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Histone deacetylase1 promotes TGF-β1-mediated early chondrogenesis through down-regulating canonical Wnt signaling

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ABSTRACT

Cartilage formation during both embryonic development and bone repairing processes involves mesenchymal stem cells (MSCs) differentiation. Wnt/β-catenin signaling pathway inhibits early chondrogenesis and is down-regulated during Transforming growth factor-β1 (TGF-β1)-induced chondrogenesis. However, the regulatory molecules that participate in the process is unknown. This study was designed to investigate the underlying mechanisms that down-regulate Wnt/β-catenin pathway during chondrogenesis. TGF-β1-induced micromass cultures of C3H10T1/2 were used as chondrocyte differentiation model. Gene expression profile was detected by realtime-PCR. Regulatory role of HDAC1 on β-catenin was investigated by luciferase assay, chromatin immunoprecipitation (ChIP) assay, co-immunoprecipitation (Co-IP) assay and *in vitro* ubiquitination assay. In this study, we showed that HDAC1 was induced and suppressed β-catenin gene expression through direct binding to its promoter. Besides, HDAC1 could also interact with deacetylate β-catenin protein through its deacetylase domain, which causes degradation of β-catenin. Our results indicate that HDAC1 plays an important role in chondrogenesis and may represent a therapeutic target for modulation of cartilage development.

1. Introduction

Endochondral ossification is a process where mesenchymal cells condense, proliferate, and differentiate into chondrocytes, producing cartilaginous matrix, and undergoing maturation and hypertrophy. Chondrocyte hypertrophy is followed by matrix mineralization and bone substitution of the cartilage [1]. Various signaling pathways including both Transforming growth factor-β1(TGF-β1)/SMAD pathway and Wnt/β-catenin pathway have been shown to be involved in this process [2–7]. The balance in the two signaling pathways determines the rate of differentiation in each step of the cascade by regulating the sequential steps of chondrogenesis, proliferation, prehypertrophy and hypertrophy [8–12].

TGF-β1 is a multifunctional growth factor produced by monocytes-macrophages [13], platelets [14], and chondrocytes [15]. It plays an important role in the development and maintenance of growth plate and articular cartilage and is a potent inducer of chondrogenesis during development and cartilaginous extra cellular matrix synthesis [16–18]. Altered TGF-β1 signaling has been associated with osteoarthritis (OA) in both mice and humans [16,19,20]. As an attempt to repair tissue damage, TGF-β1 is elevated in human rheumatoid arthritis (RA) [21], synovial fluid, and is a major growth factor for stimulating cartilage regeneration. It suppresses acute and chronic arthritis by counteracting the effects of interleukin 1 (IL-1) [22].

Wnt/β-catenin signaling is an evolutionarily conserved signaling cascade with imperative roles during multiple developmental processes including bone development [4]. Canonical Wnt signaling is mainly controlled by the regulation of β-catenin. β-catenin undergoes different types of modification, such as phosphorylation, acetylation. Acetylation of β-catenin can improve its stability [23,24]. Wnt/β-catenin signaling plays a negative role in early chondrogenesis [3], and therefore is blocked during chondrogenesis through unknown mechanisms.

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Acetylation and deacetylation seem to be critical for pathway regulation. Among the deacetylation, histone deacetylases (HDACs) are best known epigenetic regulators that promote transcriptional repression and silencing through the removal of acetyl groups from histone core proteins of target gene promoters, resulting in a less transcriptionally active state [25–29]. It is reported that HDACs regulate chondrocyte differentiation [30–33], but the underlying molecular mechanism needs to be further investigated.

In this study, we clarified that HDAC1 was up-regulated in TGF-β1-induced early chondrogenesis and further unveiled the critical function of HDAC1 in this process. It suppressed β-catenin gene expression through direct binding to its promoter. In addition, HDAC1 could also interact with β-catenin protein through its deacetylase domain, which causes degradation of β-catenin. This conclusion may provide an exciting opportunity for the drug design to treat chondrodysplasia, osteoarthritis and other bone diseases.

