

# Notch signaling represses hypoxia-inducible factor-1 $\alpha$ -induced activation of Wnt/ $\beta$ -catenin signaling in osteoblasts under cobalt-mimicked hypoxia

CHEN-TIAN LI, JIAN-XIU LIU, BO YU, RUI LIU, CHAO DONG and SONG-JIAN LI

Department of Orthopedics, Zhujiang Hospital of Southern Medical University, Guangzhou, Guangdong 510282, P.R. China

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**Abstract.** The modification of Wnt and Notch signaling pathways by hypoxia, and its association with osteoblast proliferation and apoptosis remain to be fully elucidated. To investigate Wnt-Notch crosstalk, and its role in hypoxia-induced osteoblast proliferation and apoptosis regulation, the present study investigated the effects of cobalt-mimicked hypoxia on the mouse pre-osteoblast-like cell line, MC3T3-E1, when the Notch signals were repressed using a  $\gamma$ -secretase inhibitor DAPT. The data showed that the cobalt-mimicked hypoxia suppressed cell proliferation under normal conditions, but increased cell proliferation under conditions of Notch repression, in a concentration-dependent manner. The results of western blot and reverse transcription-quantitative polymerase chain reaction analyses showed that the cobalt treatment increased the levels of activated  $\beta$ -catenin protein and the expression levels of the target genes, axis inhibition protein 2 and myelocytomatosis oncogene, under DAPT-induced Notch repression. However, no significant changes were found in the expression levels of the Notch intracellular domain protein or the Notch target gene, *hes1*. In a  $\beta$ -catenin gene-knockdown experiment, the proliferation of the MC3T3-E1 cells under hypoxia were decreased by DAPT treatment, and knockdown of the expression of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) suppressed the cobalt-induced increase in Wnt target gene levels. No significant difference in cell proliferation rate was found following DAPT treatment when the expression of HIF-1 $\alpha$  was knocked down. The results of the present study showed the opposing effects of Wnt and Notch signaling under cobalt-mimicked hypoxia, which were partially regulated by HIF-1 $\alpha$ . The results also showed that osteoblast proliferation was dependent on Wnt-Notch signal crosstalk.

## Introduction

In mammalian skeletal systems, several signaling pathways are important for bone development, including the transforming growth factor- $\beta$ /bone morphogenetic protein, Hedgehog, Wnt, Notch and mitogen-activated protein kinase signaling pathways. They control osteoblast differentiation, proliferation, maturation and cytokine secretion via a number of mechanisms. Typically, these signaling pathways have their own canonical transduction pathways, and each of these has a central factor, which identifies their pathway. However, as investigations on signaling networks have progressed, signaling crosstalk has been found to act as a critical function to achieve tight signaling regulation and maintain a balance between cell signaling pathways. In particular, the orchestral regulation of Notch and Wnt signaling activity by the pathway networks is an important method, which regulates osteoblast differentiation and proliferation procedures, and may cause specific osteoblast behaviors (1).

The current view is that the Notch-Wnt signaling crosstalk is critical in controlling cell fate, and the importance of this crosstalk between the Notch and Wnt signaling pathways has led to suggestion that it be termed 'Wntch' (2). Typically, Notch and Wnt signaling often have opposing roles in osteoblasts (3-5). If one signal pathway is disrupted, the other responds to the Notch-Wnt signaling crosstalk. Therefore, every factor that affects Notch or Wnt signaling introduces the potential to produce marked crosstalk disruption and cause multiple cell changes.

Among all the environmental factors that disrupt the Notch-Wnt signaling pathways, hypoxia and hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is of particular interest due to the frequent presentation of hypoxic conditions in the pathologic microenvironment of bones (6,7). Hypoxia not only initializes the HIF-1 $\alpha$  pathway, but also interrupts other pathways, which are vital for bone development and survival, including the Notch and Wnt pathways. According to certain studies, HIF-1 $\alpha$  induces a hypoxic condition, which can promote Notch signaling activity by upregulating the levels of Notch intracellular domain (NICD) via several direct and indirect mechanisms (8,9). However, the effect of hypoxia and HIF-1 $\alpha$  on Notch-Wnt signaling crosstalk remains an area of debate.

Although accumulating evidence indicates that an increase in Wnt signaling activity is observed in hypoxic

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*Correspondence to:* Professor Song-Jian Li, Department of Orthopedics, Zhujiang Hospital of Southern Medical University, 253 Gongye Road, Guangzhou, Guangdong 510282, P.R. China  
E-mail: lisongjian321@126.com

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conditions (10), other studies have reported contradictory conclusions. In particular, the mice osteoblast-like cell line, MC3T3-E1, showed a downregulatory trend of Wnt signaling activity in hypoxic conditions, according to previous studies (11,12). However, the regulation of the Wnt signaling pathway in hypoxia and its molecular mechanisms remain to be elucidated.

In addition, evidence has indicated that HIF-1 $\alpha$  can promote Notch signaling activity in hypoxic conditions (8); it is a reasonable hypothesis that HIF-1 $\alpha$  can modify osteoblast growth through Notch-Wnt signaling crosstalk. However, the exact role of Notch-Wnt crosstalk in hypoxic conditions remains to be fully elucidated.

To investigate the remaining questions, the present study aimed to investigate how Notch-Wnt signaling crosstalk regulates Wnt signaling and the survival of the MC3T3-E1 osteoblast-like cell line in cobalt-mimicked hypoxic conditions. The potential role of HIF-1 $\alpha$  in the underlying molecular mechanism was also investigated. The current study may provide evidence to explain the potential mechanism of how hypoxia regulates osteoblast proliferation, and elucidate a solution to regulate the osteoblast proliferation under pathological conditions, particularly in the hypoxic microenvironment.

