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Effects of histone deacetylase inhibitors on odontoblast differentiation

히스톤 탈아세틸화효소 억제제가 MDPC23세포의 상아모세포 분화에 미치는 영향

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서울대학교 대학원

치의과학과 분자유전학 전공

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지도교수 백 정 화

이 논문의 치의과학박사 학위논문으로 제출함

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ABSTRACT

Effects of histone deacetylase inhibitors on odontoblast differentiation

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Previous reports demonstrated that histone deacetylase inhibitors (HDACi) enhances osteoblast differentiation *in vitro* and new bone formation *in vivo*. However, it has not been elucidated whether HDACi regulate odontoblast differentiation. Nfic and Wnt/β-catenin signaling are involved in odontoblast differentiation and tooth root formation. Smurf1 is an E3 ubiquitin ligase that negatively regulates osteoblast differentiation and bone formation. The purpose of the study was to investigate the effect of HDACi on

odontoblast differentiation and to elucidate the regulatory roles of Wnt/β-catenin, Nfic and Smurf1 in HDACi-induced odontoblast differentiation. MDPC23 cell line derived from the mouse pulp cells was treated with SAHA and Trichostatin A. Odontoblast differentiation was evaluated by examining the expression levels of marker genes, including dentin sialophosphoprotein (Dspp), dentin matrix protein 1 (Dmp1) and Nestin using quantitative RT-PCR and Western blot analyses. Matrix mineralization was observed using Alizarin S Red staining. Chromatin immunoprecipitation, biotinylation pull down assays and luciferase reporter assays were performed to examine the regulatory role of Nfic on Dspp transcription. To examine the effect of HDACi on the transcriptional activation of Smurf1 gene, chromatin immunoprecipitation was performed using antibodies to acetylated histone H3 (Ac-H3), methylated histone H3 (H3K4me) and RNA polymerase II. Wnt3a protein levels in conditioned medium obtained from MDPC23 cells were evaluated using ELISA kit. Transcriptional activity of β-catenin/Tcf/Lef complexes was determined by TOP/FOP-flash luciferase reporter assays. Knockdown of Nfic, Smurf1, Wnt3a or β-catenin was induced by transient transfection of small interfering RNA (siRNA) mixtures specific to each gene. HDACi enhanced odontoblast differentiation and matrix mineralization. HDACi increased expression levels of Nfic, and subsequently Nfic directly bound to Dspp promoter and upregulated Dspp transcription. Nfic silencing attenuated HDACi-induced odontoblast differentiation. Overexpression of Smurf1 in MDPC23 cells inhibited odontoblast differentiation, whereas Smurf1 knockdown enhanced

odontoblast differentiation. HDACi treatment or overexpression of Nfic downregulated the expression levels of Smurf1 gene. Chromatin immunoprecipitation results demonstrated that increase in Nfic protein level via HDACi treatment or overexpression of Nfic significantly downregulated the levels of Ac-H3, H3K4me and RNA polymerase II associated with the promoter region or transcription start site of the mouse Smurf1 gene. Nfic knockdown attenuated HDACi-mediated downregulation of Smurf1 expression. Activation of Wnt/β-catenin signaling via Wnt3a treatment or β-catenin overexpression enhanced odontoblast differentiation and Nfic expression, whereas knockdown of Wnt3a or β-catenin suppressed odontoblast differentiation and Nfic expression. HDACi increased expression levels of Wnt3a mRNA and protein and enhanced TOP-flash reporter activity in MDPC23 cells. Blockade of Wnt/β-catenin signaling via silencing of Wnt3a suppressed HDACi-mediated odontoblast differentiation and Nfic expression. However, overexpression or knockdown of Nfic did not affect HDACi-mediated induction of Wnt/β-catenin signaling. These indicate that HDACi enhance odontoblast differentiation via activation of Wnt/β-catenin signaling in MDPC23 cells. In turn, increased Nfic protein upregulates odontoblast differentiation marker genes such as Dspp, while downregualting Smurf1, a negative regulator of odontoblast differenation.

Key Words: odontoblast, histone deacetylase inhibitor, Nfic, Wnt/β-catenin signaling, Smurf1

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I. LITERATURE REVIEW

I.1. Dentin

Dentin is a calcified tissue of the body, and along with enamel, cementum and pulp is one of the four major components of teeth. By weight, dentin consists of 70% the mineral hydroxyapatite, 20% organic material and 10% water. It is usually covered by enamel on the crown and cementum on the root. Dentin is necessary for the support of enamel and it is less mineralized and less brittle than enamel. The formation of dentin, known as dentinogenesis, begins prior to the amelogenesis and is initiated by the odontoblasts in the pulp. Odontoblasts are derived from the dental papilla cells of the tooth germ. After apposition of predentin and maturation into dentin, the cell bodies of the odontoblasts remain inside the tooth along the outer wall of the pulp. Unlike the enamel, dentin continues to form throughout the life, and dentin formation after tooth development can also be initiated in response to stimuli, such as tooth decay or attrition.

There are three types of dentin, the primary, secondary and tertiary (Zilberman and Smith, 2001). The primary dentin, the most prominent dentin in the tooth, lies between the enamel and pulp chamber. The secondary dentin is formed after root formation is complete when the tooth has been erupted and is functional. The secondary dentin grows much more slowly than the primary dentin, but it maintains its

incremental aspect of growth. Structure of the secondary dentin is similar to that of the primary dentin, although the deposition of secondary dentin is not always even around the pulp chamber. The growth of secondary dentin causes the decrease in the size of the pulp chamber with aging. The tertiary dentin is formed as a reaction to external stimulation such as cavity preparation. It is of two types; the reactionary dentin which is formed by pre-existing odontoblasts, and the reparative dentin generated by odontoblast-like cells which were newly differentiated from the pulpal progenitor cells due to the death of the original odontoblasts. The tertiary dentin is deposited rapidly as a reaction to the external stimuli, resulting in osteodentin which has a sparse and irregular tubular pattern and some cellular inclusions. If the stimulus is less active, it is laid down less rapidly with a more regular tubular pattern and hardly contains any cellular inclusions (Kinney et al., 2005). Tertiary dentin formation is an attempt to slow down the progress of the caries as well as to prevent the diffusion of bacteria and their metabolites into the pulp, reducing the probability of partial pulp necrosis.

I.2. Odontoblast differentiation

Odontoblast is a cell of neural crest origin whose biological function is dentinogenesis. Odontoblasts first appear at the sites of tooth development around 17–18 weeks *in utero* and maintained throughout the life span, unless killed by bacterial, chemical or physical attack. Odontoblasts and the pulpal mesenchymal cells have

similar embryological backgrounds because both cells were originally derived from the dental papilla of the tooth germ. Odontoblasts are large columnar cells, whose cell bodies are arranged along the interface between dentin and pulp, from the crown to cervix to the root apex in a mature tooth. Odontoblasts are rich in endoplasmic reticulum and Golgi complex, especially during the primary dentin formation, allowing them to have a high secretory capacity. Odontoblasts first deposit the collagenous matrix to form predentin, and then mineral levels are increased to form the mature mineralized dentin. Odontoblasts form approximately 4 μm of predentin daily during tooth development. After differentiation from the outer cells of the dental papilla, odontoblasts are polarized so that their nuclei are aligned away from the newly formed dentin. During the matrix secretion, odontoblasts move pulpally, away from the basement membrane, leaving behind the odontoblastic process within the dentin matrix.

Odontoblast differentiation is initiated by signal produced by Enamel knot (Thesleff et al., 2001). The basement membrane underlying the enamel epithelium is important for odontoblast differentiation and may serve as a reservoir of signal molecules such as bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wnt (Jernvall and Thesleff, 2000). As differentiation begins, cells differentiate into cuboidal preodontoblast expresssing BMP4 and they elongate into large columnar and polarized odontoblats expressing BMP2 (Nakashima, 1994). FGF and Wnt signals have been implicated in odontoblast differentiation and dentin formation (Unda et al.,

2000). For example, Wnt10a is expressed in enamel knot and later in the secretory odontoblasts (Yamashiro et al., 2007).

Odontoblast differentiation can be evaluated by examining the expression levels of odontoblast differentiation marker genes, including Nuclear Factor I C (Nfic), Dentin sialophosphoprotein (Dspp), Dentin matrix protein 1 (Dmp1) and Nestin. Nfic is one of the Nuclear factor I (Nfi) family transcription factors which were consisted of Nfia, Nfib, Nfic and Nfix. Among the Nfi family members, Nfic is expressed primarily in odontoblasts, but not in preodontoblasts, and is known as an important regulator for tooth root formation (Steele-Perkins et al., 2005). Nfic-deficient mice demonstrated short and abnormal root formation, especially in molar teeth (Steele-Perkins et al., 2003).

Dmp1 and Dspp are two non-collagenous extracellular matrix proteins that belong to the SIBLING (Small Integrin Binding LIgand N-linked Glycoprotein) family, a group of acidic and highly phosphorylated proteins involved in the mineralization process of dentin and bone (Deshpande et al., 2011; Qin et al., 2004). Dmp1 is involved in the initial phases of mineralized dentin formation and shown to act as a hydroxyapatite nucleator (Goldberg and Smith, 2004; Narayanan et al., 2001). Dspp also has an important role in dentin mineralization**.** In Dspp-deficient mice**,** dentin is hypomineralized during the mineralization process of the dentin matrix (Nanci et al., 2008; Sreenath et al., 2003). Dspp mutation is related with dentinogenesis imperfecta

in human (Holappa et al., 2006; Kim et al., 2004). The protein encoded by Dspp gene is proteolytically cleaved into dentin sialoprotein (Dsp) and dentin phosphoprotein (Dpp) (Feng et al., 1998). This cleavage is occurred in odontoblasts. Dsp is released into the predentin, whereas Dpp is released at the mineralization front and retained within the mineralized dentin (Prasad et al., 2010). There are synergistic interactions between Dmp1 and Dspp, both in a direct and indirect manner. The active fragments obtained from the cleavage of Dspp gene product may interact with Dmp1 within the dentin matrix (Prasad et al., 2010). In addition, Dmp1 acts as a transcription factor for Dspp expression in odontoblast (Narayanan et al., 2006)

Nestin is also a marker of differentiated odontoblasts (About et al., 2000; Terling et al., 1995). Nestin is an intermediate filament protein first identified in neuroepithelial stem cells. Nestin is predominantly expressed in the early developmental stage of the central nervous system and muscles (Wiese et al., 2004). In human and rodent tooth germs, Nestin is widely distributed in the pulp tissue, particularly in the cuspal regions (About et al., 2000; Terling et al., 1995). Nestin expression is downregulated in the pulp cells during tooth maturation, and Nestin expression is observed only in the functional odontoblasts in mature teeth (About et al., 2000; Hasegawa et al., 2007; McLachlan et al., 2003; Terling et al., 1995). Interestingly, Nestin is up-regulated in the dentin and pulp tissue under the pathologic conditions induced by caries, cavity preparation and tooth replantation (About et al.,

2000; Hasegawa et al., 2007), suggesting that Nestin plays a role as a marker primarily expressed in newly differentiated and functional odontoblasts.

I.3. Histone deacetylase (HDAC) inhibitors

DNA is packaged into chromatin, a highly organized and dynamic protein–DNA complex. Subunits of chromatin include the nucleosome, which is composed of four core histones, an H3/H4 tetramer and two H2A/H2B dimers (Ito et al., 2000; Strahl and Allis, 2000). Transcription in eukaryotic cells is influenced by the manner in which DNA is packaged (Wade, 2001): the local chromatin architecture is now generally recognized as an important factor in the regulation of gene expression. In resting cells, DNA is tightly compacted to prevent accessibility of transcription factors. During activation of gene transcription, this compact, inaccessible DNA is made available to DNA binding proteins via post-translational modifications of histone proteins (Ito et al., 2000). In general, histone hyper-acetylation is associated with increased transcriptional activity and gene expression, whereas histone hypo-acetylation is associated with decreased gene expression (Forsberg and Bresnick, 2001; Ito et al., 2000; Wade, 2001). The levels of acetylation of the core histones result from the balance between histone acetyltransferases and HDAC (Wade, 2001). HDAC removes the acetyl group from the proteins, including core histones and transcription factors. Members of the HDAC family fall into 4 different classes, namely class I (HDAC 1, 2, 3 and 8), class IIa

(HDAC 4, 5, 7 and 9), class IIb (HDAC 6 and 10), class III (SIRT 1 to 7) and class IV (HDAC 11).

HDAC inhibitors are known to block the function of HDAC. Among the potent HDAC inhibitors discovered so far are suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA) and valproic acid (VPA). Both SAHA and TSA belong to the hydroxamic acids group and VPA is one of aliphatic acids group. SAHA (Vorinostat or Zolinza™) received FDA approval as an anticancer agent in October, 2006, and its efficacy has been confirmed by clinical trials for malignant diseases such as non-Hodgkin lymphoma, acute myeloid leukemia, breast cancer and cutaneous T cell lymphoma (Marks, 2007; Munster et al., 2011; Richon et al., 2001; Stathis et al., 2011). TSA has been used as an anti-fungal agent, but later its anti-proliferatory effects in cancer cells were reported (Ganslmayer et al., 2004; Tsuji et al., 1976). VPA is a commonly prescribed anti-epileptic drug (Reynolds et al., 2007).

Different HDAC inhibitors induce cancer cell death by different mechanisms, which include changes in gene expression via alteration of acetylation in both histone and non-histone proteins. HDAC inhibitors cause changes in expression of genes involved in several biological processes such as cell cycle arrest and apoptosis (Mariadason et al., 2000). Many genes contributing to the regulation of the cell cycle and apoptosis were found to be modified by HDAC inhibition (Eyupoglu et al., 2006; Minucci and Pelicci, 2006). The most important mechanism of cell cycle arrest induced

by HDAC inhibitors is an induction of $p21^{WAF}$ which blocks the formation of dimers from cyclins and cyclin-dependent kinases. This leads to cell cycle arrest and induction of cell differentiation (Richon et al., 2000). In tumor cells exposed to HDAC inhibitors, pro-apoptotic genes involved in the extrinsic and intrinsic apoptotic pathways were up-regulated, whereas the expression of anti-apoptotic genes was reduced (Zhu et al., 2004). Some HDAC inhibitors also exert anti-angiogenic effects through the down-regulation of vascular endothelial growth factor and endothelial nitric oxide synthase (Michaelis et al., 2004; Montgomery et al., 2008).

Numerous studies now indicate that HDAC inhibitors induce osteoblast maturation *in vitro*. HDAC inhibitors increased alkaline phosphatase (Alp) production and matrix mineralization of *in vitro*-cultured osteoblastic cells and *ex vivo*-cultured calvaria (de Boer et al., 2006; Iwami and Moriyama, 1993; Jeon et al., 2006; Schroeder and Westendorf, 2005). HDAC inhibitors increased the expression of Osteopontin and Rankl in osteoblasts and accelerated osteoblast differentiation of bone marrow-derived mesenchymal cells via activating Erk signaling (Chen et al., 2007; de Boer et al., 2006; Fan et al., 2004; Sakata et al., 2004). In contrast to their positive effects on osteoblast maturation, HDAC inhibitors decreased the survival and maturation of osteoclasts. TSA inhibited osteoclasts differentiation from hematopoietic bone marrow cells and induced osteoclast apoptosis through induction of $p21^{WAF}$ expression (Rahman et al., 2003; Yi et al., 2007). It has also been reported that SAHA eliminates osteoclastogenesis via the NF-κB activation (Takada et al., 2006). However, recent

reports demonstrated that long-term VPA treatment in epileptic patients causes osteopenia/osteoporosis with increased fracture risk (Boluk et al., 2004; Guo et al., 2001; Sato et al., 2001; Vestergaard et al., 2004). The mechanism by which VPA causes bone loss is not clear but may be related to certain characteristics of epilepsy such as low physical inactivity or insufficient vitamin D or calcium intake (Guo et al., 2001).

I.4. Smurf1

Smurf1 (Smad ubiquitination regulatory factor 1) is an E3 ubiquitin ligase. Smurf1 contains a C2 domain, two WW domains (WW1 and WW2) and a HECT domain. WW domains bind to the PY motif of its substrates while HECT domains interact with both E2 and ubiquitin molecules to proceed ubiquitination. Smurf1 HECT domains may also interact with the C2 domain of another Smurf1 for auto-inhibition (Wan et al., 2011). Smurf1 is expressed during all developmental stages from embryo to adult in many organs, including bone, cartilage, heart, lungs, the nervous system and genital organs (Marin, 2010). It indicates that Smurf1 has a comprehensive role in the physiological regulation.

The transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMP) signaling pathways play vital roles in cell development, growth and

differentiation. Smurf1 controls the components of TGF-β and BMP signaling cascades. Smurf1 ubiquitinates Smad1/5 (the receptor-regulated Smads), Smad4 (the common mediator Smad) and Smad7 (the inhibitory Smad) and triggers their proteasomal degradation (Moren et al., 2005; Suzuki et al., 2002; Zhu et al., 1999). Other substrates of Smurf1 include the Runx2, Runx3, Mekk2, JunB, Tbx6 and the Traf4 (Kalkan et al., 2009; Yamashita et al., 2005; Zhao et al., 2010; Zhao et al., 2003). Smurf1-deficient mice showed an age-dependent increase in bone mineral density along with high levels of JunB and Mekk2 (Yamashita et al., 2005; Zhao et al., 2010). Down-regulation of Smurf1 increased osteogenesis whereas the up-regulation of Smurf1 protein attenuated osteogenetic activity, suggesting that Smurf1 is a negative regulator of bone formation (Cui et al., 2011; Lu et al., 2008). Having certain regulatory functions in the TGF-β and BMP pathways, Smurf1 also plays an important role in embryonic development. Overexpression of Smurf1 in the *Xenopus laevis* embryo triggered dorsalization of mesodermal tissues and differentiation of the ectodermal germ layer, whereas down-regulation of Smurf1 expression/function led to several dorsal neuroectodermal developmental defects (Alexandrova and Thomsen, 2006; Zhu et al., 1999).

Beyond cell growth and development, broader functions of Smurf1 have been identified in cell migration and epithelial-mesenchymal transition (EMT). In cellular protrusions, Smurf1 ubiquitinates a small GTPase, RhoA for proteasomal degradation (Wang et al., 2003). Down-regulation of RhoA induces tight-junction dissolution and initiates EMT (Ozdamar et al., 2005). During cell protrusion, Smurf1 induces

degradation of phosphorylated Par6 (Lu et al., 2008; Ozdamar et al., 2005). Through these actions, Smurf1 affects cell adhesion, enhances membrane protrusive behavior and promotes cell migration and invasion (Sanchez and Barnett, 2012). Based on its regulatory role in cell migration and EMT, Smurf1 may have a potential effect on tumor invasion and metastasis. Smurf1 is also associated with certain cancers, including breast cancer, gastric adenocarcinoma and pancreatic cancer. In breast cancer, down-regulation of Smurf1 suppresses tumor cell migration (Fukunaga et al., 2008). Smurf1 was chosen as a candidate enhancer in gastric adenocarcinomas (van Dekken et al., 2009). Smurf1 was shown to be highly expressed in pancreatic cancer cells, suggesting a role of Smurf1 as an oncogenic factor (Bashyam et al., 2005; Kwei et al., 2011; Suzuki et al., 2008).

I.5. Wnt/β-Catenin signaling and tooth development

There are 19 Wnt genes found in humans. Wnt proteins signal across cell membranes by interacting with receptors of the Frizzled (Fzd) family. The Wnt/β-catenin or canonical pathway is activated by Wnt ligands such as Wnt1, Wnt2, Wnt3a and Wnt10a, whereas non-canonical or Wnt/Ca^{2+} pathways are activated by Wnt ligands such as Wnt5a and Wnt11 (Gordon and Nusse, 2006). When the canonical Wnt signaling switch is off, cytoplasmic β-catenin is associated with adenomatous polyposis coli (Apc) and Axin proteins, and is phosphorylated by glycogen synthase

kinase 3β (Gsk3β) and casein kinase 1α (Ck1α), resulting in polyubiquitination by βTrcp1 or βTrcp2 complexes and targeting for proteasomal degradation (Liu et al., 1999). When the canonical Wnt ligand binds to a Fzd receptor and a low-density lipoprotein-related-receptor protein (Lrp) 5 or 6 co-receptor, Fzd interact with the cytoplasmic protein Disheveled (Dsh), resulting in Ck1α-mediated phosphorylation of Dsh and its binding to Gsk3β with the help of Frat protein. These interactions cause inactivation of the Axin/Apc/Gsk3β/Ck1α complex and stabilization of cytoplasmic β-catenin, resulting in nuclear translocation of β-catenin. Nuclear β-catenin binds to Lymphoid Enhancer Factor/T-cell factor (Lef/Tcf) and activates the transcription of target genes (Kramps et al., 2002).

Multiple secreted and intracellular inhibitors control activity of the Wnt/β-catenin signaling pathway. Among the secreted Wnt inhibitors, Dickkopf (Dkk) family members interact with Lrp5/6 co-receptors and cause endocytosis of the Wnt/Lrp5/6 complex, whereas secreted Frizzled related protein family members and Wnt inhibitory factor 1 inhibit the binding of Wnt ligands to Fzd family receptors (Kawano and Kypta, 2003).

