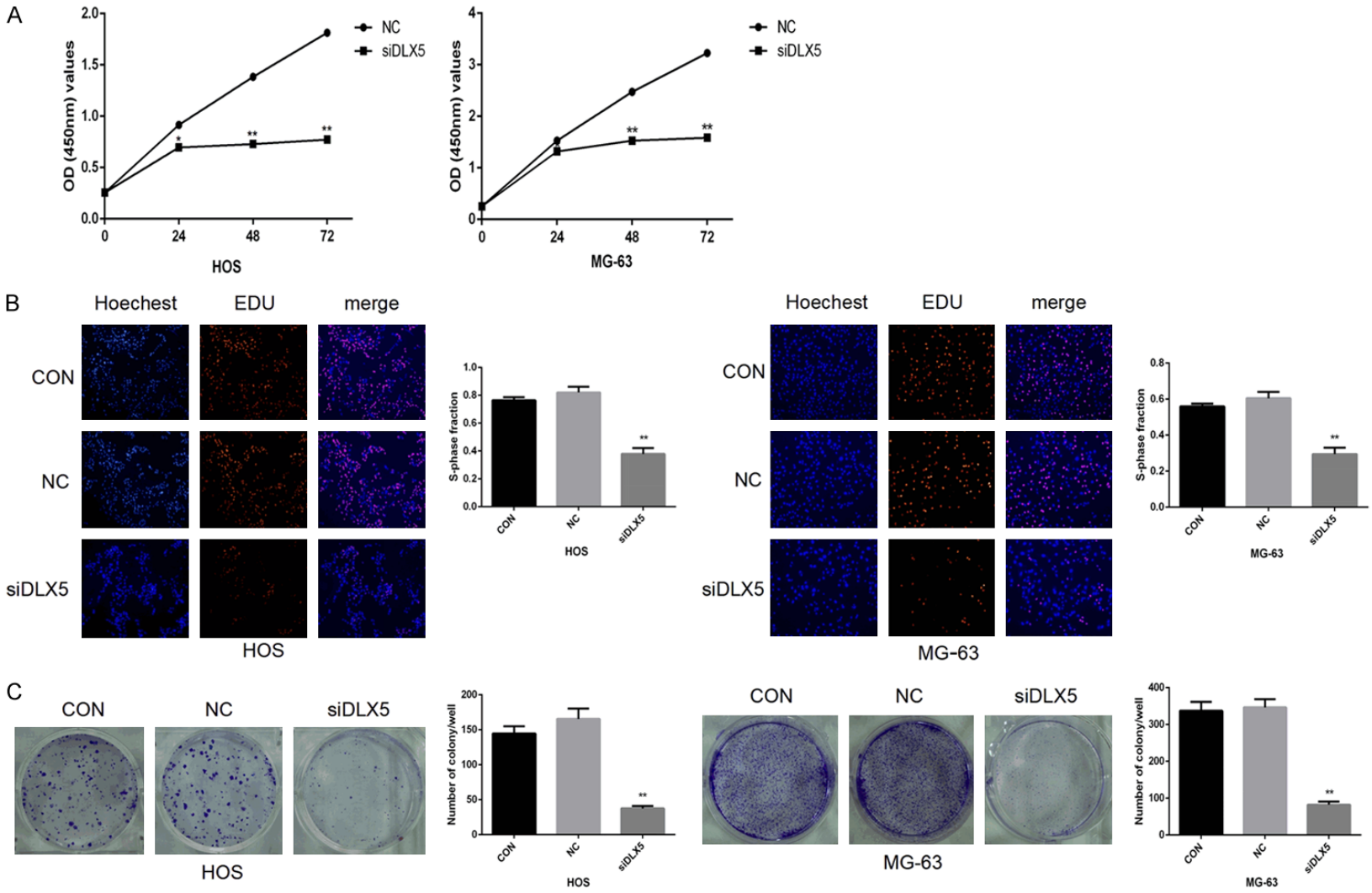
Figure 1. Differential expression of DLX5 in OS cell lines. A. qRT-PCR (left) and western blot analysis (right) of DLX5 expression in four OS cell lines and osteoblasts. The far-right bar graph is quantified of WB. qRT-PCR and western blot assays indicated that DLX5 was upregulated in HOS and MG-63 cell lines, but no difference with that of U2OS and 143B cell lines. DLX5 mRNA and protein levels were normalized to GAPDH. B. qRT-PCR and western blot assays showed that the expression of DLX5 was decreased in HOS and MG-63 cells transfected with DLX5 specific siRNA (siDLX5) compared with the untreated group (CON) and the group transfected with negative control siRNA (NC). C. qRT-PCR and western blot assays showed that the expression of DLX5 was increased in DLX5-overexpression stable cells (lv-DLX5) of U2OS and 143B compared with the negative control cell (vector). Data were presented as the mean ± s.d. of three independent experiments. ***P<0.001, **P<0.01, *P<0.05.

Knockdown of DLX5 increases OS cells apoptosis and G2/M phase percentage

We have performed flow cytometry analysis to measure cell apoptosis and cell cycle changes in OS cells after different treatments. Flow cytometry analysis showed that knockdown of DLX5 led to a significant increase in OS cell apoptosis (**Figure 3A**). The cell cycle analysis showed that a significant G2 cell cycle arrest accompanied by reduction in the fraction of cells in S phase were observed in both HOS and MG-63 cells (**Figure 3B**), suggesting that DLX5 inhibition was able to induce OS cell cycle arrest in G2 phase and inhibit DNA synthesis. These results together suggested that reduced OS cell proliferation by DLX5 inhibition may be associated with cell apoptosis induction and cell cycle arrest.

