치의과학박사 학위논문

Excessive O-GlcNAcylation inhibits osteogenic differentiation

오글루넥당화 조절이 골모세포 분화에 미치는 영향

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ABSTRACT

Excessive O-GlcNAcylation inhibits osteogenic differentiation

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Diabetes mellitus (DM) is a common metabolic disorder characterized by hyperglycemia, insulin resistance, and relative insulin deficiency. DM frequently leads to serious complications that affect the heart, blood vessels, eyes, kidneys, and nerves. DM also significantly affects bone health. Increased protein O-linked-β-N-acetylglucosamine glycosylation (O-GlcNAcylation) is directly linked to insulin resistance and to hyperglycemia-induced glucose toxicity, two hallmarks of diabetes and diabetic complications. Hyperglycemic conditions in diabetic patients can affect various cellular functions, including the modulation of osteogenic differentiation. However, the molecular mechanisms by which hyperglycemia affects osteogenic

differentiation are yet to be clarified. The purpose of the study was to investigate whether the aberrant increase in protein O-GlcNAcylation contributes to the suppression of osteogenic differentiation due to hyperglycemia. In part I, it was demonstrated that excessive O-GlcNAcylation inhibits BMP2-induced osteogenic differentiation of C2C12 cells via inhibiting transcriptional activity of Runx2. Treatment of C2C12 cells with high glucose, glucosamine or N-acetylglucosamine increased O-GlcNAcylation of Runx2, a critical transcription factor for osteoblast differentiation and bone formation, and the total levels of O-GlcNAcylated proteins, which led to a decrease in the transcriptional activity of Runx2, expression levels of osteogenic marker genes (Runx2, osterix, alkaline phosphatase, and type I collagen), and activity of alkaline phosphatase. These inhibitory effects were rescued by lowering protein O-GlcNAcylation levels by adding ST045849, an O-GlcNAc transferase (OGT) inhibitor, or by overexpressing β-N-acetylglucosaminidase. In part II, it was presented that excessive protein O-GlcNAcylation conditions attenuated osteogenic differentiation partly via inhibiting Wnt/β-catenin signaling in ST2 cells. N-acetylglucosamine downregulated Wnt3a-induced alkaline phosphatase activity and osteogenic marker gene expression. N-acetylglucosamine increased β-catenin O-GlcNAcylation as well as the total levels of O-GlcNAcylated proteins. N-acetylglucosamine downregulated Wnt3a-induced TOP-flash activity and destabilized β-catenin protein by increasing β-catenin ubiquitination in the presence of Wnt3a. The inhibitory effect of N-acetylglucosamine on Wnt3a/β-catenin signaling was rescued by addition of OGT inhibitors (ST045849, OSMI-1)

or Noni leaf extract. In the previous study, Noni leaf extract has been shown to enhance osteogenic differentiation through activation of Wnt/β-catenin signaling. In addition, bone healing of calvarial defects in type 2 diabetic mice for 7 weeks in the presence of collagen sponge impregnated with vehicle, OSMI-1, ST045849, or Noni extract demonstrated that OSMI-1 and Noni extract enhanced new bone formation. These results suggest that excessive protein O-GlcNAcylation contribute to high glucose-induced suppression of osteogenic differentiation and new bone formation partly via inhibiting transcriptional activity of Runx2 and β-catenin/TCF/LEF.

Keyword: excessive O-GlcNAcylation, osteogenic differentiation, Runx2, Wnt/β-catenin signaling, T2DM
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ABBREVIATIONS

BMP	Bone morphogenic protein
DM	Diabetes mellitus
hPDL cells	Human periodontal ligament cells
Noni	*Morinda citrifolia*
OGA, O-GlcNAcase	N-acetyl-β-glucosaminidase
O-GlcNAc	O-linked-β-N-acetylglucosamine
O-GlcNAcylation	O-GlcNAc glycosylation
OGT	O-linked N-acetylglucosamine transferase
Runx2	Runt-related transcription factor 2
T2DM	Type 2 diabetes mellitus

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I. LITERATURE REVIEW

I.1. Diabetes mellitus

Diabetes mellitus (DM) is one of the most common chronic diseases in nearly all countries, and continues to increase in numbers and significance, as economic development and urbanization lead to changing lifestyles. In 2011 there are 366 million people with diabetes, and this is expected to rise to 552 million by 2030 [1, 2]. DM is a common metabolic disorder characterized by hyperglycemia, insulin resistance, and relative insulin deficiency [3]. DM is a complex syndrome with more than one cause and is responsible for numerous complications that affect the entire body. DM frequently leads to serious complications that affect the heart, blood vessels, eyes, kidneys, and nerves.

