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Wnt/β-catenin Signaling Mediates HDAC Inhibitor-induced Osteoblast Differentiation

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서울대학교 대학원

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ABSTRACT

Wnt/β-catenin Signaling Mediates HDAC Inhibitor-induced Osteoblast Differentiation

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Histone deacetylase inhibitors (HDIs) influence a broad repertoire of biological processes, including cellular proliferation, differentiation, and cell death, via inhibiting the acetylation of both histone and non-histone proteins. Currently, HDIs are used as anti-cancer and anti-epileptic drug; however, positive effects for bone regeneration have recently been reported. Mechanisms by which HDIs are believed to stimulate osteoblast differentiation include the acetylation of Runx2 and osterix; however, a further understanding of additional mechanisms is needed. In this study, to investigate additional mechanisms of HDIs in osteoblast differentiation, it was examined the role of Wnt/β-catenin signaling, one of the key positive

signaling pathways for osteoblast differentiation, and Smurf1 expression as a negative regulator of osteoblast differentiation in response to HDIs. To this end, we used trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and MS275 as HDIs, along with cultured C2C12 cells and primary cultured mouse calvarial cells (MC cells). To elucidate the effects of HDIs on osteoblast differentiation, C2C12 cells were incubated with HDIs and a sub-minimal concentration of bone morphogenetic protein 2 (BMP2, 10 ng/mL) followed by alkaline phosphatase (ALP) staining, ALP activity assays, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for bone marker genes. C2C12 cells were treated with only HDIs for select parts of the experiment to verify the molecular mechanisms of HDIs. Experimental results demonstrated that C2C12 cells differentiated into osteoblasts when incubated with HDIs and BMP2, but not when treated with BMP2 alone. HDIs activated Wnt/β-catenin signaling, with HDIs inducing the Wnt10A gene to the highest extent. In addition, HDIs repressed Smurf1 expression and these effects were dependent on Wnt/β-catenin signaling. Taken together, these results suggest that HDIs enhance osteoblast differentiation through the increased expression of Wnt10A and the activation of Wnt/β-catenin signaling and subsequent decrease in Smurf1 expression.

Key Words: histone deacetylase inhibitor, osteogenic differentiation, Wnt10A, β-catenin, Smurf1

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CONTENTS

I. INTRODUCTION

Histone deacetylase inhibitors (HDIs) increase the acetylation levels of histone and non-histone proteins by interfering with the activity of histone deacetylases (HDACs), of which at least 15 have been identified (Wang et al., 2009). The reversible acetylation of histone within chromatin is an important signal for regulating gene expression by inducing chromatin remodeling. The acetylation of non-histone proteins also regulates gene expression via conformation changes which regulate protein-protein interactions, DNA binding, and other processes. Most HDIs target both class I and II (including class IIb) HDACs, and are named as pan-HDIs such as suberoylanilide hydroxamic acid/vorinostat (SAHA) and trichostatin A (TSA). However, some HDIs modulate a specific class of HDACs, such as MS275, which inhibits HDAC1 and 3 (Xu et al., 2007). As HDIs have effects of anti-proliferation, pro-differentiation, and pro-apoptosis, resulting in cancer-specific cell death, more than 350 clinical trials have been performed (Minucci and Pelicci, 2006). As a result, SAHA has been approved by the US Food and Drug Administration for the treatment of cutaneous T-cell lymphoma as of 2006 (Marks and Breslow, 2007).

In addition, recent studies have demonstrated that HDIs have a positive effect on osteoblast differentiation. However, to date, few mechanisms

explaining the effects of HDIs on osteoblast differentiation have been reported. SCOP402 stimulates osteoblast differentiation *in vitro*, and bone formation *in vivo*, through the increased acetylation and expression of Runx2, an essential transcription factor for osteogenesis (Jeon et al., 2006). MS275 administration has been reported to stimulate bone regeneration in a calvarial defect mouse model by inducing tissue-nonspecific alkaline phosphatase (ALP) transcription (Kim et al., 2011). TSA also enhances osteogenic differentiation of human periodontal ligament cells by the inhibition of class I and II HDACs (Huynh et al., 2016). Osterix (OSX), a critical transcription factor for osteogenic differentiation, is acetylated by CREB binding protein, and TSA and SAHA stimulate osteoblast differentiation by increasing the levels of acetylated OSX and thereby enhancing its stability and transcriptional activity (Lu et al., 2016). However, with the exception of bone morphogenetic protein (BMP) signaling, there have been no reports published on key pathways for osteoblast differentiation as a response to HDIs.

The ubiquitin proteasome system (UPS) plays a pivotal role in the degradation of major proteins in a variety of cellular processes, including cell proliferation, apoptosis, protein quality control, DNA repair, transcription, and immune activity (Eldridge and O'Brien, 2010). The UPS is regulated by enzyme cascades consisting of the activity of three enzymes: E1, E2, and

E3 (Hershko and Ciechanover, 1998). SMAD specific E3 ubiquitin protein ligase 1 (Smurf1) is one of the HECT-type E3 ubiquitin-protein ligases that tag ubiquitin on specific lysine residues of target proteins, and thereby, initiate the degradation of the ubiquitin-tagged proteins by 26S proteasomes (Metzger et al., 2012). Smurf1 has been related to various biological processes such as embryonic development, cell polarity, cell adhesion and migration, viral autophagy, and immune responses (Cao and Zhang, 2013). Smurf1 has been implicated in bone disorders (Horiki et al., 2004; Zhao et al., 2003), embryonic development (Alexandrova and Thomsen, 2006; Podos et al., 2001; Xia et al., 2010; Zhu et al., 1999), epithelial-mesenchymal transition (Ozdamar et al., 2005; Sanchez and Barnett, 2012), cancer (Kwei et al., 2011; Sahai et al., 2007), and other biological phenotypes (Bryan et al., 2005; Lee et al., 2011b; Murakami et al., 2010).

Recent reports demonstrated that Smurf1 expression is up-regulated in osteoblasts by tumor necrosis factor (TNF), promoting ubiquitination and the proteasomal degradation of Smad1 and Runx2 (Guo et al., 2008). Cadherin-1 augments the catalytic activity of Smurf1 through disrupting the auto-inhibition mediated by the Smurf1 homodimer at the C2 domain (Wan et al., 2011). The epidermal growth factor (EGF) enhances the transcription of the Smurf1 gene by activating JNK and ERK and the subsequent binding of c-Jun and Runx2 to the Smurf1 promoter (Lee et al., 2014). In addition, several

down-regulators of Smurf1 in osteoblast differentiation have recently been described. Small molecule compounds selected from *in silico* screening have been shown to interrupt Smurf1-Smad1/5 interactions by blocking WW1 domain of Smurf1 (Cao et al., 2014). Smurf1 expression is also down-regulated by miR-15b, which is highly expressed in differentiated osteoblasts and directly targets Smurf1 mRNA (Vimalraj et al., 2014), and by miR-17, of which expression is decreased in aged mice (Liu et al., 2015b). Melatonin down-regulates TNFα-induced Smurf1 expression, thereby inhibiting the degradation of Smad1 and restoring TNFα-impaired osteogenesis (Lian et al., 2016).

Although HDIs have a potential as clinical therapeutics in the prevention and treatment of bone loss, the specific mechanisms underlying these processes are yet to be unveiled. Therefore, in the present study, the underlying mechanisms of HDIs' enhancement of osteogenic differentiation was investigated by focusing on the regulatory role of HDIs on Wnt/β-catenin signaling and the Smurf1 expression using C2C12 cells and primary cultured mouse calvarial cells (MC cells).

II. MATERIALS AND METHODS

II.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium Eagle α modifications (α-MEM), and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Easy-BLUE™ and PRO-PREP™ were obtained from iNtRON Biotechnology (Sungnam, Korea). The Alkaline Phosphatase staining kit and cycloheximide were purchased from Sigma (St. Louis, MO, USA). For first-strand cDNA synthesis, AccuPower RT PreMix was purchased from Bioneer (Daejeon, Korea) and SYBR premix EX Taq was purchased from Takara (Otsu, Japan). The PCR primers were synthesized by Macrogen (Seoul, Korea). The Lipofectamine™ reagent was purchased from Invitrogen (Carlsbad, CA). Wnt10A was purchased from Biorbyt (Berkeley, CA, USA). Recombinant human BMP2 (rhBMP2) was purchased from Cowellmedi (Seoul, Korea). Antibodies to Wnt3A, Wnt10A, β-catenin, RNA polymerase II (pol II), acetyl-histone H3, tri-methylated histone H3K9 (H3K9me3), and β-actin, normal rabbit IgG, and goat horseradish peroxidase (HRP)-conjugated IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Smurf1 antibody was purchased from Invitrogen and MeCP2 antibody was purchased from Abcam (Cambridge, UK). Cy3-conjugated donkey anti-mouse IgG was obtained

from Jackson ImmunoResearch (West Grove, PA, USA). Vectashield mounting medium for fluorescence with DAPI was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). The Super Signal reagent was ordered from Thermo Scientific (Rockford, IL, USA). The T-cell factor/lymphoid enhancer factor (Tcf/Lef) reporter plasmid, TOP-flash, and negative control FOP-flash plasmids were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The Dual-Glo luciferase assay kit was purchased from Promega (Madison, WI, USA). TSA, *p*-nitrophenyl phosphate, and *p*-nitrophenol were purchased from Sigma-Aldrich (St. Louis, MO, USA). SB216763, a GSK-3β inhibitor, and SAHA were purchased from Cayman Chemical (Ann Arbor, MI, USA). MS275 was purchased from Alexis (San Diego, CA, USA).