2. Materials and methods

2.1. Cell culture and transfection

HEK293T and C3H10T1/2 cell lines were maintained in DMEM supplemented with 10% fetal calf serum at 37 °C in a humidified 5% CO_2 atmosphere. C3H10T1/2 cell lines in micromass cultures were plated at a density of 1×10^7/ml. After the cells were incubated for 2 h, the control medium or medium with TGF-β1 (The final concentration is 10 ng/ml) (Peprotech Inc., CA, USA) was added. The medium was changed every 2 d.

2.2. Antibodies

The following antibodies were used: mouse anti-HDAC1 (CST, CA, USA); rabbit anti-β-catenin (Abcam, CA, UK); normal mouse or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-FLAG, mouse anti-GFP (Sigma, CA, USA).

2.3. Transient transfection and dual luciferase reporter assays

HEK293T cells were seeded at 1×10^5 per well in a 24-well plate 24 h before transfection. Cells were transfected with the indicated vectors. HEK293T cells were plated and transfected with a reporter plasmid (0.2 μg) that carried the firefly luciferase gene, a reference plasmid prl-sv40 (0.02 μg) that carried the *Renilla* luciferase gene controlled by an SV40 enhancer and promoter and an effector protein expression plasmid (0.2 μg). Approximately after 24 h, the cells were harvested and subjected to luciferase assay according to the manufacturer's instructions. Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega Corporation, CA, USA). Relative activity was defined as the ratio of firefly luciferase activity to that of *Renilla* luciferase.

2.4. Cell fractionation assay

C3H10T1/2 cells in micromass cultures were harvested in cold phosphate buffer saline (PBS) and then centrifuged at 500×*g* for 5 min. Cells were washed by suspending the cell pellet with PBS twice. To remove and discard the supernatant, a pipette was used, leaving the cell pellet as dry as possible. A nuclear and cytoplasmic protein extraction reagent kit (Thermo Scientific, CA, USA) was used to produce the nuclear and cytoplasmic components. A protease inhibitor, phenyl methyl sulfonyl fluoride (PMSF) was added to ice-cold CER I (200 μl) before use. The resulting mixture was added to the cell pellet. To suspend the cell pellet fully, the tube was vigorously vortexed at the highest setting (or a 1 ml needle was used to produce a single cell suspension efficiently). The tube was then incubated on ice for 30 min, afterward, ice-cold CER II (11 μl) was added to the tube, which was vortexed for 15 s at the highest setting, and then incubated on ice for 10 min. The tube was vortexed for another 15 min at 12,000×*g* in an Eppendorf centrifuge. The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled tube, which was placed on ice until use or for storage. The resulting insoluble pellet fraction, which contains the nuclei, was suspended in ice-cold NER to which protease inhibitors has been added before use. The tube was vortexed for another 15 s. The sample was then placed on ice and vortexed for another 15 s at 10 min intervals, for a total of 40 min. The tube was centrifuged at 16,000×*g* in an Eppendorf centrifuge for 10 min. The supernatant (nuclear extract) fraction was immediately transferred to a clean pre-chilled tube and placed on ice. The extracts were stored at −80 °C until use.

2.5. Western blotting analyses

Forty-eight hours following transfection, the cells were washed with PBS and solubilized in lysis buffer (100 mM NaCl, 50 mM Tris, pH 7.5, 1% Triton X-100, and 2 mM EDTA) or radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 1% Np-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) with PMSF. Extracts were clarified by centrifugation at 12,000×*g* for 15 min at 4 °C. Following SDS–PAGE, the proteins were transferred to nitrocellulose membranes which were incubated with the specific primary antibody after blocking with 5% low-fat milk. The membranes were washed in Tris-Buffered Saline and Tween (TBST) and incubated for 1 h with a secondary antibody. After washing in TBST, membranes were scanned by Odyssey (LI-COR, Gene Company Limited, USA).

2.6. Chromatin immunoprecipitation (ChIP) experiments

C3H10T1/2 cells in micromass cultures were fixed with 37% formaldehyde for 10 min, followed by 20 rounds of 30 s sonication to fragment the chromatin. The chromatin was incubated with the anti-HDAC1 antibody (Abcam, CA, UK) at 4°Covernight and immunoprecipitated with protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) the following day. Purified DNA was amplified by PCR using primer pairs that include the predicted HDAC1 binding sites.