Wnt/β-catenin signaling plays essential roles in skeletal development (Chen and Alman, 2009; Takada et al., 2009; Westendorf et al., 2004). Wnt ligands have been identified as critical mediators for limb morphogenesis/development and joint formation (Takada et al., 2009). Deficiencies of non-canonical Wnt signal transducers

lead to abnormal bone formation throughout development (Fujino et al., 2003; Harada et al., 1999). Recent studies indicate that Wnt signaling is an important regulator of both osteogenic and adipogenic differentiation (Kang et al., 2007; Takada et al., 2009). Wnt signalings promote osteoblastic differentiation while suppressing adipogenesis (Glass et al., 2005; Krishnan et al., 2006). The canonical Wnt/β-catenin signaling and Runx2 act together and promote osteoblast differentiation (Bennett et al., 2005; Glass and Karsenty, 2007). The non-canonical Wnt pathway suppresses the adipogenic transcription factor PPARγ while enhancing the expression of osteogenic transcription factor Runx2 (Kang et al., 2007; Takada et al., 2007).

Tooth development proceeds through several morphogenetic stages: initiation, bud, cap, bell and cytodifferentiation stages (differentiation of the epithelial ameloblasts and mesenchymal odontoblasts which produce tooth enamel and dentin, respectively). Wnt/β-catenin signaling is active during all the stages of tooth development, and plays multiple roles in tooth morphogenesis (Jarvinen et al., 2006; Liu et al., 2008). Lef1-deficient mice revealed the arrest of tooth growth at the bud stage and similarly, epithelial overexpression of Dkk1 or mesenchymal knockout of β-catenin halted tooth development at the bud stage (Chen et al., 2009; Liu et al., 2008; van Genderen et al., 1994). It was also suggested that Wnt signaling is important in the function of the enamel knot, which is an important signaling structure at the cap stage. Mice overexpressing β-catenin developed supernumerary teeth (Jarvinen et al., 2006).

Wnt7b is expressed at the initiation stage throughout the oral epithelium and inhibits the expression of Sonic Hedgehog (Sarkar et al., 2000). Wnt10b expression is restricted to initiation site epithelium whereas Wnt5a is present in oral mesenchymal cells at the early stage (Sarkar and Sharpe, 1999). At the cap stage, Wnt3, Wnt5a, Wnt6 and Wnt10b are expressed in the enamel knot, whereas the adjacent dental papilla mesenchyme expresses Wnt5a (Peng et al., 2010b; Sarkar and Sharpe, 1999). Wnt5a expression localizes to the dental papilla at the bell stage and later to odontoblasts. It has been demonstrated that Wnt5a inhibits proliferation and migration of human dental pulp cells but stimulates their differentiation to odontoblasts with enhanced expression of Osteonectin, Osteopontin and Dmp1 (Peng et al., 2010a; Peng et al., 2010b). Wnt5a was also shown to be expressed in ameloblastomas and *in vitro* studies demonstrated that Wnt5a may regulate development of oral tumors (Sukarawan et al., 2010). Wnt6 stimulates differentiation of human dental pulp cells toward odontoblasts and increases the expression of Osteopontin, bone sialoprotein (Bsp) and Dmp1 (Wang et al., 2010). Wnt10a was expressed in polarizing odontoblasts in a pattern suggesting that it is an upstream signal for the Dspp expression (Yamashiro et al., 2007). These results indicate that multiple Wnt ligands may function in the maturation of the odontoblasst phenotype. However, the role of Wnt signaling in ameloblasts differentiation is less clear because epithelial overexpression of Wnt3 in mice resulted in a postnatal loss of ameloblasts, while LiCl, a canonical Wnt signaling activator, increased the expression of Enamelin, an ameloblasts differentiation marker

gene (Millar et al., 2003; Tian et al., 2010).

II. INTRODUCTION

Tooth is made up of enamel, dentin, cementum and pulp. Enamel is the hardest mineralized tissue in the body and functions as a frame of tooth. Dentin is also a mineralized tissue underlying enamel and cementum, and it is secreted by the odontoblasts (Tziafas, 2004). Cementum is excreted by cementoblasts and helps periodontal ligament to attach to the tooth. Pulp is soft connective tissue located in the central part of the tooth that contains blood vessels and nerves.

In human, odontoblasts first appear around 17-18 weeks *in utero* and remains throughout the life unless affected by external stimuli, such as bacterial infection, chemical attack and trauma. Odontoblasts are tall and columnar in shape and differentiated from ectomesenchymal cells originated from the neural crest (Ruch, 1998). During dentinogenesis, odontoblasts secrete the organic matrix which contains a mixture of collagenous and non-collagenous proteins and is subsequently mineralized. Odontoblasts are joined together through adherence junctions at the apical ends of cell bodies (Nishikawa and Kitamura, 1987; Sasaki and Garant, 1996). Odontoblasts produce several types of dentin, including primary dentin, secondary dentin and tertiary dentin (Butler and Ritchie, 1995). Primary dentin, the most prominent dentin in the tooth, is produced during tooth development. Secondary dentin is a layer of dentin produced after the root is completely formed and tooth is functional. Tertiary dentin is formed in response to a pathologic stimulus, such as a carious attack.

Pulpotomy is a common therapy performed in a primary molar with extensive caries. The pulpotomy procedure involves covering pulp stumps with a pulp-capping agent to promote healing or an agent to fix the underlying tissue by formation of dentin bridge (Schuurs et al., 2000). Calcium hydroxide was the first medication that induced dentin bridge formation in pulpotomy (Dos Santos et al., 1977). Unfortunately, calcium hydroxide does not adhere to dentin and lacks the ability to seal, acting as pathways for microleakage (Cox et al., 1985). Another alternative pulpotomy agent is mineral trioxide aggregate (MTA), a biocompatible material that induces thicker dentin bridge than calcium hydroxide (Holan et al., 2005). However, dentin bridge that induces by calcium hydroxide or MTA is not true dentin but is simple ectopic intrapulpal calcifications or mineralized tissue more like to bone tissue (Ricucci et al., 2014). Therefore, studies for the reagents that induce newly differentiated odontoblasts to recapitulate the secretory, defense and sensory functions of the primary odontoblasts are necessary.

Post-translational modification of histones regulates gene expression through the modulation of accessibility of chromatin remodeling complexes, transcription factors and transcription machinery. Histone acetyltransferases transfer acetyl group to lysine residues in core histones, promoting RNA polymerase II binding and gene transcription. In contrast, HDAC removes acetyl groups from acetylated lysine residues and promotes chromatin condensation, inhibiting gene transcription (Grunstein, 1997; Hassig and Schreiber, 1997; Lee et al., 1993). Non-histone proteins are also the

substrates of histone acetyltransferases/HDAC with varying functional effects. There are 18 HDACs which are classified into three groups: Class I (HDAC 1, 2, 3, 8 and 11), Class II (HDAC 4, 5, 6, 7, 9 and 10) and Class III (SIRT 1 to 7).

Several classes of HDAC inhibitors have been identified, including hydroxamic acids, cyclic peptides, short-chain fatty acids and epoxides (Rodriguez-Menendez et al., 2008). These HDAC inhibitors suppress the activity of most class I and II HDAC, but they have no effect on class III HDAC (Johnstone, 2002). Treatment of cells with HDAC inhibitors increases general acetylation level of core histones in the cells. However, HDAC inhibitors affect the expression of only a small subset of genes, leading to transcriptional activation of some genes, but repression of an equal or larger number of other genes (Vigushin and Coombes, 2004). Most HDAC inhibitors are being investigated as a therapeutic agent for cancers and parasitic and inflammatory diseases. HDAC inhibitors exert anti-tumor effects via modulating transcription of oncogenes, tumor suppressor genes and genes regulating angiogenesis and apoptosis (Chueh et al., 2014). HDAC inhibitors also have a long history of use in psychiatry and neurology as mood stabilizers and anti-epileptics (Eyal et al., 2004).

It has also been demonstrated that HDAC inhibitors increase Alp production and matrix mineralization in osteoblastic cells and *ex vivo*-cultured calvaria (de Boer et al., 2006; Iwami and Moriyama, 1993; Jeon et al., 2006; Schroeder and Westendorf, 2005). Considering the similar nature of mineralized matrix production between osteoblasts

and odontoblasts, it is suggested that HDAC inhibitors may exert a similar positive regulatory effect on odontoblast differentiation. However, effect of HDAC inhibitors on odontoblast differentiation has not been clearly elucidated.

The Nfi transcription factor family is involved in the development of several organ systems, such as the brain, lungs and skeleton (Gronostajski, 2000). Among the Nfi family members, Nfic is known to be an important regulator for odontoblast differentiation and dentinogenesis because Nfic deficient mice exhibit short and abnormal molar roots and aberrant odontoblast differentiation during tooth formation (Park et al., 2007; Steele-Perkins et al., 2003).

Among the non-collagenous proteins in dentin organic matrix, Dsp and Dpp are expressed at high level. Dsp and Dpp are cleavage products of a parent protein encoded by the Dspp gene (Yamakoshi, 2009). Dspp is expressed predominantly in odontoblasts and at low levels in osteoblasts (D'Souza et al., 1997; Qin et al., 2002). Mutations of the human DSPP gene are associated with dentinogenesis imperfecta type II and type III (Dong et al., 2005; Zhang et al., 2001). Dspp-deficient mice have teeth that display dentin mineralization defects that are similar to human dentinogenesis imperfect type III, indicating that Dspp plays a critical role in odontoblast differentiation and dentinogenesis (Sreenath et al., 2003). Dmp1 undergoes transcriptional regulation during tooth development and one of the differentiation maker genes of odontoblasts (Hao et al., 2004). Nestin has been known as a marker of neural stem cells or

progenitor cells (Lendahl et al., 1990). In tooth, Nestin is expressed during the tooth development, but disappears when development is complete (About et al., 2000). However, when the tooth is damaged by external stimulus such as caries or cavity preparation, Nestin expression is induced (About et al., 2000; About and Mitsiadis, 2001; McLachlan et al., 2003).

Smurf1, an E3 ubiquitin ligase, is expressed in many organs including bone, cartilage, heart, lung, nervous system and genital organs during all of the developmental stages from embryo to adult (Marin, 2010). Smurf1 exerts regulatory effects on intracellular signaling, cell cycle progression, differentiation and migration via the regulation of target protein degradation (Durrington et al., 2010; Guo et al., 2008). Cell adhesion, polarity and migration are important aspects of many physiological processes, including embryonic development, neurodevelopment and cancer metastasis. Smurf1 regulates cell migration via inducing degradation of RhoA, a small GTPase, in cellular protrusions (Wang et al., 2003). Smurf1 may have notable functions in immunological processes. Smurf1 degraded Traf family proteins which primarily involved in the regulation of inflammation (Li et al., 2010). Smurf1 also plays an important role as a negative regulator in BMP signaling and Mekk2 signaling pathways (Yamashita et al., 2005; Zhu et al., 1999). Smurf1 reduces cellular responses to BMP via triggering proteasomal destruction of Smad1 and Smad5, causing impaired BMP signal transduction and aberrant embryonic development in *Xenopus laevis* (Zhu et al., 1999). Smurf1 overexpression downregulates the expression of osteoblast

differentiation marker genes, and Smurf1 transgenic mice showed decreased bone volume and *in vitro* bone formation compared to normal mice (Zhao et al., 2003). Converting wild-type Smurf1 into a catalytic mutant (C699A), which loses the E3 catalytic ability, increased its osteoblastic ability (Zhao et al., 2003). The physiological role of Smurf1 *in vivo* was also characterized in Smurf1 knock-out mice which showed an increase in bone mass in aged mice (Yamashita et al., 2005). However, the regulatory role of Smurf1 in odontoblast differentiation has not been elucidated.

Wnts are the major family of morphogens which regulate cell-to-cell interactions during embryogenesis and are conserved across all metazoan species. Wnts regulate essential biological processes, including body axis patterning, cell proliferation, cell polarity and migration, stem cell renewal, cell fate specification and apoptosis (Clevers and Nusse, 2012; Logan and Nusse, 2004; Willert and Nusse, 2012). Wnt signaling pathways include the canonical and the non-canonical pathways (Nusse and Varmus, 1992; Nusse and Varmus, 2012). Canonical Wnt pathway modulates many cellular functions such as proliferation and differentiation by regulating gene transcription. Binding of Wnt ligands to Fzd receptors and Lrp family co-receptors causes β-catenin accumulation, nuclear translocation and transcriptional activation of target genes by forming β-catenin/Lef/Tcf complexes (Logan and Nusse, 2004). Without canonical Wnt ligand binding, the cytoplasmic β-catenin is tagged for ubiquitination and subsequent degradation by complexes containing Axin, Apc, protein phosphatase 2A, Gsk3β and Ck1α (Minde et al., 2011). The non-canonical pathway regulates the

cytoskeleton that is responsible for the regulation of the cell shape and motility.

It has been studied that Wnt/β-catenin signaling regulates tooth development at many levels (Jarvinen et al., 2006; Liu et al., 2008; Liu and Millar, 2010). Wnt3 ligand shows specific expression in the enamel knot, and the overexpression of Wnt3 causes progressive loss of ameloblasts and reduction of enamel in postnatal mouse incisor teeth (Millar et al., 2003). In *Lef1* knockout mice, tooth development arrests at the bud stage (van Genderen et al., 1994). Targeted epithelial overexpression of the secreted Wnt antagonist Dkk1 arrests tooth development at the lamina stage in mice, which is slightly earlier than the arrest to tooth development seen in *Lef1*-knockout mice (Liu et al., 2008). β-Catenin, as the central component of the Wnt/β-catenin pathway, has been found to have a central role in tooth development (Chen et al., 2009; Jarvinen et al., 2006). Inactivation of β-catenin in mesenchyme of developing tooth results in arrested tooth development at the bud stage, while β-catenin activation in embryogenesis or post-natal life causes ectopic tooth formation (Liu et al., 2008; Liu and Millar, 2010). Tooth development and dental repair processes share some common molecular mechanisms (Mitsiadis and Rahiotis, 2004; Smith, 2002). Therefore, β-catenin may also participate in odontoblast differentiation during reparative dentin formation.

It has been studied that HDAC inhibitors regulates Wnt/β-catenin signaling in cancer cells. HDAC inhibitors promote the expansion of breast cancer stem cells through dedifferentiation and have important clinical implications for the use of HDAC

inhibitors in the treatment of cancer (Debeb et al., 2012). In T lymphoblastic leukemi HDAC inhibitors exert profound anti-leukemic effects partly by augmentation of Wnt/β-catenin signaling and could be an attractive new strategy for the treatment of T lymphoblastic leukemia (Shao et al., 2012). However, it is not clear whether HDAC inhibitors regulate Wnt/β-catenin signaling in odontoblasts.

III. PURPOSE OF STUDY

The regulatory role of HDAC inhibitors and Smurf1 in osteoblast differentiation has been reported. In addition, the enhancing effect of HDAC inhibitors on Wnt/β-catenin signaling in cancer cells was described. However, the relationship of HDAC inhibitors and Smurf1 to odontoblast differentiation has not been clearly elucidated. In addition, the effect of HDAC inhibitors on Wnt/β-catenin signaling in odontoblast has not been demonstrated. Therefore, the purpose of this study is to investigate the relationships between HDAC inhibitors, Smurf1 and Wnt/β-catenin signaling in odontoblast differentiation as follows:

PART I: Effect of HDAC inhibitors on odontoblast differentiation and role of Nfic in HDAC inhibitor-induced odontoblast differentiation

PART II: Effect of HDAC inhibitors on Smurf1 expression and the role of Nfic as an intermediator

PART III: Effect of HDAC inhibitors on Wnt/β-catenin signaling in MDPC23 cells

PART IV: Interrelationships between Wnt/β-catenin signaling, Nfic and Smurf1 in odontoblast differentiation

IV. MATERIALS AND METHODS

IV.1. Cell culture

MDPC23 cells (Hanks et al., 1998) were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone; Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker; Walkersville, MD, USA), 100 ìg/ml streptomycin and 100 U/ml penicillin.

Odontoblastic differentiation of MDPC23 cells were induced by incubating cells in differentiation medium (growth medium supplemented with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid). When indicated, SAHA (in a concentration of 1 to 5 ìM; Cayman Chemical; Ann Arbor, MI, USA), TSA (in a concentration of 10 to 100 nM; Sigma-Aldrich; St. Louis, MO, USA), Wnt3a (in a concentration of 50 ng/ml; R&D Systems, Inc.; Minneapolis, MN, USA) or Dkk1 (in a concentration of 200 ng/ml; R&D Systems, Inc.; Minneapolis, MN, USA) were added to culture medium for the indicated periods. Stock solutions for HDAC inhibitors were prepared by dissolving SAHA and TSA in dimethyl sulfoxide (DMSO). Therefore, DMSO was used as a vehicle control for the following experiments. To inhibit proteasomal degradation, MG132 (in a concentration of 2.5 μM; Sigma-Aldrich) was added to culture medium 30 min before the addition of HDAC inhibitors and further incubated for the indicated periods. C2C12, mouse myoblast cell line, was maintained

in DMEM plus 10% FBS, and the experiments described in the Results section were performed in DMEM supplemented with 5% FBS. HEK293T cells were also cultured in DMEM plus 10% FBS.

IV.2. Cytotoxicity test

To test the cytotoxicity of SAHA, MDPC23 cells were plated at a density of 2×10^3 cells/well in 96-well tissue culture plates, and treated with SAHA for 48 h. Cell viability was then examined using Ez-CyTox (Daeil Lab Service; Seoul, Korea) according to the manufacturer's instructions.

IV.3. Mineralization assays

After MDPC23 cells were cultured in differentiation medium for 14 days, matrix mineralization was determined by Alizarin red S staining. At the end of culture period, the cells were fixed with 70% ethanol, washed with phosphate-buffered saline (PBS) and stained with Alizarin red S solution. To quantitatively determine the mineral content, the stain was eluted using 0.5 N HCl containing 5% sodium dodecyl sulfate (SDS), and the absorbance was measured at 415 nm.

IV.4. Reverse transcription-polymerase chain reaction (RT-PCR)

To evaluate mRNA expression, semi-quantitative or quantitative RT-PCR was performed in the range of linear amplification. Total RNA was isolated using easy-BLUE™ RNA Extraction Reagents (iNtRON Biotechnology; Sungnam, Korea). Complementary DNA was synthesized from total RNA using AccuPower™ RT PreMix (Bioneer; Daejeon, Korea) and subsequently subjected to PCR amplification using *Star*Taq™ polymerase (iNtRON Biotechnology). The PCR products were electrophoresed in 1.2% agarose gel and visualized under UV light by ethidium bromide staining. Mouse genes and their primer sequences for PCR reaction were as followed: Nestin-*f* 5'-CCCTGAAGTCGAGGAGCTG-3', Nestin-*r* 5'-CTGCTGCACCTCTAAGCGA-3'; Alp-*f* 5'-AGGCAGGATTGACCACGG-3', Alp-*r* 5'-TGTAGTTCTGCTCATGGA-3'; Dmp1-*f* 5'-AACTGGAAGTGATGAGGAC-3', Dmp1-*r* 5'-TTTAGATTCTTCCGACCTGA-3'and Gapdh-*f* 5'-TCACCATCTTCCAGGAGCG-3', Gapdh-*r* 5'-CTGCTTACCACCTTCTTGA-3'.

Quantitative real time-PCR was also performed using SYBR premix EX Taq (Takara; Otsu, Japan) in an AB7500 Fast Real-time system (Applied Biosystem; Foster City, CA, USA). Mouse genes and their primer sequences for quantitative real time-PCR were as follows: Nfic-*f* 5'-GACCTGTACCTGGCCTACTTTG-3', Nfic-*r* 5'-CACACCTGACGTGACAAAGCTC-3'**;** Dspp-*f* 5'-ATTCCGGTTCCCCAGTTAGTA-3', Dspp-*r* 5'-CTGTTGCTAGTGGTGCTGTT-3';

Nestin-*f* 5'-CCCTGAAGTCGAGGAGCTG-3', Nestin-*r* 5'-CTGCTGCACCTCTAAGCGA-3'; Dmp1-*f* 5'- CATTCTCCTTGTGTTCCTTTGGG-3', Dmp1-*r* 5'-TGTGGTCACTATTTGGCTGTG-3'; β-catenin-*f* 5'-ATGGAGCCGGACAGAAAAGC-3', β-catenin-*r* 5'-CTTGCCACTCAGGGAAGGA; Wnt3a-*f* 5'-CATGCACCTCAAGTGCAAATG-3′, Wnt3a-*r* 5′-TGAGGAAATCCCCGATGGT-3′ and Gapdf-*f* 5'-TCAATGACAACTTTGTCAAGC-3', Gapdh-*r* 5'-CCAGGGTTTCTTACTCCTTGG-3'. For quantification, Gapdh was used as the reference for the normalization of each sample.

IV.5. Western blot analysis and immunoprecipitation

At the end of culture periods, the cells were washed with PBS, lysed using PRO-PREP™ (iNtRON Biotechnology) and sonicated briefly. Samples containing equal amounts of protein were subjected to sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electro-transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. The membrane was incubated with the indicated primary antibody followed by incubation with HRP-conjugated secondary antibody. Immune complex were visualized with the Sensi-view™ Pico ECL Reagent (Lugen Sci Inc.; Bucheon, Korea) and detected with a MicroChemi (DNR; Jerusalem, Israel).

For immunoprecipitation, cells were washed twice with ice-cold PBS ad scraped into immunoprecipitation buffer consisting of 20 nM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 2% Brij35, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μM aprotinin, 1 μM leupeptin and 1 μM pepstatin, and sonicated briefly. Samples containing equal amounts of protein were incubated with 4-6 μg of primary antibody overnight at 4°c with gentle agitation. The bead pellets were washed five times with immunoprecipitation buffer, denatured by boiling in 2X SDS sample buffer, and then subjected to SDS-PAGE and immunoblot analysis.