DM also significantly affects bone health. Reportedly, DM-associated hyperglycemia modulates osteoblast gene expression, function, and bone formation, thereby causing diabetic bone loss in a mouse model of insulin-dependent DM [4]. At a structure level, enhanced formation of advanced glycation end (AGE) products in the bone matrix contributes to reduced bone strength and increased fracture risk in type 2 diabetes mellitus (T2DM) [5]. Human osteoblasts treated with high level glucose and AGEs show a reduced expression of *RUNX2* and *SP7* genes [6]. Increasing evidence has demonstrated that patients with DM have increased cortical porosity and bone metabolism disorder, leading to reduced bone strength and defective bone healing [7-9]. It has also been demonstrated that high glucose

concentrations inhibit osteogenic differentiation of bone marrow mesenchymal stem cells by attenuating the effect of bone morphogenetic protein 2 (BMP2) [10]. A recent study demonstrated that dramatically decreased expressions of osteogenic transcription factors *Runx2* and *Sp7* and the inhibited capacity of bone formation under T2DM condition, which was in line with the alkaline phosphatase (ALP) staining result [11]. Hyperglycemia induces osteopenia, increases bone fragility and unbalances in the coupling of osteoblasts and osteoclasts [12]. Liu et al. demonstrated that hyperglycemia elevated the expression of C/EBP-homologous protein (CHOP) and finally led to osteoporosis. Furthermore, it has been reported that hyperglycemia increases sclerostin expression by enhancing reactive oxygen species and tumor necrosis factor alpha production in osteoblasts and osteocytes [13], suggesting that increased sclerostin expression contributes to DM-associated osteopenia in diabetic patients. The influence of DM on osteoclasts remains obscure and controversial. Hyperglycemia plays a role in aberrant osteoclast differentiation leading to an increased capacity for bone resorption [14].

DM has also been linked to periodontitis, which is a common chronic inflammatory disease characterized by alveolar bone resorption and supporting tissue destruction around the teeth [15]. There is emerging evidence to support the existence of a two-way relationship between diabetes and periodontitis, with diabetes increasing the risk for periodontitis, and periodontal inflammation negatively affecting glycemic control. Poorer glycemic control leads to both an increased risk for alveolar bone loss and more severe progression over those without T2DM [16,

17]. Several studies have investigated the mechanisms how high glucose affect the proliferation, osteogenic differentiation, and apoptosis of periodontal ligament cells [18-21].

I.2. O-GlcNAcylation

O-linked-β-N-acetylglucosamine (O-GlcNAc) modification is an important post-translational modification of nucleocytoplasmic proteins [22]. O-GlcNAc glycosylation (O-GlcNAcylation) involves the covalent attachment of N-acetylglucosamine to serine (Ser) or threonine (Thr) residues of proteins [23, 24]. O-GlcNAcylation and phosphorylation cross talk to regulate protein function [25]. Since both modifications can occur on the same sites and thus, might entail a direct competition between the two. In addition, these two modifications can coexist at adjacent or even proximal sites that may impose a steric hindrance on each other [26, 27]. For example, Thr58 on the transactivation domain of c-Myc is modified by both O-GlcNAcylation and phosphorylation [28, 29], and elevating O-GlcNAcylation in human colon cell lines drastically reduced phosphorylation at Thr41, a key residue of the D box responsible for β-catenin stability [30]. Unlike phosphorylation, which is regulated by hundreds of kinases and phosphatases, cycling of O-GlcNAc residues on intracellular proteins is known to be controlled by two highly conserved enzymes; O-linked N-acetylglucosamine transferase (OGT), which transfers N-acetylglucosamine from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc)

to protein substrates, and N-acetyl-β-glucosaminidase (OGA, O-GlcNAcase), which removes the O-GlcNAc modification. Together, these enzymes dynamically alter the post-translational state, translocation and function of proteins in response to cellular signals [22, 26, 31].

In the cell, glucose and glucosamine imported via a glucose transporter can be converted to uridine diphosphate (UDP)-GlcNAc through the hexosamine biosynthetic pathway (HBP) [22]. More than 4000 nuclear and cytoplasmic proteins belonging to almost every functional class of proteins, including transcription factors, cytoskeletal proteins, RNA polymerases, cell cycle regulators, phosphatases, and kinases, are modified by O-GlcNAc [32]. O-GlcNAcylation is highly dynamic in response to intracellular and extracellular signals, nutrient availability, and stresses, and aberrant O-GlcNAcylation has been implicated in the progression of diseases such as cancer, neurodegeneration, and DM [33, 34]. Increased O-GlcNAcylation is directly linked to insulin resistance and to hyperglycemia-induced glucose toxicity, two hallmarks of diabetes and diabetic complications [35]. Therefore, it has been suggested that global cellular O-GlcNAcylation levels must remain within an optimal zone in the various fluctuations of cellular environments in order to preserve normal cellular function [36]. OGT deletion results in embryonic lethality in mice, reflecting the significance of protein O-GlcNAcylation in development and survival [37]. In addition, OGA deletion is perinatally lethal in mice [38]. Reportedly, the OGA gene *MGEA5* is a DM-susceptibility locus in humans [39, 40], and Goto-Kakizaki rats harboring an exon 8 deletion in *Mgea5* spontaneously develop DM

[41], suggesting that aberrant upregulation of O-GlcNAcylation is involved in the pathogenesis and/or complications of DM. Protein O-GlcNAcylation increases in diabetic tissues and hyperglycemia drives excessive chronic O-GlcNAcylation of proteins, including those involved in insulin signaling in DM [42, 43].