2.7. Lentivirus-mediated shRNA knockdown of HDAC1 in C3H10T1/2 cells

Pre-synthesized shRNAs against mouse HDAC1 were synthesized and tested. The constructed transfer vectors, pLSLG, VSVG and Δ8.9 were co-transfected into HEK293FT cells at a molar ratio of 15:6:9 and the culture supernatant contained the viral particles was harvested 48 h after transfection and clarified with a 0.45 μm membrane filter (BD Biosciences, CA, USA). The resulting supernatant was then condensed by PEG-8000 (1:4) resuspended in the cell culture medium, and stored at 4 °C. The medium with the viral particles was used directly to infect C3H10T1/2 cells. A lentivirus preparation that carried a non-target "non-sense" shRNA (scrambled shRNA) was used as the control.

2.8. Alcian blue staining

C3H10T1/2 cells in micromass cultures 1, 2, 3, 4, 5, and 6 days were rinsed with PBS twice, fixed with 4% paraformaldehyde for 15 min, and then stained with 1% Alcian blue 8GX (pH2.5) for 0.5 h. The stained cells were washed with PBS thrice, and photographed. Experiments were performed in triplicate.

2.9. Quantitative real-time PCR

Total RNA isolated from C3H10T1/2 and KEK293T cells using 1 mL TRIzol reagent kit (Invitrogen, CA, USA) was dissolved in 0.1% diethylpyrocarbonate water and quantified by nanodrop at an absorbance of 260 nm (absorbance). cDNA was synthesized from 1 μg total RNA through reverse transcription using the RevertAid H minus First Strand cDNA synthesis kit (Fermentas, CA, Canada). The sequences for primers used to amplify cDNA were as follows:

GAPDH	PF:AGGTCGGTGTGAACGGATTTG
	PR:TGTAGACCATGTAGTTGAGGTCA
Sox9	PF:GAGCCGGATCTGAAGAGGGA
	PR:GCTTGACGTGTGGCTTGTTC
β-catenin	PF:ATGGAGCCGGACAGAAAAGC
	PR:CTTGCCACTCAGGGAAGGA
HDAC1	PF:AGTCTGTTACTACTACGACGGG
	PR:TGAGCAGCAAATTGTGAGTCAT

Quantitative real-time PCR was carried out using SYBR Green (TAKARA, CA, Japan) in 384-well plates in a LightCycler rapid thermal cycler system (Roche, CA, Switzerland) according to the manufacturer's instructions. PCR products were subjected to melting curve analysis, with the data analyzed and quantified using RotorGene 6.0 analysis software (Roche, CA, Switzerland). Relative quantification of each gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We used the $2^{-\Delta\Delta Ct}$ (cycle threshold) method to calculate relative gene expression levels. For related samples, results are presented as fold changes in gene expression normalized to GAPDH, and compared with control conditions (untreated cultures). Analyses of the results were based on triplicate (or more) experiments.

2.10. Statistical analysis

Results are presented as mean ± standard deviation (SD). Statistical differences were determined using Student's *t*-test. Significance was accepted at $P < 0.01$ or $P < 0.05$.

3. Results

3.1. Expression of HDAC1 and β-catenin during TGF-β1-induced chondrogenesis in C3H10T1/2 micromass cultures

The micromass culture system is a classical model used to investigate embryonic limb development and endochondral ossification *in vitro*. In this culture system, E11.5 limb bud derived mesenchymal cells can spontaneously differentiate into mature chondrocytes and recapitulate the endochondral ossification *in vitro*. C3H10T1/2 cell line is a remarkable representative of mesenchymal stem cells because of similar capacity. We performed Alcian blue (Fig. 1A upper panel) and Toluidine blue staining (Fig. 1A, lower panel) to confirm the upregulation of Col II and aggrecan after micromass culture. Gene expression analysis by quantitative RT-PCR revealed that HDAC1 gene expression was highly induced after 2 days of TGF-β1 induction. In contrast, β-catenin decreased to a very low level at the same time (Fig. 1B). At the protein level, HDAC1 increased from the first day after induction, whereas β-catenin decreased from the first day (Fig. 1C). β-Catenin not only participates in canonical Wnt signal transduction in chondrocytes, but also mediates cell adhesion. When canonical Wnt signaling is activated, β-catenin translocates into nucleus. To determine whether the down-regulated total β-catenin level as mentioned above indicates inhibiting of canonical Wnt signaling by HDAC1, we separately detected β-catenin