IV.6. Biotin pull-down assay

A biotin pull-down assay was performed to analyze the *in vitro* binding of Nfic to biotinylated oligonucleotides whose sequence was composed of -1030 to -1007 bp of the mouse Dspp gene promoter. MDPC23 cells were transiently transfected with pcDNA or Nfic expression plasmids and incubated in the presence or absence of SAHA for 48 h. Nuclear proteins (300 mg) were isolated and incubated with 5′-biotinylated double-stranded oligonucleotides (1 mg), followed by incubation with streptavidin-agarose beads. When indicated, non-biotinylated wild-type oligonucleotides or non-biotinylated mutant oligonucleotides containing nucleotide substitutions in the Nfic binding element (CCAAT→CTGAT) were added to the

reaction mixture in a 50-fold molar excess just prior to the addition of the biotinylated probe. The complex was pulled down, and proteins in the complex were dissociated and analyzed by Western blotting with anti-Nfic antibody. The DNA sequences of oligonucleotides were as follows: wild-type *f* 5′-GGAACCTGTCCAATCCCTACCTAC-3′ and mutant *f* 5′-GGAACCTGTC*tg*ATCCCTACCTAC-3′.

IV.7. Plasmid construction and luciferase reporter assays

The Nfic expression plasmid and the luciferase reporter constructs Dspp-luc and Nfic-luc containing the mouse Dspp promoter (1.5 kb) and Nfic promoter (1.6 kb), respectively, were kindly provided by Prof. JC Park at the Seoul National University (Lee et al., 2009). The construction of expression vectors for Smurf1 and â-catenin and luciferase reporter constructs Bsp-luc and Ocn-luc containing the mouse Bsp and Osteocalcin (Ocn) promoter, respectively, has been previously described (Benson et al., 1999; Cho et al., 2012; Jeon et al., 2006; Jun et al., 2010). Function-defective reporters construct (Dspp-MT-luc) that bears mutations in the putative Nfic binding site at -1021 to -1017 bp (CCAAT→CTGAT) was produced by Cosmogenetech (Seoul, Korea). The DNA sequence of the reporter constructs was confirmed by a sequencing analysis.

For luciferase reporter assays, MDPC23 cells were seeded at a density of 1×10^4

cells/well in a 96-well plate. After an overnight culture, the cells were transiently transfected with the indicated plasmids using LipofectAMINE™ reagent (Invitrogen; Carlsbad, CA, USA) and incubated for 24 h in the presence or absence of 5 μM SAHA. In each transfection, 0.2 μg of reporters (pGL3-basic, Nfic-luc, Bsp-luc, Ocn-luc, Dspp-luc, Dspp-MT-luc, TOP FLASH or FOP FLASH) and 0.07 μg of *Renilla* luciferase plasmids were used. Then, the cells were harvested after 24 h and luciferase activity was measured using the Dual-Glo™ luciferase assay kit (Promega; Madison, WI, USA) according to the manufacturer's instructions. Briefly, firefly luminescence was measured after equilibrated Dual-Glo® Reagent was added to each well. Then, equal volumes of Dual-Glo® Stop and Glo® Reagent were added, and *Renilla* luminescence was measured. The relative luciferase activity was calculated by dividing firefly luciferase activity by *Renilla* luciferase activity to normalize the transfection efficiency.

To examine the transcriptional activity of â-catenin, MDPC23 cells were plated in 24-well plates and incubated overnight at 37°C. On the following day, the cells were transfected with 1 μg of β-catenin expression plasmid and Lef/Tcf-sensitive (TOP) or Lef/Tcf-insensitive (FOP) reporter vector using Lipofectamine 2000 according to the manufacturer's instructions. On the following day, cells were washed with PBS and treated with HDAC inhibitors for 24 h. Luciferase activities were then determined as described above.

IV.8. Chromatin immunoprecipitation (ChIP) assays

Cells were cross-linked with 1% formaldehyde, lysed and sonicated to get DNA fragments of 200-800 bp. After pre-clearing with blocked protein G agarose, immunoprecipitation was carried out with the indicated antibody or equivalent concentrations of IgG (Santa Cruz, Ca, USA) from the same species with the indicated antibody as a negative control. Anti-Nfic, anti-acetyl-H3 and anti-RNA polymerase II (Pol II) antibodies were purchased from Cell Signaling Technology (Dancers, MA, USA) and anti-H3K4me (detecting both H3K4me2 and H3K4me3) antibody was obtained from Abcam (Cambridge, MA, USA). DNA was then purified and subjected to semi-quantitative or quantitative PCR to amplify the indicated promoter region of the mouse Dspp, Smurf1, Wnt3a and β-catenin gene. Primer sequences used for PCR were as follows: Dspp *f* 5'-GCAAAGTGTTTACTATGGAAG-3' and *r* 5-CACTATCCAGCAACAGCATC-3'; Smurf1 site 1 *f* 5'-GCACATCCGACACTCGATCA-3', *r* 5'-GCACATCCGACACTCGATCA-3' and Smurf1 site 2 *f* 5'-GCAGGAACCTTGCAGCTC-3', *r* 5'-ACGGGCTGGAACCGTAGAA-3'; Wnt3a *f* 5'- GCCTCAAAGTTGCCAAGAGC-3', Wnt3a *r* 5'- GGATGTGCCTAGGTGTGCAT and β-catenin *f* 5'-GCCCGCTCTCGATTCCTTTA-3', β-catenin *r* 5'-TTCACAGGACACGAGCTGAC.

IV.9. Gene knockdown by small interfering RNA

Small interfering RNAs (siRNA) to mouse Nfic, Smurf1, Wnt3a or β-catenin and non-targeting control siRNA (ON-TARGETplus Non-targeting siRNA #2 D-001810-02-5) were purchased from Dharmacon (Lafayette, CO, USA). Transient transfection of siRNA was performed using DharmaFECT 4 Transfection Reagent (Dharmacon) according to the manufacturer's instructions. The siRNAs (TARGETplus SMARTpool) used in this study are a mixture of four siRNAs targeting independent sequences of the target mRNA. The sequences of each siRNA are as follows: Nfic siRNA, 5'-GCAGAAGAFFCUCGAGGGA-3', 5'-UCUGAAGGGUUCCAGCGUAA-3', 5'-GCUCGAUGGAAGAGGACGU-3' and 5'-GCACAGAUGGUGAGCGGCU-3'; Smurf1 siRNA, 5'-CCAAAUAGUGGUCAGUUUA-3', 5'-AAACUAAAUCCCUCAGAUA-3', 5'-CAAGAUCCGUCUGACAGUA-3' and 5'-UAGGUGGGCUGGAUAAGAU-3'; â-catenin siRNA, 5'-AAGCUGACCUGAUGGAGUU-3', 5'-CAGCAAAUCAUGCGCCUUU-3', 5'-GCGCUUGGCUGAACCAUCA-3' and 5'-GUGAAAUUCUUGGCUAUUA-3' and Wnt3a siRNA, 5'-GGGCAUGACAGCCUCGUAU-3', 5'-CUGUCUGGGAUACGGGUUU-3', 5'-ACUCAGAUCUCCCGGGAAA-3' and 5'-ACUUCAAGGUGCCGACAGA-3'. The efficacy of knockdown was assessed by real-time PCR and Western blot analysis.

IV.10. Statistical Analysis

All of the quantitative data are represented as the mean ± SD. Each experiment was performed with a sample number of 3 to 4 and repeated at least twice. The results from the representative experiment were presented. Statistical significance was analyzed by Student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

V. RESULTS

V.1. SAHA enhances odontoblast differentiation through increasing Nfic expression

V.1.1. SAHA increases odontoblast differentiation

Before evaluating the odontogenic effect of SAHA, it was examined whether SAHA shows cytotoxicity in MDPC23 cells. Cells were incubated for 48 h in the presence or absence of SAHA (1, 3 and 5 ìM) and cytotoxicity was evaluated. SAHA did not show any cytotoxic effects at the concentrations used in this study (Fig. 1A). Furthermore, significant cell death was not observed even when MDPC23 cells were incubated with 5 ìM SAHA for 14 days under differentiation conditions (data not shown).

The effect of SAHA on odontoblast mineralization was evaluated. MDPC23 cells were cultured in differentiation medium for 14 days, and matrix mineralization was determined by Alizarin red S staining. SAHA enhanced matrix mineralization in a dose-dependent manner (Figs. 1B). Elution of Alizarin red S stain and determination of optical density demonstrated that mineral contents deposited in the matrix significantly increased in a dose-dependent manner (Fig. 1C). To examine whether the matrix mineralization effect of SAHA is cell line-specific, the same experiments were performed with OD11 cells, another murine pre-odontoblast cell line. Similar to the

results from MDPC23 cells, SAHA enhanced matrix mineralization of OD11 cells (Fig. 1D). Furthermore, TSA also exhibited stimulatory effect on matrix mineralization of MDPC23 cells at the concentration of 50 nM (Fig. 1E). These results suggest that HDAC inhibitors have enhancing effect on matrix mineralization of odontoblasts at the sub-cytotoxic concentrations.

To verify that SAHA-enhanced matrix mineralization is associated with odontoblast differentiation, mRNA levels of odontoblast differentiation marker genes were examined. Total RNA was prepared from the cells incubated for 7 days in differentiation medium. Consistent with Alizarin red S staining results, SAHA increased the mRNA levels of Nestin, Alp, Dmp1 and Dspp (Fig. 2). Stimulatory effect of SAHA on the expression of odontoblast differentiation marker genes was most noticeable at a concentration of 5 ìM. Therefore, SAHA at a concentration of 5 ìM was used for the following experiments. These results indicate that SAHA stimulates matrix mineralization through the enhancement of odontoblastic differentiation of MDPC23 cells but not by cytotoxicity and non-specific mineral deposition.

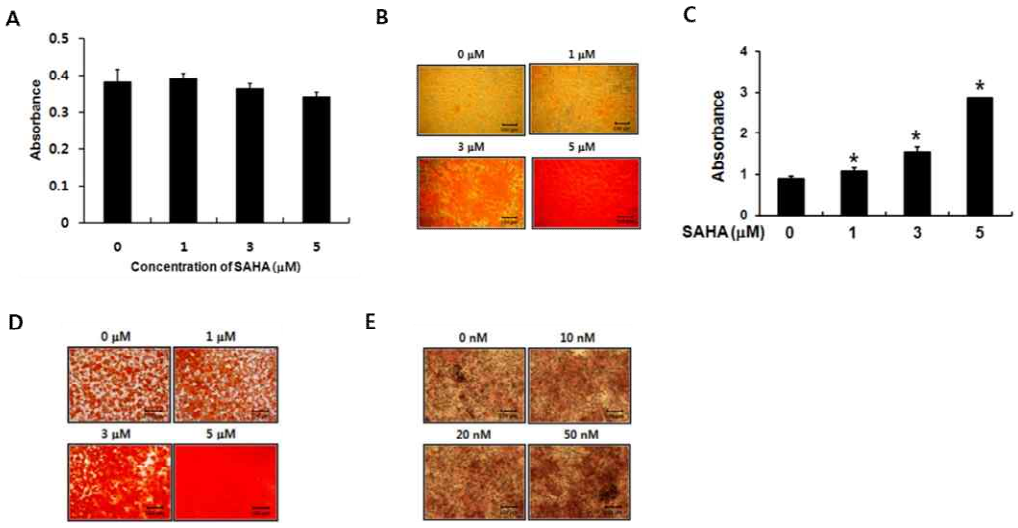

Fig. 1. SAHA stimulated odontoblast differentiation. (A) MDPC23 cells were incubated in the presence of SAHA for 48 h, and cytotoxicity was evaluated. SAHA does not induce cytotoxic effects in MDPC23 cells at the concentrations used in this study. (B, C) MDPC23 cells were cultured in odontoblast differentiation medium for 14 days with SAHA at the indicated concentrations. (D) OD11 cells were incubated in odontoblast differentiation medium for 7 days in the presence or absence of SAHA. (E) MDPC23 cells were cultured in odontoblast differentiation medium for 14 days in the presence or absence of TSA. Matrix mineralization was examined by Alizarin red S staining (B-E). Bar indicates 100 ìm. For quantitative determination of mineral content, the stain was eluted, and the absorbance was measured at 415 nm (C). Data represent the mean + SD. *$p<0.05$, significantly different from vehicle control.

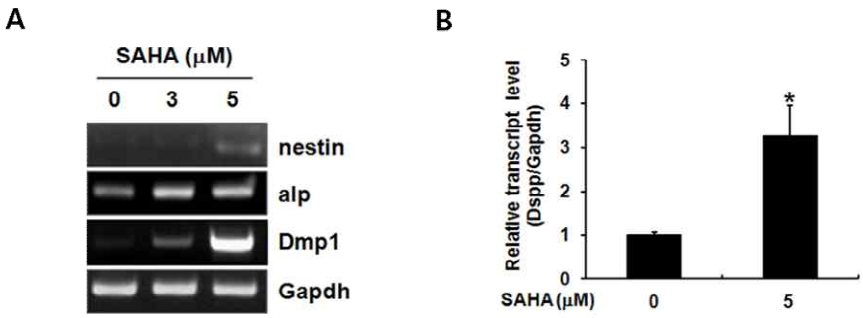

Fig. 2. SAHA increased the mRNA levels of odontoblast differentiation marker genes. MDPC23 cells were cultured in odontoblast differentiation medium for 7 days in the presence or absence of SAHA. Total RNA was prepared, and semi-quantitative (A) or quantitative (B) RT-PCR for odontoblast differentiation marker genes was performed. Data are presented as the mean + SD of triplicates. *$p<0.05$, compared to vehicle control.

V.1.2. SAHA increases the acetylation levels of H3 and Runx2 proteins in MDPC23 cells

Previous reports demonstrated that HDAC inhibitors enhance osteoblast differentiation via increasing acetylation and transcriptional activity of Runx2 (Jeon et al., 2006; Schroeder et al., 2004). To confirm that SAHA shows sufficient HDAC inhibitor activity in MDPC23 cells, the acetylation levels of histone H3 and Runx2 proteins were determined. MDPC23 cells were transiently transfected with myc-tagged Runx2 expression vector and incubated in the presence or absence of SAHA for 24 h. The level of acetyl-H3 was examined by Western blot analyses with anti-acetyl H3 and anti-H3 antibodies (Fig. 3A). The level of acetyl-Runx2 was examined by immunoprecipitation with anti-myc antibody and subsequent Western blot analyses using anti-acetyl-lysine and anti-myc antibodies (Fig. 3B). SAHA increased the acetylation levels of H3 and myc-Runx2 compared to the control, suggesting that SAHA exert significant inhibitory effect on HDACs in MDPC23 cells at a concentration of 5 ìM.

Because the Ocn and Bsp promoters contain Runx2 binding elements, the regulatory effect of SAHA on the transcriptional activity of Runx2 was also evaluated using these promoter reporters in HEK293T cells (Jeon et al., 2006; Roca et al., 2005). SAHA significantly increased the transcriptional activity of Runx2 on both Ocn and Bsp promoter reporters in HEK 293T cells (Fig. 3C). However, when Bsp-luc reporter

assays were performed in MDPC23 cells, SAHA significantly reduced both basal and Runx2-induced Bsp-luc reporter activity (Fig. 3D). In addition, overexpression of Runx2 did not increase Dspp-luc activity in MDPC23 cells but SAHA significantly increased Dspp promoter activity regardless of Runx2 overexpression (Fig. 3E). These results suggest that SAHA enhance Dspp promoter activity through the regulation of the target proteins other than Runx2 in MDPC23 cells.

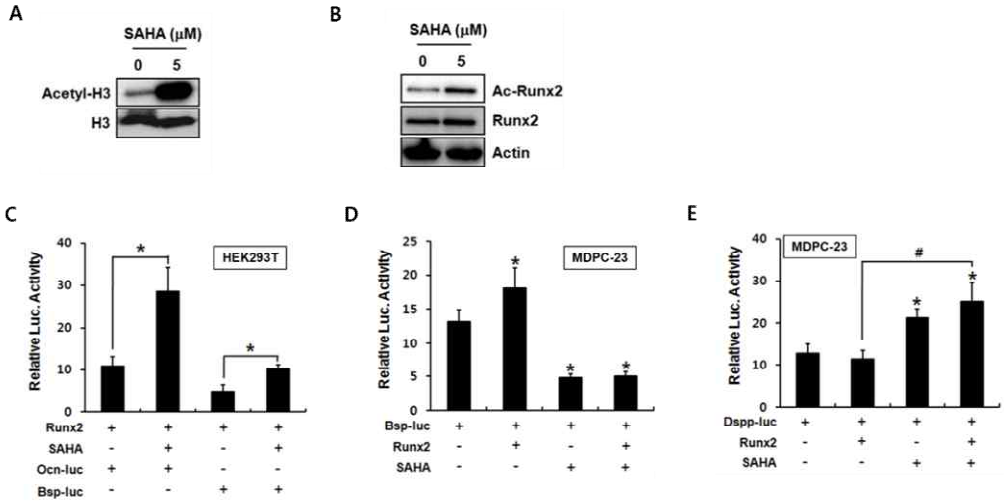

Fig. 3. SAHA increased the acetylation levels of histone H3 and Runx2 in MDPC23 cells. (A, B) MDPC23 cells were transiently transfected with myc-tagged Runx2 expression vectors and incubated in the presence or absence of SAHA (5 ìM) for 24 h. Western blot analysis was then performed with anti-acetyl H3 and anti-H3 antibodies (A), or immunoprecipitation was performed with an anti-myc antibody, followed by Western blot analyses with anti-acetyl-lysine (Ac-Runx2) or anti-myc (Runx2) antibodies (B). Actin bands were presented as an input control. (C-E) HEK293T (C) and MDPC23 (D, E) cells were transiently transfected with the indicated plasmids and incubated in the presence or absence of SAHA for 24 h. Luciferase activities were then measured with the Dual-Glo® luciferase assay kit. Transfection efficiency was normalized by dividing firefly luciferase activity to *Renilla* luciferase activity. Data represent the mean + SD (N = 6). When it is not specified, asterisk indicates significant difference compared to Bsp-luc or Dspp-luc alone (D, E).

V.1.3. SAHA increases Nfic expression in MDPC23 cells

To determine the molecular mechanism by which SAHA enhances odontoblast differentiation, regulation of Dspp expression was determined because Dspp is a representative odontoblast marker gene. Because a previous report has shown that Nfic overexpression increases Dspp expression (Lee et al., 2009), regulatory effect of SAHA on Nfic expression was evaluated. Consistent with the previous report, Nfic overexpression significantly increased Dspp expression in MDPC23 cells (Fig. 4A). Treatment of cells with 5 ìM SAHA for 48 h increased Nfic expression at both mRNA and protein levels (Figs. 4B, 4C). Furthermore, SAHA also increased the activity of luciferase reporter which contains the mouse Nfic promoter sequence, indicating that SAHA stimulates Nfic transcription in MDPC23 cells (Fig. 4D). These results suggest that SAHA may up-regulate Dspp expression via enhancing Nfic expression.

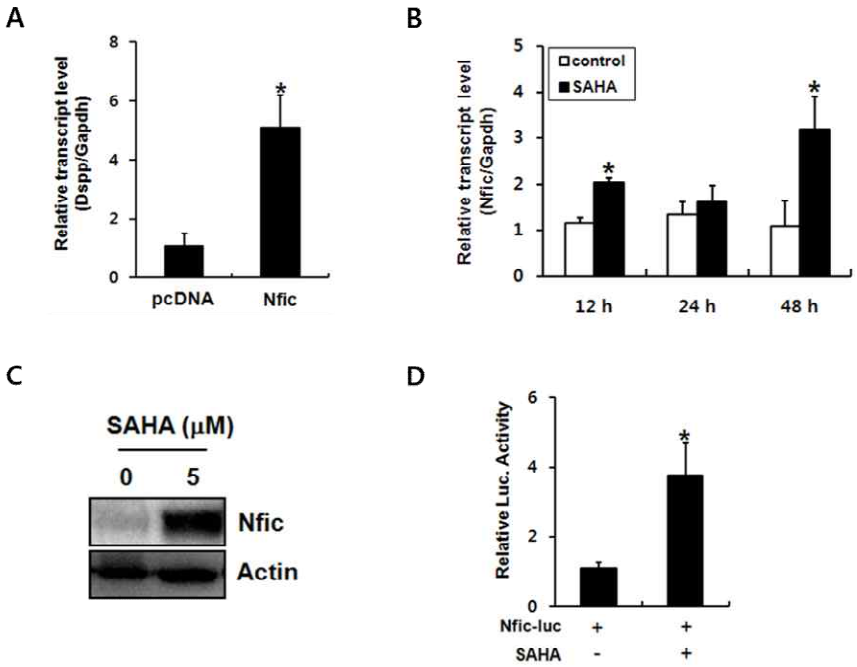

Fig. 4. SAHA increased Nfic expression in MDPC23 cells. (A) MDPC23 cells were transiently transfected with pcDNA or Nfic expression plasmids and incubated for 24 h. Dspp mRNA expression levels were examined by real-time PCR. (B) MDPC23 cells were incubated for the indicated time periods in the presence or absence of 5 ìM SAHA. Nfic mRNA levels were examined by real-time PCR. (C) MDPC23 cells were incubated for 48 h in the presence or absence of 5 ìM SAHA. Nfic protein levels were examined by Western blot analysis. (D) SAHA increased Nfic promoter activity. MDPC23 cells were transiently transfected with the Nfic promoter reporter plasmid containing the 1.6-kb mouse Nfic promoter (Nfic-luc) and incubated for 24 h in the presence or absence of 5 ìM SAHA. Subsequently, the luciferase reporter assay was performed. Data are shown as firefly luciferase activity relative to *Renilla* luciferase activity. Data represent the mean + SD (*$p<0.05$).