Overexpression of DLX5 promotes OS cell growth in vitro

To further validate the oncogenic role of DLX5 in OS, U2OS and 143B stable cell lines were established by infection with lentiviral-DLX5 or vector control (**Figure 1C**). CCK-8 assay was conducted and the results showed that overexpression of DLX5 significantly enhanced the viability of OS cells compared with that of the control group (**Figure 4A**). Next, we conducted the EDU staining assay to examine the effect of DLX5 on the proliferation ability of cells, and cells with DLX5 overexpression showed stron-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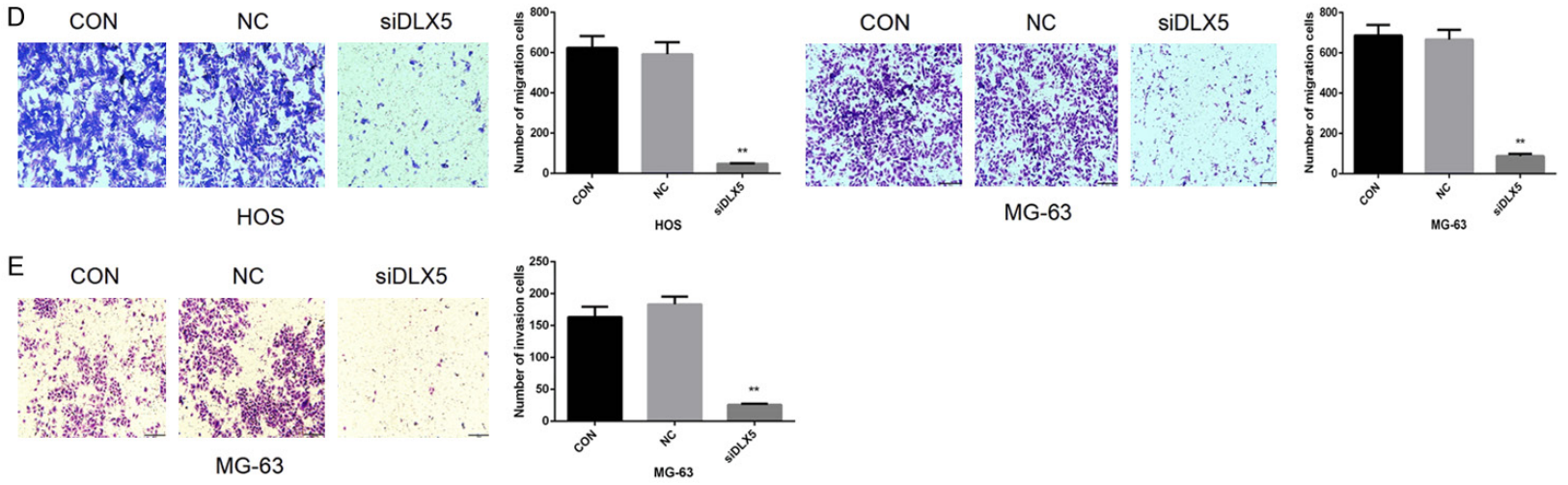
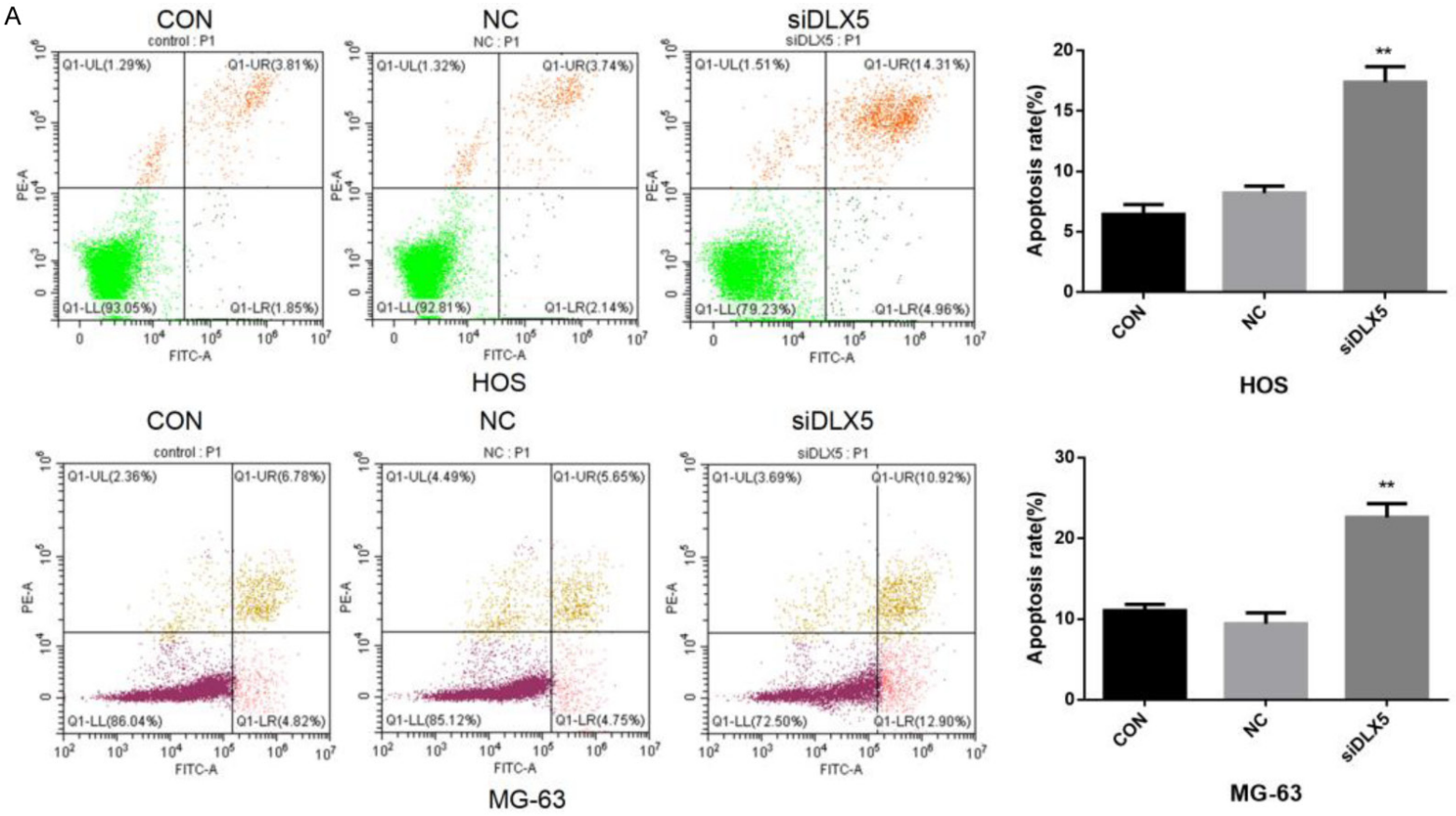


Figure 2. Knockdown of DLX5 inhibits OS cell growth in vitro. Cells were transfected with DLX5-siRNA (siDLX5) to knockdown DLX5, NC-siRNA (NC) and untreated (CON) cells were used as negative controls. A. Cell Counting Kit-8 assays indicated that the viability of HOS and MG-63 cells was inhibited following DLX5 knockdown. B. EDU assay showed that DLX5 knockdown reduced the EDU incorporation rate in HOS and MG-63 cells. The EDU staining (red) cells showed proliferative activity. C. Colony formation assay indicated that the clonogenicity was weakened following DLX5 silencing in HOS and MG-63 cells. D. Transwell assay showed that DLX5 knockdown reduced cell migration of HOS and MG-63 cells. E. Transwell assay also showed that DLX5 knockdown reduced MG-63 cell invasion. Data were presented as the mean ± s.d. of three independent experiments. $^{**}P<0.01$, $^{*}P<0.05$.