Reportedly, overall cellular O-GlcNAcylation levels change during adipogenic, chondrogenic, and osteogenic differentiation of mesenchymal stem cells [44]. Previous reports have demonstrated that global O-GlcNAcylation levels increase during osteoblastic differentiation of MC3T3-E1 and bone marrow mesenchymal stem cells, and that further increases in O-GlcNAcylation levels by OGA inhibition promotes osteoblast differentiation [45-47]. Increased O-GlcNAcylation of Runx2, a critical transcription factor for osteoblast differentiation and bone formation, enhances transcriptional activity of Runx2 and mRNA expression levels of Runx2 target genes, including ALP and osteocalcin, contributing to osteoblast differentiation [45, 47].

I.3. Runx2

Runt-related transcription factor 2 (Runx2) is a transcription factor critical for the progression of osteoblast differentiation. Runx2 is post-translationally modified downstream of a diverse set of signaling pathways, whose coordinated action controls osteoblast differentiation and bone development [48]. Runx2 is O-GlcNAcylated and its transcriptional activity was shown to be increased by O-

GlcNAcylation inducers [45, 47]. Nagel and Ball reported novel sites of O-GlcNAc modification and methylation on human type II Runx2 protein, and also observed three sites of O-GlcNAc modification on murine Runx2 at Ser32, Ser33, and Ser371, which reside proximal to regulatory phosphorylation sites [47].

I.4. Wnt signaling

Wnts, a large family of secreted glycoproteins, are involved in controlling cell proliferation, cell-fate specification, gene expression, and cell survival [49-53]. Cells recognize canonical Wnt ligands with 10 Frizzled receptors (Fzd) and Lrp molecules (Lrp5/6 and potentially Lrp4). The large number of ligands and receptors creates great combinatorial diversity and contributes to widely variable cellular responses depending on the molecules present. Wnts were historically classified as either “canonical” or “non-canonical” based on their ability to activate β-catenin, however, in reality the distinction is not so clear because some Wnts stimulate both pathways depending on the cellular context.

The Wnt signaling pathway is a key regulator of cellular differentiation and of crucial importance in skeletal development, bone mass maintenance and remodeling and has therefore gained much attention from the research community. Wnt signaling pathways have been the focus of intense research activity in bone biology field because of their importance in skeletal development, bone mass maintenance, and therapeutic potential for regenerative medicine [52, 53]. The best

known is the Wnt/β-catenin pathway [52]. β-Catenin is a cytoplasmic and nuclear protein encoded by the *Ctmnb1* gene. It is a key link in numerous signaling cascades, including the canonical Wnt pathway, is essential for embryonic development, and is hyperactivated by mutations in many cancers. Increased O-GlcNAcylation correlated with β-catenin and E-cadherin expression in colorectal cancer [54]. High glucose can target different components of the canonical Wnt pathway to induce β-catenin destabilization in mouse osteoblastic cells [55].

Wnt and bone morphogenic protein (BMP) signaling pathways are evolutionarily conserved and essentially independent signaling mechanisms, which, however often regulate similar biological processes [56]. BMP has dual roles in Wnt signaling. On one hand, BMP inhibits Wnt/β-catenin signaling by increasing Wnt antagonist Dkk1 and Sost expression and by preventing β-catenin nuclei translocation [57]. On the other hand, BMP promotes Wnt/β-catenin signaling by forming co-transcriptional complex with β-catenin/TCF/LEF/Runx2, by increasing Wnt expression, by antagonizing Dvl function, and by decreasing β-transducin repeat-containing protein (β-TrCP expression [57]. A recent study showed that blocking Wnt/β-catenin signaling exerted a more potent inhibitory effect on osteogenic differentiation and even on key factors of BMP/SMAD signaling pathway [58]. Overall, there are numerous osteogenic differentiation and bone formation processes that involve BMP, Wnt, and β-catenin, yet there are conflicting data on the role and interaction of these factors. However, RUNX2 is regarded as a common downstream target gene or crucial mediator of Wnt/β-catenin and

BMP/SMAD signaling during osteogenic differentiation [57, 58].

I.5. Osteogenic differentiation

Osteogenic differentiation of mesenchymal stem cells and periodontal ligamental stem cells can be induced by tri-component combination of ascorbic acid, β-glycerophosphate, and dexamethasone. Osteogenic differentiation of C2C12 pre-myoblasts is effectively induced by BMP2, and Wnt3a efficiently stimulates osteogenic differentiation of C3H10T1/2 mesenchymal progenitor cells and ST2 bone marrow stromal cells [59].

I.6. *Morinda citrifolia* (Noni)

Morinda citrifolia (Noni) is one of the most important traditional Polynesian medicinal plants for over 2000 years, and the leaf is available during all seasons in tropical countries [60, 61]. A number of major components such as scopoletin, octanoic acid, potassium, vitamin C, terpenoids, alkaloids, anthraquinones (such as nordamnacanthal, morindone, rubiadin, rubiadin-1-methyl ether, and anthraquinone glycoside), b-sitosterol, carotene, vitamin A, flavone glycosides, linoleic acid, Alizarin, amino acids, acubin, L-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin, kaempferol-3-O-rutinoside, and a putative proxeronine have been identified in the Noni plant [61]. Recent scientific research has supported the indigenous uses of Noni leaf, which include enhancing wound

healing and bone regeneration [62]. Noni fruit extract increased osteoblast activity, enhanced matrix mineralization, and restrained osteoclast activity [63]. However, excessive intake of Noni fruit extract may cause liver toxicity [64]. A recent report demonstrated that Noni leaf extract enhanced osteogenic differentiation through activation of Wnt/β-catenin signaling [65].