V.1.4. Nfic knockdown suppresses SAHA-mediated induction of odontoblast differentiation

To examine the importance of Nfic in SAHA stimulation of odontoblast differentiation and Dspp expression, Nfic was silenced using siRNA (Fig. 5). Knockdown efficiency of Nfic siRNA was verified by examining the expression levels of Nfic mRNA and protein in both vehicle- and SAHA-treated cells (Figs. 5D, 5E). Knockdown of Nfic suppressed SAHA enhancement of odontogenic marker gene expression, including Dspp, Dmp1, and Nestin (Figs. 5A-5C). Nfic knockdown also prevented SAHA stimulation of Dspp protein expression and Dspp-luc reporter activity (Figs. 5E, 5F). These results further demonstrate that Nfic plays a role as a mediator for SAHA stimulation of Dspp expression.

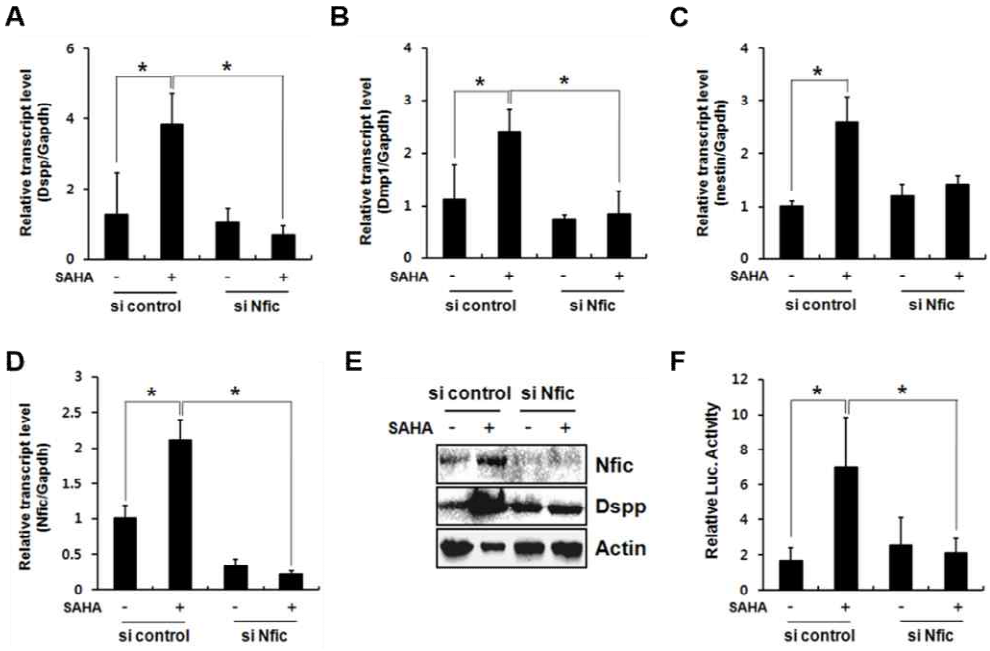

Fig. 5. Nfic knockdown suppressed SAHA stimulation of odontoblast differentiation marker gene expression in MDPC23 cells. (A-E) MDPC23 cells were transiently transfected with Nfic siRNA or control siRNA and incubated in the presence or absence of 5 μM SAHA for 48 h. The effect of Nfic knockdown on the expression of Dspp (A, E), Dmp1 (B), and Nestin (C) was analyzed by real-time PCR and Western blot analysis. The knockdown efficiency of Nfic siRNA was confirmed by real-time PCR (D) and Western blot analysis (E). Data are presented as the mean + SD of triplicates (*$p<0.05$). (F) MDPC23 cells were transiently transfected with Nfic siRNA or control siRNA and incubated overnight. Then, the cells were transfected with Dspp-luc and *Renilla* luciferase reporters and incubated for an additional 24 h in the presence or absence of 5 μM SAHA, followed by a luciferase reporter assay. Data are shown as firefly luciferase activity relative to *Renilla* luciferase activity. Data represent the mean + SD of quadruplicates (*$p<0.05$).

V.1.5. SAHA-induced Nfic increases Dspp transcription via direct binding to the mouse Dspp promoter

To examine whether SAHA enhances Dspp expression through the transcriptional activation by Nfic, *in silico* analysis was performed using the Transcription Element Search System to find Nfic binding elements in the Dspp promoter. Although the dyad symmetric consensus sequence for Nfic binding was not found within the 1.5 kb mouse Dspp promoter, three putative half-sites were present, at -1021 to -1017 (site 1), -857 to -853 (site 2) and -174 to 170 (site 3) bp (Fig. 6A). To determine the functionality of these putative binding sites, ChIP assay was performed. Because Nfic overexpression did not up-regulate the luciferase activity of Dspp promoter reporter that contained -791 to +54 bp of the moue Dspp gene in a previous report (Lee et al., 2009), site 3 (-174 to -170 bp) was excluded in this study. MDPC23 cells were transiently transfected with pcDNA or an Nfic expression plasmid and incubated for 24 h in the presence or absence of SAHA. DNA fragments were then immunoprecipitated with anti-Nfic antibody or control IgG. PCR amplification of the Dspp promoter regions containing the putative Nfic binding site 1 or 2 revealed that Nfic bound to site 1 (Fig. 6B) but not site 2 (data not shown). PCR amplification of DNA fragments immunoprecipitated with control IgG did not produce any amplified DNA bands, suggesting that the PCR reactions are specific. Binding of Nfic to site 1 was slightly increased by Nfic overexpression or SAHA treatment. The combination of SAHA treatment with Nfic overexpression further increased DNA binding by Nfic (Fig.

6B).

To verify the direct binding of Nfic to the Dspp promoter, biotin pull-down assay was performed. Consistent with the ChIP assay results, Nfic bound to the biotinylated oligonucleotides containing the Nfic-binding site 1 in the Dspp promoter in an Nfic-protein amount-dependent manner (Fig. 6C, upper panel). Nfic binding to biotinylated probes was abolished by wild-type competitors but not by mutant competitors (Fig. 6C, lower panel). These results further indicate that Nfic directly bind to the Dspp promoter in a sequence-specific manner.

To further confirm that Nfic transactivates Dspp through binding on site 1, Dspp promoter reporter assays was performed using Dspp-luc and Dspp-MT-luc (Fig. 6D). Nfic over-expression significantly increased the reporter activity of Dspp-luc but not Dspp-MT-luc (Figs. 6E, 6F). These results indicate that Nfic directly binds to the site 1 of the mouse Dspp promoter which, in turn, induces the expression of the Dspp gene. Similar to the ChIP assay result, SAHA increased basal Dspp-luc reporter activity and further enhanced Nfic-induced Dspp-luc activity (Fig. 6E). In addition, SAHA-induced Dspp promoter activity was not observed in Dspp-MT-luc reporter (Fig. 6F).These results indicate that SAHA increases Dspp expression, at least in part, through the enhancement of Nfic expression and direct binding to the Dspp promoter.

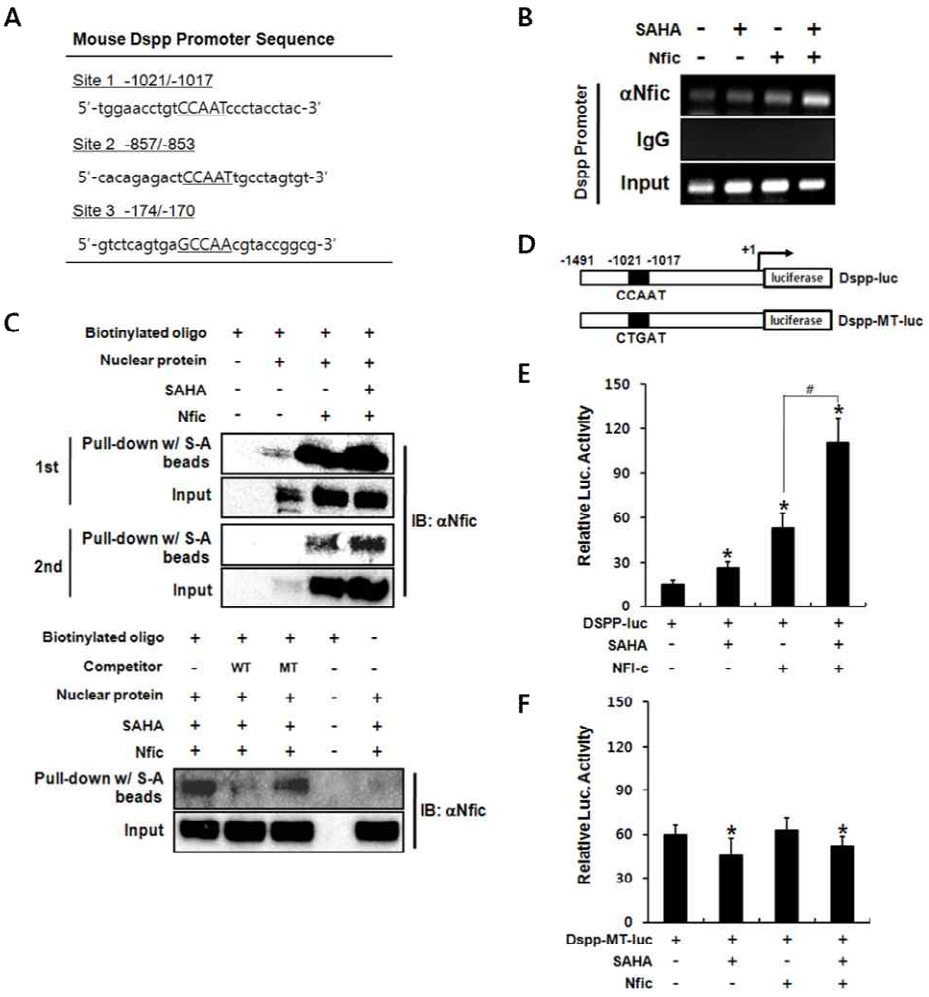

Fig. 6. SAHA increased Dspp promoter activity in an Nfic-dependent manner. (A) Three putative NF-binding half-consensus sequences are present in the 1.5-kb mouse Dspp promoter. Nuclear factor (Nf) binding consensus sequences at -1021 to -1017 (site 1), -857 to -853 (site 2), and -174 to -170 (site 3) bp are underlined. (B) MDPC23 cells were transiently transfected with pcDNA or an Nfic expression plasmid and incubated in the presence or absence of 5 μM SAHA for 24 h. Cellular DNA fragments were immunoprecipitated with anti-Nfic antibody or normal IgG, followed by PCR

amplification of the region containing Nfic binding site 1. (C) Nuclear proteins were prepared from the cells which were transfected with pcDNA or Nfic expression plasmids and incubated in the presence or absence of 5 μM SAHA for 48 h. Nuclear proteins were incubated with 5′-biotinylated double-stranded oligonucleotides that contain the Nfic-binding element on the mouse Dspp promoter (-1030 to -1007 bp). When indicated, nonbiotinylated wild-type (WT competitor) or mutant oligonucleotides (MT competitor) were added to the reaction mixture in a 50-fold excess. The mutant competitor includes nucleotide substitutions in the Nfic-binding element (CCAAT → CTGAT). Biotinylated DNA-protein complexes were then pulled down with streptavidin-agarose (S-A) beads, and bound Nfic was detected by Western blotting with anti-Nfic antibody. The upper panel shows repeated experimental results from the independently prepared samples. (D) Schematic illustration of the Dspp promoter reporter, Dspp-luc and its mutant Dspp-MT-luc. (E, F) MDPC23 cells were transiently transfected with the indicated plasmids and incubated for 24 h in the presence or absence of 5 μM SAHA, followed by a luciferase reporter assay. Data are shown as firefly luciferase activity relative to *Renilla* luciferase activity. Data represent the mean + SD (*$p<0.05$, compared with Dspp-luc or Dspp-MT-luc only; #$p<0.05$, Nfic *vs*. Nfic+SAHA).

V.2. HDAC inhibitor-induced Nfic downregulates Smurf1 and subsequently Smurf1 suppresses odontoblast differentiation

V.2.1. SAHA and TSA inversely regulates the expression levels of Smurf1 and Nfic in MDPC23 cells and C2C12 cells

Inhibitory effect of Smurf1 on osteoblast differentiation is well known (Yamashita et al., 2005; Zhao et al., 2003). Interestingly, a previous report has demonstrated that Smurf1 binds to and ubiquitinates phosphorylated Nfic, resulting in downregulation of Nfic protein (Lee et al., 2011). Because SAHA increased Nfic expression in both mRNA and protein levels (Fig. 4), it was next examined whether HDAC inhibitors decrease Smurf1 expression in MDPC23 cells. MDPC23 cells were incubated in the presence or absence of SAHA (3 μM) and TSA (100 nM) for 12, 24, 48 and 72 h. Real time-PCR and Western blotting results confirmed that both SAHA and TSA increased Nfic expression levels at all the time points examined (Figs. 7A, 7C). Interestingly, Smurf1 mRNA levels were significantly downregulated by SAHA and TSA at all the time points examined (Fig. 7B). These inhibitory effects on Smurf1 were also observed at the protein level (Fig. 7C). These results suggest that expression levels of Smurf1 are inversely correlated with those of Nfic.

To examine whether inverse regulation of Nfic and Smurf1 by HDAC inhibitors is also observed in other cell types, C2C12 cells were treated with SAHA and TSA in osteogenic medium. Similar to the results obtained from MDPC23 cells, SAHA and

TSA increased Nfic expression while decreasing Smurf1 expression in both mRNA and protein levels (Fig. 8). These results indicate that inverse expression pattern of Nfic and Smurf1 is not restricted to odontoblast lineage cells and that HDAC inhibitor-induced Nfic may be involved in the downregulation of Smurf1 expression by HDAC-inhibitor.

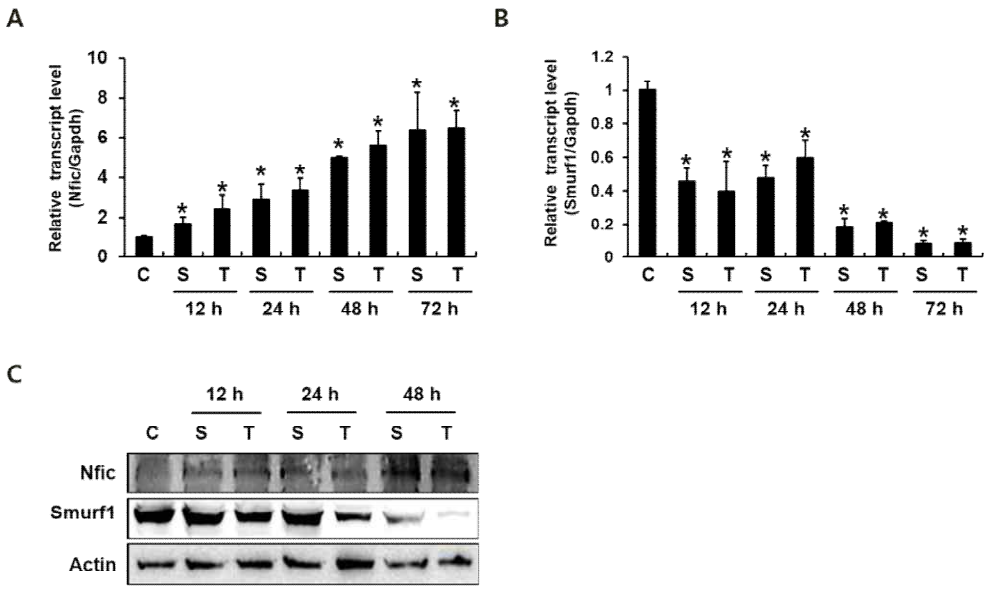

Fig. 7. SAHA and TSA inversely regulated the expression levels of Smurf1 and Nfic in MDPC23 cells. MDPC23 cells were cultured in the presence of SAHA or TSA for the indicated time periods. C, vehicle-treated control; S, 3 μM SAHA; T, 100 nM TSA. (A, B) Total RNA was then isolated and mRNA levels of Nfic and Smurf1 were evaluated by quantitative real-time PCR. Data are presented as the mean + SD of triplicates. *$p<0.05$, compared with vehicle-treated control. (C) Western blot analyses were performed using Nfic and Smurf1 antibodies.

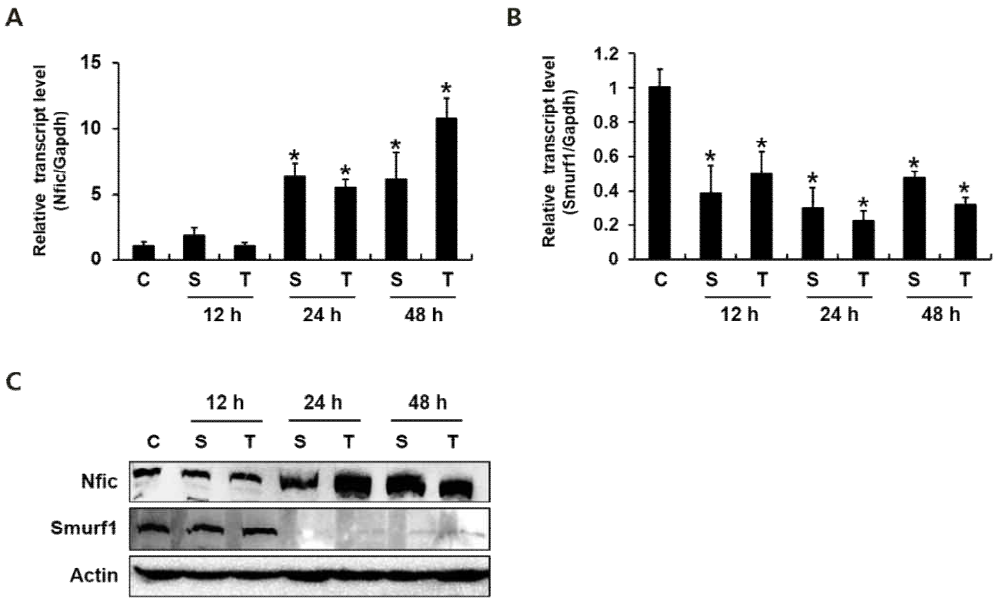

Fig. 8. SAHA and TSA inversely regulated the expression levels of Smurf1 and Nfic in C2C12 cells. C2C12 cells were cultured in the presence of SAHA or TSA for the indicated time periods. C, vehicle-treated control; S, 3 μM SAHA; T, 100 nM TSA. (A, B) Total RNA was then isolated and mRNA levels of Nfic and Smurf1 were evaluated by quantitative real-time PCR. Data are presented as the mean + SD of triplicates. *$p<0.05$, compared with vehicle-treated control. (C) Western blot analyses were performed using Nfic and Smurf1 antibodies.

V.2.2. Nfic overexpression decreases Smurf1 expression, whereas Nfic knockdown prevents HDAC inhibitor-mediated suppression of Smurf1 expression

To verify the relationship between Nfic and Smurf1, Nfic was overexpressed in MDPC23 cells and Smurf1 expression was evaluated. Transient transfection of Nfic expression vector increased Nfic mRNA expression to the levels observed in the cells treated with SAHA or TSA (Fig. 9A). Similar to the results in Fig. 8, Smurf1 expression was downregulated by Nfic overexpression as well as by SAHA and TSA (Fig. 9B). These phenomena were also observed in the protein level (Fig. 9C).

To confirm the inhibitory effect of Nfic on Smurf1 expression, Nfic was silenced in MDPC23 cells and the cells were treated with SAHA and TSA. Nfic siRNA-mediated decrease in the levels of Nfic mRNA and protein was confirmed by real time-PCR and Western blotting (Figs. 9D, 9F). Nfic siRNA also blocked HDAC inhibitor-mediated induction of Nfic expression. In Nfic-silenced cells, expression levels of Smurf1 were significantly higher than those in control siRNA-tranfected cells, regardless of the presence of HDAC inhibitors (Figs. 9E, 9F).

Regulatory effect of Nfic on Smurf1 expression was also examined in C2C12 (Fig. 10) and HEK293T cells (Fig. 11). Similar to the results from MDPC23 cells, Nfic overexpression was sufficient to suppress Smurf1 expression in C2C12 (Figs. 10B, 10C) and HEK 293T cells (Figs. 11B, 11C). Nfic silencing increased basal Smurf1 expression and prevented HDAC inhibitor-mediated decrease in Smurf1 expression in

C2C12 cells (Figs. 10E, 10F). These results indicate that HDAC inhibitor-induced Nfic is a mediator that negatively regulates Smurf1 expression.