A
CON
control : P1
Q1-UL(1.29%)
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Q1-UR(3.74%)
Q1-LL(92.81%)
Q1-LR(2.14%)
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siDLX5 : P1
Q1-UL(1.51%)
Q1-UR(14.31%)
Q1-LL(79.23%)
Q1-LR(4.96%)
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FITC-A
HOS
Apoptosis rate(%)
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Q1-UR(6.78%)
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Q1-LR(4.82%)
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Q1-LR(4.75%)
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siDLX5 : P1
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Apoptosis rate(%)
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NC
siDLX5
MG-63

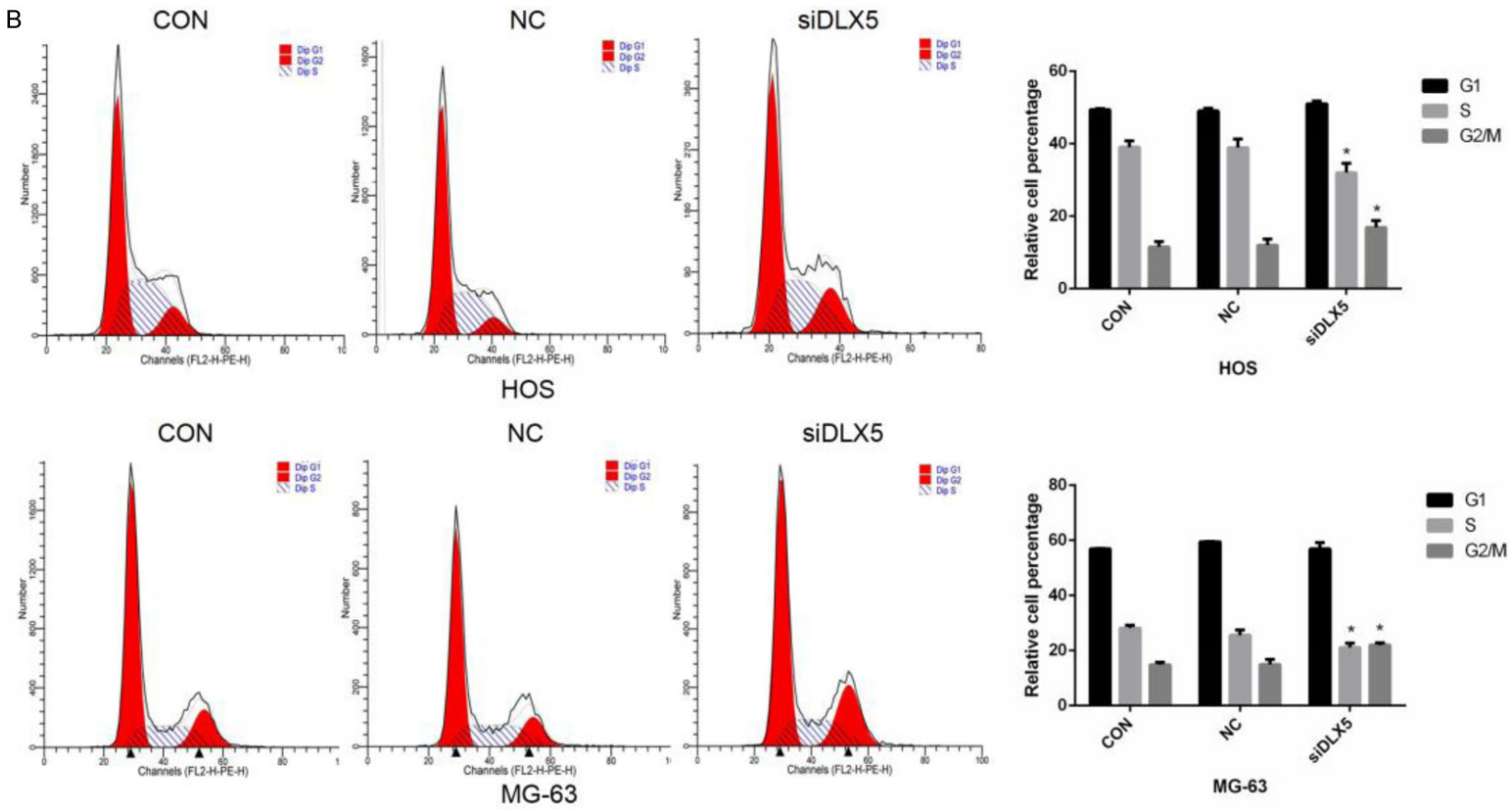


Figure 3. Knockdown of DLX5 increased OS cell apoptosis and G2/M phase percentage. A. Representative FACS images (left) and quantification bar plots (right) demonstrated an increase in the proportion of cell apoptosis following DLX5 knockdown in HOS and MG-63 cells. B. Cell populations in the G1, S and G2/M phases were analyzed by flow cytometry and showed in representative FACS images (left) and quantification bar plots (right). The number of S phase cells was decreased while the number of G2/M phase cells was increased following DLX5 knockdown in HOS and MG-63 cells. Data were presented as the mean ± s.d. of three independent experiments. $^{**}P<0.01$, $^{*}P<0.05$.