II.2. Cell culture

C2C12 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). To induce osteoblast differentiation, C2C12 cells were incubated with a sub-minimal concentration of rhBMP2 (10-30 ng/mL) in the presence or absence of the indicated HDIs. MC cells were prepared as previously described (Kim et al., 2003) and maintained in α-MEM supplemented with 10% FBS and antibiotics.

Stock solution of HDIs was prepared by dissolving them in dimethyl sulfoxide (DMSO) to make the following concentrations: TSA (100 μM), SAHA (5 mM), and MS275 (1 mM).

II.3. ALP staining and activity assays

ALP staining of C2C12 cells was performed using an Alkaline Phosphatase staining kit according to the manufacturer's instructions. The ALP assay was performed as previously described (Boonanantanasarn et al., 2012). Whole cell lysates of C2C12 cells were clarified by centrifugation and 20 μL of cell extracts were added to each well of a 96-well plate containing 100 μL of *p*-nitrophenyl phosphate. After incubation for 10–30 min at 37°C, depending on the ALP activity in the extracts, ALP activity was determined by measuring absorbance at 405 nm. *p*-Nitrophenol was used as a standard. The total protein amount in each sample was determined using a protein assay dye reagent obtained from Bio-Rad (Berkeley, CA, USA). ALP activity was then normalized to the protein content in the samples.

II.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

To evaluate mRNA expression, qRT-PCR was performed as follows. Total RNA was extracted using easy-BLUE™ RNA extraction reagents.

Complementary DNA was synthesized from total RNA with the AccuPower™ RT PreMix. Real time-PCR was performed using SYBR premix EX Taq using an AB 7500 Fast Real-Time system (Applied Biosystems; Foster City, CA, USA). Each sample was analyzed in triplicate, and target genes were normalized to the reference housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase. Fold differences were then calculated for each treatment group using C_T values normalized to the control. The PCR primer sequences used for real time-PCR are presented in the Table 1.

II.5. Western Blot Analysis

To obtain the whole cell protein, cells were washed with phosphate-buffered saline (PBS) and lysed using PRO-PREP™ reagent. The lysate was briefly sonicated and centrifuged at 13,000 g for 10 min. Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequently, electro-transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween20, incubated with the indicated primary antibody, and subsequently incubated with HRP-conjugated secondary antibody. Immune complexes were visualized using the Super Signal reagent and luminescence was detected with a MicroChemi imaging system (DNR; Jerusalem, Israel) and the GeneGenome XRQ system (Syngene, UK).

Table 1. Primer sequences used for qRT-PCR

Genes	Forward (5′-3′)	Reverse (5′-3′)
Smurf1	AGCATCAAGATCCGTCTGACA	CCAGAGCCGTCCACAACAAT
Wnt3A	GCCCCTTTCCAGTCCTGGTGTA	CCCTTGAAGAAGGGGTGCAGAG
Wnt7A	TGGATGCCCGGGAGATC	CCGACCCGCCTCGTTATT
Wnt7B	TTCTGGAGGACCGCATGA	GGTCCAGCAAGTTTTGGTGGTA
Wnt10A	CATGAGTGCCAGCATCAGTT	ACCGCAAGCCTTCAGTTTAC
β-catenin	GGTGCTGACTATCCAGTTG	GGCAGAGTAAAGTATTCACCC
Runx2	TTCTCCAACCCACGAATGCAC	CAGGTACGTGTGGTAGTGAGT
ALP	CCAACTCTTTTGTGCCAG	GGCTACATTGGTGTTGAGCTTTT
OSX	CCCACCCTTCCCTCACTC	CCTTGTACCACGAGCCAT
OCN	CTGACAAAGCCTTCATGT	GCGCCGGAGTCTGTTCAC
GAPDH	TCAATGACAACTTTGTCAAGC	CCAGGGTTTCTTACTCCTTGG

ALP, tissue non-specific alkaline phosphatase; OSX, osterix; OCN, osteocalcin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

II.6. Immunofluorescence staining

C2C12 cells were plated on an 8-well cell culture slide (SPL; Incheon, Korea) at a density of 2 x 10^3 cells/well. At the end of the culture period, the cells were washed extensively with PBS and fixed with 10% formaldehyde in PBS for 10 min. After additional washing, the cells were blocked with 5% normal goat serum in PBS for 1 h. The cells were incubated with β-catenin antibody overnight at 4°C, and then washed and incubated with a Cy3-conjugated secondary antibody for 1 h, followed by thorough washing. For nuclei staining, the cells were mounted with Vectashield, which contains DAPI in the mounting solution, and observed using an Olympus BX51 fluorescence microscope (Olympus Optical; Tokyo, Japan).

II.7. Luciferase reporter assays

C2C12 cells were plated into a 96-well plate at a density of 2 x 10^4 cells/well. For luciferase reporter assays, the cells were transiently transfected with the indicated plasmids using the Lipofectamin™ reagent and incubated for 24 h in the presence or absence of HDIs or Wnt10A at the indicated concentration. In each transfection, 0.2 μg of reporter plasmids (pGL3-basic, TOP-flash, or FOP-flash), 0.2 μg of β-catenin expression plasmid, and 0.07 μg of *Renilla* luciferase plasmids were used. The cells were then harvested after 48 h and luciferase activity was measured using

the Dual-Glo™ luciferase assay kit according to the manufacturer's instructions. The relative luciferase activity was calculated by dividing firefly luciferase activity by *Renilla* luciferase activity to normalize the transfection efficiency.

II.8. Gene knockdown by small interfering RNA (siRNA)

siRNAs to mouse Smurf1, Wnt10A, and non-targeting control siRNA (ON-TARGETplus Non-targeting siRNA #2D-001810-02-5) were purchased from Dharmacon (Lafayette, CO, USA). Transient transfection of siRNA was performed using DharmaFECT 1 transfection reagent (Dharmacon) according to the manufacturer's instructions. The siRNAs (TARGETplus SMART-pool) used in this study are a mixture of two siRNAs targeting independent target mRNA sequences. These sequences of each siRNA are listed in the Table 2. The efficacy of the knockdown was assessed by qRT-PCR and Western blot analysis.

II.9. Chromatin immunoprecipitation (ChIP) assays

C2C12 cells were incubated for 24 h in the presence or absence of TSA (100 nM) and ChIP assays were performed as previously described (Lee et al., 2011a). Briefly, cells were cross-linked with 1% formaldehyde, lysed, and sonicated to obtain DNA fragments of 200-800 bp. After pre-clearing with

blocked Protein G agarose, immunoprecipitation was carried out with antibodies to polymerase II, acetyl-H3, H3K9me3, or MeCP2 (10 μg). Equivalent concentrations of goat and rabbit IgG were used as a negative control. Semi-quantitative or quantitative PCR was then performed with the purified DNA using the primers listed in Table 3. The Wnt10A promoter sequence was referenced from a previous study (Jing et al., 2016).

II.10. Statistical analysis

All quantitative data are represented as the mean ± SD. Statistical significance was analyzed by Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

Table 2. Primer sequences for siRNAs

siRNAs	Forward (5′-3′)	Reverse (5′-3′)
Smurf1 siRNA	CCAAAUAGUGGUCAGUUUA	AAACUAAAUCCCUCAGAUA
	CAAGAUCCGUCUGACAGUA	CAAGAUCCGUCUGACAGUA
Wnt10A siRNA	CGGAUGUGGGCUUCGGAGA	GGUCAGCACCCAACGACAU
	GAGAGAGUGCUUUCGCUA	AAACUGAAGGCUUGCGGUU

Table 3. Primers for ChIP assays

Target sites	Forward (5′-3′)	Reverse (5′-3′)
Smurf1 promoter (-150 to +124 bp)	GCAGGAACCTTGCAGCTC	ACGGGCTGGAACCGTAGAA
Wnt10A promoter (-7 to +140 bp)	TCCTTCCTTCACCCTCTGCAT	TAGTGTCTAAGGGTTCTACCCC AAGT

III. RESULTS

III.1. HDIs stimulate osteogenic differentiation of C2C12 cells

Osteogenic differentiation of C2C12 cells could be induced by culturing the cells with BMP2 at a dose higher than 50 ng/mL. To confirm that HDIs enhance osteogenic differentiation in our culture condition, C2C12 cells were treated with sub-minimal concentrations of rhBMP2 (10, 20, and 30 ng/mL) in the presence or absence of TSA (100 nM). After incubation for 48 h, ALP staining and activity assays were performed. ALP staining result demonstrated that BMP2 alone could not sufficiently induce ALP activity; however, TSA highly increased ALP activity in a BMP dose-dependent manner (Fig. 1A). ALP activity assays also showed the similar results (Fig. 1B).

In the similar experimental conditions, the expression levels of bone marker genes, including Runx2, ALP, and osteocalcin (OCN), were not significantly changed by BMP2 treatment alone, but were increased by a combination with TSA (Fig. 1C). However, the expression of OSX gene was significantly induced by sub-minimal concentrations of rhBMP2, and TSA showed the tendency to increase OSX expression, even though it is not significant (Fig. 1C). These data indicate that TSA can enhance osteogenic

differentiation of C2C12 cells in the presence of a sub-minimal concentration of BMP2, which is not sufficient to induce osteogenic differentiation.