## Materials and methods

**Cell line and cell culture.** The MC3T3-E1 cells were provided by the Cell bank of the Chinese Academy of Sciences (Shanghai, China). All cells were incubated with  $\alpha$ -MEM, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The culture medium was replaced every 2 days. All cells were incubated in a cell incubator with 5% CO<sub>2</sub> at 37°C. For all of the following assessments, the cells were seeded in 6-well plates at 6x10<sup>3</sup> cells/cm<sup>2</sup>, unless specifically mentioned otherwise.

**DAPT and CoCl<sub>2</sub> treatment.** The  $\gamma$ -secretase inhibitor, DAPT (Selleck Chemicals, Shanghai, China), was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) at a 10 mM final concentration. To inhibit Notch signaling activity, the MC3T3-E1 cells in each treatment group were treated with 10, 20, 30 or 40  $\mu$ M DAPT, which was added into the culture medium, according to the different experiment requirements. To eliminate the interruption effect of DMSO, the final concentration of DMSO in the different groups was adjusted to ensure equal quantities by adding an additional DMSO into the culture medium.

To establish a model of chemical hypoxia *in vitro*, the cell lines were treated with 100  $\mu$ M CoCl<sub>2</sub> (cat. no. C8661; Sigma-Aldrich), which was added directly into the culture medium to mimic a hypoxic condition. In the DAPT+CoCl<sub>2</sub> treatment group, CoCl<sub>2</sub> and DAPT were administered simultaneously.

The medium containing DAPT and CoCl<sub>2</sub> to incubate the cell lines was replaced every 2 days. The treatment was confirmed effective by performing western blotting.

**Small interfering (si)RNA transfection, and knockdown of the gene expression of HIF-1 $\alpha$  and  $\beta$ -catenin (Ctnnb1).** For the

gene expression knockdown experiments, the mRNA expression of Ctnnb1 and HIF-1 $\alpha$  were inhibited by performing siRNA transfection. The siRNAs were purchased from ViGene Biosciences, Inc. (Rockville, MD, USA). The siRNA oligoconstruction used in the experiments were as follows: siRNA for HIF-1 $\alpha$ , sense 5'-UCAUCCAAGGAGCCUUAATT-3' and antisense 5'-GUUAAGGCUCCUUGGAUGATT-3'; and siRNA for ctnnb1, sense 5'-AGGACAAGCCACAGGAUUATT-3' and antisense 5'-UAAUCCUGUGGCUUGUCCUTT-3'.

The cells were transfected using 3-5  $\mu$ g siRNA mixed with Lipofectamine 2000 (Life technologies; Grand Island, NY, USA) in a 6-well plate at a density of 5x10<sup>5</sup>/well. All transfection procedures were performed in strict accordance with the Lipofectamine 2000 reagent protocol. The gene knockdown efficiency was confirmed by a reverse transcription-polymerase chain reaction (RT-qPCR) assay, and the rates of protein production were determined using western blotting.

**Cell viability assessment using a cell counting kit-8 (CCK-8).** The cells were seeded into a 96-well plate at a density of 6x10<sup>3</sup>/well, and each treatment group (n=5) was treated with a different concentration of DAPT. The normal oxygen tension (normoxia; Nx) groups were treated with 0, 10, 20, 30 and 40  $\mu$ M DAPT, respectively, and the cobalt mimicked-hypoxia (Hx) groups were treated with 0, 10, 20, 30 and 40  $\mu$ M DAPT, respectively. All Hx groups received 100  $\mu$ M of CoCl<sub>2</sub>. The CCK-8 assays were performed, according to the manufacturer's protocol. Following treatment for 24, 48 and 72 h at 37°C, the medium was replaced with 100  $\mu$ l fresh  $\alpha$ -MEM and 10  $\mu$ l CCK-8 solution (cat. no. CK04-500; Dojindo Laboratories, Kumamoto, Japan) and incubated. The absorbance value was assayed using an ELISA reader at 450 nm following 2 h incubation at 37°C.

**Acridine orange (AO) staining and fluorescence microscopy.** To assess cell viability under the microscope, the cells seeded in a 24-well plate were divided into four groups (n=4): i) Normal oxygen tension groups (Nx groups); ii) 100  $\mu$ M cobalt-mimicked hypoxic conditions (Co groups); iii) 20  $\mu$ M DAPT in normoxic conditions (Nx + DAPT groups); and iv) 20  $\mu$ M DAPT in 100  $\mu$ M cobalt-mimicked hypoxic conditions (Co + DAPT groups). Following treatment for 48 h at 37°C, the cells were stained using AO fluorescent dye (cat. no. A9231; Sigma-Aldrich). Images were captured using a fluorescence microscope (Olympus Corporation, Tokyo, Japan) with 488 nm laser emission and 515 nm laser filter at 200x magnification.

**Cell apoptosis assays using flow cytometry.** The analysis of apoptosis was performed on the following four groups: i) Control; ii) inhibitor group with 30  $\mu$ M DAPT; iii) chemical hypoxia group with 100  $\mu$ M CoCl<sub>2</sub> and iv) hypoxia and inhibitor group with 100  $\mu$ M CoCl<sub>2</sub> and 30  $\mu$ M DAPT. The cells in each group were incubated in the treatment medium for 48 h at 37°C. The ratio of cell viability was detected using an Annexin-V/Propidium iodide staining kit (Invitrogen; Thermo Fisher Scientific, Inc.) and profiling was performed using flow cytometry (FACSVerse, BD Biosciences, Franklin Lakes, NJ, USA).