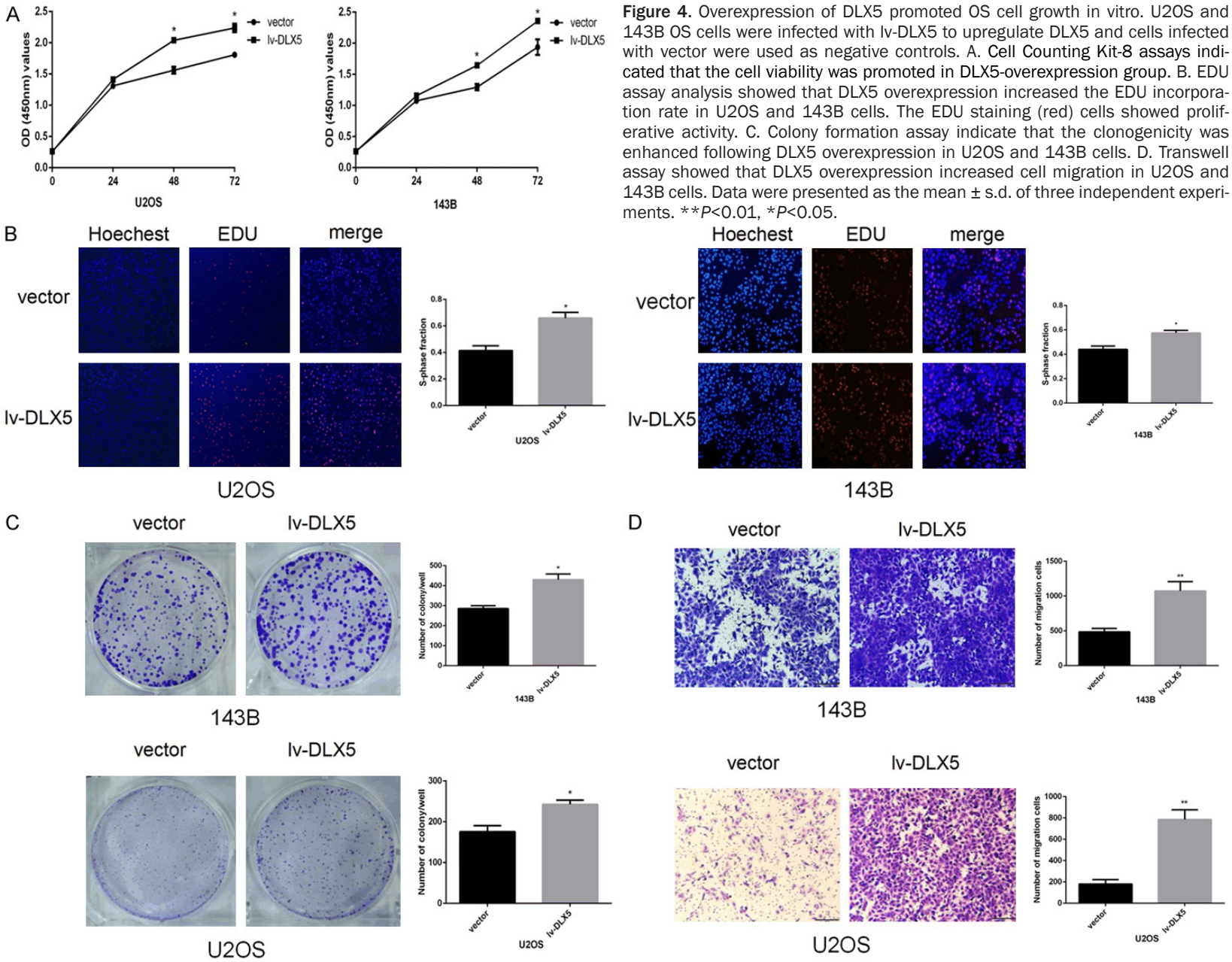


Figure 4. Overexpression of DLX5 promoted OS cell growth in vitro. U2OS and 143B OS cells were infected with lv-DLX5 to upregulate DLX5 and cells infected with vector were used as negative controls. A. Cell Counting Kit-8 assays indicated that the cell viability was promoted in DLX5-overexpression group. B. EDU assay analysis showed that DLX5 overexpression increased the EDU incorporation rate in U2OS and 143B cells. The EDU staining (red) cells showed proliferative activity. C. Colony formation assay indicate that the clonogenicity was enhanced following DLX5 overexpression in U2OS and 143B cells. D. Transwell assay showed that DLX5 overexpression increased cell migration in U2OS and 143B cells. Data were presented as the mean ± s.d. of three independent experiments. $^{**}P<0.01$, $^{*}P<0.05$.

ger proliferation ability than that of the vector control (**Figure 4B**). Meanwhile, DLX5 overexpression in these cells led to increased colony formation and enhanced proliferation ability in anchorage-dependent growth conditions (**Figure 4C**). To determine the migratory response of OS cells to DLX5 overexpression, a transwell assay was performed, and increased number of migrated cells were observed in cells infected with lentiviral-DLX5 compared with that transfected with lentiviral vector (**Figure 4D**).

DLX5 activates NOTCH1 by directly binding to its promoter

It was previously reported that DLX5 induced NOTCH1 expression is associated with keratinocytes differentiation [32]. We anticipated that NOTCH1 may be a key downstream target of DLX5 to promote tumorigenesis. To validate if DLX5 controls the expression of NOTCH1 in OS cells, we performed qRT-PCR to determine the levels and correlation between DLX5 and NOTCH1 expression. As shown in **Figure 5A**, DLX5 knockdown led to decreased NOTCH1 expression in mRNA level (**Figure 5A**). Meanwhile, DLX5 knockdown resulted in decreased expression of NOTCH1, NICD, and HES1 in protein level (**Figure 5B**). In contrast, DLX5 overexpression led to increased NOTCH1 and HES1 in 143 and U2OS cells (**Figure 5B**). The results demonstrated that DLX5 was indeed involved in the regulation of NOTCH1 and the signaling pathway. To explore the underlying mechanisms, we analyzed 2000 bp of the *NOTCH1* promotor using the web-based software JASPAR and found two potential DLX5 binding sites (**Figure 5C**). To determine which binding site of DLX5 might be functional, three vectors containing different *NOTCH1* promoter sequences (P1-P3) upstream of the luciferase gene were constructed (P1: full length of *NOTCH1* promotor; P2: cut off the first binding sites; P3: cut off the second binding sites). The results of luciferase reporter assay showed that the relative luciferase activities of the plasmids containing P1 or P2 promoter were both increased in the presence of DLX5 (**Figure 5D**). These results indicated that DLX5 promoted NOTCH1 expression and possibly the downstream signaling pathway via the two binding sites within the *NOTCH1* promotor.

NOTCH1 overexpression rescued the effect of DLX5 silencing on OS cells in vitro

Given that DLX5 promotes OS cell viability, proliferation and migration and that NOTCH1 is a potential target of DLX5, we next investigated whether DLX5 plays its oncogenic role through NOTCH signaling pathway. HOS and MG-63 cells were first transfected with siRNA-DLX5. Furthermore, we also overexpressed NOTCH1 in DLX5 knockdown cells by transfection with NICD expression plasmid and investigated the effects of NOTCH1 overexpression on DLX5 inhibition. As expected, NOTCH1 overexpression significantly attenuated the effects of DLX5 inhibition on cell viability (**Figure 6A**), proliferation (**Figure 6B**), migration (**Figure 6C**) and colony formation (**Figure 6D**).

DLX5 inhibition reduces OS progression in vivo via the NOTCH signaling pathway

To determine the impact of DLX5 on OS development *in vivo*, we established a xenograft tumor model in nude mice with negative control HOS or DLX5-knockdown HOS cells injection subcutaneously and orthotopically and evaluated the tumor progression of these cells. The results showed that DLX5 knockdown significantly reduced tumor size and weight (**Figure 7A**, **7B**). H&E-stained sections of mouse tumor tissue showed the tumor tissue morphology in different groups (**Figure 7C**). Tumors in nude mice in the negative control had typical morphological characteristics of malignant tumor cells: large nuclei, deep staining, multiple nucleoli, visible tumor giant cells, obvious nuclear abnormalities etc. Pathological nuclear fissions were uncommon and the number of tumor cells showed moderate decrease in DLX5 knockdown group. Next, we examined the expression of DLX5 (**Figure 7D**), NICD (**Figure 7E**) and NOTCH1 (**Figure 7F**) using IHC analysis. As shown, the brownish yellow or deep brownish yellow staining of DLX5, NOTCH1, NICD all diminished in DLX5 knockdown group compared with the negative control group. The IHC assay of OS tissue revealed that knockdown of DLX5 decreased the expression of NOTCH1 and NICD and inhibited OS cell progression in vivo.