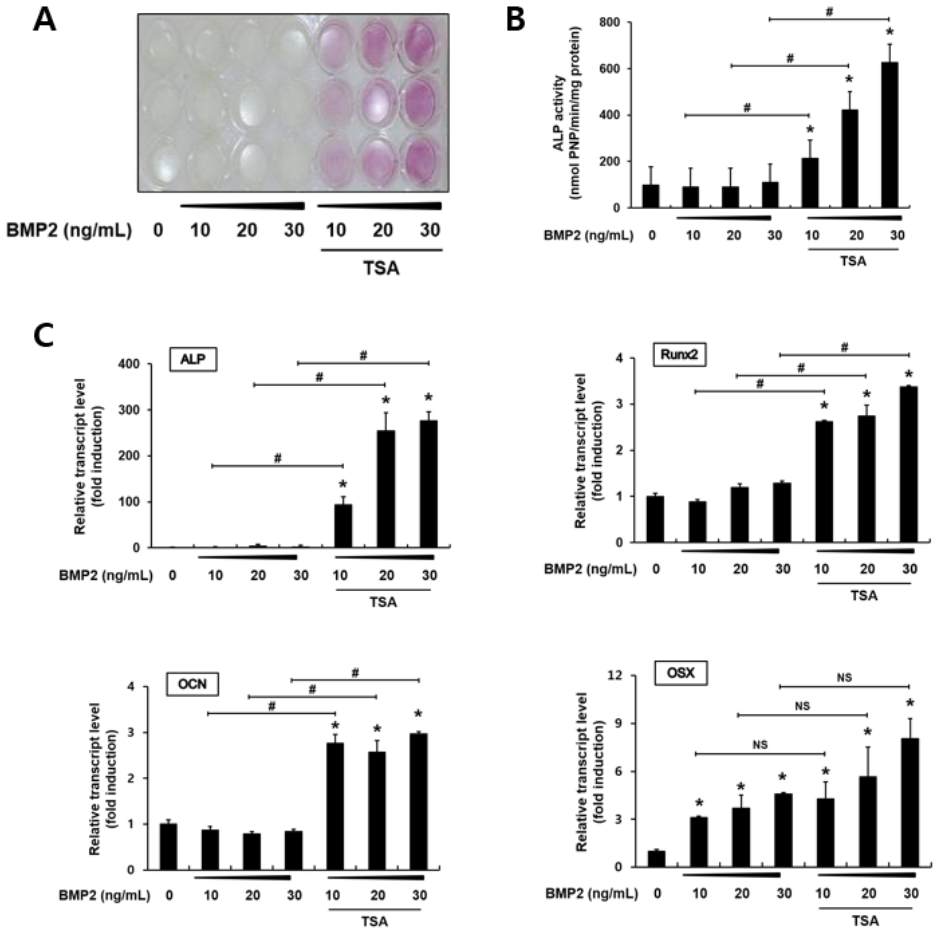

Fig. 1. HDIs enhanced osteogenic differentiation of C2C12 cells. C2C12 cells were incubated for 48 h in the presence or absence of BMP2 of the indicated concentrations and TSA (100 nM). ALP staining **(A)**, ALP activity assays **(B)**, and quantitative RT-PCR **(C)** were then performed. Quantitative data were presented as the mean ± SD. An * indicates that $p < 0.05$ compared to the non-treatment control; # indicates that $p < 0.05$ in the

indicated pair. ALP activity presents the normalized amount of *p*-nitrophenol produced in the reaction per unit time (μmol/μg total protein/min). BMP2, bone morphogenetic protein 2; TSA, trichostatin A; ALP, alkaline phosphatase; OCN, osteocalcin; OSX, osterix

III.2. HDIs increase the expression levels of canonical Wnt signaling-related genes and transcriptional activity of β-catenin

Previous studies have demonstrated that HDIs increase apoptosis and decrease proliferation of cancer cells through enhancing the Wnt/β-catenin signaling (Bordonaro and Lazarova, 2016; Bordonaro et al., 2007; Lazarova et al., 2004; Lazarova et al., 2013; Shao et al., 2012). Therefore, it was examined whether HDIs increase Wnt/β-catenin signaling in the context of osteoblast differentiation. C2C12 cells were treated with three types of HDIs (7 μM SAHA, 100 nM TSA, 4 μM MS275) for 24 h, and then, mRNA levels were examined.

Results showed that HDIs up-regulated mRNA levels of genes in Wnt signaling cascades, including Wnt3A, Wnt7A, Wnt7B, Wnt10A, β-catenin, Lrp5, and Tcf (Fig. 2A). Notably, the Wnt10A gene demonstrated the highest increase in mRNA expression from TSA. In addition, protein levels of Wnt3A, Wnt10A, and β-catenin were all increased by three HDIs (Fig. 2B). To further examine whether this induction of the Wnt signaling cascade causes β-catenin to translocate into the nucleus from the cytosol and bind to Tcf/Lef, we performed TOP/FOP-flash assay. Luciferase activity showed that these HDIs significantly increased TOP-flash activity; however, luciferase activity was highest in TSA sample, compared to those in SAHA or MS

275 samples (Fig. 2C). These results indicate that HDIs increase Wnt/β-catenin signaling cascade gene expression and activate Wnt/β-catenin signaling.

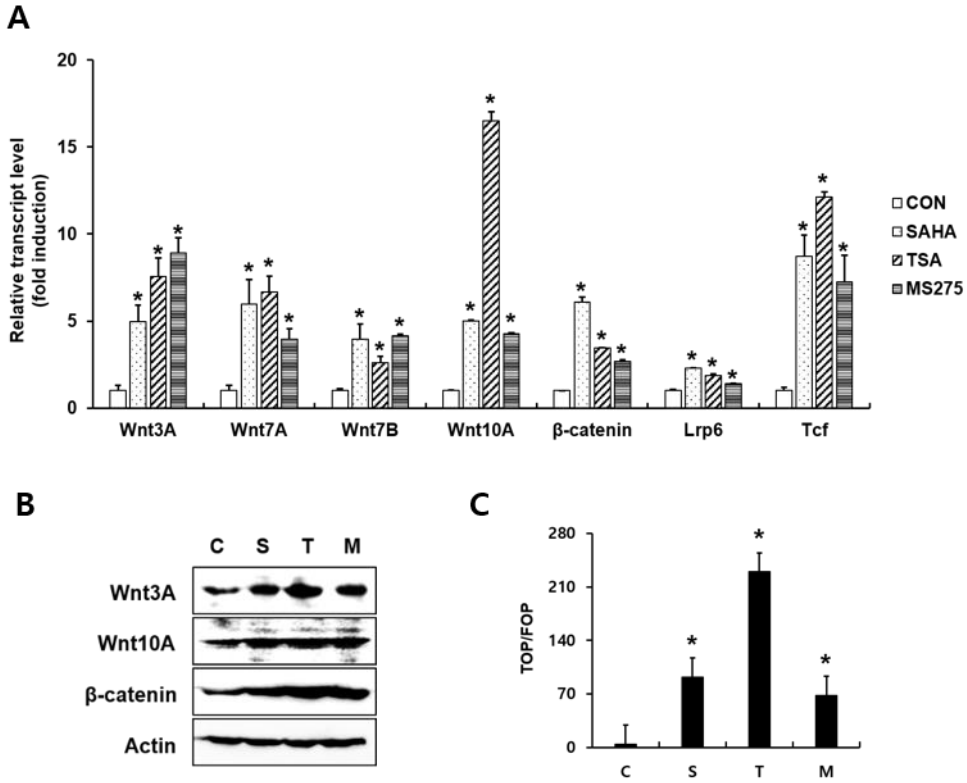

Fig. 2. HDIs increased the expression levels of the genes involved in the Wnt/β-catenin signaling cascade. (A, B) C2C12 cells were cultured in the presence or absence of HDIs (SAHA, 7 μM; TSA, 100 nM; MS275, 6 μM) for 24 h. Quantitative RT-PCR **(A)** and Western blot analysis **(B)** were then performed. **(C)** C2C12 cells were transiently transfected with TOP-Flash or FOP-Flash luciferase reporter plasmids in combination with β-catenin expression plasmid and *Renilla* plasmid, and were incubated for 48 h in the presence or absence of HDIs (S, 7 μM SAHA; T, 100 nM TSA; M, 6 μM

MS275). At the end of the culture period, dual luciferase assays was performed. FOP-Flash served as a negative control and *Renilla* served as an internal control. TOP-Flash and FOP-Flash activities were normalized to *Renilla* activity, and the data are presented as the ratio of TOP-Flash activity to FOP-Flash activity. * $p < 0.05$ *vs*. untreated control cells (CON or C)

III.3. TSA increases Wnt10A transcription via inducing chromatin remodeling in the *Wnt10a* gene

Because the Wnt10A gene demonstrated the highest increase in mRNA expression after exposure to TSA, the following experiments were performed using TSA alone. C2C12 cells were incubated with TSA (100 nM) for 4, 8, 12, 24, and 48 h and the mRNA levels of Wnt10A were determined by qRT-PCR. The Wnt10A mRNA levels were increased by TSA in a time-dependent manner (Fig. 3A).

HDIs regulate gene expression through the regulation of the acetylation/methylation of histone proteins and DNA methylation on their promoter regions (Strahl and Allis, 2000). Therefore, ChIP assays were performed to investigate whether TSA changes the acetylation/methylation of histone proteins and the recruitment of pol II on the mouse Wn10A promoter region. The sequence for the Wnt10A promoter region was referenced from a previous study (Jing et al., 2016), which was designed with about 200 bp, including transcription start sites. C2C12 cells were treated with TSA for 24 h, and DNA-protein complexes were immunoprecipitated with Pol II, acetylated-H3, H3K9me3, and MeCP2 antibodies. TSA increased the level of acetylated-H3, and by contrast, decreased the levels of H3K9me3 and MeCP2. Pol II recruitment to the Wnt10A promoter was also increased

by TSA treatment (Fig. 3C, left panel). Quantitative PCR data also confirmed these results, even though TSA-induced decrease in MeCP2 binding was statistically insignificant (Fig. 3C, right panel). These data suggest that these regulations, including the recruitment of Pol II, acetylation/methylation of histones, and DNA methylation on the Wnt10A promoter, contribute to the increased expression of Wnt10A.