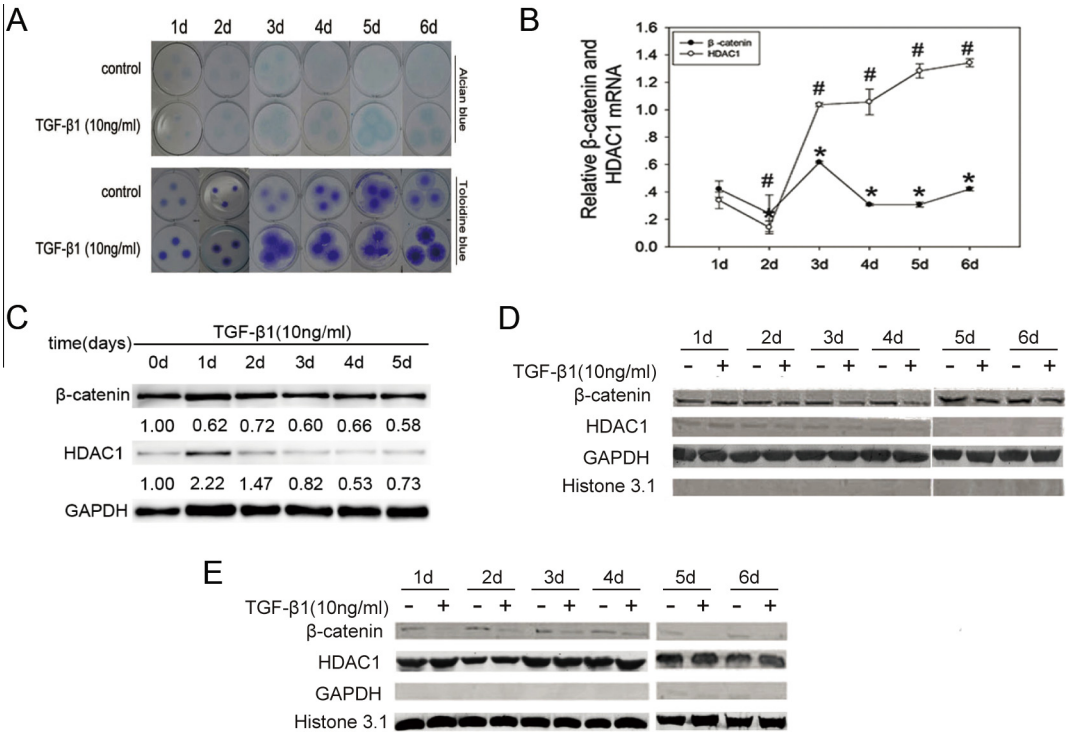

Fig. 1. Expression of HDAC1 and β-catenin during TGF-β1-induced chondrogenesis in C3H10T1/2 micromass cultures. (A) C3H10T1/2 in micromass cultures were untreated (the first and third panels) or treated (the second and fourth panels) with TGF-β1 at different times. Cultures were stained with Alcian blue and toluidine blue. Treated groups showed more intense staining. (B) The expression level of β-catenin and HDAC1 presented the opposite pattern from day 2. (C) The protein level of HDAC1 was increased from the first day after TGF-β1 induction, whereas β-catenin was decreased. The relative expression levels of protein are shown at the bottom of the bands as normalized by the GAPDH level. (D) The concentration of HDAC1 in the cytoplasm was very low and β-catenin unchanged. (E) HDAC1 increased in the nucleus but β-catenin significantly decreased from the first day after TGF-β1 induction. All of the gene expressions were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are shown as mean ± SD (*$P < 0.05$; **$P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

levels in cytoplasm and nucleus. In the cytoplasm, β-catenin remained unchanged, whereas HDAC1 maintained at a low expression level (Fig. 1D). In the nucleus, β-catenin decreased significantly from the first day and HDAC1 increased from the fifth day (Fig. 1D). These results suggest that HDAC1 is a negative regulator of canonical Wnt/β-catenin signaling in TGF-β1-induced chondrogenesis.