Discussion

DLX5 is a family member of the distal-less homeobox protein that has previously been

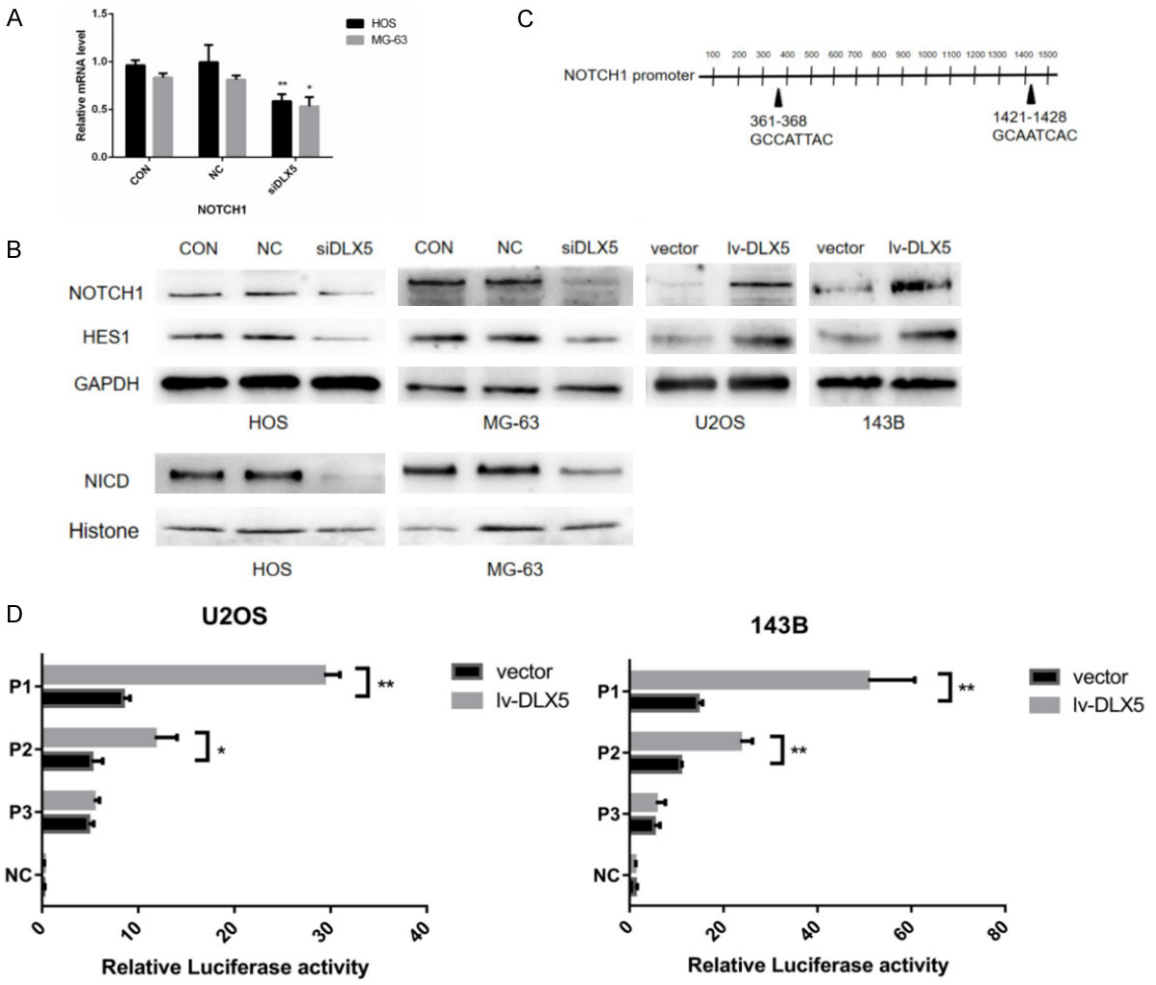


Figure 5. DLX5 activates NOTCH1 transcription and the signaling pathway by directly binding to its promoter. A. Expression of NOTCH1 was inhibited in DLX5 knockdown group as measured by qRT-PCR in the HOS and MG-63 cells. B. NOTCH1, NICD and HES1 protein levels were decreased with DLX5 knockdown in HOS and MG-63 cells. Meanwhile, NOTCH1 and HES1 protein levels were increased when DLX5 was overexpressed in U2OS and 143B cells. NOTCH1 and HES1 protein levels were normalized to GAPDH. NICD protein levels were normalized to Histone. C. Schematic diagram depicting the positions of the two binding sites of DLX5 in the *NOTCH1* promoter by JASPAR software prediction. D. Dual Luciferase assays showed luciferase activities were increased following transfection with plasmids containing P1 and P2 promoter as indicated in DLX5-overexpression cell lines of U2OS and 143B. Data were presented as the mean ± s.d. of three independent experiments. $^{**}P<0.01$, $^{*}P<0.05$.

implicated roles both in bone and in cancer development [35, 36]. DLX5 has also been shown to interact with RUNX2 and the NOTCH signaling pathway that are known to play essential roles during bone and cancer formation [11-13, 19, 20, 33, 34]. We therefore surmised that DLX5 may play a role in OS, the primary malignant bone cancer, via mechanisms relating to the NOTCH signaling pathway.

In this study, we examined the mRNA and protein levels of DLX5 in four OS cell lines HOS, MG-63, U2OS and 143B. Compared with human osteoblasts cell line hFOB1.19, OS cell lines HOS and MG-63 showed much higher level of DLX5 expression, but not significantly different in other two OS cell lines U2OS and 143B, suggesting an involvement of DLX5 in the pathogenesis of OS. Indeed, by knocking down of DLX5 in HOS and MG-63 cells, obviously decreased cell viability, proliferation and migration ability were observed in DLX5 interference group compared with the control group. Meanwhile, over-expression of DLX5 in U2OS and 143B cells confirmed its oncogenic function. Moreover, knockdown of DLX5 in