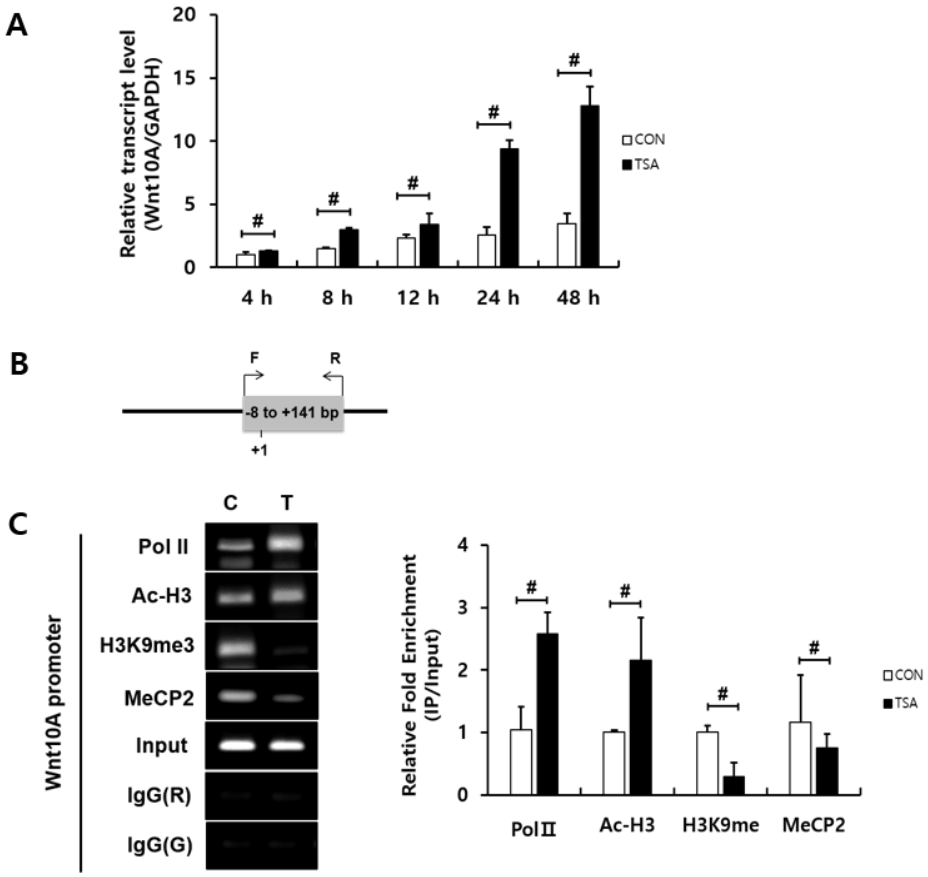

Fig. 3. TSA increased Wnt10A transcription. (A) C2C12 cells were incubated in the presence or absence of TSA (100 nM) for the indicated periods, and the Wnt10A mRNA levels were determined by quantitative RT-PCR. **(B)** Schematic illustration of the *wnt10a* DNA region which was amplified in the ChIP assays. **(C)** C2C12 cells were incubated in the presence or absence of TSA (100 nM) for 24 h, followed by ChIP assays using the indicated antibodies. Left panel shows conventional PCR results

and right panel demonstrate quantitative PCR results obtained from ChIP assays. # indicates that $p < 0.05$ in the indicated pair. Pol II, RNA polymerase II; Ac-H3, acetyl histone H3; H3K9me3, histone H3 with trimethylated K9 residue; MeCP2, methyl-CpG binding protein 2; IgG (R), rabbit IgG; IgG (G), goat IgG

III.4. Wnt10A induces accumulation of β-catenin and increases TOP-flash reporter activity

To confirm the role of Wnt10A as a canonical Wnt ligand in C2C12 cells, immunofluorescence staining of β-catenin and TOP/FOP-flash reporter assays were performed. C2C12 cells were treated with the recombinant Wnt10A at the concentrations of 20, 50, 100, 200, and 300 ng/mL for 3 h, and stained with β-catenin and DAPI. Overall, β-catenin staining was stronger in the presence of Wnt10A in a dose-dependent manner (Fig. 4A). In addition to the induction of β-catenin accumulation, Wnt10A significantly increased TOP-flash luciferase activity in concentrations of 200 and 300 ng/mL (Fig. 4B). These results confirmed that Wnt10A also induces β-catenin activation in C2C12 cells.

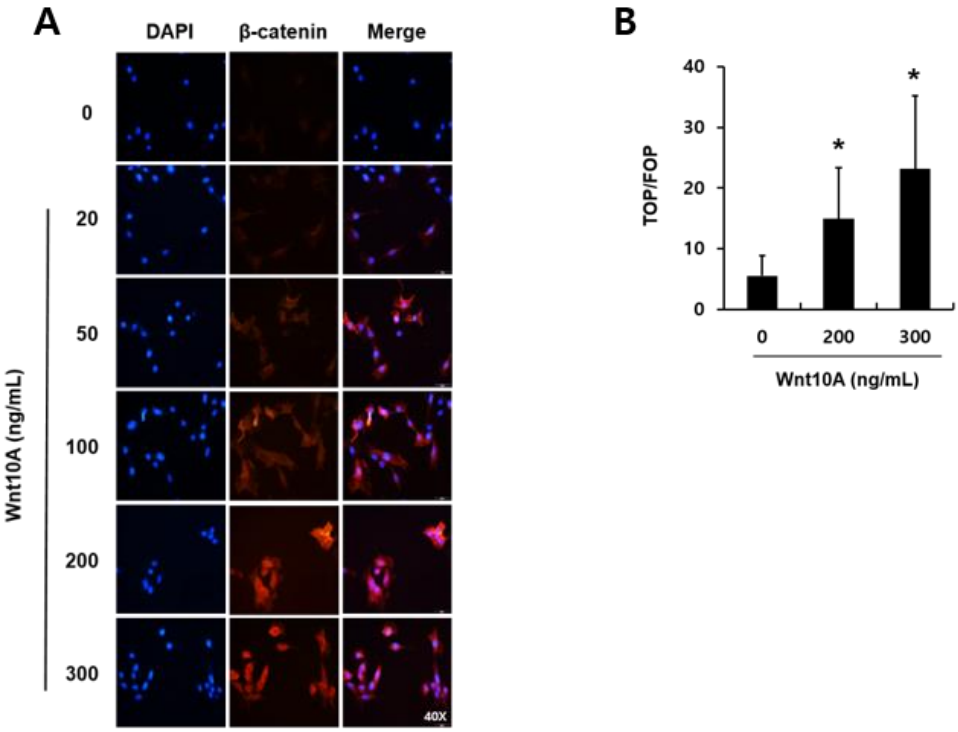

Fig. 4. Wnt10A induced accumulation and transcriptional activity of β-catenin protein. (A) C2C12 cells were incubated for 3 h in the presence or absence of Wnt10A at the indicated concentrations. Immunofluorescence staining was then performed using β-catenin antibody (red), followed by counterstaining with DAPI to show the cell nucleus (blue). **(B)** C2C12 cells were incubated for 48 h in the presence or absence of Wnt10A and TOP-Flash/FOP-Flash reporter assays were then performed. *$p < 0.05$ *vs*. untreated control cells.

III.5. Wnt10A knockdown blocks TSA-induced osteogenic differentiation

Because TSA highly induced Wnt10A mRNA expression by enhancing its transcription, it was next examined whether Wnt10A mediates HDIs-induced activation of Wnt/β-catenin signaling by knocking down Wnt10A in C2C12 cells. In C2C12 cells transiently transfected with Wnt10A siRNA, the levels of Wnt10A mRNA were significantly lower than those in control cells (Fig. 5C). Knockdown of Wnt10A faded the increase in ALP staining (Fig. 5A), ALP activity (Fig. 5B), and expression levels of osteoblast differentiation marker genes (Fig. 5C) compare to TSA treated cells which were transfected with non-targeting siRNA. These results support that HDIs increase Wnt10A expression, and this induction contributes to osteogenic differentiation of C2C12 cells.