**Western blot analysis.** To detect changes in protein levels in the MT3C3-E1 cells, the cells from the different treatment groups were harvested and 1 ml of 1X radioimmunoprecipitation assay buffer (Cell Signaling Technology, Inc., Beverly, MA, USA; cat. no. 9806S) was used for protein extraction. The protein concentrations were measured using a Bicinchoninic Acid Protein assay kit (cat. no. 23225; Pierce Biotechnology, Inc., Rockford, IL, USA). Equal quantities of cellular proteins (30  $\mu$ g) were separated using 10% SDS-PAGE. The proteins were transferred onto PVDF membranes, and the membranes were blotted with the following primary antibodies: NICD (1:1,000; cat. no. 2421; Cell Signaling Technology, Inc.); activated  $\beta$ -catenin (1:1,000; cat. no. 05-665; EMD Millipore, Billerica, MA, USA); HIF-1 $\alpha$  (1:1,000; cat. no. ab2185; Abcam, Cambridge, UK). Following overnight incubation at 4°C with the primary antibodies at a recommended dilution, the membranes were washed with phosphate-buffered saline with Tween (PBST) and then incubated with secondary antibodies at 20°C; for active- $\beta$ -catenin, goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP) antibody (1:10,000; cat. no. BS12478; Bioworld Technology, Inc., St. Louis, MN, USA) was used, for HIF-1 $\alpha$  and NICD, goat anti-rabbit IgG (H+L)-HRP (1:5,000; cat. no. AP307P; EMD Millipore) was used. Following 90 min incubation, the membranes were washed with PBST four times and ECL substrate (cat. no. WBKLS0500; EMD Millipore) was used to develop protein signals, according to the manufacturer's protocol.

The processed films were scanned, and the band intensity was quantified as integrated optical density values using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

**RT-qPCR assay.** RNA was extracted from the MC3T3-E1 cells, following the treatments described above, using TRIzol reagent (Invitrogen Life Technologies; Thermo Fisher Scientific, Inc.). First strand cDNA was prepared using SuperScript II reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China). The qPCR analysis was performed using Power SYBR Green PCR master mix (Takara Biotechnology Co., Ltd.). All procedures were performed according to the manufacturer's protocol.

Each 2  $\mu$ l cDNA sample was added to a well of PCR plate with 0.25  $\mu$ l forward and reverse primers, 10  $\mu$ l SYBR Premix Ex Taq solution and 7.5  $\mu$ l DEPC water. The qPCR reactions were run on an Illumina-Eco qPCR monitor (Illumina, Inc., San Diego, CA, USA) using the following cycling parameters: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 30 sec at 60-64°C. A melting curve was added to the end of the program to confirm specific amplification. The quantification cycle (Cq) was determined from the average of triplicate samples. Calculations were based on the ' $\Delta\Delta Cq$  method' using the following equation: ratio =  $2^{-\Delta\Delta Cq}$ . The results were standardized using the housekeeping gene,  $\beta$ -actin.

The target mRNA and primer sequences were as follows: hairy and enhancer of split 1 (Hes1), forward 5'-CTAACGCAGTGTCACCTTCC-3' and reverse 5'-CTAGGGACTTTACGGGTAGCA-3'; axis inhibition protein 2 (Axin2), forward 5'-ACAGCGAGTTATCCAGCGACG-3' and reverse 5'-GTGGGTCTCGGAAAATGAGGTAG-3'; myelocytomatosis oncogene (Myc) forward 5'-CAGGACTGTATGTGGAGCGGT-3'

and reverse 5'-TGCTGTTCGTTGAGCGGGTA-3'; vascular endothelial growth factor-A (VEGF-A), forward 5'-AGAGAA GACACGGTGGTGGAA-3' and reverse 5'-TGGGAAGGG AAGATGAGGAA-3'; hypoxia-inducible factor-1  $\alpha$  (HIF-1 $\alpha$ ), forward 5'-TAAGGCATCAGCATAACAGTGG-3' and reverse 5'-GATTCAAAGTGGCAGACAGGT-3';  $\beta$ -actin, forward 5'-TGAGAGGGAAATCGTGCCTGAC-3' and reverse 5'-GCTCGTTGCCAATAGTGATGACC-3'.

**Statistical analysis.** All results were evaluated using SPSS v.13 (SPSS, Inc., Chicago, IL, USA). The data are presented as the mean  $\pm$  standard deviation. The statistical differences were examined using a one-way analysis of variance Bonferroni correction for multiple comparisons or a Student's t-test for comparison of two independent samples.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cobalt-mimicked hypoxia and its association with osteoblast proliferation, Wnt and Notch signaling.** As several studies have reported, hypoxia may effect cell proliferation with multiple cell signaling pathway changes (13-15). The present study examined the effect of the hypoxia-mimicking agent, cobalt, on osteoblast proliferation and the regulation of Wnt-Notch signaling pathway coupling. The data showed that, following 48 h culture in the cobalt medium, osteoblast proliferation decreased (Fig. 1A and B) and the apoptotic rate increased (Fig. 1C). This showed the negative effect of cobalt-mimicked hypoxia on osteoblast growth.