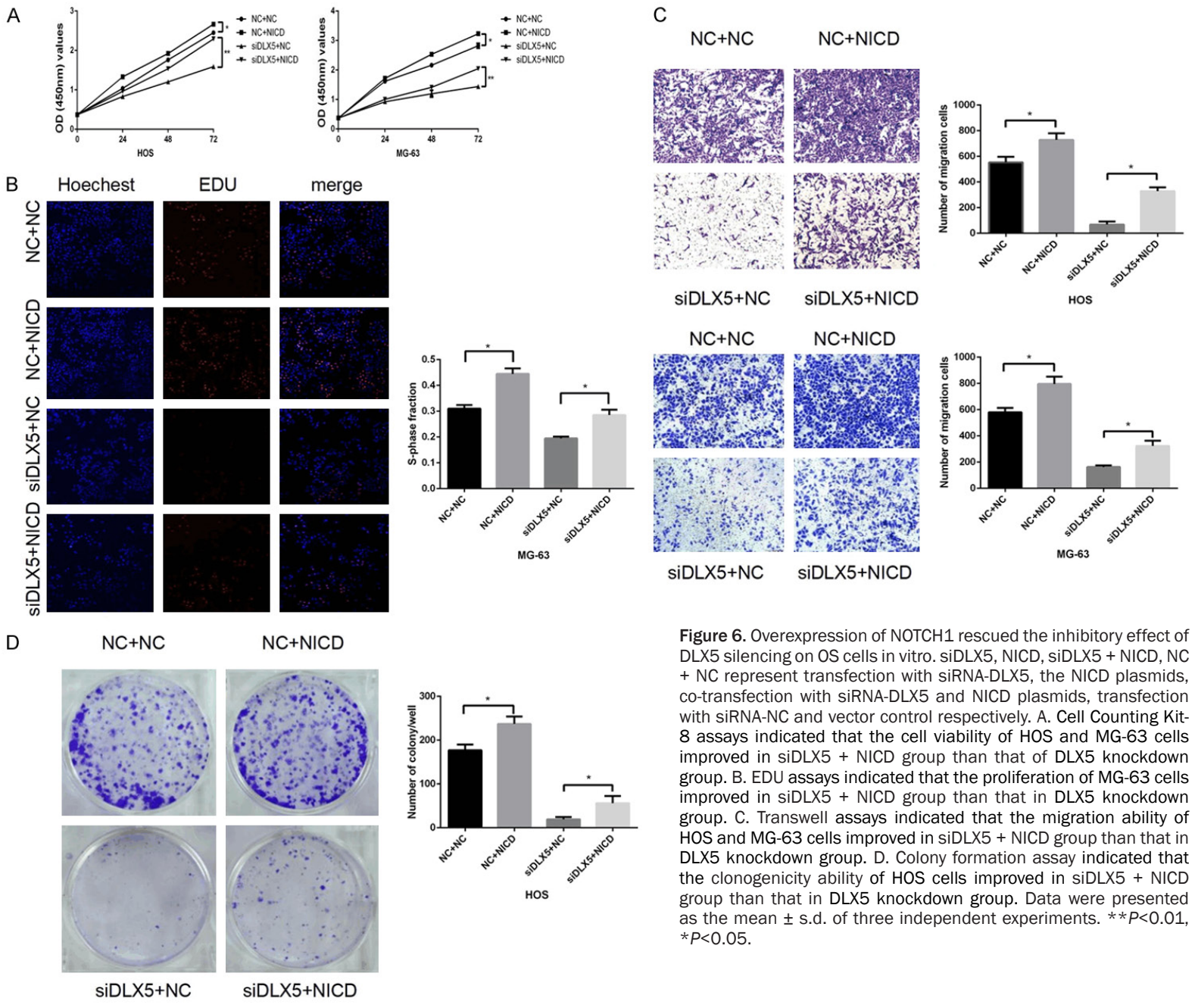
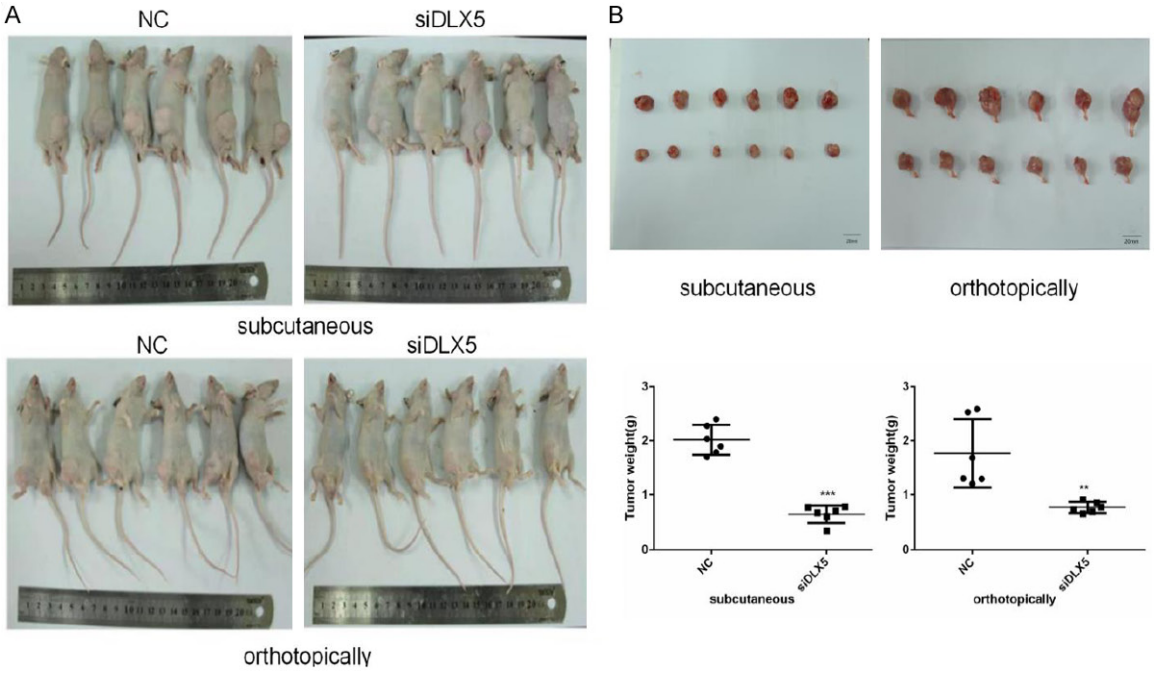