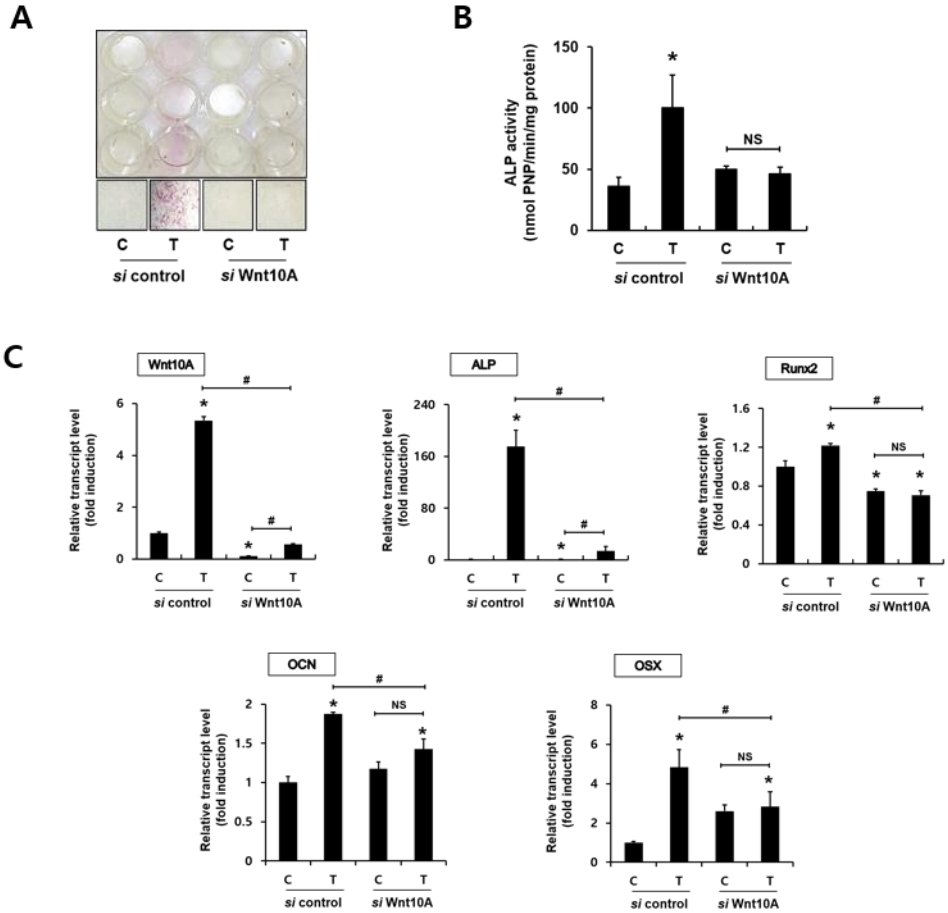

Fig. 5. Wnt10A knockdown suppressed TSA-induced osteogenic differentiation. C2C12 cells were transiently transfected with non-targeting control siRNA (*si* control) or Wnt10A siRNA (*si* Wnt10A), followed by incubation for 48 h in the presence or absence of TSA (100 nM). ALP staining **(A)**, ALP activity assays **(B)**, and quantitative RT-PCR **(C)** were then

performed. *$p < 0.05$ *vs. si* control-transfected, untreated control cells. NS, not significant.

III.6. HDIs Decrease Smurf1 expression

It was next examined whether HDIs regulate the expression level of Smurf1, a negative regulator of osteogenic differentiation. C2C12 cells were treated with three HDIs for 24 h at the following concentration ranges: TSA (10-100 nM), SAHA (1-7 μM), and MS275 (1-6 μM). Three types of HDIs were found to lead to the diminution of Smurf1 mRNA and protein expression in a dose-dependent manner (Fig. 6A).

To further determine the expression profiles of Smurf1 mRNA in the presence of HDIs at different time points, C2C12 cells were treated with 100 nM TSA, 5 μM SAHA, or 4 μM MS275 for 0, 4, 8, 12, 24, and 48 h and harvested at the same time. Although Smurf1 mRNA levels were significantly down-regulated by HDIs at all the time points examined, the reduction effects of HDIs were most constant and highest after treatment for 24 h (Fig. 6B).

It was then examined whether HDIs downregulate Smurf1 transcription by regulating chromatin regulation on the mouse *smurf1* gene. Primers were designed to amplify the Smurf1 promoter regions, including the transcription start site (-150 to + 124 bp). In contrast with the trend of the Wnt10A promoter

(Fig. 3C), TSA treatment decreased acetylated H3 level and pol II recruitment in the Smurf1 promoter (Fig. 6D). In addition, TSA increased H3K9me3 and MeCP2 recruitment in the Smurf1 promoter (Fig. 6D). These data indicate that TSA suppress Smurf1 transcription by regulation of chromatin structure.

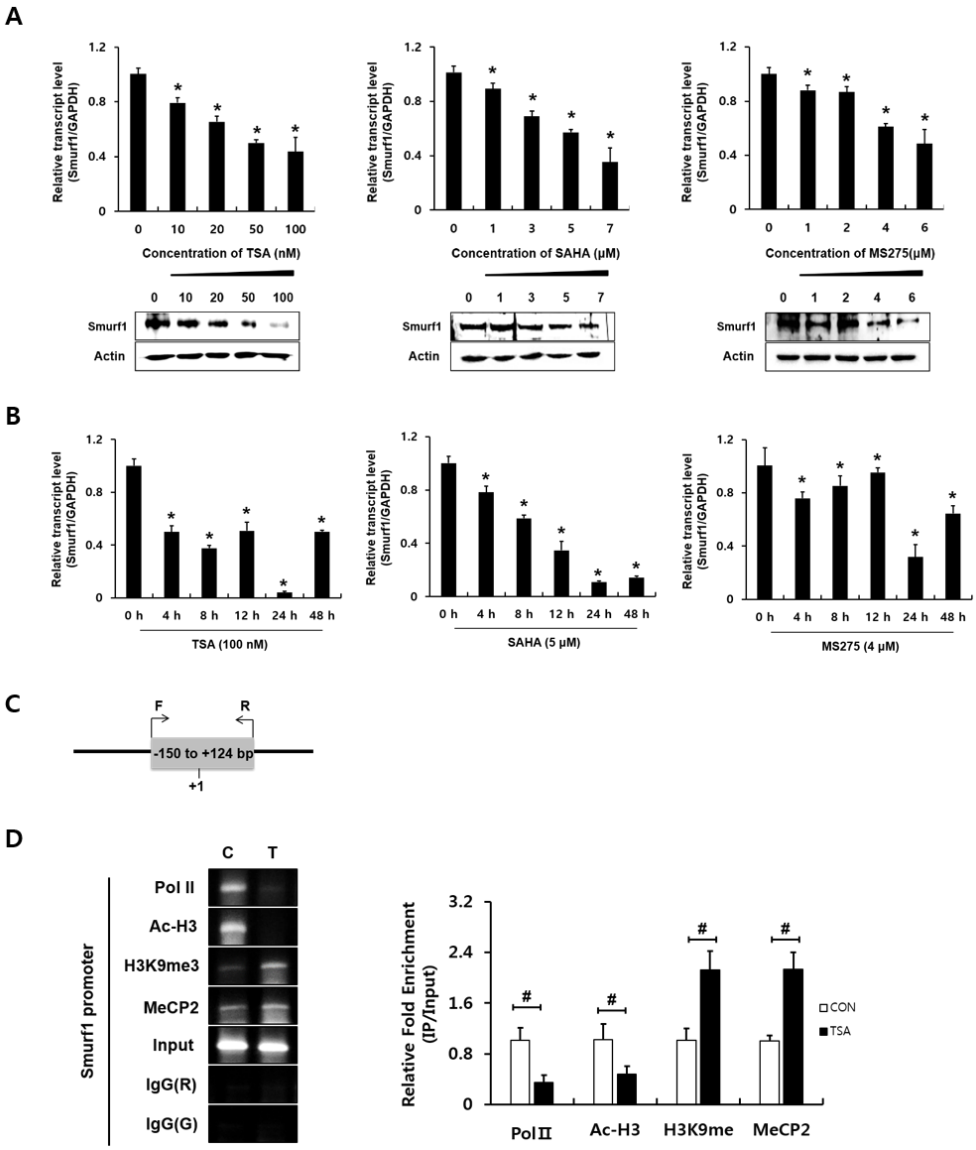

Fig. 6. HDIs down-regulated Smurf1 transcription. (A) C2C12 cells were treated with SAHA, TSA, or MS275 at the indicated concentrations for 24 h, followed by quantitative RT-PCR **(A, upper panel)** and Western blot analysis

(A, lower panel). **(B)** C2C12 cells were incubated in the absence or presence of indicate HDIs (SAHA, 7 μM; TSA, 100 nM; MS275, 6 μM) for the indicated time periods, and quantitative RT-PCR was performed. **(C)** Schematic illustration of the *smurf1* DNA region which was amplified in the ChIP assays. **(D)** C2C12 cells were incubated in the presence or absence of TSA (100 nM) for 24 h, followed by ChIP assays using the indicated antibodies. Left panel shows conventional PCR results and right panel demonstrate quantitative PCR results obtained from ChIP assays. An * indicates that $p < 0.05$ compared to the non-treatment control; # indicates that $p < 0.05$ in the indicated pair.

III.7. Wnt/β-catenin signaling down-regulates the expression of Smurf1

To determine the correlation between Wnt/β-catenin signaling and Smurf1 expression with regard to HDIs, we examined whether Wnt/β-catenin signaling can suppress Smurf1 expression. C2C12 cells were treated for 24 h with SB216763, a GSK3β inhibitor, which stabilizes β-catenin protein by inhibiting the phosphorylation of β-catenin, and finally, activates Wnt/β-catenin signaling (Aberle et al., 1997). At the end of the culture period, mRNA and protein levels of Smurf1 were detected using qRT-PCR and Western blot analysis. Smurf1 mRNA expression was decreased by SB216763 (Fig. 7A). In addition, Smurf1 protein level was reduced in a dose-dependent manner, as opposed to β-catenin protein (Fig. 7B). Down-regulation of Smurf1 protein levels by SB216763 was evident even after 4 h-incubation, while decrease in Smurf1 mRNA levels was significant after 8 h-incubation (Fig. 7C, 7D). The inhibitory effect on Smurf1 expression was sustained until 48 h. These results suggest that HDIs-induced Wnt/β-catenin signaling mediates the downregulation of Smurf1 expression.