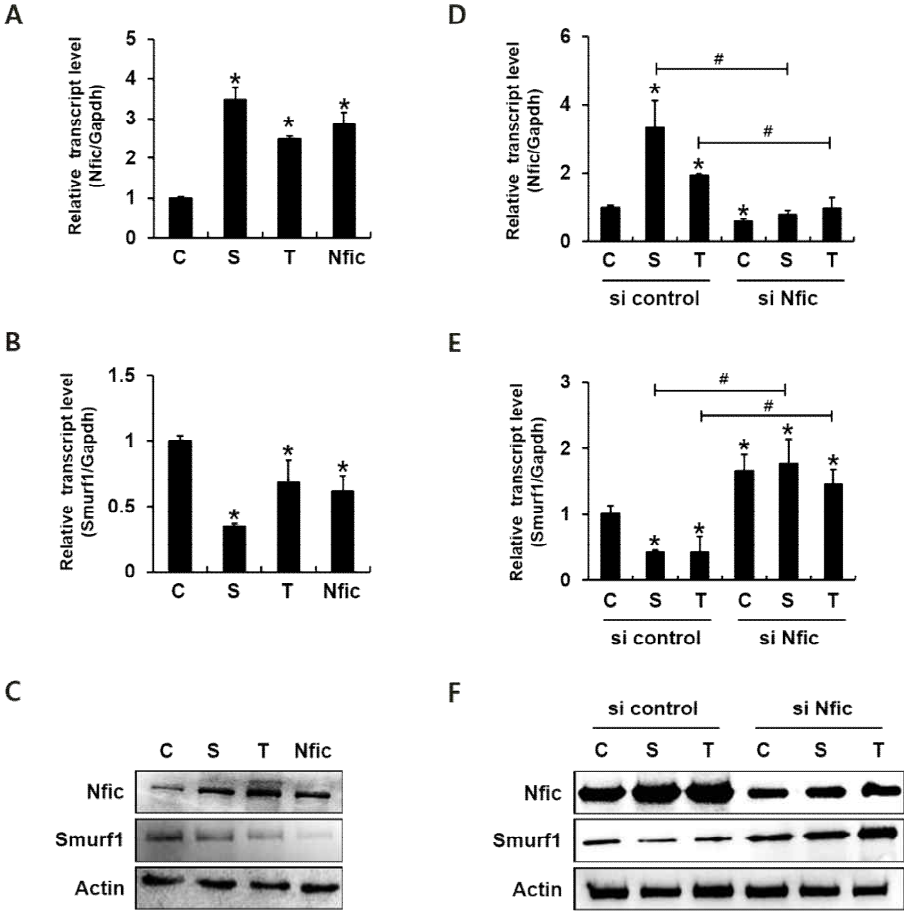

Fig. 9. Nfic negatively regulated Smurf1 expression in MDPC23 cells. MDPC23 cells were transiently transfected with Nfic expression plasmids (A-C) or Nfic siRNA(D-F) and incubated in the presence or absence of SAHA and TSA for 48 h. Then quantitative RT-PCR (A, B, D, E) or Western blot analyses (C, F) were performed. Quantitative data are presented as the mean + SD of triplicates. *$p<0.05$, compared to vehicle-treated control (A, B) or vehicle-treated si control cells (D, E); #$p<0.05$, for the indicated pairs.

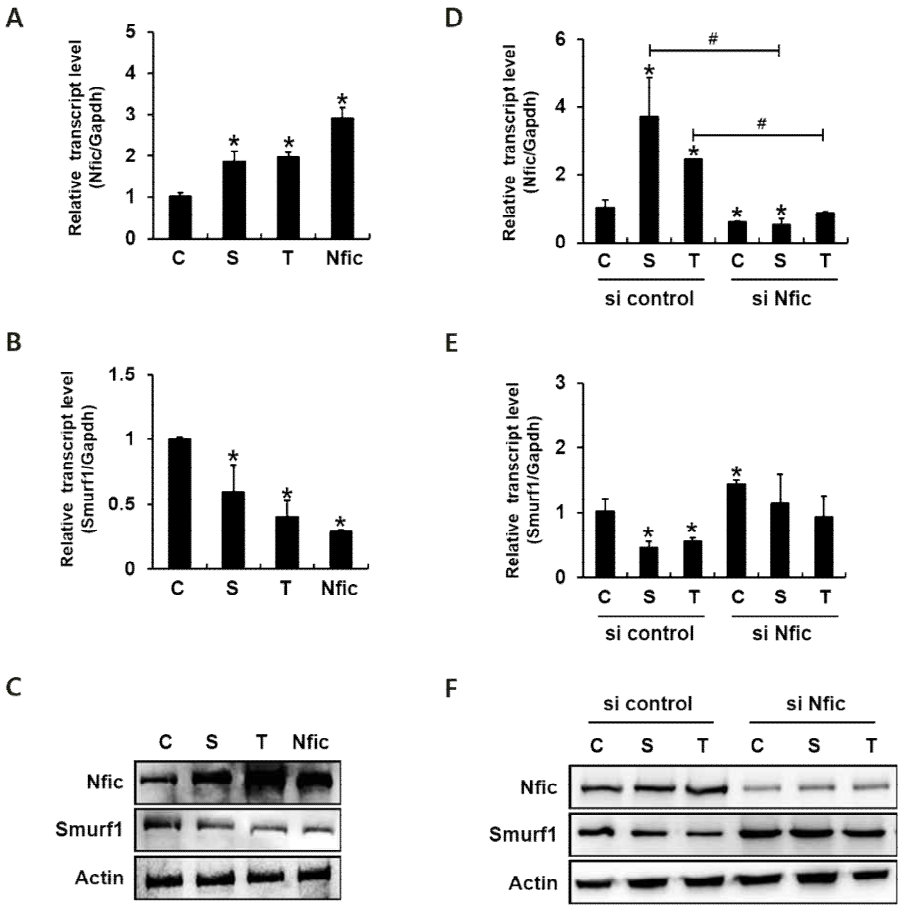

Fig. 10. Nfic negatively regulated Smurf1 expression in C2C12 cells. C2C12 cells were transiently transfected with Nfic expression plasmids (A-C) or Nfic siRNA(D-F) and incubated in the presence or absence of SAHA and TSA for 48 h. Then quantitative RT-PCR (A, B, D, E) or Western blot analyses (C, F) were performed. Quantitative data are presented as the mean + SD of triplicates. *$p<0.05$, compared to vehicle-treated control (A, B) or vehicle-treated si control cells (D, E); #$p<0.05$, for the indicated pairs.

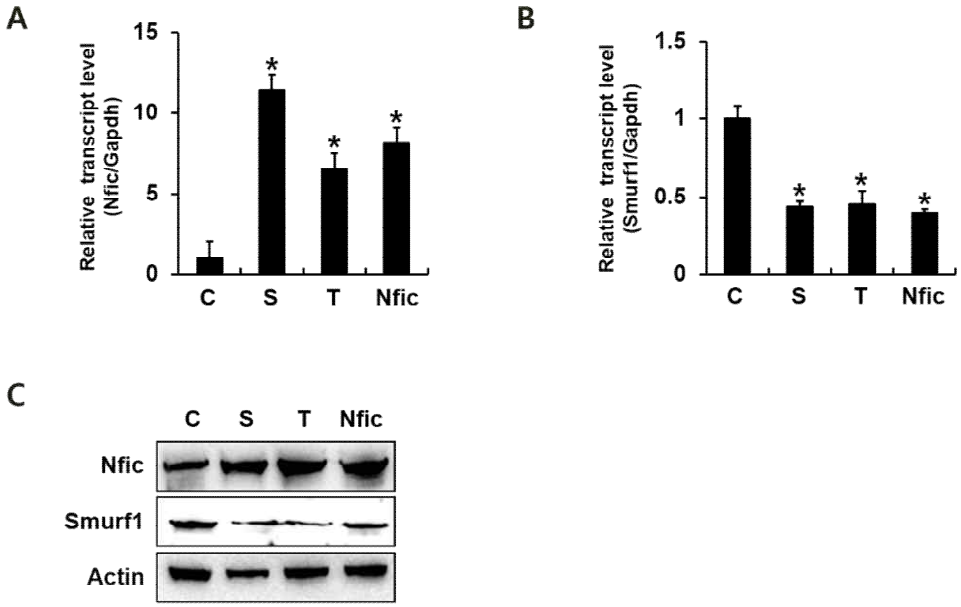

Fig. 11. Overexpression of Nfic suppressed Smurf1 expression in HEK293T cells. HEK293T cells were transiently transfected with Nfic expression plasmid and incubated in the presence or absence of SAHA and TSA for 48 h. Expression levels of Smurf1 and Nfic were evaluated by real-time PCR (A, B) and Western blot analysis (C). Data are presented as the mean + SD of triplicate. *$p<0.05$, compared to vehicle-treated control.

V.2.3. HDAC inhibitor-induced Nfic decreases mRNA levels of Smurf1 via downregulating the levels of Ac-H3, H3K4me and RNA polymerase II bound to the mouse Smurf1 gene in MDPC23 cells

To examine the mechanisms by which Nfic decrease Smurf1 mRNA level, computer-aided analysis was performed to find putative Nfic binding elements in the mouse Smurf1 promoter region. However, it was not detected within 2 kb region of the Smurf1 promoter. Chromatin remodeling by HDAC inhibitors is well documented to correlate with recruiting Pol II and other transcription factors to various promoters. Therefore, it was next examined whether an increase in Nfic protein level via either HDAC inhibitor treatment or Nfic overexpression induce changes in the chromatin structure around the Smurf1 gene to prevent Smurf1 transcription. Acetylation of H3 (Ac-H3) or methylation of H3 on the lysine 4 (H3K4me) are the representative modifications linked with chromatin structures permissible to the transcription machinery and transcription factors (Pan et al., 2007). Therefore, ChIP assays were performed to observe the change in histone modifications and binding of RNA polymerase II in the mouse Smurf1 promoter region. MDPC23 cells were treated with HDAC inhibitors or transiently transfected with Nfic expression plasmids, followed by incubation for 24 h. DNA-protein complexes were immunoprecipitated with specific antibodies, anti-Ac-H3, anti-H3K4me and anti-Pol II antibodies. Primers were designed to amplify the Smurf1 promoter regions including transcription start site (Site 1: -150 to +124 bp) and upstream promoter region (Site 2: -463 to -273 bp) (Fig. 12A).

Consistent with the results from RT-PCR and Western blot analyses, SAHA/TSA treatment and Nfic significantly decreased the levels of Ac-H3 and H3K4me associated with the Smurf1 promoter site 1 (Figs. 12B, 12C) and site 2 (Figs. 12E, 12F). Pol II recruitment to the Smurf1 promoter was also inhibited by SAHA/TSA treatment and Nfic (Figs. 12D, 12G). These data indicate that HDAC inhibitor-induced Nfic modifies the modification pattern of core histones associated with the Smurf1 promoter region and inhibits the recruitment of Pol II, resulting in suppression of Smurf1 transcription.

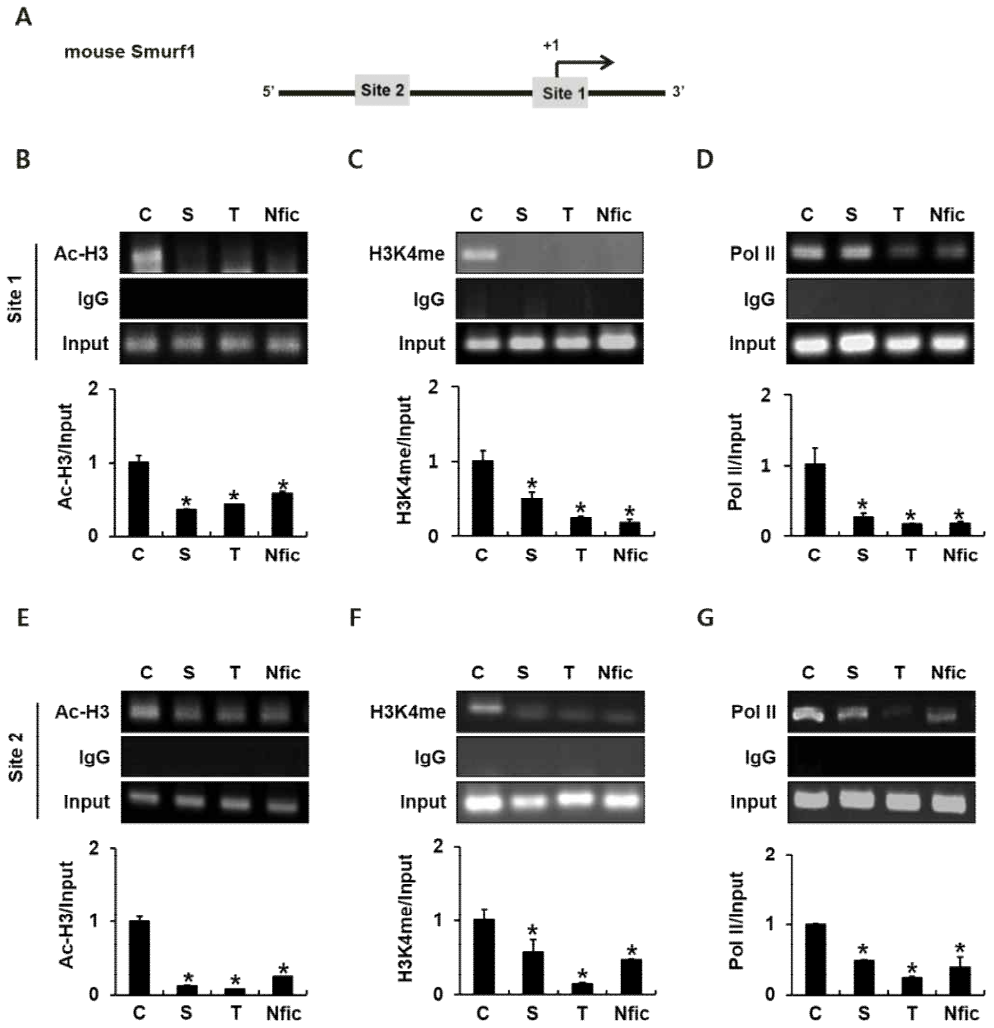

Fig. 12. SAHA/TSA-induced Nfic decreased the levels of Ac-H3, H3K4me and Pol II bound to the Smurf1 promoter. (A) Schematic illustration of the mouse Smurf1 DNA region amplified in ChIP assays. Site 1: -150 to +124 bp, Site 2: -463 to -273 bp. (B-G) MDPC23 cells were transiently transfected with Nfic or incubated in the presence or absence of SAHA/TSA. Cellular DNA fragments were immunoprecipitated with anti-Aceylhistone H3 (Ac-H3), anti-methylhistone H3 (H3K4me), anti-RNA Polymerase II (Pol II) or normal IgG and quantitative PCR was

performed using the primers specific to each site (lower panels). Amplified products were also observed in agarose gel (upper panels). Data represent the mean + SD. *$p<0.05$, compared to vehicle-treated control cells.

V.2.4. Smurf1 negatively regulates odontoblast differentiation in MDPC23 cells

Although Smurf1 is known to be a negative regulator for osteoblast differentiation, the role of Smurf1 in odontoblast differentiation has not been elucidated. Therefore, to evaluate the regulatory role of Smurf1 in odonoblast differentiation, Smurf1 overexpression was induced in MDPC23 cells, followed by incubation in differentiation medium. Overexpression of Smurf1 significantly decreased matrix mineralization and the expression levels of odontoblast differentiation marker genes, including Dspp, Dmp1 and Nestin (Fig. 13). Because SAHA and TSA significantly suppressed expression levels of Smurf1 in MDPC23 cells, it was examined whether overexpression of Smurf1 blocks SAHA/TSA-induced odontoblast differentiation. Alizarin red S staining results and quantitative RT-PCR results clearly demonstrated that overexpression of Smurf1 prevented SAHA/TSA-mediated induction of odontoblast differentiation (Fig. 13). These results indicate that suppression of Smurf1 expression is an important step to produce HDAC inhibitor-induced odontoblast differentiation.

To further confirm the role of Smurf1 downregulation in HDAC inhibitor-induced odontoblast differentiation, the effect of Smurf1 knockdown was compared with those of HDAC inhibitor treatment. In MDPC23 cells transiently transfected with Smurf1 siRNA, the levels of Smurf1 mRNA and protein were significantly lower than

those in control cells (Figs. 14B, 14F). Interestingly, Smurf1 knockdown alone produced significant increase in matrix mineralization (Fig. 14A) and expression levels of odontoblast marker genes (Figs. 14B-14F). Stimulatory effect of Smurf1 silencing on odontoblast differentiation was similar to those of SAHA and TSA. These results further support an importance of Smurf1 downregulation in SAHA/TSA-induced odontoblast differentiation.

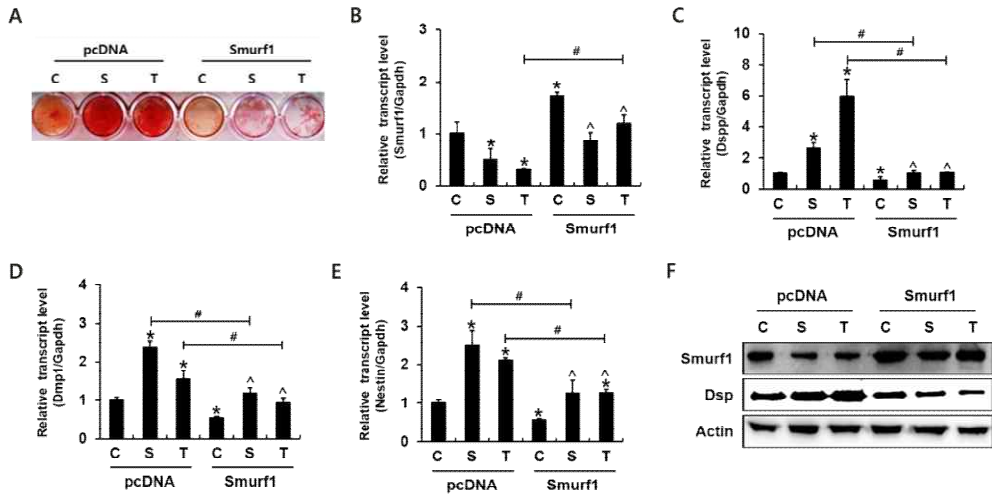

Fig. 13. Overexpression of Smurf1 suppressed both basal and HDAC inhibitor-induced odontoblast differentiation in MDPC23 cells. MDPC23 cells were transiently transfected with pcDNA or Smurf1 expression plasmid, followed by incubation in differentiation medium for 12 days (A) or 48 h (B-F) in the presence or absence of SAHA and TSA. At the end of culture periods, Alizarin red S staining (A), real time-PCR and Western blot analyses (F) were performed. (B-E) Data are presented as the mean + SD of triplicate. *p<0.05, compared to vehicle-treated pcDNA cells; ^p<0.05, compared to vehicle-treated Smurf1 cells; #p<0.05, for the indicated pairs.

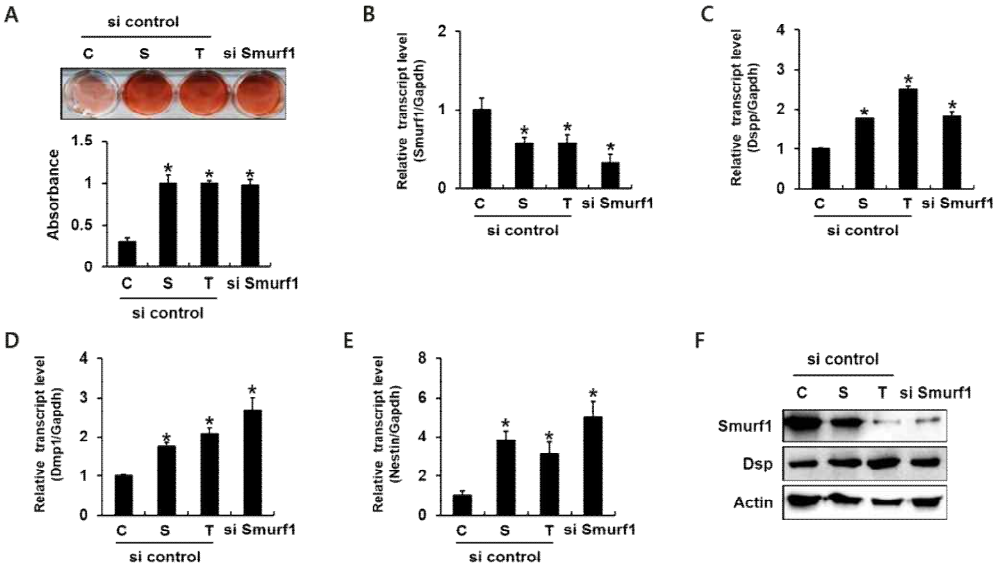

Fig. 14. Smurf1 knockdown enhanced matrix mineralization and odontoblast differentiation marker gene expression to the levels similar to those in HDAC inhibitor-treated cells. MDPC23 cells were transiently transfected with control siRNA (si control) or Smurf1 siRNA (si Smurf1), followed by incubation in differentiation medium for 12 days (A) or 48 h (B-F) in the presence or absence of SAHA and TSA. At the end of culture periods, Alizarin red S staining (A), real time-PCR (B-E) and Western blot analyses (F) were performed. For quantitative determination of mineral content, the stain was eluted, and the absorbance was measured at 415 nm (A, lower panel). (B-E) Data are presented as the mean + SD of triplicate. *$p<0.05$, compared to vehicle-treated si control cells.

V.3. Wnt/β-catenin signaling mediates HDAC inhibitors-induced odontoblast differentiation

V.3.1. SAHA and TSA increases expression levels of Wnt3a and β-catenin in MDPC23 cells

It has been demonstrated that Wnt/β-catenin signaling is important for differentiation of both osteoblast and odontoblast (Wang and Xu, 2010; Zhang et al., 2013). Wnt/β-catenin signaling plays multiple roles in various stages of tooth morphogenesis (Liu and Millar, 2010). Wnt3a, a representative canonical Wnt ligand, induces translocation of β-catenin into the nucleus and subsequently interaction of β-catenin with Tcf/Lef transcription factors (Sato et al., 2001). Because HDAC inhibitors induce Wnt/β-catenin signaling in cancer cells (Debeb et al., 2012; Shao et al., 2012), the effect of SAHA and TSA on the activation of Wnt/β-catenin signaling was examined. When MDPC23 cells were incubated in the presence of SAHA and TSA, Wnt3a mRNA levels started to increase after incubation for 24 h and reached a peak level after 48 h incubation (Fig. 16A). Secretion of Wnt3a protein was significantly higher in SAHA- and TSA-treated cells (Fig. 16B). Then, ChIP assays were performed to examine whether increased Wnt3a mRNA level is correlated with increased histone acetylation by HDAC inhibitors. ChIP assays using Ac-H3 antibody demonstrated that SAHA and TSA significantly increased H3 acetylation in the Wnt3a promoter region (-2443 to -2202 bp), compared to vehicle-treated control cells (Fig.15C). These results

suggest that SAHA and TSA increase Wnt3a expression via enhancing transcription in MDPC23 cells.