3.2. Down-regulating HDAC1 rescued β-catenin protein level during TGF-β1-induced chondrogenesis

To further detect the relationship between HDAC1 and β-catenin, we used TSA, a HDAC inhibitor, to treat the cell line in micromass cultures and examined the effects of HDACs on chondrogenesis. We found that the expression of β-catenin was greatly increased under the treatment of TSA (Fig. 2A). Besides, chondrogenesis was blocked as indicated by the inhibited expression of Sox9 by TSA treatment (Fig. 2A and B). As TSA is an ubiquitous HDAC inhibitor, we next constructed lentivirus-shRNA to confirm the specific role of HDAC1. The knockdown efficiency was conformed in Fig. 2C. Similar to TSA treatment, knock-down of HDAC1 rescued down-regulated β-catenin level during chondrogenesis, but decreased the protein level of Sox9 (Fig. 2D). These results suggest that HDAC1 promotes chondrogenesis through negatively regulating canonical Wnt/β-catenin pathway during TGF-β1-induced chondrogenesis.

3.3. HDAC1 inhibited ctnnb1 promoter activity

Next we investigated the molecular mechanisms of down-regulated β-catenin level by HDAC1. As we observed down-regulated β-catenin at both mRNA and protein levels, we postulated that HDAC1 might function at the transcriptional and post-translational level. We first tested the effect of HDAC1 on *ctnnb1* promoter. Luciferase assay indicated that over-expressed HDAC1 (HDAC1-EGFP) greatly inhibited *ctnnb1* promoter activity with or without TGF-β1 treatment, which could be rescued by shHDAC1 (Fig. 3A). Chromatin immunoprecipitation assay (ChIP) assay revealed the binding of HDAC1 to *ctnnb1* promoter at the proximal element (Fig. 3B). These results indicate that HDAC1 inhibits *ctnnb1* promoter activity and β-catenin transcription through direct binding.

3.4. HDAC1 down-regulates β-catenin protein at post-translational level through its deacetylase domain

To confirm whether post-translational regulation exists in HDAC1-mediated down-regulation of β-catenin, we performed TOP/FOP flash assay. HEK293T cells were co-transfected with TOP-flash or FOP-flash, β-catenin expression plasmid and HDAC1-EGFP expression plasmid. We found that HDAC1 could significantly inhibit luciferase activity (Fig. 4A). Treatment with shHDAC1 partially rescued the luciferase activity (Fig. 4A), indicating that HDAC1 down-regulated β-catenin protein level. Next, we constructed expression plasmids with full-length HDAC1 and its different truncated mutants (Fig. 4B) to detect the effects of different HDAC1 domains on β-catenin protein. Western blot showed that full-length HDAC1 as well as HDAC1-deacetylase domain could down-regulate β-catenin protein level (Fig. 4C). Consistently, TOP/FOP reporter assay showed that both full length and HDAC1-deacetylase domain could reduce TOP/FOP relative luciferase activity, whereas HDAC1-C-terminus domain couldn't (Fig. 4D). These results indicate that HDAC1 down-regulates β-catenin protein level through its deacetylase domain.