II. EXPERIMENTAL TECHNIQUES

II.1. Cell cultures and reagents

C2C12 cells and ST2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100-U/mL penicillin and 100-μg/mL streptomycin). To induce osteogenic differentiation, C2C12 cells were incubated in DMEM supplemented with 5% FBS and BMP2 (50 ng/mL) for 48 h. DMEM, FBS, antibiotics, and other reagents for cell culture were purchased from HyClone Laboratories (GE Healthcare Life Sciences, South Logan, UT, USA). Recombinant human BMP2 was purchased from Cowellmedi (Seoul, Korea). To induce osteogenic differentiation, ST2 cells were incubated in Wnt3a-conditioned medium. L-Wnt3a cells were purchased from ATCC (Manassa, VA, USA) and Wnt3a-conditioned medium was prepared according to the manufacturer's instructions. Human periodontal ligament (hPDL) cells were purchased from ScienCell™ Research Laboratories (Carlsbad, CA, USA) and maintained in alpha-minimum essential medium (αMEM) supplemented with 10% FBS and antibiotics. Osteogenic differentiation of hPDL cells was induced by osteogenic supplements (50 μg/mL ascorbic acid, 10 mM β-glycerophosphate and 0.1 μM dexamethasone). Glucose concentration in DMEM and αMEM was 5 mM. To induce excessive *O*-GlcNAcylation, C2C12, ST2 cells and hPDL cells were exposed to the indicated glucose, glucosamine, or *N*-acetylglucosamine concentrations. Expression plasmids for human OGT and OGA were kindly

provided by Prof. Jin Won Cho from Yonsei University and were transiently transfected into C2C12 cells using Lipofectamine 2000 reagent (Invitrogen, Waltham, MA, USA) [66].

II.2. Cell cytotoxicity test

Cells were plated in a 96-well plate, and treated with indicated reagents. After 2 or 3 days, cytotoxicity was examined using EZ-Cytox Cell Viability Assay Kit (Daeil Lab Service; Seoul, Korea) according to the manufacturer's instructions.

II.3. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA extraction and qRT-PCR was performed as previously described [67]. Target genes were normalized to the reference housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The mouse PCR primer sequences used for qRT-PCR were as follows: *Alpl* (ALP), (f) 5′-CCA ACT CTT TTG TGC CAG-3′ and (r) 5′-GGC TAC ATT GGT GTT GAG CTT TT-3′; *Runx2*, (f) 5′-TTC TCC AAC CCA CGA ATG CAC-3′ and (r) 5′-CAG GTA CGT GTG GTA GTG AGT-3′; *Sp7* (Osterix), (f) 5′-CCC ACC CTT CCC TCA CTC-3′ and (r) 5′-CCT TGT ACC ACG AGC CAT-3′; *Bglap2* (Osteocalcin), (f) 5′-CTG ACA AAG CCT TCA TGT CCA A-3′ and (r) 5′-GCG CCG GAG TCT GTT CAC TA-3′; *Col1a1* (f) 5′-GCT CCT CTT AGG GGC CAC T-3′ and (r) 5′-CCA CGT CTC ACC

ATT GGG G-3′; *Ogt*, (f) 5′-CTG TCA CCC TTG ACC CAA AT-3′ and (r) 5′-ACG AAG ATA AGC TGC CAC AG-3′; *Mgea5*, (f) 5′-TGG AAG ACC TTG GGT TAT GG-3′ and (r) 5′-TGC TCA GCT TCT TCC ACT GA-3′; *β- catenin* (f) 5′-GGT GCT GAC TAT CCA GTT G-3′ and (r) 5′- GGC AGA GTA AAG TAT TCA CCC-3′; and *Gapdh*, (f) 5′-TCA ATG ACA ACT TTG TCA AGC-3′ and (r) 5′-CCA GGG TTT CTT ACT CCT TGG-3′. The human PCR primer sequences used for qRT-PCR were as follows: *ALPL*, (f) 5′-AAC TTC CAG ACC GGC TTG A-3′ and (r) 5′-TTG CCG CGT GTC TT-3′; *RUNX2*, (f) 5′-CAG ATG GGA CTG TGG CTG T-3′ and (r) 5′-GTG AAG ACG GTT ATG AAG G-3′; *SP7*, (f) 5′-ACC TAC CCA TCT GAC TTT GCT-3′ and (r) 5′-CCA CTA TTT CCC ACT GCC TTG-3′; *DLX5* (f) 5′-CAA CTT TGC CCG AGT CTT C-3′ and (r) 5′-GTT GAG AGC TTT GCC ATA GG-3′; *BGLAP* (Osteocalcin) (f) 5′-GTG CAG AGT CCA GCA AAG GT-3′ and (r) 5′-CGA TAG GCC TCC TGA AAG C-3′; and *GAPDH*, (f) 5′-CCA TCT TCC AGG AGC GAG ATC-3′ and (r) 5′-GCC TTC TCC ATG GTG GTG AA-3′.