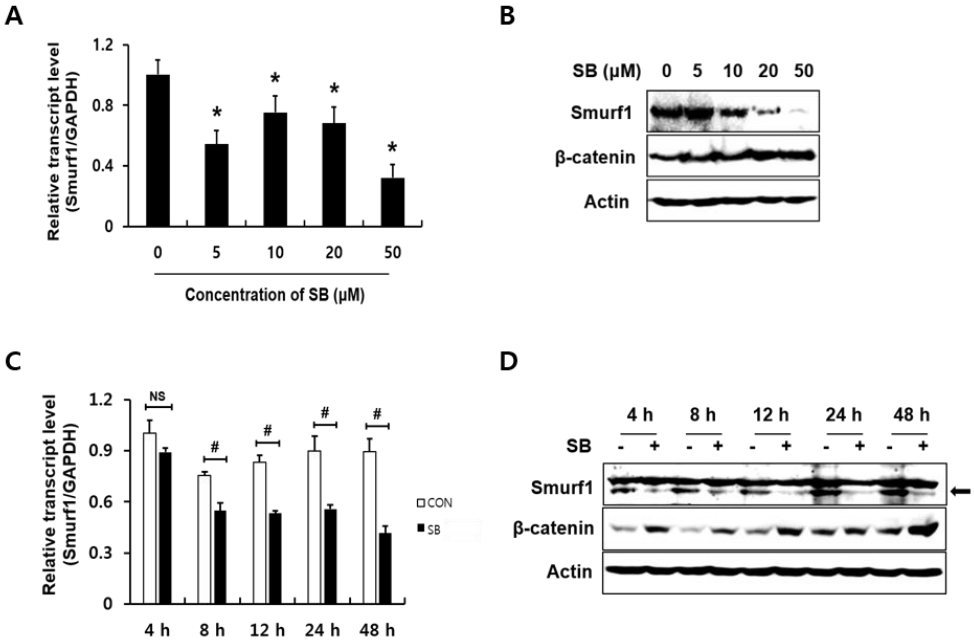

Fig. 7. Wnt/β-catenin signaling induced by the GSK3-beta inhibitor downregulated Smurf1 expression. (A, B) C2C12 cells were incubated for 24 h in the presence or absence of SB216763 (SB), a GSK3β inhibitor, at the indicated concentrations, and gene expression levels were evaluated by quantitative RT-PCR **(A)** and Western blot analysis **(B)**. **(C, D)** C2C12 cells were incubated for the indicated periods of time in the presence or absence of SB (10 μg/mL), followed by quantitative RT-PCR **(C)** and Western blot analysis **(D)**. An * indicates that $p < 0.05$ compared to the non-treatment control; # indicates that $p < 0.05$ in the indicated pair.

III.8. Wnt10A down-regulates the expression of Smurf1

It was next verified whether Wnt10A represses Smurf1 expression. C2C12 cells were incubated in the presence or absence of Wnt10A at the indicated concentrations for 24 h. Smurf1 mRNA and protein were detected using RT-PCR and Western blot analysis, respectively. Consistent with the results of SB216763 treatment, Smurf1 expression was suppressed by Wnt10A, and Smurf1 protein levels were downregulated by Wnt10A in a dose-dependent manner (Fig. 8A, 8B). In addition, Smurf1 downregulation by Wnt10A was significant after incubation for 12 h (Fig. 8C, 8D). These results indicate that stimulation of canonical Wnt signaling decrease Smurf1 expression, and downregulation of Smurf1 expression by HDIs is associated with enhanced Wnt10A expression.

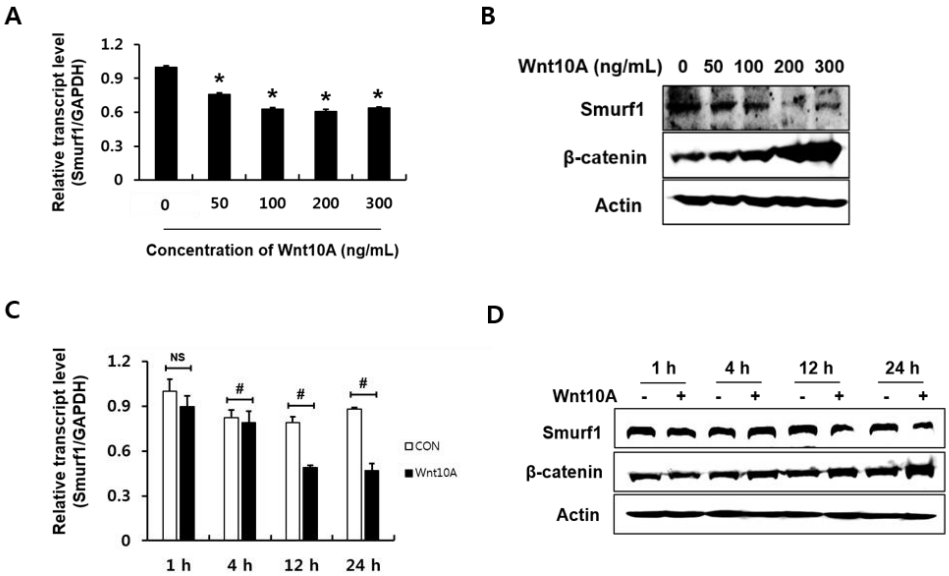

Fig. 8. Wnt10A downregulated the expression of Smurf1. (A, B) C2C12 cells were incubated for 24 h in the presence or absence of Wnt10A at the indicated concentrations, and gene expression levels were evaluated by quantitative RT-PCR **(A)** and Western blot analysis **(B)**. **(C, D)** C2C12 cells were incubated for the indicated periods of time in the presence or absence of Wnt10A (200 ng/mL), followed by quantitative RT-PCR **(C)** and Western blot analysis **(D)**. An * indicates that $p < 0.05$ compared to the non-treatment control; # indicates that $p < 0.05$ in the indicated pair.

III.9. HDIs down-regulate Smurf1 expression in a β-catenin-dependent manner

Because SB216763 and Wnt10A attenuated Smurf1 expression, it was further verified whether the repression of Smurf1 expression by HDIs is dependent on Wnt/β-catenin signaling by knocking down β-catenin. C2C12 cells were transiently transfected with non-targeting control siRNA or siRNA targeting β-catenin, and further incubated in the presence or absence of TSA for 24 h. Silencing efficiency was verified by qRT-PCR and Western blot analysis (Fig. 9A, 9B). Knockdown of β-catenin increased basal Smurf1 expression and diminished the effect of HDIs on Smurf1 expression (Fig. 9A, 9B).

To confirm the results from the knockdown experiment, overexpression of β-catenin was induced and expression levels of Smurf1 were examined in the presence and absence of TSA. Overexpression of β-catenin decreased basal Smurf1 expression, which was further reduced by TSA addition (Fig. 9C, 9D). These results indicate that Wnt/β-catenin signaling mediates the repression of Smurf1 by HDIs.

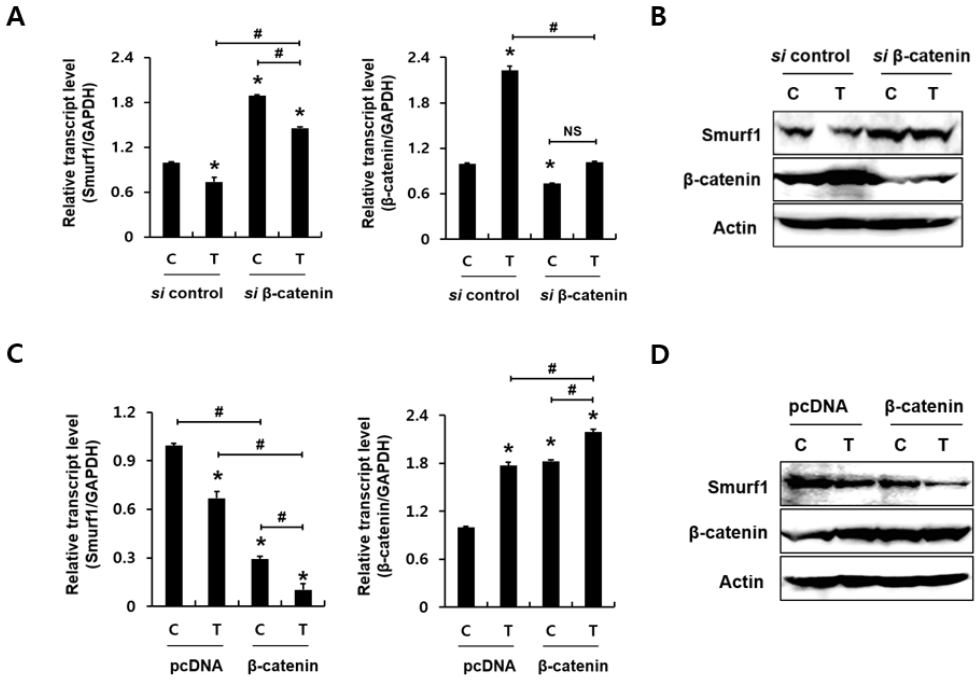

Fig. 9. TSA downregulated Smurf1 expression in a β-catenin-dependent manner. (A, B) C2C12 cells were transiently transfected with non-targeting control siRNA (*si* control) or β-catenin siRNA (*si* β-catenin), followed by incubation for 24 h in the presence or absence of TSA (T, 100 nM). **(C, D)** C2C12 cells were transiently transfected with pcDNA or β-catenin expression plasmids, followed by incubation for 24 h in the presence or absence of TSA. Expression levels of β-catenin and Smurf1 mRNA and protein were then examined using qRT-PCR **(A, C)** and Western blot analysis **(B, D)**. *$p < 0.05$ *vs*. *si* control (or pcDNA)-transfected, untreated control cells (C); # indicates that $p < 0.05$ in the indicated pair.

III.10. Wnt10A is necessary for TSA-induced Smurf1 downregulation

The above results demonstrated that HDIs strongly increased Wnt10A expression. To evaluate if the Wnt10A gene is require for this stimulation of osteoblast differentiation on HDIs, it was examined the role of endogenous Wnt10A in HDI-repressed Smurf1 expression. Wnt10A knockdown was induced by the transient transfection of C2C12 cells with control siRNA and Wnt10A siRNA. Similar to the results from β-catenin-silenced cells, Wnt10A silencing blocked the repression of Smurf1 expression in response to HDIs (Fig. 10). These results suggest that Wnt10A is required for the downregulation of Smurf1 expression by HDIs.