4. Discussion

Articular cartilage development is a highly conserved complex biological process. It is dynamic and robust in nature, which proceeds well without incident or failure in all joints of most young growing individuals. In this process, it is very important to under-

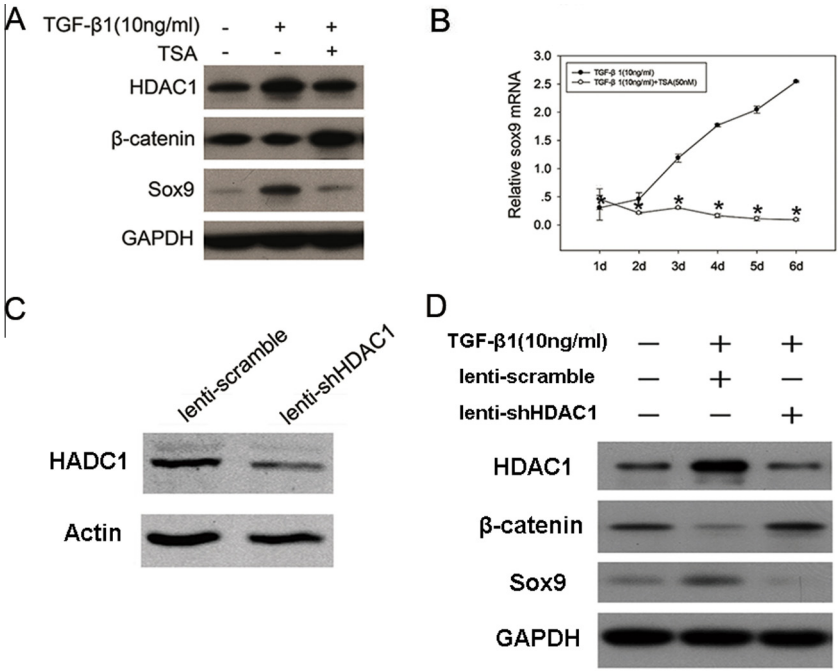

Fig. 2. Down-regulating HDAC1 rescued β-catenin protein level during TGF-β1-induced chondrogenesis. (A) C3H10T1/2 cells were treated as indicated for 3 days. The protein level of β-catenin was increased under TSA treatment, whereas Sox9 was decreased. (B) Sox9 expression level was decreased by TSA treatment during TGF-β1-induced chondrogenesis. (C) The knoch-down efficiency of lenti-shHDAC1. (D) Knock-down of HDAC1 rescued the down-regulated β-catenin level during chondrogenesis, but down-regulated Sox9 level. All of the gene expressions were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are shown as mean ± SD (*$P < 0.05$; **$P < 0.01$).