II.4. Western Blot Analysis and Immunoprecipitation

Cell lysates for Western blot analysis were prepared using PRO-PREP™ (iNtRON Biotechnology, Sungnam, Korea) and were briefly sonicated. To examine cellular global protein *O*-GlcNAcylation patterns, samples containing equal amounts of protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline

containing 0.1% Tween 20. The membrane was serially incubated with O-GlcNAc antibody, HRP-conjugated secondary antibody, and Sensi-view^{TM} Pico ECL Reagent (Lugen Sci. Inc., Buncheon, Korea), followed by the detection of chemiluminescence using a MicroChemi system (DNR; Jerusalem, Israel).

For immunoprecipitation, cell lysates were prepared using buffer containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (pH 8.0), 2% Brij-35, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1-μg/mL aprotinin, 1 μM leupeptin, and 1 μM pepstatin. Each 1 mg of protein sample was mixed with the Runx2 antibody and protein G agarose beads. The washed bead pellet was denatured by boiling in 2× SDS sample buffer, followed by SDS-PAGE and immunoblot analysis with *O*-GlcNAc and Myc antibodies. Antibodies used for immunoprecipitation and Western blot analyses were as follows: *O*-GlcNAc antibody purchased from BioLegend (San Diego, CA, USA); Myc, Runx2, and actin antibodies purchased from Santa Cruz Biotechnology (Dallas, TX, USA); and HRP-conjugated secondary antibodies purchased from Thermo Fisher Scientific (Waltham, MA, USA).

To examine the levels of ubiquitin-modified β-catenin, ST2 cells were transiently transfected with plasmids encoding HA-tagged ubiquitin and incubated for 24 h in the presence of the indicated reagents and proteasome inhibitor MG132 (10 μM), followed by immunoprecipitation with HA antibody and immunoblotting with β-catenin antibody.

II.5. ALP Staining, ALP Activity Assays, and Alizarin Red S Staining

At the end of the culture period, ALP staining was performed by employing an ALP staining kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. ALP activity was assessed using a QuantiChrom™ ALP assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. ALP activity was normalized to the total protein amount in the samples. Matrix mineralization was examined by Alizarin Red S staining. hPDL cells were incubated in the osteogenic medium for 21 days in the presence or absence of *N*-acetylglucosamine (2.5 mM). At the end of the culture period, the cells were fixed with 70% ethanol and stained with 2% Alizarin red S solution for 10 min at room temperature. After thorough rinsing with distilled water, the calcium deposition was quantified by the elution of the stain with 0.5 N HCl containing 5% SDS and the measurement of the optical density at 415 nm.

II.6. Luciferase Reporter Assays

Cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and, after overnight culture, the cells were transiently transfected with Myc-Runx2 expression plasmids/Runx2 reporter plasmids (OSE-luc) or β-catenin expression plasmids/TOP-flash or FOP-flash reporter plasmids using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA). In each transfection, 0.5 μL of LipofectAMINE

2000, 0.2 μg each of indicated expression plasmids and reporter plasmids, and 0.07 μg of Renilla luciferase plasmids were used. Luciferase activity was measured after an additional incubation for 24 h in the presence or absence of *O*-GlcNAcylation inducers and OGT inhibitor [68]. When indicated, OGT or OGA expression plasmids were co-transfected with Myc-Runx2 expression plasmids. Luciferase assays were performed using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) and the GloMax-Multi Detection System (Promega). The relative luciferase activity was calculated by dividing firefly luciferase activity by Renilla luciferase activity to normalize the transfection efficiency.

II.7. Preparation of Noni leaf aqueous extract

Noni leaf aqueous extract was prepared as previously described (Boonanantanasarn et al., 2012). Briefly, fresh Noni leaves were collected from Chonburi Province, Thailand. After being washed with water, air dried, and powdered with an electric blender, the leaf powder was imported to the Department of Molecular Genetics at the Seoul National University. Noni leaf powder was suspended in sterilized distilled water at 4°C. After 24 h, the Noni leaf mixture was filtered using a fine muslin cloth and was then centrifuged at 14,240 *g* for 10 min at 4°C. The supernatant was filtered through a Whatman filter paper No. 1 to remove remaining debris, freeze dried with a lyophilizer (FD-8512; ilShinBioBase, South Korea), and stored at -80°C. One gram of freeze-dried extract was obtained from 10 gram of Noni leaf powder. Freeze-dried Noni leaf extract was dissolved in a culture

medium, filtered through a 0.22-μm cellulose acetate membrane filter (Pall Corporation, MI, USA), and sterilized by UV light for 30 min before use.