The effect of SAHA and TSA on β-catenin expression was also examined. Similar to the effect on Wnt3a expression, both SAHA and TSA increased β-catenin mRNA levels but significant induction was only observed after incubation for 48 h, suggesting that β-catenin induction is indirect event (Fig. 16A). Increase in β-catenin protein level was also observed in the cells treated with SAHA or TSA for 48 h compared to control cells (Fig. 16B). Consistent with these data, ChIP assay results showed that both SAHA and TSA induced histone hyperacetylation of histone in the mouse β-catenin promoter region (-2132 to -1854 bp) (Fig. 16C). These results indicate that SAHA and TSA increase the expression levels of Wnt3a and β-catenin in MDPC23 cells, suggesting a potential role of canonical Wnt/β-catenin signaling in HDAC inhibitor-induced odontoblast differentiation.

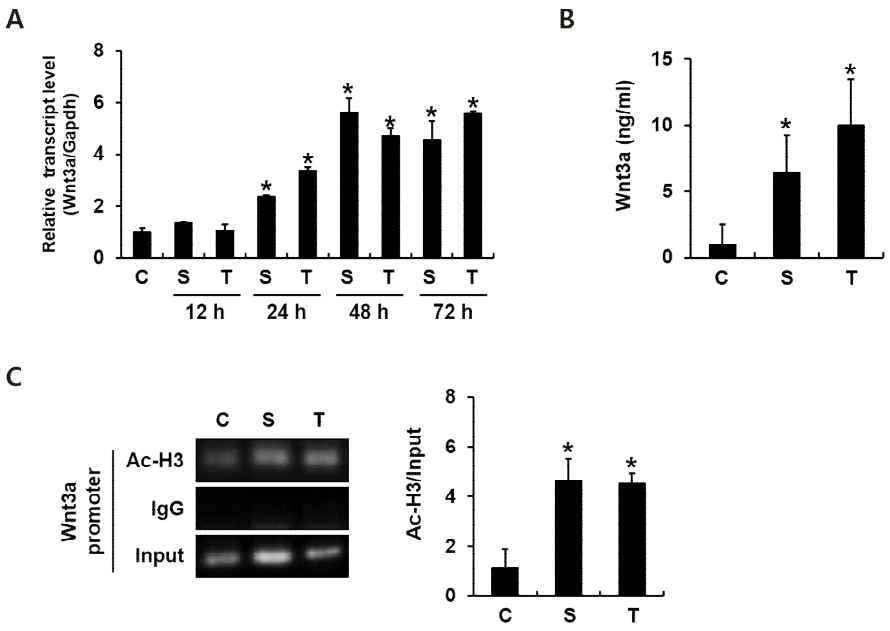

Fig. 15. SAHA and TSA increased Wnt3a expression in MDPC23 cells. (A) MDPC23 cells were incubated in the presence or absence of SAHA and TSA for the indicated periods and Wnt3a mRNA levels were determined by real-time PCR. (B) MDPC23 cells were incubated in the presence or absence of SAHA and TSA for 48 h and the levels of Wnt3a protein secreted to the culture medium were determined using Wnt3a ELISA kit. (C) MDPC23 cells were incubated in the presence or absence of SAHA and TSA for 48 h and ChIP assays were performed using anti-Ac-H3 antibody and primers that specifically bind to the mouse Wnt3a promoter region (-2443 to -2202 bp). Quantitative PCR results were presented in the right panel. Data are presented as the mean + SD. *$p<0.05$, compared to vehicle-treated control.

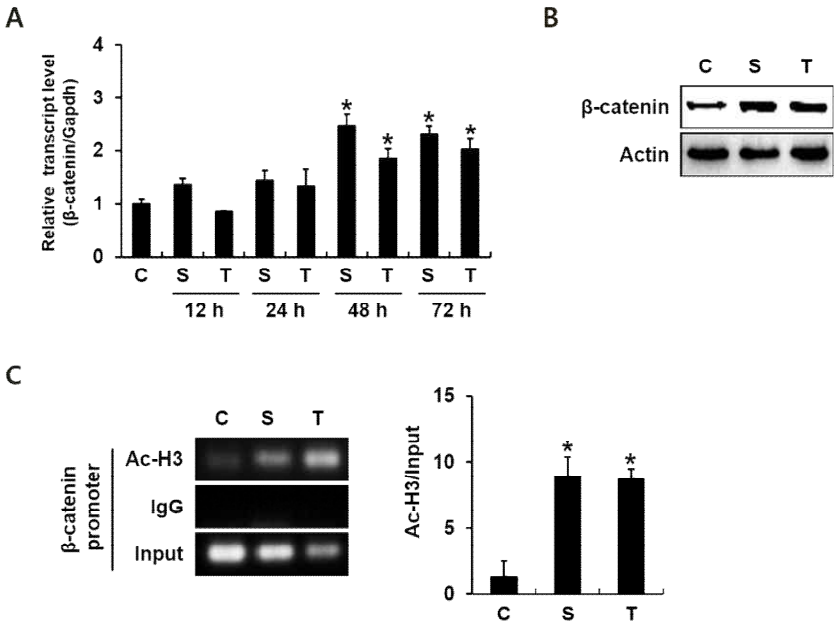

Fig. 16. SAHA and TSA increased β-catenin expression in MDPC23 cells. (A) MDPC23 cells were incubated in the presence or absence of SAHA and TSA for the indicated periods and β-catenin mRNA levels were determined by real-time PCR. (B) MDPC23 cells were incubated in the presence or absence of SAHA and TSA for 48 h and the levels of β-catenin protein were examined using Western blot analysis. (C) MDPC23 cells were incubated in the presence or absence of SAHA and TSA for 48 h and ChIP assays were performed using anti-Ac-H3 antibody and primers that specifically bind to the mouse β-catenin promoter region (-2132 to -1854 bp). Quantitative PCR results were presented in the right panel. Data are presented as the mean + SD. *$p<0.05$, compared to vehicle-treated control.

V.3.2. SAHA and TSA increases transcriptional activity of β-catenin/Tcf/Lef complex in MDPC23 cells

Activation of canonical Wnt/β-catenin signaling increases the transcriptional activity of β-catenin/Tcf/Lef complexes to regulate the expression of target genes. Therefore, to confirm whether SAHA- and TSA-mediated increase in Wnt3a and β-catenin levels results in transcriptional activation of β-catenin/Tcf/Lef complexes, TOP-flash and FOP-flash luciferase reporter assays were performed. MDPC23 cells were transiently transfected with TOP-flash/FOP-flash and β-catenin expression plasmids and subsequently were incubated in the presence or absence of SAHA/TSA for 24 h. Although SAHA and TSA significantly increased both FOP- and TOP-flash activity, induction folds were much higher in TOP-flash compared to FOP-flash, suggesting that SAHA- and TSA-induced TOP-flash activity reflect the activation of β-catenin/Tcf/Lef complex (Fig. 17A, right panel). To confirm the stimulatory effect of these HDAC inhibitors on TOP-flash activity, the same experiments were performed in HEK 293T cells. Similar to the results from MDPC23 cells, SAHA and TSA also increased TOP-flash activity in HEK 293T cells (Fig. 17B). Next, it was examined whether Wnt3a is necessary for HDAC inhibitor-induced TOP-flash activity. Knockdown of Wnt3a in MDPC23 cells significantly downregulated both basal and SAHA/TSA-induced TOP-flash activities (Fig. 17C). These results suggest that Wnt3a plays an important role in transcriptional activation of β-catenin/Lef/Tcf complex in MDPC23 cells and that SAHA/TSA-induced Wnt3a expression contribute to

transcriptional activation of β-catenin/Lef/Tcf complex.

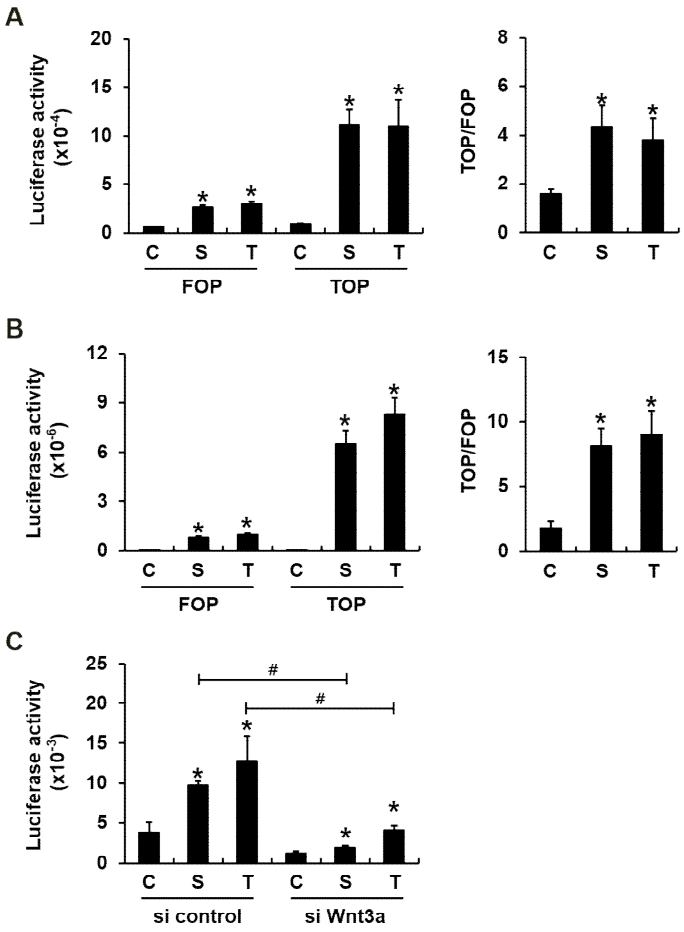

Fig. 17. SAHA and TSA increased transcriptional activity of β-catenin/Tcf/Lef complex. Luciferase assays were performed using TOP-flash and FOP-flash reporter plasmids in MDPC23 cells (A, C) and HEK 293T cells (B). (A, B) Cells were transiently transfected with the indicated reporter plasmids and β-catenin expression vectors and incubated in the presence or absence of SAHA and TSA for 24 h. (C) Wnt3a knockdown was induced using Wnt3a siRNA and then reporter assays were

performed as described above. Data represent the mean + SD. *p<0.05, vehicle-treated control vs SAHA- or TSA-treated cells. #p<0.05, for the indicated pairs.

V.3.3. Wnt3a silencing or Dkk1 treatment attenuates SAHA/TSA-induced β-catenin stabilization

Wnt3a is a canonical Wnt ligand that stabilizes the β-catenin protein through the inhibition of its phosphorylation by Gsk3β and Ck1α (Mikels and Nusse, 2006). Because the above results demonstrated that SAHA and TSA increases transcriptional activity of β-catenin, it was next examined whether these HDAC inhibitors stabilize β-catenin in MDPC23 cells. To rule out the effect of HDAC inhibitors on β-catenin transcription, overexpression of flag-tagged β-catenin was induced. MG132, a proteasome inhibitor, increased flag-tagged β-catenin protein levels, suggesting that β-catenin protein is continuously degraded through the proteasomal pathway in MDPC23 cells. In the absence of MG132, SAHA and TSA increased protein levels of flag-tagged β-catenin (Fig. 18). However, in the presence of MG132, these enhancing effects on β-catenin protein were abolished, suggesting that SAHA and TSA increase β-catenin protein levels via inhibition of β-catenin degradation as well as stimulation of its transcription.

To further verify the role of Wnt3a in SASA/TSA-mediated β-catenin stabilization, Wnt3a silencing was induced in MDPC23 cells. Consistent with the results from Top-flash assays, Wnt3a silencing reduced both basal and SAHA/TSA-induced β-catenin protein levels (Fig. 18B). Addition of Dkk1 also decreased both basal and SAHA/TSA-induced β-catenin protein levels similar to those in Wnt3a-

silenced cells (Fig. 18C). These results further support the critical role of Wnt3a in SAHA/TSA-induced activation of Wnt/β-catenin signaling in odontoblasts.

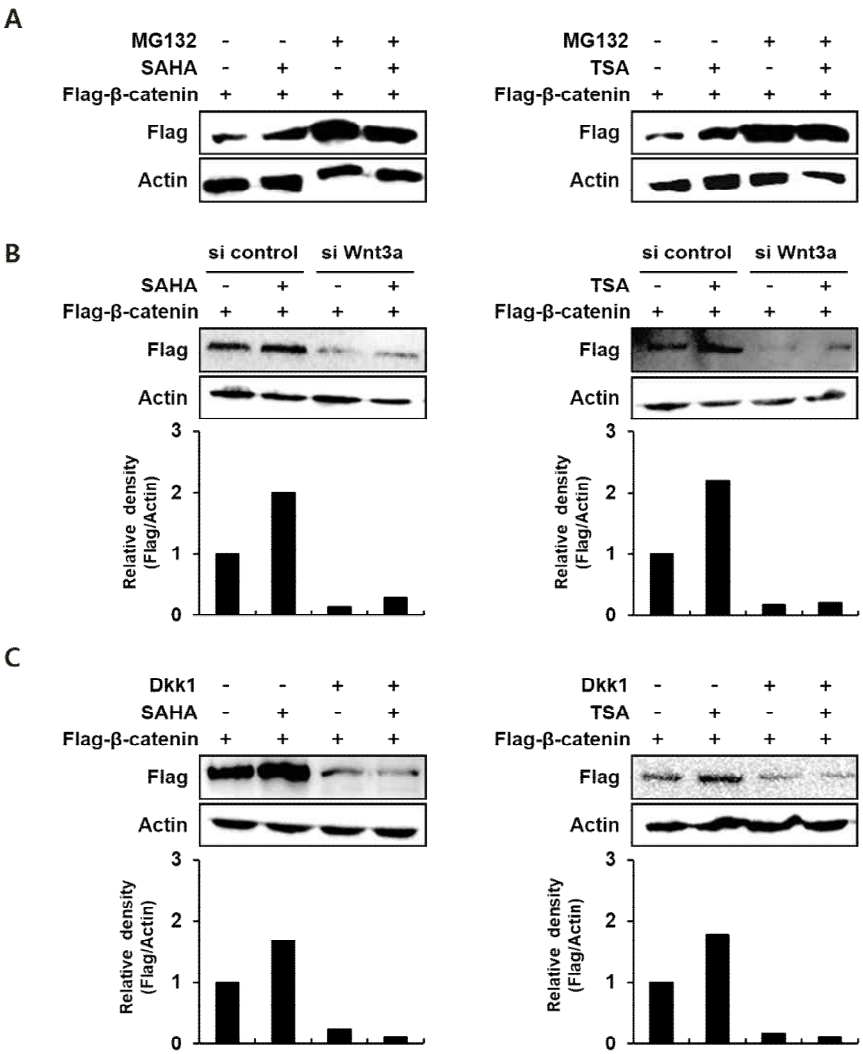

Fig. 18. SAHA/TSA stabilized flag-tagged β-catenin which was abolished by Wnt3a silencing or addition of Dkk1 in culture medium. Flag-tagged β-catenin was overexpressed in MDPC23 cells, and the cells were incubated for 48 h in the presence or absence of the indicated reagents. Western blot analyses were then performed. (A) When indicated, MG132 (2.5 μM) was added to culture medium 30 min before the treatment with SAHA or TSA. (B) Control siRNA or Wnt3a siRNA and flag-tagged β-

catenin expression plasmids were transiently transfected. (C) Dkk1 was added to culture medium when SAHA and TSA were treated. Densitometric analyses were performed using Image J software. Flag-tagged β-catenin band density was normalized to that of Actin band, and the relative protein density was presented as the ratio to that of vehicle-control cells.

V.3.4. Overexpression of β-catenin enhances, but silencing of β-catenin suppresses SAHA/TSA-induced odontoblast differentiation in MDPC23 cells

To evaluate the role of Wnt/β-catenin signaling in HDAC inhibitor-induced odontoblast differentiation, MDPC23 cells were transiently transfected with pcDNA or β-catenin expression plasmid and further incubated in differentiation medium for 12 days in the presence or absence of SAHA/TSA. At the end of the culture period, matrix mineralization was examined by Alizarin red S staining. Overexpression of β-catenin significantly increased matrix mineralization compared to pcDNA-transfected control group (Fig. 19A). SAHA and TSA enhanced both basal- and β-catenin-induced matrix mineralization. To further verify the effects on odontoblast differentiation, expression levels of odontoblast differentiation marker genes were examined in the cells incubated in differentiation medium for 48 h. The levels of β-catenin mRNA in β-catenin-transfected cells were similar to those in SAHA- or TSA-treated cells (Fig. 19B). Consistent with the results of matrix mineralization, overexpression of β-catenin or treatment with HDAC inhibitors upregulated the mRNA expression of Dmp1, Dspp and Nestin to the similar levels (Figs. 19C-19E). Combination of HDAC inhibitors with β-catenin overexpression further increased the expression of Dspp and Nestin. Western blot analyses data further confirmed that SAHA/TSA treatment and β-catenin overexpression increased levels of β-catenin and Dsp proteins in MDPC23 cells (Fig. 19F).

To further verify the role of endogenous β-catenin, β-catenin knockdown was induced. Alizarin red S staining of MDPC23 cells cultured for 12 days demonstrated that β-catenin knockdown significantly decreased matrix mineralization in control siRNA-transfected cells (Fig. 20A). Furthermore, β-catenin knockdown blocked SAHA/TSA-induced matrix mineralization mediated odontoblast mineralization. Knockdown efficiency of β-catenin siRNA was confirmed in both mRNA and protein levels (Figs. 20B, 20F). RT-PCR and Western blot analyses results also demonstrated the inhibitory effects of β-catenin knockdown on both basal and SAHA/TSA-induced odontoblast differentiation (Figs. 20C-20F). These results indicate that activation of Wnt/β-catenin signaling are important in both basal and HDAC inhibitor-induced odontoblast differentiation and matrix mineralization.

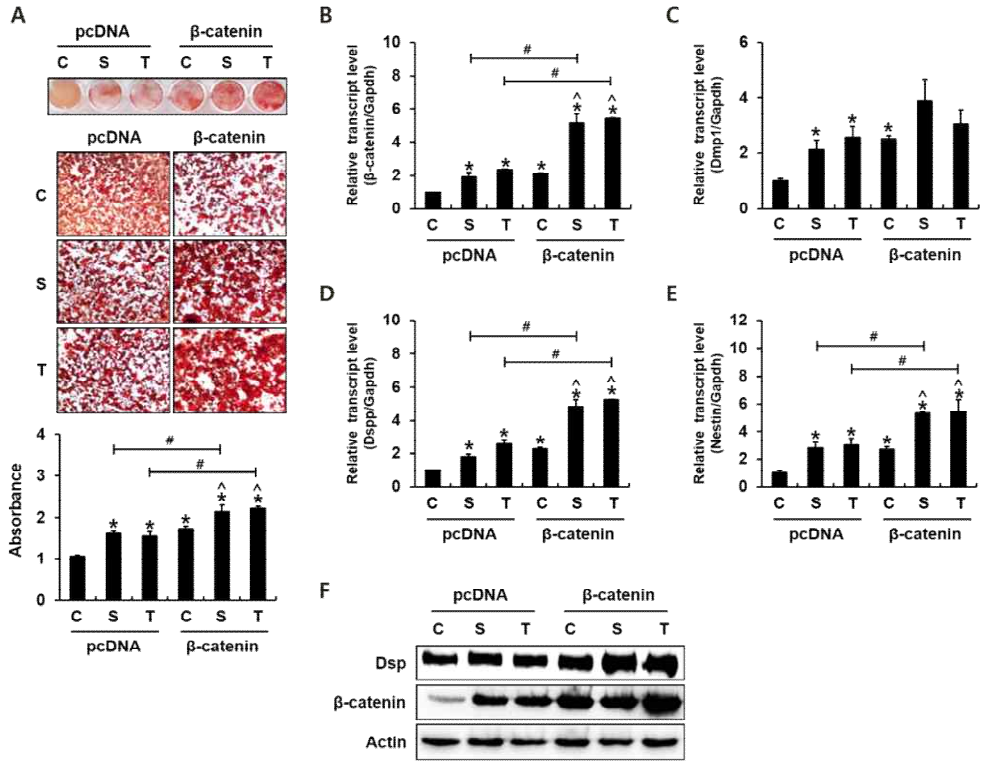

Fig. 19. Overexpression of β-catenin enhanced both basal and SAHA/TSA-induced odontoblast differentiation in MDPC23 cells. MDPC23 cells were transiently transfected with pcDNA or β-catenin expression plasmids and cultured in differentiation medium for 12 days (A) or 48 h (B-F) in the presence or absence of SAHA/TSA. At the end of culture periods, odontoblast differentiation was evaluated by Alizarin red S staining (A), RT-PCT (B-E) and Western blot analyses (F). Alizarin red S stained cells were observed under the microscope (A, middle panel, x40). For quantitative determination of mineral content deposited in the matrix, the stain was eluted and the absorbance was measured at 415 nm (A, lower panel). Quantitative data are presented as the mean + SD. *p<0.05, compared to pcDNA-transfected vehicle control. ^p<0.05, compared to β-catenin-overexpressed vehicle control. #p<0.05, for the indicated pairs.