To understand the molecular mechanism of cobalt-induced osteoblast growth inhibition, the present study focused predominantly on the Notch and Wnt signaling pathways, and examined changes in their target genes under this condition. An active component of Notch signaling, NICD (Fig. 2A and B), and the target gene of Notch signaling, Hes1 (Fig. 2C), were markedly increased in the cobalt-mimicked hypoxic conditions. However, the Wnt signaling active component,  $\beta$ -catenin (Fig. 2A and B), was not sensitive to the cobalt-mimicked hypoxia. The target genes, Axin2 and Myc, were also found to decrease (Fig. 2C).

**Cobalt-mimicked hypoxia and osteoblast proliferation.** As Notch signaling was elevated under hypoxic conditions (Fig. 2), the present study examined whether Notch was involved in osteoblast proliferation under cobalt-mimicked hypoxic conditions.

To assess the function of Notch signaling under hypoxic conditions, the Notch signal was inhibited using the  $\gamma$ -secretase inhibitor, DAPT, and the effect of cobalt-mimicked hypoxia on the osteoblasts following Notch signaling suppression was examined. Coupled with the results from the AO staining (Fig. 1A), CCK-8 assay (Fig. 1B) and analysis of apoptosis (Fig. 1C), cobalt-induced hypoxia increased osteoblast proliferation following Notch suppression, and this occurred in a concentration-dependent manner.

To further clarify the mechanism underlying the low concentration hypoxia rescue of cell survival by DAPT, the Notch signaling messenger and alterations in target genes were analyzed using western blot analysis (Fig. 2A and B) and qPCR analysis (Fig. 2C). The data from these analyses showed no