Figure 6. Overexpression of NOTCH1 rescued the inhibitory effect of DLX5 silencing on OS cells in vitro. siDLX5, NICD, siDLX5 + NICD, NC + NC represent transfection with siRNA-DLX5, the NICD plasmids, co-transfection with siRNA-DLX5 and NICD plasmids, transfection with siRNA-NC and vector control respectively. A. Cell Counting Kit-8 assays indicated that the cell viability of HOS and MG-63 cells improved in siDLX5 + NICD group than that of DLX5 knockdown group. B. EDU assays indicated that the proliferation of MG-63 cells improved in siDLX5 + NICD group than that in DLX5 knockdown group. C. Transwell assays indicated that the migration ability of HOS and MG-63 cells improved in siDLX5 + NICD group than that in DLX5 knockdown group. D. Colony formation assay indicated that the clonogenicity ability of HOS cells improved in siDLX5 + NICD group than that in DLX5 knockdown group. Data were presented as the mean ± s.d. of three independent experiments. $^{**}P<0.01$, $^{*}P<0.05$.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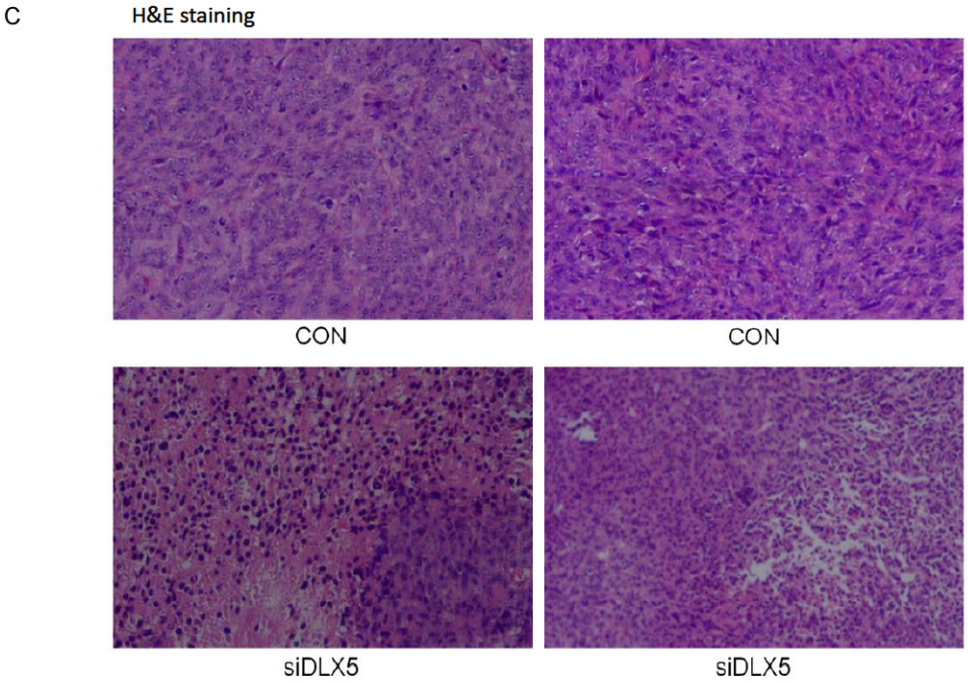
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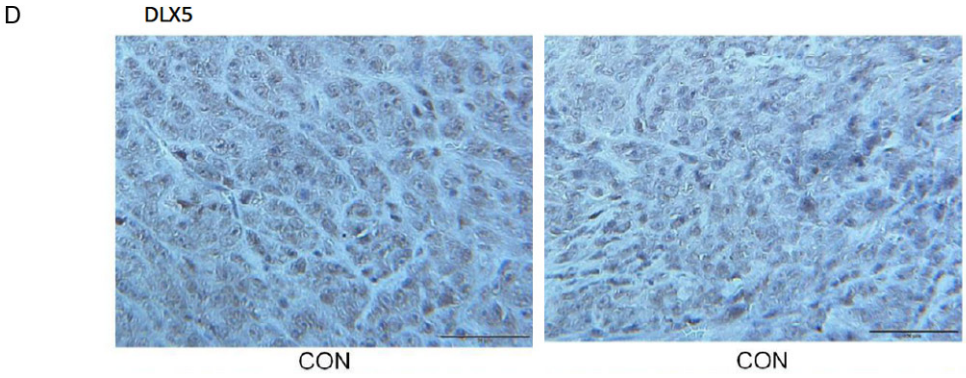
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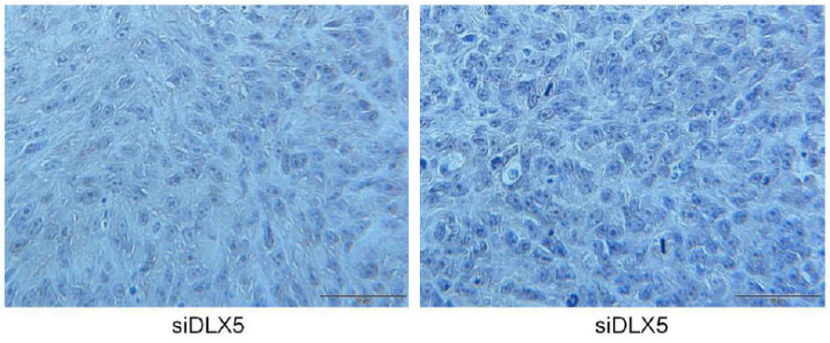
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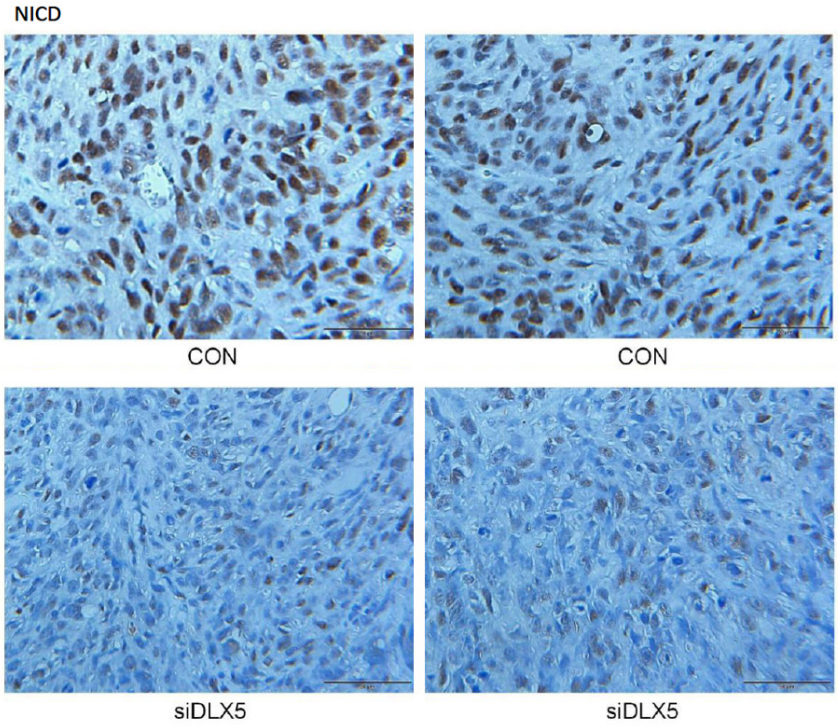
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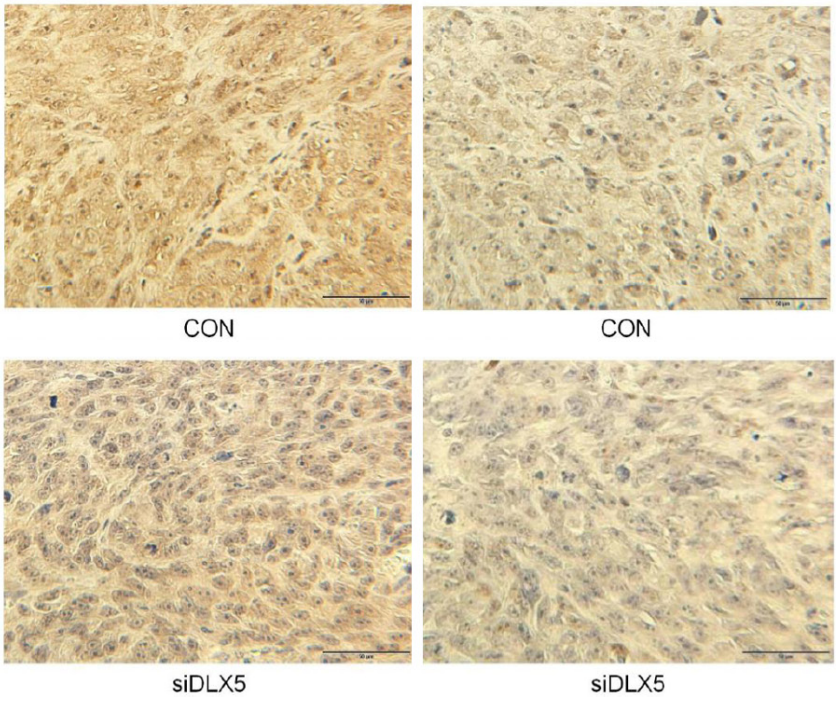
NOTCH1
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Figure 7. Knockdown of DLX5 inhibits OS cell tumorigenicity in vivo via regulation of NOTCH1 signaling pathway. (A) After tumor formation with HOS cells injection subcutaneously and orthotopically (n = 6 in each group), the gross tumor sizes of each group of mice were obviously decreased in DLX5 knockdown group (siDLX5) compared with the negative control (NC) group. (B) The tumor tissue images (upper: NC; under: siDLX5) and tumor weight graph formed in the subcutaneous and orthotopical injection groups showed that the tumor tissue size and tumor weight was reduced in DLX5 knockdown group compared with negative control. (C) H&E staining of tumor tissue showed obvious morphological characteristics of tumor tissue in NC group than that in DLX5 knockdown group (×200). (D-F) IHC assay was performed to confirm the decreased protein expression of DLX5 (D), NICD (E), and NOTCH1 (F) following DLX5 interference. (left: subcutaneous; right: orthotopical) (×400). ***P<0.001, *P<0.05.