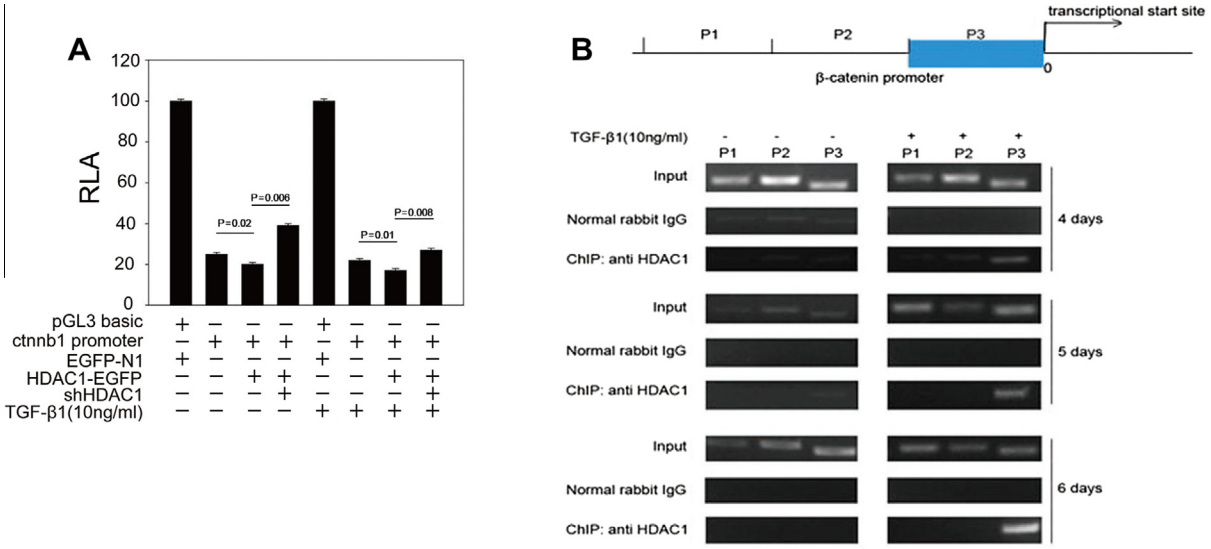

Fig. 3. HDAC1 inhibited β-catenin at the transcriptional level through directly binding to its promoter. (A) HEK293T cells were transfected with indicated plasmids and treated as indicated. Over-expressed HDAC1 (HDCA1-EGFP) greatly inhibited luciferase activity compared with control (EGFP-N1), which was rescued by shHDAC1. (B) ChIP assay using HDAC1 antibody detected the interactions of the endogenous HDAC1 protein with the β-catenin locus under the treatment of TGF-β1 at indicated time points. The blue region corresponds to the proximal promoter to which HDAC1 preferentially binds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