II.8. Animal experiments

Six-week-old male C57BL/6 mice were obtained from Orient Bio (Gyeonggi-do, South Korea) (Fig. 13A). Mice were fed either normal diet (ND) or 60% kcal high fat diet (HFD) for 4 weeks, then injected with vehicle (0.1 M sodium citrate buffer at pH 4.5) or streptozotocin (STZ, 40 mg/kg, in 0.1 M sodium citrate buffer at pH4.5; Sigma, USA) intraperitoneally. A week after the injection, fasting blood glucose levels were assessed using a glucometer and mice with blood glucose over 16.7 mM were considered diabetic. Glucose tolerance test (GTT) were performed after overnight fasting. Mice were injected with glucose (1 g/kg) intraperitoneally. Blood was collected from tail vein at 0, 15, 30, 60, 90, 120 min after injection. Mice weight and blood glucose levels were measured until sacrifice. After diabetes confirmation, diabetic mice were divided into 4 groups (8 mice/group) for the surgical procedures. Mice were anesthetized with intraperitoneal injections of Zoletil (80 mg/kg) and rompun (10 mg/kg) mixture according to Plumb's Vet Drug Handbook. After shaving the scalp hair, a longitudinal incision was made in the middle line of the cranium and the periosteum was elevated to expose the surface of the parietal bones. Using the trephine bur and a low speed handpiece drill, 2 mm diameter defects were produced in each parietal bone. The calvarial defects were filled with collagen sponge loaded with 0.3 μL of phosphate-buffered saline (PBS),

OSMI-1 (40 μg/mL), ST045849 (45 μg/mL), or noni extract (0.1 g/mL). The incisions were closed using 6-0 nylon sutures. All the animal experiments were approved by the Institutional Animal Care and Use Committee at Seoul National University (SNU-180312-4-3).

II.9. MicroCT analysis

For the *in vivo* microCT follow-up of bone regeneration within calvarial defects, mice were anesthetized with isofluorane and scanned using Quantum GX (PerkinElmer, USA). Follow-up *in vivo* microCT scans were made directly after the surgery and at 2, 5 and 7 weeks after the surgery to monitor bone structure. At 7-week post-surgery, the calvarial bones were removed, fixed in 4% paraformaldehyde for 24 h, and then scanned using Skyscan1272 microCT (Bruker, Belgium). The images were processed using NRecon software (Bruker). New bone volume (mm^3) and new bone surface (%) were analyzed using CTAn software and then visualized using CTvol software (Bruker).

II.10. Histological analysis

The calvarial bones were decalcified in 10% EDTA for 7 days, dehydrated through a series of ethanol, and embedded in paraffin. Then 5 μm thick coronal sections were cut along the defect and calvarial bone. Sections were stained with hematoxylin and eosin, and then examined by light microscopy.

II.11. Statistical analysis

All quantitative data are presented as mean ± standard deviation. Statistical significance was analyzed by ANOVA with Tukey's multiple comparison tests using Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). A p value < 0.05 was considered statistically significant.

III. PART I

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Introduction

DM is a chronic metabolic disease characterized by hyperglycemia due to the inability of insulin-dependent cells to effectively take up glucose. DM frequently leads to serious complications that affect the heart, blood vessels, eyes, kidneys, and nerves. DM also significantly affects bone health. Reportedly, DM-associated hyperglycemia modulates osteoblast gene expression, function, and bone formation, thereby causing diabetic bone loss in a mouse model of insulin-dependent DM [4]. Further, enhanced formation of advanced glycation end products in the bone matrix contributes to reduced bone strength and increased fracture risk in type 2 DM [5]. Although hyperglycemic conditions in diabetic patients significantly affect bone health, the molecular mechanisms underlying the inhibition of osteogenic differentiation and bone formation remain unclear.

O-GlcNAcylation, which involves the covalent attachment of N-acetylglucosamine to serine or threonine residues of proteins, is a unique post-translational modification [23, 24]. More than 4000 nuclear and cytoplasmic proteins belonging to almost every functional class of proteins, including transcription factors, cytoskeletal proteins, RNA polymerases, cell cycle regulators, phosphatases, and kinases, are modified by O-GlcNAc [32]. The dynamics of O-GlcNAcylation are unique among sugar modifications, being cycled on a shorter time scale than protein

turnover [34]. O-GlcNAcylation is highly dynamic in response to intracellular and extracellular signals, nutrient availability, and stresses, and aberrant O-GlcNAcylation has been implicated in the progression of diseases such as cancer, neurodegeneration, and DM [33]. Therefore, it has been suggested that global cellular O-GlcNAcylation levels must remain within an optimal zone in the various fluctuations of cellular environments in order to preserve normal cellular function [36].

Protein O-GlcNAcylation is reversibly catalyzed by OGT and OGA. OGT deletion results in embryonic lethality in mice, reflecting the significance of protein O-GlcNAcylation in development and survival [37]. In addition, OGA deletion is perinatally lethal in mice [38]. Reportedly, the OGA gene *MGEA5* is a DM-susceptibility locus in humans [39, 40], and Goto-Kakizaki rats harboring an exon 8 deletion in *Mgea5* spontaneously develop DM [41], suggesting that aberrant upregulation of O-GlcNAcylation is involved in the pathogenesis and/or complications of DM. Protein O-GlcNAcylation increases in diabetic tissues and hyperglycemia drives excessive chronic O-GlcNAcylation of proteins, including those involved in insulin signaling in DM [42, 43].