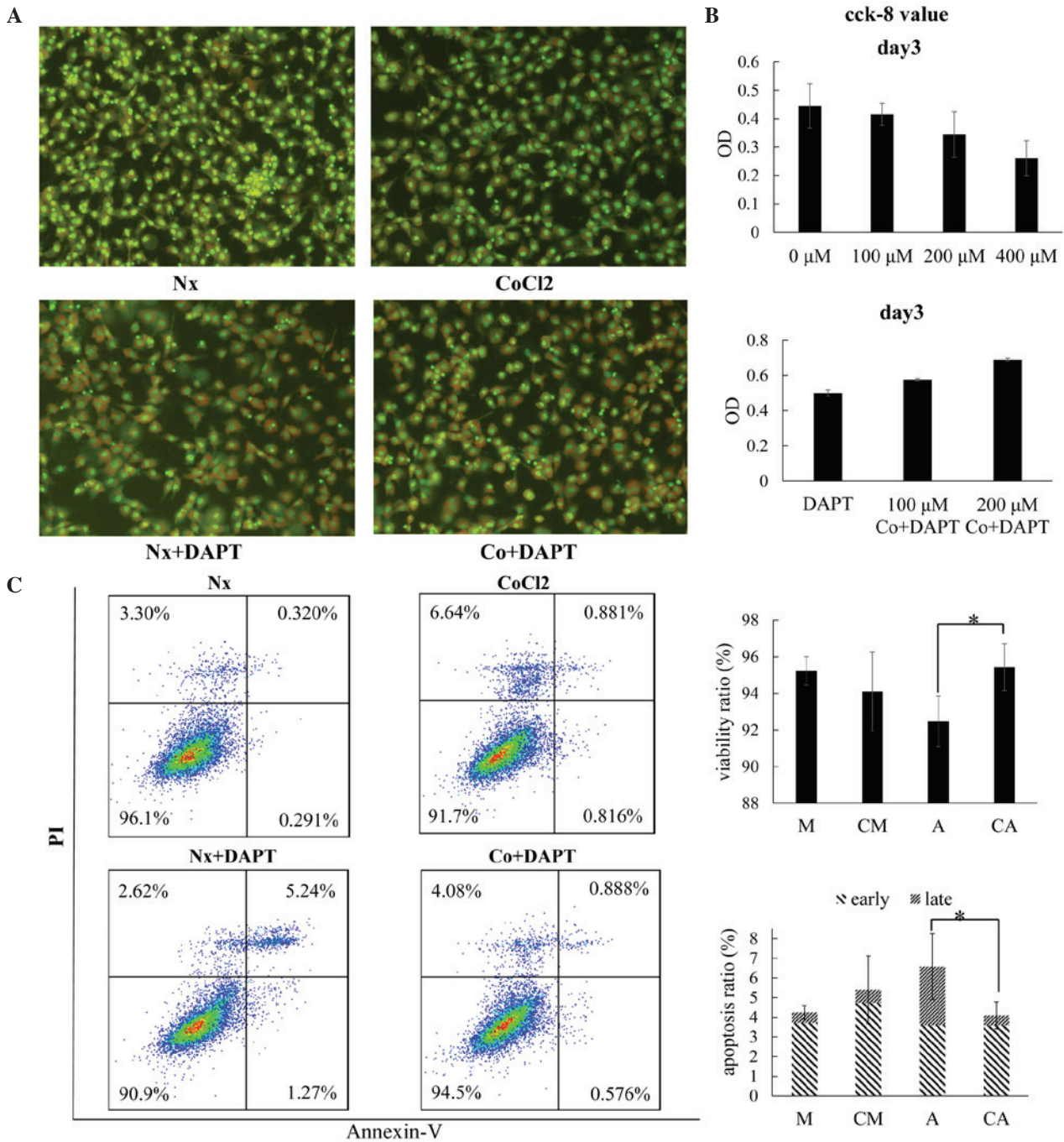


Figure 1. Inhibition of osteoblast proliferation in cobalt-mimicked hypoxia corresponds with Wnt signaling downregulation and Notch signaling upregulation. (A) Fluorescence images of cells following acridine orange staining (magnification, x200). The MC3T3E1 cells were treated in normoxic conditions (Nx), 100  $\mu$ M cobalt-mimicked hypoxic conditions (Co), 20  $\mu$ M DAPT in normoxic conditions (Nx+DAPT) or 20  $\mu$ M DAPT in 100  $\mu$ M cobalt-mimicked hypoxic conditions (Co+DAPT). (B) CCK-8 assay for cell viability following 3 days of treatment. The cells were treated with different concentration of cobalt (0, 100, 200 and 400  $\mu$ M) for 3 days (upper figure), or with 20  $\mu$ M DAPT and different concentrations of cobalt (0, 100 and 200  $\mu$ M; lower figure). (C) Detection of cell viability and apoptotic ratio by flow cytometry under Annexin-V/PI staining following 48 h treatment. The data are presented as the mean  $\pm$  standard deviation of three independent experiments. \*P<0.05. PI, propidium iodide; OD, optical density. CCK-8, cell counting kit-8.

differences in the expression level of NICD or the Hes1 gene between the normoxic and hypoxic conditions under Notch signaling suppression. This showed that the cobalt-mimicked hypoxia rescued cell survival in a Notch-independent manner.

*Cobalt-mimicked hypoxia promotes osteoblast proliferation by increasing  $\beta$ -catenin and Wnt target gene expression in conditions of Notch signaling suppression.* To evaluate the changes in

Wnt signaling induced by cobalt hypoxia under Notch suppression, the levels of active  $\beta$ -catenin (Fig. 2A and B) and Wnt target gene (Fig. 2C) were examined. Unlike Notch signaling, the levels of  $\beta$ -catenin and the Wnt target gene increased, which indicated activation of the Wnt signaling pathway under hypoxic conditions and Notch suppression. The levels of active  $\beta$ -catenin and the Wnt target gene, Myc, increased simultaneously, suggesting that Wnt signaling activation enhanced osteoblast survival.

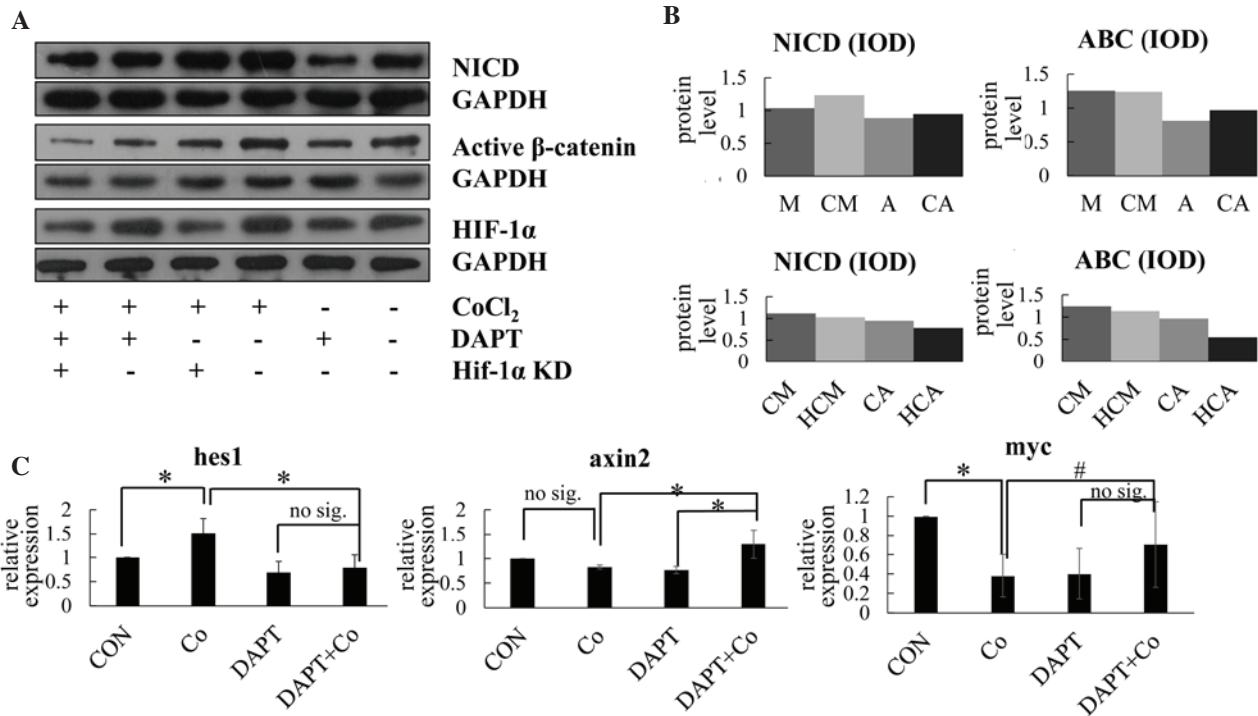


Figure 2. Notch signaling is inhibited by DAPT in cobalt-mimicked hypoxia promoted osteoblast proliferation and enhanced Wnt signaling pathway. (A) Western blotting for NICD, ABC and HIF-1α under different treatment conditions. (B) Relative protein expression levels of NICD and ABC under normoxia (M) or hypoxia (CM), under normoxia with DAPT (A) or hypoxia with DAPT (CA), and under hypoxia with HIF-1α knockdown (HCM) or under hypoxia with DAPT and HIF-1α knockdown (HCA). (C) Relative expression levels of Hes-1, Axin2 and Myc under different treatment conditions, determined using reverse transcription-quantitative polymerase chain reaction analysis. The data are presented as the mean ± standard deviation of three independent experiments. \*P<0.05 for two-tailed test; #P<0.05 for one-tailed test; no sig, P>0.05. NICD, cleaved Notch1; ABC, active-β-catenin; HIF-1α, hypoxia-inducible factor-1α; Hes1, hairy and enhancer of split 1; Axin2, axis inhibition protein 2; Myc, myelocytomatosis oncogene; IOD, integrated optical density; CON, control; Co, cobalt-mimicked hypoxia.