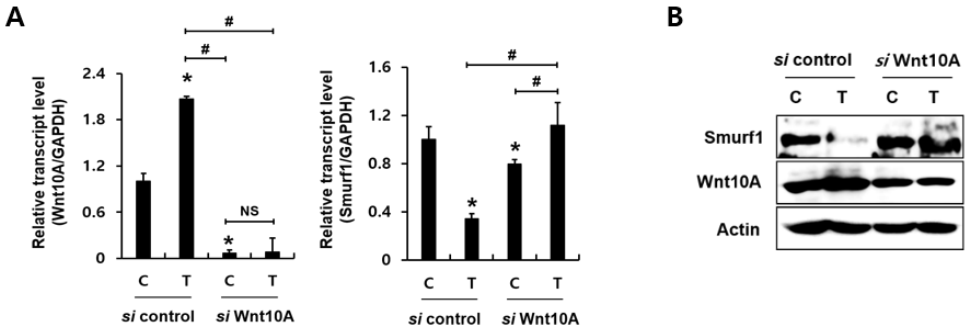

Fig. 10. Wnt10A is necessary for TSA-induced Smurf1 downregulation. C2C12 cells were transiently transfected with non-targeting control siRNA (*si* control) or Wnt10A siRNA (*si* Wnt10A), followed by incubation for 24 h in the presence or absence of TSA (T, 100 nM). Expression levels of Wnt10A and Smurf1 mRNA and protein were then examined using qRT-PCR **(A)** and Western blot analysis **(B)**. *$p < 0.05$ *vs*. *si* control-transfected, untreated control cells (C); # indicates that $p < 0.05$ in the indicated pair.

III.11. HDIs stimulates Wnt/β-catenin signaling and downregulates Smurf1 expression in MC cells

It was next examined whether HDIs activate Wnt/β-catenin signaling and reduce Smurf1 expression in primary culture MC cells. Similar to the response in C2C12 cells, three types of HDIs decreased Smurf1 expression, and conversely, increased genes of the Wnt/β-catenin signaling cascade (Fig. 11).

To examine the regulatory role of Wnt/β-catenin signaling and Wnt10A in HDI-mediated repression of Smurf1 in MC cells, overexpression of β-catenin or knockdown of Wnt10A or β-catenin were induced in MC cells. In these cells, it was confirmed the effects of TSA on Smurf1 expression. Similar to the results in C2C12 cells, overexpressed β-catenin led to reduced Smurf1 expression, whereas the knockdown of Wnt10A or β-catenin diminished the effect of TSA on Smurf1 expression (Fig. 12). These results demonstrate that HDIs repress Smurf1 expression and that this effect is dependent on canonical Wnt signaling, especially Wnt10A ligand, in MC cells as well.

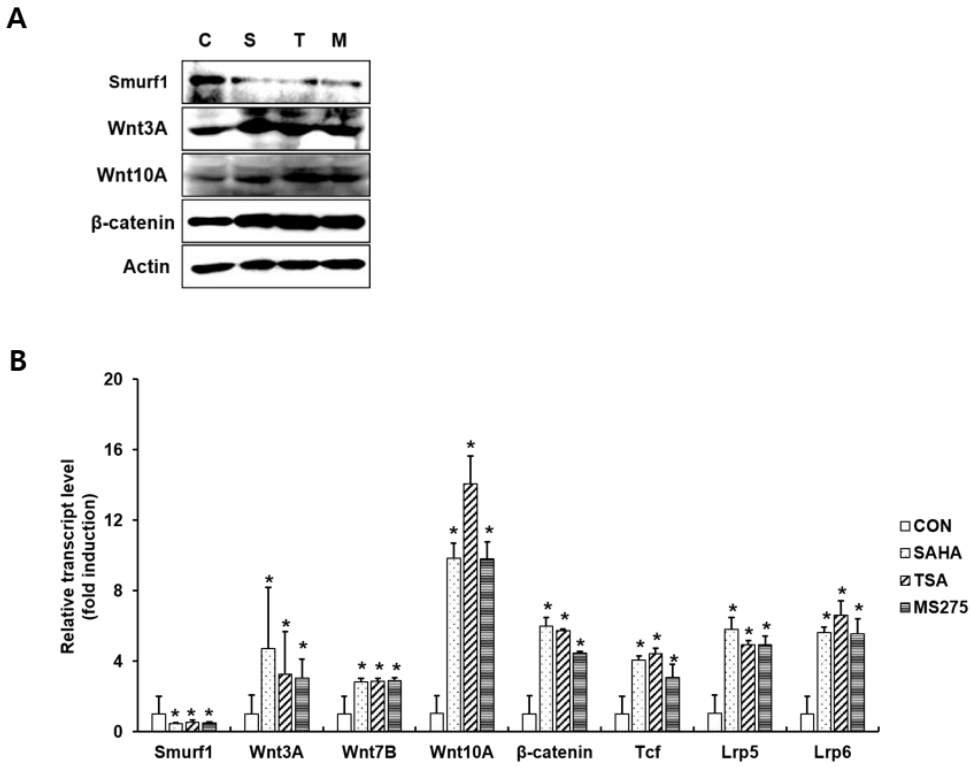

Fig. 11. HDIs increased the expression levels of the genes involved in the Wnt/β-catenin signaling cascade in primary cultured mouse calvarial cells. Mouse calvarial (MC) cells were cultured in the presence or absence of HDIs (S, 7 μM SAHA; T, 100 nM TSA; M, 6 μM MS275) for 24 h. Western blot analysis **(A)** and quantitative RT-PCR **(B)** were then performed. * $p < 0.05$ *vs*. untreated control cells (CON).

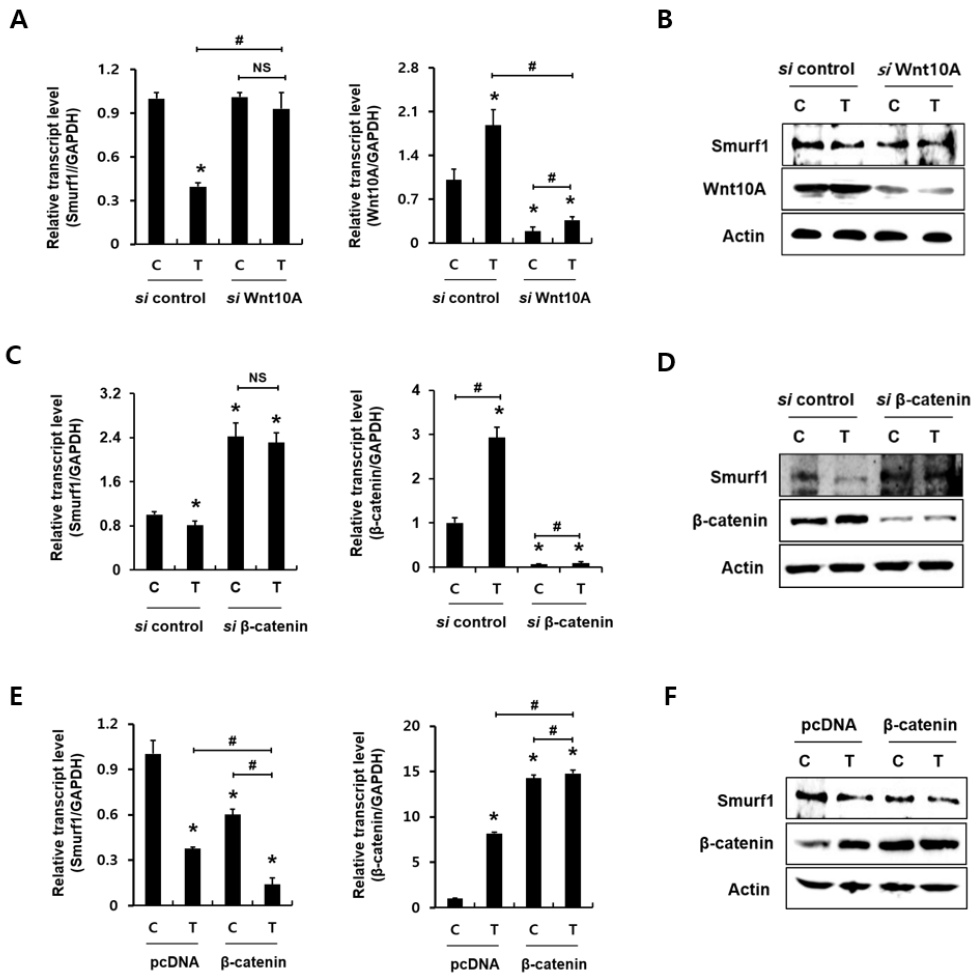

Fig. 12. TSA down-regulated Smurf1 expression in Wnt10A- and β-catenin-dependent manner in MC cells. (A-D) MC cells were transiently transfected with non-targeting control siRNA (*si* control), Wnt10A siRNA (*si* Wnt10A), or β-catenin siRNA (*si* β-catenin), followed by incubation for 24 h in the presence or absence of TSA (T, 100 nM). **(E, F)** MC cells were

transiently transfected with pcDNA or β-catenin expression plasmids, followed by incubation for 24 h in the presence or absence of TSA. Expression levels of Wnt10A, β-catenin, and Smurf1 mRNA and protein were then examined using qRT-PCR **(A, C, E)** and Western blot analysis **(B, D, F)**. *$p < 0.05$ *vs. si* control (or pcDNA)-transfected, untreated control cells (C); # indicates that $p < 0.05$ in the indicated pair.