Reportedly, overall cellular O-GlcNAcylation levels change during adipogenic, chondrogenic, and osteogenic differentiation of mesenchymal stem cells (reviewed in Reference [44]). Previous reports have demonstrated that global O-GlcNAcylation levels increase during osteoblastic differentiation of MC3T3-E1 and bone marrow mesenchymal stem cells, and that further increases in O-

GlcNAcylation levels by OGA inhibition promotes osteoblast differentiation [45-47]. Increased O-GlcNAcylation of Runx2, a critical transcription factor for osteoblast differentiation and bone formation, enhances transcriptional activity of Runx2 and mRNA expression levels of Runx2 target genes, including ALP and osteocalcin, contributing to osteoblast differentiation [45, 47].

However, considering that hyperglycemia increases protein O-GlcNAcylation and that aberrant upregulation of O-GlcNAcylation is involved in the pathogenesis of human diseases such as DM, it cannot be ruled out that excessive, aberrant O-GlcNAcylation of proteins induced by hyperglycemia may contribute to attenuated osteoblast differentiation and bone formation phenotypes observed in diabetic animal models or diabetic patients. Therefore, it was hypothesized that although protein O-GlcNAcylation is necessary for the progression of osteogenic differentiation, excessive O-GlcNAcylation of proteins, including Runx2, would negatively regulate osteogenic differentiation. To test the hypothesis, it was examined the effect of excessive O-GlcNAcylation inducers, including high concentrations of glucose (high glucose), glucosamine, and N-acetylglucosamine, or OGT overexpression on the osteogenic differentiation of C2C12 cells induced by treatment with BMP2. In the present study, it has demonstrated that excessive protein O-GlcNAcylation-inducing conditions inhibit BMP2-induced osteogenic differentiation of C2C12 cells and transcriptional activity of Runx2.

Results

Excessive O-GlcNAcylation-Inducing Conditions Suppressed BMP2-Induced ALP Activity and Osteogenic Marker Gene Expression

C2C12 is a mouse myoblast cell line that can differentiate into osteoblasts in the presence of BMP2, thereby resulting in the induction of ALP activity and osteocalcin production [69]. To examine the effect of protein O-GlcNAcylation inducers on osteoblast differentiation, C2C12 cells were incubated in the presence of 50 ng/mL BMP2 and O-GlcNAcylation inducers. With the initiation of osteogenic differentiation, C2C12 cells were exposed to varying glucose (GC, 10, 20, 40 or 60 mM), glucosamine (GS, 1, 2.5 or 5 mM), or N-acetylglucosamine (GN, 1, 2.5 or 5 mM) concentrations for 6 h. Protein O-GlcNAcylation was assessed using immunoblot analysis with O-GlcNAc antibody. Similar to previous reports demonstrating that osteogenic stimuli increase global O-GlcNAcylation levels [45-47], BMP2 treatment moderately increased O-GlcNAcylation levels in C2C12 cells (Fig. 1A). All three O-GlcNAcylation inducers further increased overall protein O-GlcNAcylation in C2C12 cells (Fig. 1A).

Next, the progression of osteoblast differentiation was assessed after 48 h of incubation under osteogenic conditions. ALP staining demonstrated that BMP2-induced ALP activity was suppressed by glucose at concentrations of 40 and 60 mM (Fig. 1B). Furthermore, glucosamine and N-acetylglucosamine strongly suppressed BMP2-induced ALP activity in a dose-dependent manner, and ALP staining was very

weak in the presence of 2.5–5 mM glucosamine or 5 mM N-acetylglucosamine (Fig. 1B). Therefore, subsequent experiments were performed using 40 mM glucose, 1 mM glucosamine, and 2.5 mM N-acetylglucosamine. To further confirm the effects on osteoblast differentiation, qRT-PCR of osteoblast marker genes was performed: ALP, type I collagen, and osteocalcin, early and late osteoblast markers, and Runx2 and osterix, critical transcription factors for osteoblastic differentiation. The expression levels of these genes were increased by BMP2 but were significantly suppressed by O-GlcNAcylation inducers (Fig. 1C). These results suggest that conditions inducing abnormally high levels of protein O-GlcNAcylation inhibit BMP2-induced osteogenic differentiation of C2C12 cells.

Inhibition of OGT Activity Rescued BMP2-Induced Osteogenic Differentiation that was Downregulated by O-GlcNAcylation Inducers

To confirm whether the inhibitory effect of O-GlcNAcylation inducers on osteogenic differentiation depends on excessive protein O-GlcNAcylation, it was investigated whether an OGT inhibitor rescues BMP2-induced osteogenic differentiation in the presence of O-GlcNAcylation inducers. The addition of STO45849 (1 μM), a specific OGT inhibitor, rescued BMP2-induced ALP activity downregulated by O-GlcNAcylation inducers (Fig. 2A, B). Furthermore, qRT-PCR results also demonstrated that STO45849 rescued BMP2-induced mRNA expression levels of ALP, Runx2, and osterix, which were decreased by N-acetylglucosamine (Fig. 2C). These results indicated that OGT activity is required for the inhibitory effect of O-GlcNAcylation inducers on osteogenic differentiation.