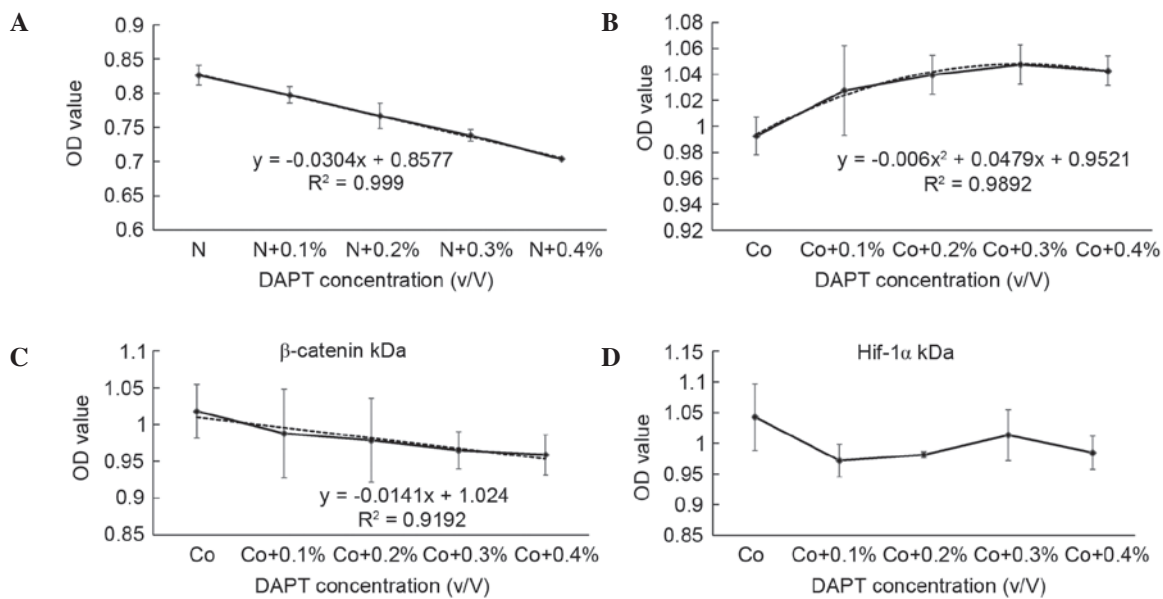


Figure 3. CCK-8 test and curve fitting. CCK-8 assay results for the different treatment groups following treatment for 48 h and curve fitting. The cells were treated with (A) different concentrations of DAPT (10 mM DAPT at 0, 0.1, 0.2, 0.3 and 0.4% V/V) in normoxic conditions, (B) different concentrations of DAPT with 100 μM CoCl<sub>2</sub>, (C) different concentrations of DAPT with 100 μM CoCl<sub>2</sub> following β-catenin knockdown, and (D) different concentrations of DAPT with 100 μM CoCl<sub>2</sub> following HIF-1α knockdown. The data are presented as the mean ± standard deviation. HIF-1α, hypoxia-inducible factor-1α; OD, optical density. CCK-8, cell counting kit-8; N, normoxia; Co, cobalt-mimicked hypoxia.

In order to evaluate the associations between Wnt signaling activation and osteoblast survival under different conditions, the present study used siRNA to knock down the expression

of β-catenin. The proliferation and apoptotic rates of the β-catenin-knockdown osteoblasts and wild-type (WT) osteoblasts under Notch-suppression and hypoxia were compared.