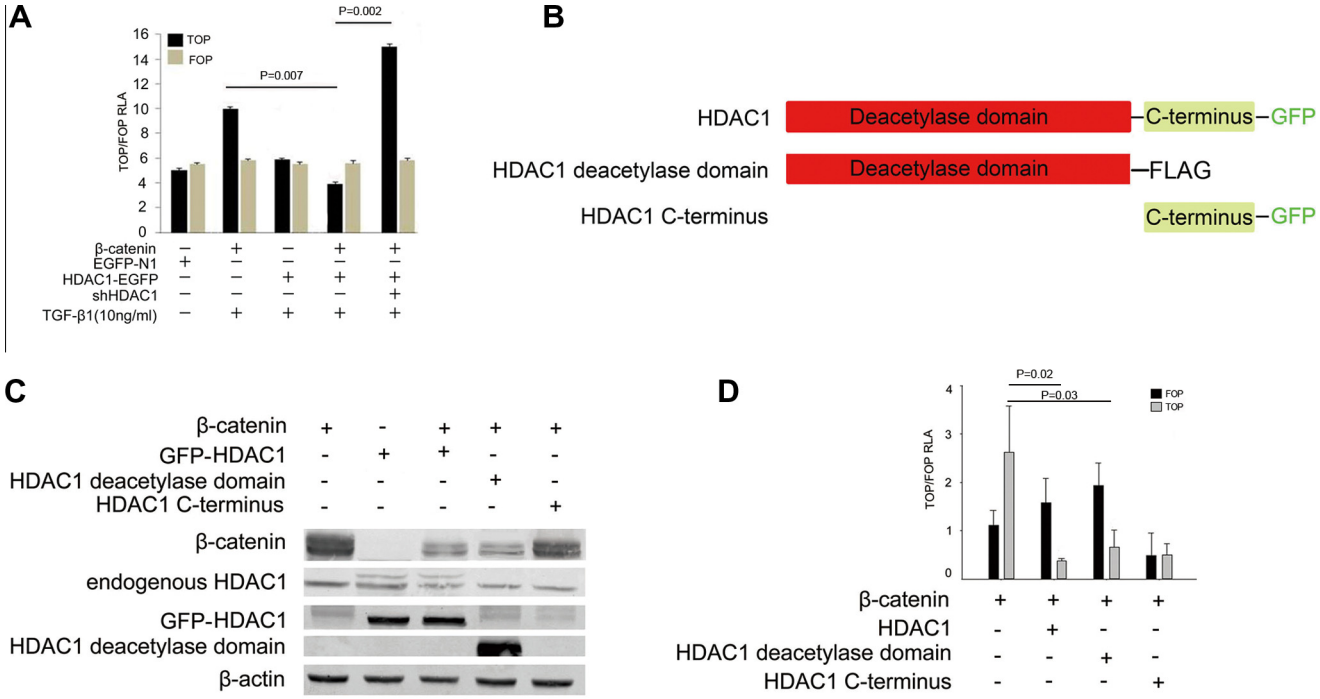

Fig. 4. HDAC1 promotes degradation of β-catenin protein through its deacetylase domain. (A) TOP-flash or FOP-flash was co-expressed with indicated plasmids in HEK293T cells. HDAC1 inhibited TOP-flash luciferase activity compared with EGFP-N1 control, which was rescued by shHDAC1. (B) A schematic diagram shows constructed plasmids with full length HDAC1 as well as different truncated mutants. (C) Indicated HDAC1 constructs were co-transfected with β-catenin. Full length HDAC1 as well as HDAC1 deacetylase domain significantly deceased β-catenin protein level. (D) TOP/FOP assay was performed using HEK293T cells. Full length HDAC1 and the HDAC1-deacetylase domain negatively regulated TOP/FOP relative luciferase activity to varying degrees.

stand the roles of TGF-β1 and associated signaling molecules, BMPs, and molecules of the Wnt/β-catenin system in chondrogenesis. The cartilage damage is often accompanied by bone lesions. Several local factors regulate the physiological remodeling of cartilage. The disequilibrium of these factors lead to a higher cartilage catabolism. Several cytokines secreted by bone cells can induce chondrocyte differentiation. Recently, molecules of the Wnt pathway have emerged as key regulators of bone and cartilage. Activation of Wnt/β-catenin induces an imbalance in cartilage homeostasis. Therefore regulation of this pathway during chondrogenesis has great important roles. But the specific mechanisms are not fully investigated. Using *In vitro* chondrogenesis model with C3H10T1/2 cells, we found both mRNA and protein level of β-catenin was inhibited, whereas HDAC1 expression was elevated. The opposite expression pattern between β-catenin and HDAC1 indicates a possible regulatory mechanism between the two factors.

Chondrogenesis occurs in a spatial and temporal manner. It is regulated by interplay of multiple integrated genetic and epigenetic factors. HDAC1 and 2 are highly similar enzymes that help regulate the chromatin structure as the core catalytic components of corepressor complexes. Although the tissue-specific deletion of HDAC1 and HDAC2 has demonstrated functional redundancy, the germ-line deletion of HDAC1 in the mouse causes early embryonic lethality, whereas HDAC2 does not [27,28,34–36]. HDAC1 plays important roles in various signaling pathways, such as Wnt/β-catenin [28], NF-κb [32] and Hedgehog [37–39] in different cell types through similar or different mechanisms.

In this study, we used an *in vitro* chondrogenesis model to functionally characterize the role of HDAC1 in chondrogenesis. We found that HDAC1 is involved in TGF-β1-induced chondrogenic differentiation by negatively regulating β-catenin both at the transcriptional and posttranslational level. Knockdown of HDAC1 leads to increased β-catenin level and inhibited chondrogenic differentiation in C3H10T1/2 micromass cultures, suggesting that HDAC1 promotes chondrogenic differentiation by inhibiting canonical Wnt/β-catenin signaling via disrupting the function of β-catenin. In addition, we determined that HDAC1 can bind to *ctnnb1* promoter and inhibit β-catenin mRNA transcription. Therefore HDAC1 is an essential factor to regulate canonical Wnt signaling during chondrogenesis.

Considering the known important function of the Wnt signaling pathway in cartilage, bone development and the role of HDAC1 in chondrogenesis presented in this study, HDAC1 may represent a therapeutic target for modulation of cartilage development. It is apparent that HDAC1 and its associated deacetylation activity are regulated in a very complicated process. As such, the regulation of HDAC1 activity and manipulating of HDAC1 should be further elucidated before effective treatments. Future studies are therefore warranted to determine the mechanism of targeting HDAC1 in a selective and tissue-specific manner. This concept may provide an exciting opportunity for the development of disease modifying drugs to treat with chondrodysplasia, OA and other bone diseases.

Authors' contributions

XJH participated in conception and design of the study, analysis and interpretation of the data, drafting of the article and critical revision of the article for important intellectual content; JJX, MJH and JL provide study materials and technical support; XLZ and KRD conceived of the study, participated in its design and coordination, helped to draft the manuscript, and final approved of the article.

Competing interest statement

All authors declare no conflict of interest.

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