HOS cells resulted in decreased OS tumor growth in xenograft tumor model in nude mice. To best of our knowledge, these results demonstrated for the first time, that DLX5 might be a key regulator of OS progression. Previous studies have indicated that knockdown of DLX5 resulted in reduced proliferation of ovarian cancer cells due to inhibition of cell cycle progression [37]. Notably, it has also been pointed out that DLX5 methylation, which downregulated DLX5 expression, correlated with MDS (myelodysplastic syndromes) transformation to leukemia, and worse prognosis of AML (acute myeloid leukemia), being consistent with the antiproliferative and proapoptotic effects of DLX5 on MDS derived AML cell line [38]. These findings suggest heterogenous mechanisms of DLX5 in progression of a variety of tumors.

As to OS pathogenesis, it is well known that OS is the most common and malignant bone cancer characterized by abnormal bone formation and aberrant osteoblast activity as its pathological features [1]. DLX5 may regulate OS progression via mechanism relating to bone formation. Indeed, previous studies revealed that Dlx5 plays an essential role in the regulation of chondrocyte differentiation as well as osteoblast differentiation during endochondral ossification [39-42]. More importantly, it has been shown that Dlx5 controls Runx2 expression and osteogenic differentiation in cranial facial bone development [43]. Dlx5 can upregulate Runx2 expression via direct interaction with its distal promoter, which is associated with BMP-2 signaling [44, 45]. This is intriguing, as RUNX2 has been found to be upregulated in human OS samples and in OS mouse models [46-50]. Given its close correlation with RUNX2, which plays significant roles both in bone and cartilage development and in OS pathogenesis [51, 52], DLX5 may synergistically work with RUNX2 together to influence OS development. However, we have performed expression profiling of following marker genes *COL10A1, RUNX2, SOX2* and *SOX9* in DLX5 knockdown HOS and MG-63 OS cell lines. We failed to detect significant change of these marker genes (data not shown), suggesting involvement of alternative mechanism regarding DLX5's oncogenic role in OS.

As a member of the NOTCH family, NOTCH1 is involved in many cellular processes, including cell proliferation, migration, differentiation, apoptosis, and determination of cell fate [53, 54]. NOTCH1 has also been implicated roles in many types of cancers, such as breast cancer (especially triple-negative breast cancer), leukemia, brain tumors, etc [55-58]. The association between DLX5 and NOTCH1 was first reported in human keratinocytes, which suggested that DLX5 is a direct positive regulator of NOTCH1 expression [32]. However, how DLX5 regulation of NOTCH1 signaling affects tumorigenesis of multiple cancers remains elusive. In this study, we observed that downregulation of DLX5 inhibited the mRNA and protein expression of NOTCH1, NICD and HES1 in OS cell lines. NOTCH1 and NICD were decreased in xenograft tumor tissues after DLX5 knockdown and as detected by IHC assay. Moreover, DLX5 inhibition in OS cells resulted in reduced cell proliferation and migration, whereas overexpression of NOTCH1 rescued the phenotypes caused by DLX5 downregulation. Further bioinformatics analysis and dual-luciferase reporter assay revealed that DLX5 may directly interact with NOTCH1 and control NOTCH1 transcription and the downstream target genes, thereby play its oncogenic role. Our findings provided new insights into the relationship between DLX5 and NOTCH1 in OS progression. However, one of the limitations of this study could be that there might exist alternative mechanism of DLX5's role in OS development, which needs further exploration.

Conclusion

In summary, we have for the first time identified DLX5 as an important oncogenic factor in OS,

and indicated its ability to promote OS cell proliferation, migration in vitro and tumor growth in vivo. We have also provided evidence that DLX5 interacts with the *NOTCH1* promoter and influences its expression, suggesting that DLX5 plays its oncogenic role in OS, at least in part via targeting the NOTCH1 signaling pathway.

Acknowledgements

This study was supported by the NSFC grants (No. 81672229, Q.Z; 81702111 to Q.W; 31671546 and 31871492 to C.C.), the support from Jiangsu Provincial key research and development program (BE2020679 to Q.Z.), the Shenzhen Science and Technology Program (Grant No. KQTD20170810154011370, Q.Z.), and the support from Dongguan Social Science and Technology Development (key) Project (2019507101163 to C.C.).

Disclosure of conflict of interest

None.

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