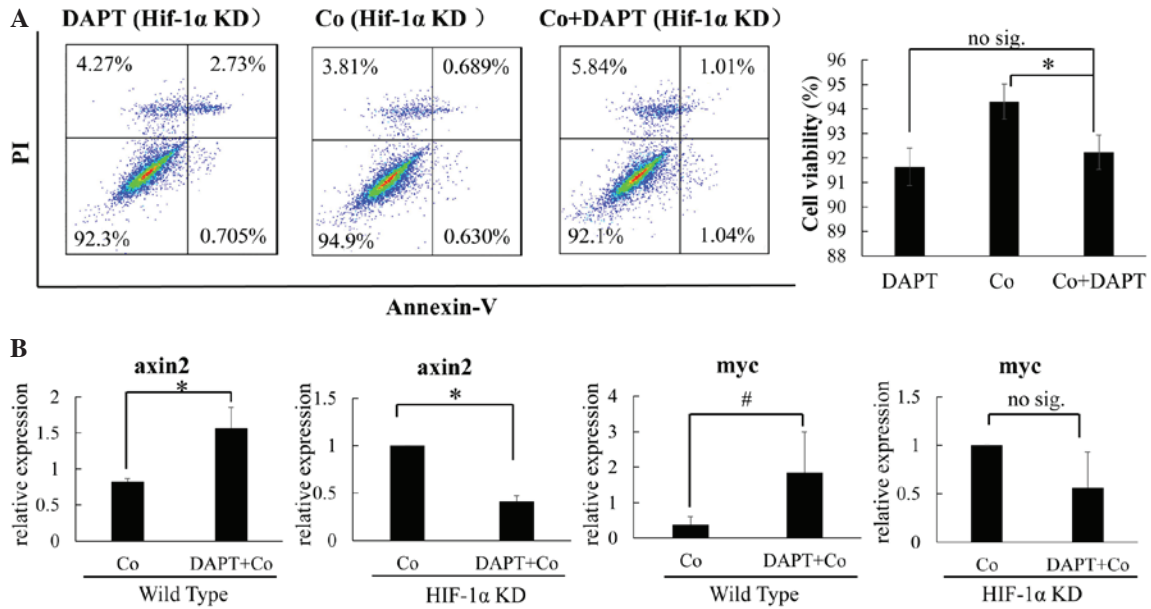


Figure 4. HIF-1 $\alpha$  is critical in the regulation of Notch signaling activity and Notch-Wnt signaling crosstalk in hypoxia. (A) Detection of cell viability and apoptosis ratios using flow cytometry with Annexin-V/PI staining following treatment for 48 h. (B) Reverse transcription-quantitative polymerase chain reaction analysis was performed to determine expression of Axin2 and Myc in HIF-1 $\alpha$ -knockdown cells. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \*P<0.05 for two-tailed test; #P<0.05 for one-tailed test; No sig., P>0.05. HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; Axin2, axis inhibition protein 2; Myc, myelocytomatosis oncogene; Co, cobalt-mimicked hypoxia; PI, propidium iodide.

By contrast with the inhibition of osteoblast proliferation caused by increasing dose of DAPT under normal oxygen condition (Fig. 3A), the proliferation rate (Fig. 3B) of the WT cells increased, whereas proliferation (Fig. 3C) was inhibited in the  $\beta$ -catenin-knockdown cells under Notch-suppression and hypoxia

*Cobalt-mimicked hypoxia requires HIF-1 $\alpha$  to maintain osteoblast proliferation and the expression of Wnt signaling target genes under Notch suppression.* To understand the molecular mechanism of cobalt-mimicked hypoxia, the HIF-1 $\alpha$ , a critical protein induced by hypoxia, was detected (Fig. 2A and B), and the effects of HIF-1 $\alpha$  on ‘Wnt-Notch’ signaling cross talk and the proliferation of osteoblasts were assessed by HIF-1 $\alpha$  knockdown using siRNA (Fig. 4).

The results showed that, when HIF-1 $\alpha$  was knocked down under hypoxic conditions, the cobalt no longer maintained Wnt signaling under Notch suppression. The level of active  $\beta$ -catenin decreased more markedly when treated with DAPT (Fig. 2B) and, although no significant differences in the cell viability ratio (Fig. 4A) were observed in the HIF-1 $\alpha$ -knockdown cells under the different conditions, the expression levels of the target genes, which were increased by DAPT (Fig. 2C), were markedly decreased (Fig. 4B) when HIF-1 $\alpha$  was knocked down. This showed the critical role of HIF-1 $\alpha$  in cobalt-induced Wnt signaling activation and cell growth. As cobalt-mimicked hypoxia was unable to induce Wnt activation under Notch suppression without HIF-1 $\alpha$  (Fig. 2B and 4B), it was confirmed that Wnt activation was dependent on the effect of HIF-1 $\alpha$ .

Furthermore, in comparing the WT cells and HIF-1 $\alpha$  knockdown cells under cobalt-mimicked hypoxic conditions, the critical Notch signaling component, NICD,

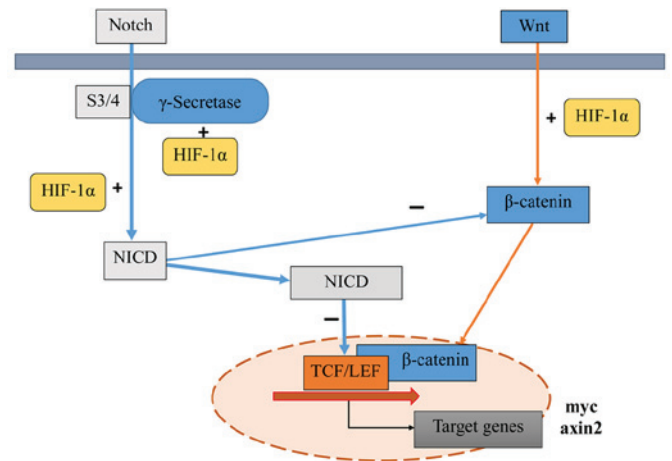


Figure 5. Schematic diagram of potential pathways through which HIF-1 $\alpha$  interacts with Wnt and Notch signaling in hypoxia. Hypoxia induces the activation of NICD and other cytokines, activating Notch signaling. Subsequent repression of Wnt signaling activity leads to decreased expression of Myc, and the subsequent inhibition of osteoblast proliferation. HIF-1 $\alpha$  activates Wnt to increase the expression levels of Myc and other cytokines, leading to cell cycle-associated gene expression. Notch signaling is activated by hypoxia, resulting in the negative regulation of Wnt signaling. NICD, cleaved Notch1; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; TCF, T-cell factor; LEF, lymphoid enhancer factor; Myc, myelocytomatosis oncogene; Axin2, axis inhibition protein 2.

was markedly inhibited by HIF-1 $\alpha$  knockdown (Fig. 2A and B), which indicated that HIF-1 $\alpha$  activated Notch signaling under hypoxia.