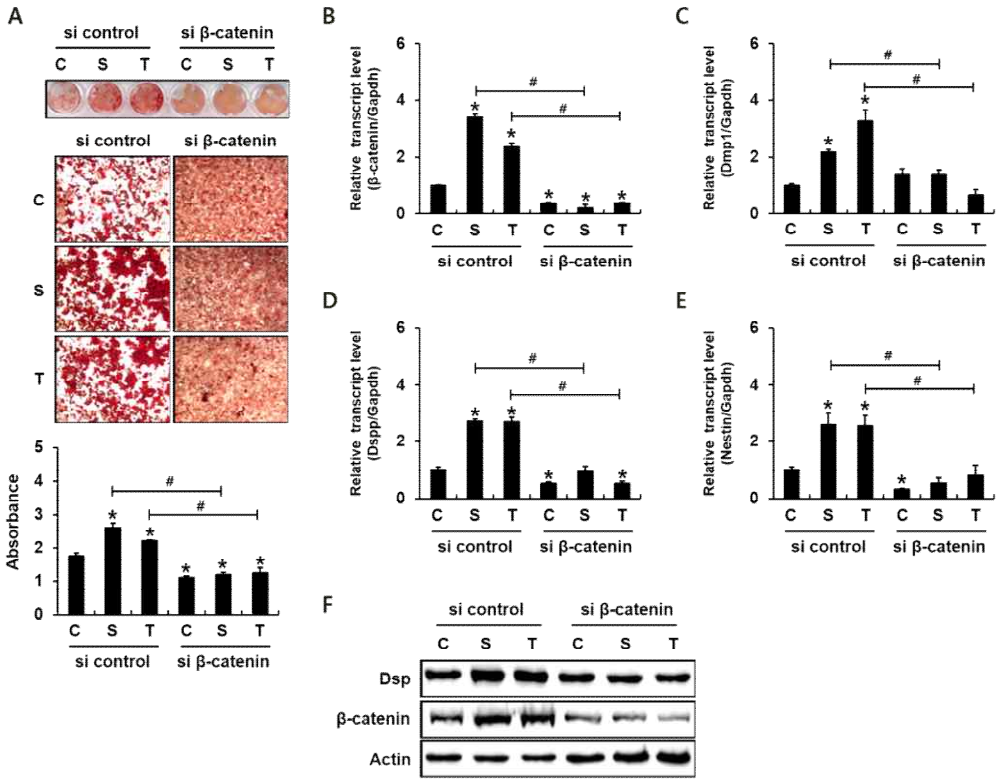

Fig. 20. Knockdown of β-catenin downregulated both basal and SAHA/TSA-induced odontoblast differentiation in MDPC23 cells. MDPC23 cells were transiently transfected with control siRNA or β-catenin siRNA and cultured in differentiation medium for 12 days (A) or 48 h (B-F) in the presence or absence of SAHA/TSA. At the end of culture periods, odontoblast differentiation was evaluated by Alizarin red S staining (A), RT-PCT (B-E) and Western blot analyses (F). Alizarin red S stained cells were observed under the microscope (A, middle panel, x40). For quantitative determination of mineral content deposited in the matrix, the stain was eluted and the absorbance was measured at 415 nm (A, lower panel). Quantitative data are presented as the mean + SD. *$p<0.05$, compared to control siRNA-transfected vehicle control. #$p<0.05$, for the indicated pairs.

V.3.5. Wnt3a upregulates odontoblast differentiation whereas Wnt3a knockdown suppresses SAHA/TSA-induced odontoblast differentiation in MDPC23 cells

To directly demonstrate whether Wnt3a upregulates odontoblast differentiation, MDPC23 cells were treated with Wnt3a (50 ng/ml) for 48 h, and odontoblast marker gene expression levels were examined. Similar to the effect of SAHA and TSA, Wnt3a significantly increased expression levels of Dspp, Dmp1 and Nestin (Figs. 21C-21F). Furthermore, Wnt3a significantly stimulated the expression of Wnt3a and β-catenin in MDPC23 cells (Figs. 21A, 21B). These results suggest that Wnt3a enhances odontoblast differentiation and that Wnt3a further increases Wnt/β-catenin signaling via inducing the expression of Wnt3a and β-catenin in MDPC23 cells.

To further examine the role of endogenous Wnt3a in HDAC inhibitor-induced odontoblast differentiation, Wnt3a knockdown was induced by transient transfection of MDPC23 cells with Wnt3a siRNA. Similar to the results from β-catenin-silenced cells, Wnt3a silencing inhibited both basal and SAHA/TSA-induced matrix mineralization and differentiation marker gene expression (Fig. 22). Wnt3a knockdown efficiency was verified by RT-PCR analysis (Fig. 22B). Wnt3a silencing also reduced β-catenin expression (Fig. 22G). These results indicate that induction of Wnt3a expression is necessary for SAHA/TSA-induced odontoblast differentiation.

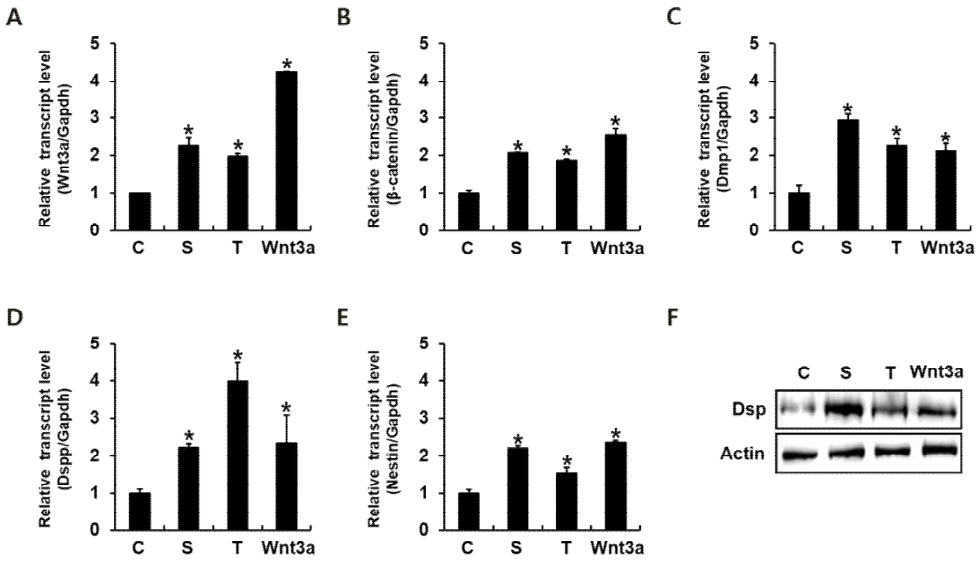

Fig. 21. Wnt3a enhances odontoblast differentiation marker genes expression. MDPC23 cells were incubated in differentiation medium for 48 h in the presence of the indicated reagents. Wnt3a was added in a concentration of 50ng/ml. RT-PCR data are presented as the mean + SD. *$p<0.05$, compared to control.

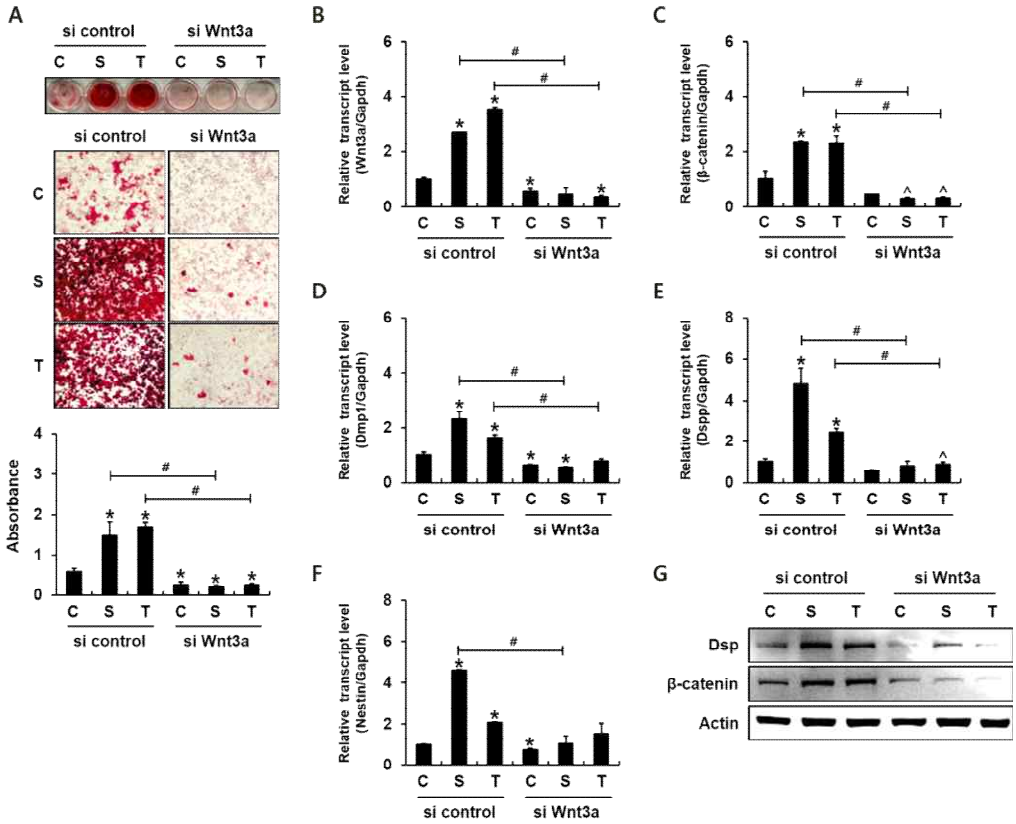

Fig. 22. Wnt3a silencing suppresses SAHA/TSA-induced odontoblast differentiation in MDPC23 cells. The cells were transiently transfected with control siRNA or Wnt3a siRNA and incubated in differentiation medium for 12 days (A) or 48 h (B-G). At the end of culture periods, odontoblast differentiation was evaluated by Alizarin red S staining (A), RT-PCT (B-F) and Western blot analyses (G). Alizarin red S stained cells were observed under the microscope (A, middle panel, x40) and the stain was quantified (A, lower panel). Quantitative data are presented as the mean + SD. *p<0.05, compared to control siRNA-transfected vehicle control. ^p<0.05, compared to Wnt3a siRNA-transfected vehicle control. #p<0.05, for the indicated pairs.

V.4. HDAC inhibitors-induced Wnt/β-catenin signaling upregulates Nfic expression in MDPC23 cells

V.4.1. Changes in Nfic expression level do not influence the expression levels of β-catenin

Because HDAC inhibitors-induced odontoblast differentiation requires induction of both Wnt/β-catenin and Nfic expression, interrelationships between Wnt/β-catenin signaling and Nfic were next examined. When overexpression of Nfic was induced in MDPC23 cells, expression levels of β-catenin did not change regardless of the presence of HDAC inhibitors (Figs. 23A, 23B). Furthermore, knockdown of Nfic did not exert any regulatory effect on SAHA/TSA-induced β-catenin mRNA expression (Figs. 23C, 23D). These results suggest that Nfic is not an upstream regulator of Wnt/β-catenin signaling in MDPC23 cells.

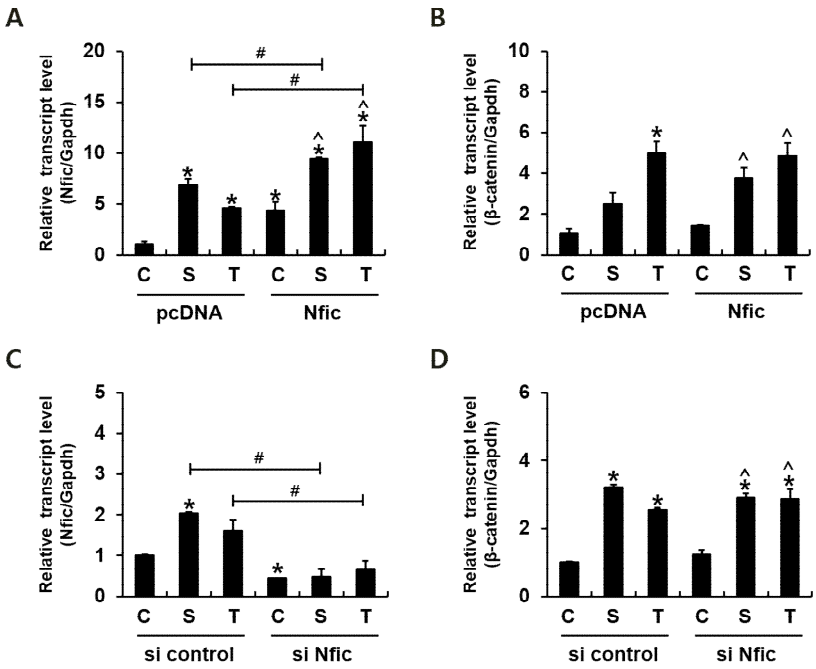

Fig. 23. Overexpression or silencing of Nfic did not regulate expression levels of basal and SAHA/TSA-induced β-catenin. MDPC23 cells were transiently transfected with pcDNA or Nfic expression plasmid (A, B) or with control siRNA or Nfic siRNA (C, D), followed by incubation for 48 h in differentiation medium. Expression levels of Nfic and β-catenin mRNA were then determined using real-time PCR. Data are presented as the mean + SD. *$p<0.05$, compared to pcDNA- or control siRNA-transfected vehicle control. ^$p<0.05$, compared to Nfic expression plasmid- or Nfic siRNA-transfected vehicle control. #$p<0.05$, for the indicated pairs.

V.4.2. Wnt3a/β-catenin signaling mediates SAHA/TSA induction of Nfic expression in MDPC23 cells

Because Nfic did not exert any regulatory effect on β-catenin mRNA levels, the effect of Wnt/β-catenin signaling on Nfic expression was examined. Addition of Wnt3a in culture medium significantly increased Nfic expression in both mRNA and protein levels (Figs. 24A, 24B). Furthermore, Wnt3a also decreased the expression levels of Smurf1, a downstream target of Nfic (Figs. 24A, 24B). Similar to the effect of Wnt3a, overexpression of β-catenin increased both basal and SAHA/TSA-induced Nfic expression while decreasing both basal and SAHA/TSA-downregulated Smurf1 expression (Figs. 24C, 24D). Then, knockdown of β-catenin was induced to further examine the regulatory role of Wnt/β-catenin signaling in HDAC inhibitors-induced Nfic expression. Knockdown of β-catenin suppressed SAHA/TSA-induced Nfic expression while inducing expression levels of Smurf1 in both control and SAHA/TSA-treated cells (Figs. 24E, 24F). These results indicate that Nfic is a downstream target of Wnt/β-catenin signaling and that SAHA/TSA-mediated induction of Nfic expression requires Wnt/β-catenin signaling in MDPC23 cells.

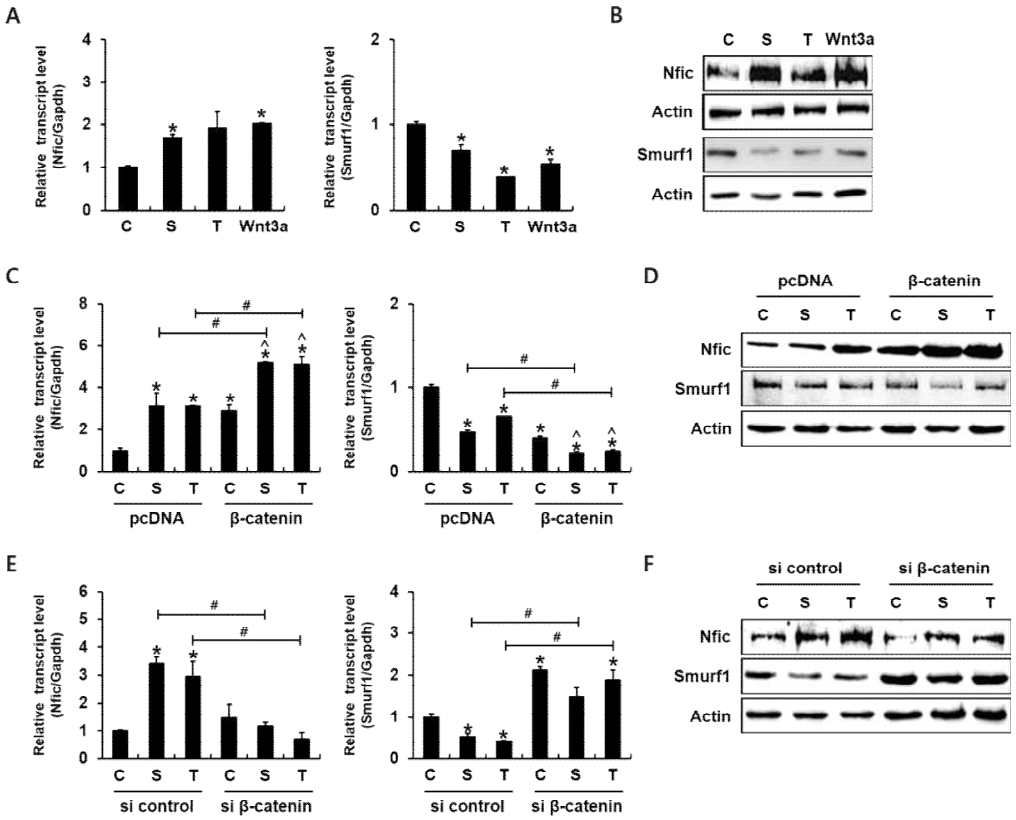

Fig. 24. Wnt3a/β-catenin signaling increased Nfic expression while suppressing Smurf1 expression. MDPC23 cells were incubated in differentiation medium for 48 h in the presence of indicated reagents. When indicated, the cells were transiently transfected with pcDNA or β-catenin expression plasmid (C, D) or with control siRNA or β-catenin siRNA (E, F) 24 h before incubation in differentiation medium. At the end of culture period, real-time PCR (A, C, E) and Western blot analyses (B, D, F) were performed. Quantitative data are presented as the mean + SD. *$p<0.05$, compared to pcDNA- or control siRNA-transfected vehicle control. ^$p<0.05$, compared to β-catenin-transfected vehicle control. #$p<0.05$, for the indicated pairs.

VI. DISCUSSION

PART I: SAHA enhances odontoblast differentiation through increasing Nfic expression

In this study, SAHA was chosen among the several HDAC inhibitors because it is clinically relevant and FDA-approved to treat cutaneous T cell lymphoma (Mann et al., 2007). In this study, SAHA showed significant stimulatory effects on matrix mineralization and odontoblast marker gene expression in MDPC23 cells. Previous reports have demonstrated that HDAC inhibitor stimulate osteoblast differentiation through the enhanced acetylation of Runx2, a critical transcription factor for osteoblast differentiation (Jeon et al., 2006; Schroeder et al., 2004). Runx2 acetylation increases the protein stability and transcriptional activity of Runx2. On the other hand, Runx2 is known to upregulate Dspp expression in pre-odontoblast while downregulating Dspp expression in mature odontoblast (Chen et al., 2005). Transgenic expression of Runx2 in odontoblast has also been demonstrated to inhibit the terminal differentiation of odontoblast while inducing transdifferentiation of odontoblast into osteoblasts (Miyazaki et al., 2008). Therefore, SAHA-induced matrix mineralization may result from enhanced-Runx2 acetylation and consequent osteoblastic transdifferentiation of MDPC23 cells. To confirm that SAHA shows sufficient HDAC inhibitor activity in MDPC23 cells, acetylation levels of histone H3 and Runx2 was evaluated. In the presence of 5 ìM SAHA, the acetylation of H3 and myc-tagged Runx2 increased

compared with the control. Because the Ocn and Bsp promoters contain Runx2 binding elements, regulatory effect of SAHA on the transcriptional activity of Runx2 was examined using these promoter reporters in HEK293T cells (Jeon et al., 2006; Roca et al., 2005). Consistent with the previous reports, SAHA significantly enhanced the transcriptional activity of Runx2 on both Ocn and Bsp promoter reporters in HEK293T cells. However, SAHA significantly decreased both basal and Runx2-induced Bsp promoter activities in MDPC23 cells. These results suggest that the increased expression levels of odontoblast differentiation-associated genes did not result from the SAHA-induced non-specific increases in histone acetylation and that the effect of SAHA in odontoblasts might have other target molecules in addition to Runx2. In this study, Runx2 overexpression did not increase Dspp promoter activity in MDPC23 cells. Because 1.5 kb mouse Dspp promoter reporter was used, this result was consistent with the previous report showing that Runx2 binding elements present at -2415 to -2410, -2409 to -2404 and -1796 to -1791 bp in the mouse Dspp promoter (Chen et al., 2005). However, SAHA increased Dspp promoter activity regardless of Runx2 overexpression in these cells, suggesting the presence of other transcription factors which mediates the stimulatory effect of SAHA on Dspp promoter.