IV. DISCUSSION

In this study, it was aimed to identify additional mechanisms involved in the osteoblast differentiation in response to HDIs. Briefly, HDIs enhanced osteoblast differentiation under the conditions of sub-minimal concentrations of BMP2, which alone could not enhance osteoblast differentiation. For this mechanism, HDIs increased Wnt/β-catenin signaling and the Wnt10A ligand expression, but decreased Smurf1 expression dependent on Wnt/β-catenin signaling, leading to the stimulation of osteoblast differentiation.

It has been demonstrated that HDIs promote bone formation *in vivo* and *in vitro* (Huynh et al., 2016; Kim et al., 2011; Schroeder and Westendorf, 2005). To date, the proposed mechanisms of these effects include the inhibition of class I HDAC, the increase in expression levels and transcriptional activity of Runx2 and OSX via enhancing their acetylation, and the stimulation of ALP transcription (Huynh et al., 2016; Jeon et al., 2006; Kim et al., 2011; Lu et al., 2016). However, the identification of other key pathways mediating osteoblast differentiation in response to HDIs has been limited.

In cancer studies, hyperactivation of Wnt/β-catenin signaling mediates the effect of HDIs on the induction of apoptosis and the repression of cell

proliferation (Bordonaro and Lazarova, 2016; Bordonaro et al., 2007; Lazarova et al., 2004; Lazarova et al., 2013; Shao et al., 2012). In addition, it has been reported that HDACs inhibit the transcriptional activity of β-catenin by interrupting the formation of β-catenin/Tcf complexes (Bradley et al., 2015; El-Hage et al., 2015; Liu et al., 2015a). Therefore, it was hypothesized that HDIs stimulate osteogenic differentiation by activating Wnt/β-catenin signaling in osteoblast precursor cells or mesenchymal stem cells. In the present study, HDIs increased the expression levels of genes of the Wnt/β-catenin signaling cascade, leading to activation of Wnt/β-catenin signaling. These results indicate that HDIs activate canonical Wnt signaling in osteoblast lineage cells.

BMP2 plays a critical role in inducing osteogenic differentiation of C2C12 cells. BMP2 converts the fate of myogenic C2C12 cells into the osteogenic lineage at the concentration of 300 ng/mL (Aoyama et al., 2011). However, BMP2 at the low concentrations such as 10-20 ng/mL could not induce osteogenic differentiation of C2C12 cells in the present study. Interestingly, combination of HDIs with BMP2 of sub-minimal concentrations strongly enhances osteogenic differentiation of C2C12 cells. It has been previously demonstrated that Wnt/β-catenin signaling is an upstream activator of BMP2 expression in various osteoblasts or osteoblast precursor cell lines (Zhang et al., 2013). Therefore, it is suggested that HDI-induced Wnt/β-catenin

signaling enhances BMP2 signaling in C2C12 cells, and thereby HDIs stimulates osteogenic differentiation of C2C12 cells in the presence of exogenous BMP2 of a sub-minimal concentration in the present study.

Patients with certain genetic diseases, such as Odonto-onycho-dermal dysplasia, Sxhopf-Schulz-Passarge syndrome, and tooth agenesis, have mutations in *WNT10A* (Kantaputra et al., 2014; Kroigard et al., 2016; Nawaz et al., 2009; Petrof et al., 2011; Yang et al., 2015). Based on these reports, Wnt10A has recently been identified as a regulator of cell fate that inhibits adipogenesis and stimulates osteoblastogenesis (Cawthorn et al., 2012; Chen et al., 2016). Some reports have demonstrated that Wnt10A gene expression is increased by the inhibition of DNA methylation on the promoter region or by binding of Bmal1 protein to the promoter region (Chatterjee et al., 2013; Chen et al., 2016; Jing et al., 2016; Yang et al., 2016). 5-Aza-2′-deoxycytidine, a DNA methylation inhibitor, stimulates Wnt10A gene expression by inducing DNA demethylation in the *Wnt10A* promoter, leading to the inhibition of adipogenesis and the promotion of osteogenesis (Chen et al., 2016). In a similar manner, the inhibition of EZH2 decreases the levels of H3K27me3 in the promoter of Wnt10A gene, and thereby shifts the differentiation fate of mesenchymal stem cells toward osteogenic lineage (Jing et al., 2016). Considering the previous reports, it is suggested that HDIs are candidates as regulators of anti-adipogenesis and pro-osteogenesis by

targeting for Wnt10A. In the present study, the ChIP assay data demonstrate that HDIs increase Wnt10A transcription via increasing histone acetylation and pol II recruitment and decreasing the methylation of histone H3K9 and DNA. These results suggest that HDIs induce Wnt10A expression via regulation of chromatin structure in the promoter region of *Wnt10A* gene. In addition, Wnt10A knockdown blocked TSA-induced osteoblast differentiation, indicating that Wnt10A is necessary to the effects of HDIs on osteoblast differentiation.

Smurf1 acts as a negative regulator for bone remodeling by targeting many of the components of the osteogenic signaling molecules, such as Smads, MEKK2, Runx2, and JunB (Yamashita et al., 2005; Zhao et al., 2010; Zhu et al., 1999). It is important to clarify the molecules and mechanisms that regulate Smurf1 in order to understand Smurf1-associated biological networks. Currently, a broad range of intracellular regulators of Smurf1 have been identified. In osteoblasts, Smurf1 has been known to be upregulated by TNFα, Cdh1, EGF, and miR-497 approximately 195 cluster (Grunhagen et al., 2015; Guo et al., 2008; Lee et al., 2014; Wan et al., 2011). On the contrary, miR-17 downregulates Smurf1 by directly targeting 3'UTR of Smurf1 mRNA (Liu et al., 2015b). Melatonin down-regulates the TNFα-induced expression of Smurf1 (Lian et al., 2016). However, the specific mechanism of this downregulation by melatonin has not been identified. In the present study, it

was demonstrated for the first time that HDIs repress Smurf1 transcription via activation of Wnt/β-catenin signaling. However, further studies are needed to understand the mechanisms that link the Wnt/β-catenin signaling to the inhibition of Smurf1 transcription.

Taken together, the results of the present study demonstrate that HDIs increase Wnt/β-catenin signaling partly through the upregulation of Wnt10A expression, and subsequently decrease Smurf1 expression. HDI-induced Wnt/β-catenin signaling leads to enhancement of osteoblast differentiation.

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VI. KOREAN ABSTRACT

히스톤탈아세틸화효소 저해제의 조골세포 분화 촉진 기전 연구

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서재란

히스톤탈아세틸화효소 저해제(histone deacetylase inhibitors, HDIs)는 히스톤 뿐 아니라 비히스톤 단백질의 탈아세틸화 저해를 통해 세포 증식과 분화, 세포사 등 다양한 생물학적 과정에 영향을 끼친다. 현재 HDIs는 항암제, 항간질제로 사용되고 있으나 근래의 보고들은 HDIs가 뼈 재생에도 긍정적인 효과를 보일 수 있음을 제시하고 있다. HDIs의 조골세포 분화 촉진 효과와 관련하여 현재까지 알려진 기전은 주로 조골세포 분화의 핵심 전사인자인 Runx2, osterix 단백질의 아세틸화 증가로 인한 전사활성 증가, 히스톤 아세틸화 증가를 통한 유전자 발현 조절 등이 주로 보고되어 왔으며 아직 추가적인 연구가 더 필요한 상황이다. 따라서 본 연구에서는 HDIs의 추가적인 조골세포 분화 촉진 기전을 규명하고자 조골세포 분화 조절의 대표적 신호계의 하나인 Wnt/β-catenin 신호계, 조골세포 분화 억제 효과를 보이는 Smurf1에 대한 효과를 관찰하였다.

HDIs로 trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA),

MS275 세 가지를 사용하였고, 조골세포 분화 유도를 위해 C2C12 세포와 일차배양한 생쥐 두개관 세포를 사용하였다. HDIs의 조골세포 분화 촉진 효과를 확인하기 위하여 C2C12세포에 저농도의 bone morphogenetic protein 2 (BMP2, 10-30 ng/mL)를 HDIs와 같이 처리한 후, 알칼리성인산분해효소(ALP) 활성, 조골세포 분화 표지 유전자 발현 정도를 비교하였다. HDIs의 분자 기전을 확인하는 일부 실험에서는 HDIs만 단독 처리하여 그 효과를 관찰하였다.

저농도 BMP2 단독으로는 C2C12세포의 조골세포 분화를 유도할 수 없었으나, HDIs를 추가한 경우 조골세포 분화가 촉진되었다. 그 기전을 확인하고자 HDIs가 조골세포 분화의 대표적 신호전달계의 하나인 Wnt 신호계에 미치는 영향을 관찰한 결과 HDIs는 Wnt/β-catenin 활성을 증가시켰고, 여러 Wnt ligand 중 특히 Wnt10A 유전자 발현 증가폭이 가장 크게 나타났다. 또한 HDIs는 조골세포 분화 억제에 관여하는 E3 ligase인 Smurf1의 발현을 저해하였으며 이러한 효과는 Wnt/β-catenin 활성의존적으로 나타났다. 이상의 결과는 HDIs가 Wnt10A의 발현 증가 및 Wnt/β-catenin 신호계 활성화를 통해 직접 조골세포 분화표지 유전자의 발현 증가를 유도할 뿐 아니라 Smurf1 발현 감소를 야기하여 조골세포 분화를 촉진함을 시사하였다.

주요어: 히스톤탈아세틸화효소 저해제, 조골세포, Wnt10A, ß-catenin, Smurf1

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