*$\gamma$ -secretase inhibition and osteoblast proliferation.* The CCK-8 assay was also applied to evaluate the effect of DAPT at different concentrations on osteoblast proliferation (Fig. 3). The CCK-8 value was marginally increased when the DAPT



concentration was elevated under cobalt-mimicked hypoxic conditions (Fig 3B); the highest OD value was observed in the cells treated with 0.2% DAPT in the 100  $\mu$ M cobalt-mimicked hypoxic condition. This showed that the proliferation induced by DAPT occurred in a concentration-dependent manner.

## Discussion

In several orthopedic diseases, including bone fractures, arthritis, osteonecrosis and bone tumors, hypoxic conditions represent a common pathologic microenvironment, which affects local cells (7) and causes significant biological cell alterations. Among these diseases, the osteoblast is one of the most important cell types, which may be affected most by the hypoxic conditions. Investigating the regulation of cell signal transduction in osteoblasts under hypoxic conditions may assist in developing current understanding of different mechanisms of bone disease. Among the signaling pathways that regulate osteoblast behavior, the Notch and Wnt signaling pathways have been found to be important in hypoxic conditions (3,16,17).

The associations between hypoxia and the Notch or Wnt signaling pathways in cells have been confirmed in various studies. Commonly, hypoxia causes activation of the HIF-1 $\alpha$  pathway via stabilization of the HIF-1 $\alpha$  protein by disabling prolyl hydroxylase domain activity (18). Earlier studies have found that increasing the level of HIF-1 $\alpha$  in cells promotes the level of NICD (8), and the same effects have been observed in bone marrow mesenchymal stem cells (19). Furthermore, multiple studies have shown that the Wnt signaling pathway is regulated by hypoxia in several cell types, including osteoblasts (10-12). However, few studies have revealed the function of the Notch-Wnt signaling interaction in osteoblasts.

In a number of cells, Wnt-Notch signaling always results in interactions on multiple levels (1), and Notch signaling can inhibit Wnt signaling pathway activity via several mechanisms. Typically, canonical Notch signaling activation causes elevations in the level of NICD and Notch target genes. Several studies have also found that NICD can directly inhibit  $\beta$ -catenin binding to the T-cell factor (TCF)/lymphoid enhancer factor (LEF), and inhibits the transcription activity of TCF/LEF (20). Notch target genes, including *Hes1* and hairy/enhancer-of-split related with YRPW motif protein 1, are also suggested to have a repressive effect on Wnt signaling activity (3). Certain studies have suggested that uncleaved Notch receptor truncation inhibits Wnt signaling, which can occur in a Notch signaling activation-independent manner (21-23). The above studies indicate the multi-level modification of Wnt signaling by Notch signaling crosstalk.

Although several studies have discussed the function of hypoxia on the proliferation and survival of different cell types, the results from the studies are varied and complex. As several studies have been performed in osteoblasts, hypoxia may regulate cell proliferation via Wnt signaling (10-12). However, the effect of hypoxia on osteoblast growth has been debated among reports, with certain studies showing hypoxia to have the opposite effect on cell proliferation (10), and others reporting negative conclusions (11,12). The primary difference between these studies was the variable level of Wnt signal activity that was induced.

The results of the present study demonstrated that HIF-1 $\alpha$  induced the activation of Wnt signaling. In addition, hypoxia induced the activation of Notch signaling, inhibiting Wnt signaling activity and cell proliferation at the same time. When Notch signaling was activated, the Wnt signaling activity was relatively inhibited. The inhibition of Notch signaling activation rescued the hypoxia-induced downregulation of Wnt signaling. This revealed a novel effect of Wnt-Notch signaling pathway crosstalk on osteoblast proliferation under hypoxic conditions (Fig. 5).

As shown in a previous study (24) and the present study, severe hypoxic conditions or cobalt-mimicked hypoxia with a concentration >100  $\mu$ M caused a decrease in osteoblast survival with downregulation of the Wnt signaling pathway. Although several mechanisms have been suggested to explain why hypoxia caused a downregulation of Wnt, the function of Notch-Wnt crosstalk has not been discussed. In the present study, it was shown that Notch-Wnt crosstalk was important in osteoblasts under hypoxic conditions in at least three ways: i) Hypoxia activated Notch and Wnt signaling in different conditions, resulting in increased Notch-Wnt crosstalk under hypoxic conditions in the regulation of cell biology; ii) the activation of Notch signaling by hypoxia inhibited the Wnt signaling activity and prevented Wnt-induced proliferation in the osteoblasts; iii) agents that target  $\gamma$ -secretase regulated Notch signaling, causing the Wnt-Notch crosstalk effect to enhance osteoblast proliferation under hypoxic conditions.

According to the mechanism revealed in the present study, regulation of Notch signaling may affect Notch-Wnt signal crosstalk and rescue HIF-1 $\alpha$ -induced Wnt signal activation, thus promoting osteoblast proliferation. To confirm the clinical significance of this mechanism, osteoblasts were treated with different concentrations of DAPT under the cobalt-mimicked hypoxic condition, which showed potential value in rescuing osteoblast growth under hypoxic conditions.

In conclusion, the present study revealed novel findings of an interaction between HIF-1 $\alpha$  and Wnt-Notch signaling pathway crosstalk. To the best of our knowledge, the present study represents the first to demonstrate this in osteoblast-like cells. Furthermore, the present study confirmed the critical role of HIF-1 $\alpha$  in this specific signaling interaction, indicating, at least partially, a novel mechanism in the Notch-Wnt signaling interaction. Further investigations are required to focus on the molecular mechanism of protein interactions between the HIF-1 $\alpha$  and the Notch and Wnt signaling regulators, and the biological significance of such interactions.

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