A previous report has shown that Nfic overexpression increases Dspp expression while suppressing Bsp expression in odontoblast (Lee et al., 2009). Because SAHA demonstrated a similar effect as Nfic overexpression, it was investigated whether SAHA regulates Nfic expression. A previous report demonstrated that Nfic

overexpression increases Dspp expression whereas Nfic knockdown diminishes Dspp expression (Lee et al., 2009). However, the results from the Dspp promoter assays demonstrated that Nfic overexpression suppressed Dspp promoter activity whereas Nfic knockdown enhanced Dspp promoter activity in the same study. Therefore, the mechanism of Nfic-induced Dspp expression remained unclear. Nfi proteins are known to bind to the dyad symmetric consensus sequence TTGGC(N5)GCCAA as a dimer or to bind to individual half sites as a monomer (Gronostajski, 2000). *In silico* analysis of the mouse Dspp promoter demonstrated that three putative half sites were present at -1021 to -1017 (site 1), -857 to -853 (site 2) and -174 to 170 (site 3) bp in the 1.5 kb mouse Dspp promoter. In the present study, ChIP assay and Dspp promoter reporter assay showed that Nfic transactivates Dspp gene by direct binding to site 1. SAHA increased the expression levels of Nfic and Dspp. Furthermore, SAHA enhanced the binding of Nfic to the Dspp promoter and increased Nfic-induced Dspp promoter reporter activity. These results indicate that SAHA increases Dspp expression, at least in part, through the enhancement of Nfic binding to the Dspp promoter. In addition, a previous report demonstrated that Dmp1 transactivates the Dspp gene during early odontoblast differentiation (Narayanan et al., 2006). Because SAHA significantly increased Dmp1 expression in MDPC23 cells, Dmp1 may play some role in SAHA enhancement of Dspp expression.

In summary, the data presented in this study demonstrate that SAHA enhances odontoblast differentiation and that SAHA increases Dspp expression partly through

the up-regulation of Nfic expression. It was also demonstrated that Nfic stimulates Dspp transcription through the direct binding to the Dspp promoter. Although SAHA is mainly used as an anticancer agent, these results suggest that SAHA has therapeutic potential as an adjunctive ingredient to conventional pulp-capping materials to enhance reparative dentin formation. To investigate the therapeutic potential of SAHA in the dental field, further studies, including *in vivo* experiments using an animal model system, are necessary.

PART II: HDAC inhibitor-induced Nfic downregulates Smurf1 and subsequently Smurf1 suppresses odontoblast differentiation

TSA is the first natural hydroxamate found to inhibit HDAC (Yoshida et al., 1990). It was also previously demonstrated that TSA promotes proliferation and odontoblast differentiation of human dental pulp stem cells *in vitro* and enhances dentin formation and odontoblast differentiation *in vivo* during tooth development (Jin et al., 2013).

Previously, it was presented that Smurf1 interacts with Runx2, a key regulator for osteoblast differentiation, and mediates Runx2 degradation (Zhao et al., 2003). Inflammatory cytokine TNF-α induces Smurf1 expression and reduces osteoblast differentiation by promoting ubiquitination of Runx2 by Smurf1 (Guo et al., 2008).

TNF-α-induced bone loss is inhibited in *Smurf1*-null mice. HDAC mediates deacetylation of Runx2 and subsequently deacetylated Runx2 is ubiquitinated by Smurf1, allowing Runx2 degradation (Jeon et al., 2006). Therefore, the inhibition of HDAC increases osteoblast differentiation through stabilizing acetylated-Runx2. In odontoblast, MAPK activation by TGF-β signaling increases the interaction of phosphorylated Nfic and Smurf1/2, resulting in Nfic degradation (Lee et al., 2011). However, it has not been studied yet the regulatory effect of HDAC inhibitors on Smurf1 expression and its molecular mechanism in odontoblast differentiation.

In this study, SAHA/TSA showed inhibitory effect on Smurf1 expression in MDPC23 cells and C2C12 cells. Interestingly, Smurf1 was inversely regulated with Nfic by SAHA/TSA in both MDPC23 cells and C2C12 cells. Nfic overexpression downregulated Smurf1 and Nfic silencing up-regulated Smurf1 expression indicates that Nfic has a regulatory role in Smurf1 expression in HDAC inhibitor-induced odontoblast differentiation.

HDAC inhibitors play important roles in the modulation of chromatin topology and the regulation of gene transcription. It has been suggested that H3K4me is correlated with transcriptional activation and that high level of H3K4me is associated with Pol II occupancy, high transcription rate and histone acetylation (Ruthenburg et al., 2007; Santos-Rosa et al., 2002). In this study, SAHA/TSA and Nfic decreased the levels of Ac-H3 and H3K4me associated with the Smurf1 promoter, indicating

SAHA/TSA-induced Nfic reduced Smurf1 transcription by inhibiting open chromatin structure. RNA Pol II initiates transcription via accumulating at exons during transcription and Pol II-ChIP is preferable to measure actual transcriptional level. Pol II-ChIP has an additional advantage to analyses showing transcription-associated changes in chromatin (Brodsky et al., 2005; Sandoval et al., 2004). Pol II recruitment on the Smurf1 promoter was also impaired by HDAC inhibitors and Nfic overexpression. These results indicate that SAHA/TSA-induced Nfic prevents Pol II binding to the Smurf1 promoter, resulting in downregulation of Smurf1 transcription.

Considering the results showing that SAHA increased the total levels of acetylated histone in MDPC23 cells (Fig. 3A), it is interesting result. Treatment of cells with HDAC inhibitors increases general acetylation level of core histones in the cells. However, HDAC inhibitors affect the expression of only a small subset of genes, leading to transcriptional activation of some genes, but repression of an equal or larger number of other genes (Vigushin and Coombes, 2004). For example, in tumor cells exposed to HDAC inhibitors, pro-apoptotic genes involved in the extrinsic and intrinsic apoptotic pathways were upregulated, whereas the expression of anti-apoptotic genes was reduced (Zhu et al., 2004). Therefore, reduction in Ac-H3 associated with the Smurf1 promoter may not be the direct results of HDAC inhibition but may be mediated by increased Nfic protein. Although the putative Nfic binding elements were not found in the Smurf1 promoter, increased Nfic protein somehow recruited HDACs or prevented the recruitment of HATs and transcription machinery to

the Smurf1 promoter.

Smurf1 plays an important role as a negative regulator in BMP signaling and Mekk2 signaling pathway (Yamashita et al., 2005; Zhu et al., 1999). It reduces cellular responses to BMP via triggering proteasomal destruction of Smad1 and Smad5, causing impaired BMP signal transduction (Zhu et al., 1999). Aside from BMP and Mekk2 signaling, Smurf1 also negatively regulates Wnt/β-catenin signaling by non-proteolytic polyubiquitination of Axin, suggesting that Smurf1 executes a broader range of functions than was expected (Fei et al., 2013). In the present study, although Smurf1 clearly showed inhibitory effects on odontoblast differentiation, SAHA/TSA-mediated downregulation of Smurf1 did not exert any effect on protein levels of Smad1 and Smad5 which were overexpressed in MDPC23 cells (data not shown). Furthermore, overexpression of Smurf1 did not significantly influence the protein levels and transcriptional activity of exogenously expressed β-catenin in MDPC23 cells (data not shown). These results suggest that the regulatory mechanisms of Smurf1 in inhibition of odontoblast differentiation seem to differ from those in osteoblast differentiation.

In summary, Smurf1 exerted inhibitory effect on odontoblast differentiation. SAHA/TSA-induced Nfic downregulated expression levels of Smurf1. Nfic decreased Smurf1 transcription via inducing repressive chromatin structure in the Smurf1 promoter and transcription start site. Further studies are necessary to clarify the

mechanisms how Nfic regulates chromatin structure of the Smurf1 gene and/or transcription of Smurf1 and to investigate the target molecules that mediate inhibitory effect of Smurf1 on odontoblast differentiation.

PART III: Wnt/β-catenin signaling mediates HDAC inhibitors-induced odontoblast differentiation

Previous studies have shown that VPA inhibits Gsk3â, resulting in stimulation â-catenin pathway with human bone marrow cells and that VPA increases Lef-luc activity and nuclear â-catenin level in cerebellar granule cells (Chen et al., 1999; Leng et al., 2008). In human breast cancer cells, HDAC inhibitors upregulated â-catenin expression and significantly increased Wnt receptor activity (Debeb et al., 2012). Gene profile analysis of osteoblast genes demonstrated that HDAC inhibitors downregulated Fzd-1, a negative regulator of bone formation, while upregulating Fzd-4, a receptor for Wnts, which promotes osteoblast differentiation (Schroeder et al., 2007). These results indicate that HDAC inhibitors activate Wnt/â-catenin signaling in many different types of cells.

Wnt/β-catenin signaling plays essential roles in general skeletal development (Chen and Alman, 2009; Takada et al., 2009; Westendorf et al., 2004). Deficiencies of non-canonical Wnt signal transducers lead to abnormal bone formation throughout

development (Fujino et al., 2003; Harada et al., 1999). Recent studies indicate that Wnt signaling is crucial in regulating both osteogenic and adipogenic lineage-specific differentiation (Kang et al., 2007; Takada et al., 2009). Wnt/β-catenin signaling is also dynamically active during tooth development. *Lef1*-null mouse revealed the arrest of tooth growth at the bud stage (van Genderen et al., 1994). Epithelial overexpression of Dkk1 or mesenchymal knockout of β-catenin halted tooth development at the bud stage (Chen et al., 2009; Liu et al., 2008). Specific Wnts are reported to be expressed in specific tooth developmental stages and play a role in tooth formation. Wnt7b is expressed at the initiation stage throughout the oral epithelium, and functions to inhibit the expression of Shh, limiting Shh expression to tooth sites (Sarkar et al., 2000). In contrast, Wnt10b expression is restricted to initiation site epithelium while Wnt5a is present in oral mesenchymal cells at this stage (Sarkar and Sharpe, 1999). Wnt3, Wnt5a, Wnt6, and Wnt10b have a role in tooth shape determination, and Wnt5a is related with adjacent dental papilla mesenchyme and functions to stimulate human dental pulp stem cell differentiation (Peng et al., 2010a; Peng et al., 2010b; Sarkar and Sharpe, 1999). These studies indicate that activation of Wnt/β-catenin signaling in odontoblast lineage cells may stimulate odontoblast differentiation. Several studies reported that Wnt/β-catenin signaling deficiency blocks odontoblast differentiation during tooth root development and that Wnt/β-catenin signaling in the dental mesenchyme is required to differentiate odontoblasts for root formation (Kim et al., 2011; Zhang et al., 2013). It was also demonstrated that β-catenin is expressed during

reparative dentin formation (Han et al., 2014). However, it was also reported that Wnt/β-catenin signaling inhibits differentiation of dental pulp stem cells (Scheller et al., 2008). It is similar to some paradoxical studies showing that Wnt1 and Wnt3a inhibits osteoblast differentiation in mesenchymal stem cells (Boland et al., 2004; de Boer et al., 2004). Therefore, further studies are required to clarify the role of Wnt/β-catenin signaling pathway in regulation of odontoblast differentiation. In addition, the effect of HDAC inhibitors on Wnt/β-catenin signaling in odontoblast lineage cells has not been defined yet.

In the present study, SAHA/TSA significantly increased mRNA expression and secretion of Wnt3a in MDPC23 cells. Although there was a delay in induction time compared to those of Wnt3a, SAHA/TSA also increased â-catenin expression. SAHA/TSA-mediated increase in Wnt3a and â-catenin mRNA was associated with increased acetylation of H3 in the Wnt3a and â-catenin promoter. In addition to an increase in expression levels of Wnt3a and â-catenin, there was a significant increase in TOP-flash reporter activity in SAHA/TSA-treated cells compared to vehicle control cells. It implies that SAHA/TSA activates Wnt/β-catenin signaling in MDPC23 cells. Considering the results showing that SAHA/TSA induction of â-catenin was delayed compared with that of Wnt3a and that Wnt3a treatment or overexpression of â-catenin further increased â-catenin expression, SAHA/TSA-induced â-catenin expression seems to be an indirect event following the increased Wnt3a production and subsequent activation of Wnt/â-catenin signaling.

In the present study, Wnt3a treatment or overexpression of â-catenin significantly enhanced odontoblast differentiation of MDPC23 cells. Furthermore, suppression of Wnt/â-catenin signaling via knockdown of â-catenin or Wnt3a minimized both basal and SAHA/TSA-induce matrix mineralization and odontoblast marker genes. These results support the previous reports showing that Wnt/â-catenin signaling is required for odontoblast differentiation from dental mesenchymal cells. Furthermore, these results indicate that Wnt/â-catenin signaling is a critical mediator for HDAC inhibitors-induced odontoblast differentiation. Considering the previous report showing that β-catenin is expressed during reparative dentin formation (Han et al., 2014), it is suggested that HDAC inhibitors are good candidate chemicals to be used for inducing reparative dentin formation.

PART IV: HDAC inhibitors-induced Wnt/β-catenin signaling upregulates Nfic expression in MDPC23 cells

Nfic regulates odontoblast differentiation during root formation, and Nfic-knockout mice exhibit short abnormal molar roots (Steele-Perkins et al., 2003). Conditional inactivation of β-catenin gene resulted in the tooth phenotypes characterized by molars erupted but lack roots and thin incisors with disruption of odontoblast differenitation (Kim et al., 2013). These finding indicates that Nfic-knockout mice and β-catenin knock-out mice shows similar root formation defect,

suggesting that there are some correlation between Nfic and Wnt/â-catenin signaling in odontoblasts. However, it has not been clarified the relationship between Nfic and Wnt/â-catenin signaling in odontoblasts.

In this study, Wnt3a treatment enhanced Nfic expression while suppressing Smurf1 expression. Similarly, overexpression enhanced Nfic and suppressed Smurf1 expression, wherease â-catenin silencing reduces Nfic and induces Smurf1 expression. However, overexpression or silencing of Nfic did not regulate â-catenin expression levels. Therefore, Wnt/â-catenin signaling is likely to be an upstream regulator of Nfic in MDPC23 cells.

In PART I, Nfic was a target molecule that mediates SAHA-enhanced odontoblast differentiation. In the present study, Wnt3a treatment or â-catenin overexpression increased Nfic expression. These results indicate that SAHA/TSA induces Wnt/â-catenin signaling and in turn Wnt/â-catenin signaling upregulates Nfic expression. Considering the results showing that Nfic knockdown blocked SAHA-induced odontoblast differentiation, Nfic may play a critical role in mediating HDAC inhibitor-Wnt/â-catenin signaling-induced odontoblast differentiation. However, it has also been reported that β-catenin enhances odontoblast differentiation by Runx2 activation in dental pulp cells (Han et al., 2014). Runx2 induced Dspp expression in preodontoblast cells but reduced its expression in mature odontoblast cells (Chen et al., 2005; Glinka et al., 1998). Therefore, it is suggested that Runx2 is another candidate

gene that mediates HDAC inhibitor-Wnt/â-catenin signaling-induced odontoblast differentiation, necessitating further studies.

VII. CONCLUSION

In this study, it was demonstrated using culture of MDPC23 cells that HDAC inhibitors enhance matrix mineralization and expression levels of odontoblast differentiation marker genes, including Dspp, Dmp1 and Nestin. HDAC inhibitors activated Wnt/β-catenin signaling through the upregulation of Wnt3a and β-catenin expression. In turn, Wnt/β-catenin signaling increased the expression levels of Nfic, a positive regulator for odontoblast differentiation. Subsequently, Nfic downregulated the expression of Smurf1 which inhibits odontoblast differentiation. In conclusion, I suggest a model relaying HDAC inhibitors to Wnt/â-catenin signaling to Nfic and downregulated Smurf1 to odontoblast differentiation (Fig. 25).

HDAC inhibitors are currently being used in cancer therapy. Systemic administration of HDAC inhibitors exerts potent cytotoxic activity, limiting systemic use of HDAC inhibitors to enhancing bone formation. However, topical application of HDAC inhibitors to induce odontoblast differentiation and reparative dentin formation might be beneficial with minimized systemic cytotoxicity. To prove practical value of HDAC inhibitors in dental treatment, further studies should be followed employing human dental pulp stem cells and *in vivo* animal experiments.

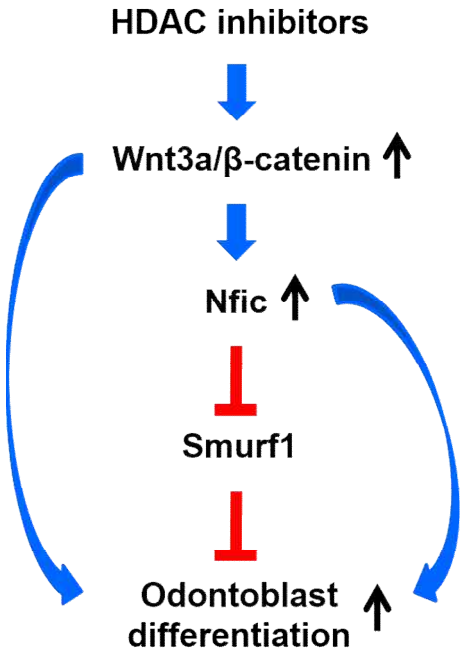

Fig. 25. Schematic illustration of the regulatory mechanisms of HDAC inhibitors employed to stimulate odontoblast differentiation.

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IX. KOREAN ABSTRACT

히스톤 탈아세틸화효소 억제제가 MDPC23 세포의 상아모세포 분화에 미치는 영향

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권 아 랑

기존의 보고에서 히스톤 탈아세틸화효소 억제제(HDACi)가 조골세포 분화와 신생골 형성을 촉진한다고 알려져 있으나 상아모세포 분화에 미치는 영향은 아직 명확히 규명되지 못 하였다. Nfic 와 Wnt/β-catenin 신호는 치아발생과정에서 상아모세포 분화에 관여하여 치근 형성을 조절한다는 보고가 있었고 E3 ubiquitin ligase 인 Smurf1 은 조골세포의 분화와 골 형성을 감소시키는 것으로 알려져 있다. 따라서 본 논문에서는 HDACi 가 상아모세포 분화에 미치는 영향을 알아보고 그 과정에서 Wnt/β-catenin, Nfic, Smurf1 의 조절 및 역할을 연구하고자 하였다. HDACi 로는 SAHA 와 Trichostatin A 를 사용하였고 상아모세포 분화 연구시스템으로 생쥐 치수에서 유래된 MDPC23 세포주를 활용하였다. 상아모세포 분화의 평가를 위해 dentin sialophosphoprotein (Dspp), dentin matrix protein 1 (Dmp1), Nestin 같은

분석을 시행하여 확인하였고, 기질의 석회화 정도는 Alizarin S Red 염색을 통해 확인하였다. Nfic 에 의한 Dspp 유전자의 전사조절을 확인하기 위해 chromatin immunoprecipitation 과 biotinylation pull down 실험을 시행하였고, Dspp 프로모터 서열이 포함된 luciferase reporter 벡터를 활용하여 리포터 분석을 시행하였다. HDACi 가 Smurf1 유전자의 크로마틴 구조 및 전사활성화 정도에 미치는 영향을 보기 위해 아세틸화된 히스톤 H3(Ac-H3), 메틸화된 히스톤 H3(H3K4me), RNA 합성효소 II 에 대한 항체를 활용하여 chromatin immunoprecipitation 을 시행하였다. HDACi 에 의한 Wnt3a 분비에 미치는 영향은 ELISA 를 통해 확인하였고, β-catenin/Tcf/Lef 의 전사활성은 TOP/FOP-flash luciferase 활성을 분석하여 확인하였다. 특정유전자의 발현억제를 유도하기 위해 유전자 특이적 small interfering RNA (siRNA) 혼합물을 주문하여 사용하였다. HDACi 는 MDPC23 세포에 의한 기질 석회화를 증가시키고 상아모세포 분화표지 유전자의 발현을 증가시켰다. HDACi 는 특히 Nfic 의 발현을 증가시켰고, 증가된 Nfic 단백질이 Dspp 프로모터에 직접 결합하여 Dspp 전사를 증가시켰다. Nfic siRNA 를 처리한 세포에서는 HDACi 에 의한 상아모세포 분화 촉진현상이 차단되었다. MDPC23 세포에 Smurf1 과발현을 유도하면 상아모세포 분화가 억제되었고, 반대로 Smurf1 siRNA 를 처치하면 상아모세포 분화가 증가되었다. 또한 MDPC23 세포에 HDACi 를 처치하거나 Nfic 를 과발현 시키면 Smurf1 발현이 감소하였으며 Smurf1 유전자의 프로모터와 전사시작 부위에서 Ac-H3, H3K4me 의 수준이 감소하고 RNA 합성효소 II 의 부착이 감소하였다. Nfic

siRNA 는 HDACi 에 의한 Smurf1 발현저해를 완화시켰다. MDPC23 세포에 Wnt3a 를 처리하거나 β-catenin 과발현을 유도하면 상아모세포 분화가 촉진되고 Nfic 발현이 증가되었으나 Wnt3a 또는 β-catenin 의 발현을 저해하면 상아모세포 분화가 억제되었고 Nfic 발현이 감소되었다. HDACi 는 MDPC23 세포에서 Wnt3a mRNA 와 단백질 발현을 증가시키고 TOP-flash 리포터 활성을 증가시켰다. Wnt3a siRNA 를 처리한 경우 HDACi 에 의한 MDPC23 세포 분화촉진 현상 및 Nfic 발현증가가 소실되었다. Nfic 의 과발현 또는 발현저해는 HDACi 에 의한 Wnt/β-catenin 신호촉진에는 별다른 영향을 미치지 않았다. 이상의 결과를 종합하면 HDACi 는 MDPC23 세포에서 Wnt/β-catenin 신호를 증가시키고 그 하위에서 Nfic 의 발현을 증가시키며 그 결과 Smurf1 의 발현을 감소시키는 효과를 나타냈고, 이러한 조절 작용을 통해 결과적으로 MDPC23 세포의 상아모세포 분화 및 기질석회화를 촉진함을 시사하였다

주요어: 상아모세포, 히스톤 탈아세틸화효소 억제제, Nfic, Wnt/β-catenin 신호, Smurf1

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