OGT Overexpression Inhibited BMP2-Induced Osteogenic Differentiation, whereas OGA Overexpression Rescued N-Acetylglucosamine-Inhibited Osteogenic Differentiation

To further verify whether excessive O-GlcNAcylation inhibits osteogenic differentiation, it was next examined the effect of OGT overexpression on BMP2-induced ALP activity and osteogenic marker gene expression. OGT overexpression blocked BMP2-induced ALP activity and osteogenic gene expression (Fig. 3A, B). OGT overexpression was confirmed by RT-PCR (Fig. 3B). BMP2 did not change basal OGT mRNA levels, but significantly increased OGT mRNA expression under conditions of OGT overexpression (Fig. 3B).

It was then examined whether OGA overexpression can rescue osteogenic differentiation under conditions of excessive O-GlcNAcylation. OGA overexpression significantly suppressed BMP2-induced ALP activity (Fig. 4A) but completely rescued BMP2-induced ALP activity and ALP mRNA expression in the presence of N-acetylglucosamine (Fig. 4A, B). N-acetylglucosamine-inhibited expression of Runx2, and osterix mRNA was only partially rescued by OGA overexpression (Fig. 4B). OGA overexpression was confirmed by RT-PCR (Fig. 4B). N-acetylglucosamine also increased OGA mRNA expression, which was further increased by OGA transfection (Fig. 4B). These results indicated that the inhibitory effect of N-acetylglucosamine on BMP2-induced expression of osteoblast marker genes depends on protein O-GlcNAcylation.

Excessive O-GlcNAcylation Suppressed Runx2 Transcriptional Activity, Which Was Restored by OGT Inhibitor Addition or OGA Overexpression

Although previous reports have demonstrated that Runx2 O-GlcNAcylation enhanced its transcriptional activity [45, 47], the above results showed that excessive O-GlcNAcylation-inducing conditions inhibited BMP2-induced osteogenic differentiation. Therefore, it was further examined whether O-GlcNAcylation inducers regulate Runx2 transcriptional activity in this system. To confirm Runx2 O-GlcNAcylation, Myc-tagged Runx2 was expressed in C2C12 cells and incubated in the presence of high glucose, glucosamine, or N-acetylglucosamine for 24 h. The results of the immunoblot analysis showed that Runx2 O-GlcNAcylation was induced by all three O-GlcNAcylation inducers (Fig. 5A). Next, luciferase reporter assays were performed to examine whether increased Runx2 O-GlcNAcylation leads to changes in Runx2 transcriptional activity. C2C12 cells were transfected with the OSE-luc reporter and Myc-Runx2 expression plasmids and incubated for 24 h in the presence of 40 mM glucose, 1 mM glucosamine, or 2.5 mM N-acetylglucosamine. The results demonstrated that all three O-GlcNAcylation inducers significantly decreased Runx2 transcriptional activity (Fig. 5B). Similarly, OGT overexpression blocked Myc-Runx2-mediated induction of luciferase activity, and the effect of OGT overexpression was attenuated by adding STO45849 (Fig. 5C). Furthermore, OGA overexpression rescued Runx2 transcriptional activity, which was downregulated by N-acetylglucosamine (Fig. 5D). However, OGA

overexpression itself suppressed Runx2 transcriptional activity in the absence of N-acetylglucosamine (Fig. 5D). These results indicated that excessive O-GlcNAcylation inducers inhibit Runx2 transcriptional activity, but that basal O-GlcNAcylation levels are necessary for Runx2 transcriptional activity.

Excessive O-GlcNAcylation Attenuated Osteogenic Differentiation and Matrix Mineralization of hPDL Cells

To ensure that excessive O-GlcNAcylation inducers inhibit osteogenic differentiation, it was examined the effect of N-acetylglucosamine on osteogenic differentiation of primary cultured hPDL cells. With the initiation of osteogenic differentiation, hPDL cells were exposed to varying concentrations of N-acetylglucosamine (GN, 0.5, 1, 2.5, 5, or 10 mM) for 6 h. Increased global protein O-GlcNAcylation levels was induced by treatment with N-acetylglucosamine at the concentrations of 2.5, 5, and 10 mM (Fig. 6A).

Next, the progression of osteoblast differentiation was assessed after seven days of incubation under osteogenic conditions. ALP staining demonstrated that ALP activity was suppressed by N-acetylglucosamine at concentrations of 2.5–10 mM (Fig. 6B). qRT-PCR of early osteoblast marker genes demonstrated that the expression levels of ALP, RUNX2, and DLX5 genes were increased by osteogenic stimuli, but were significantly suppressed by N-acetylglucosamine (Fig. 6C).

To further examine the effect of N-acetylglucosamine on matrix mineralization, hPDL cells were incubated for 21 days, followed by Alizarin Red S staining (Fig. 6D). N-acetylglucosamine significantly suppressed osteogenic stimuli-induced matrix mineralization, which was rescued by the addition of STO45849. However, STO45849 treatment alone inhibited matrix mineralization. These results suggest that conditions inducing abnormally high levels of protein O-GlcNAcylation inhibit osteogenic differentiation of hPDL cells.

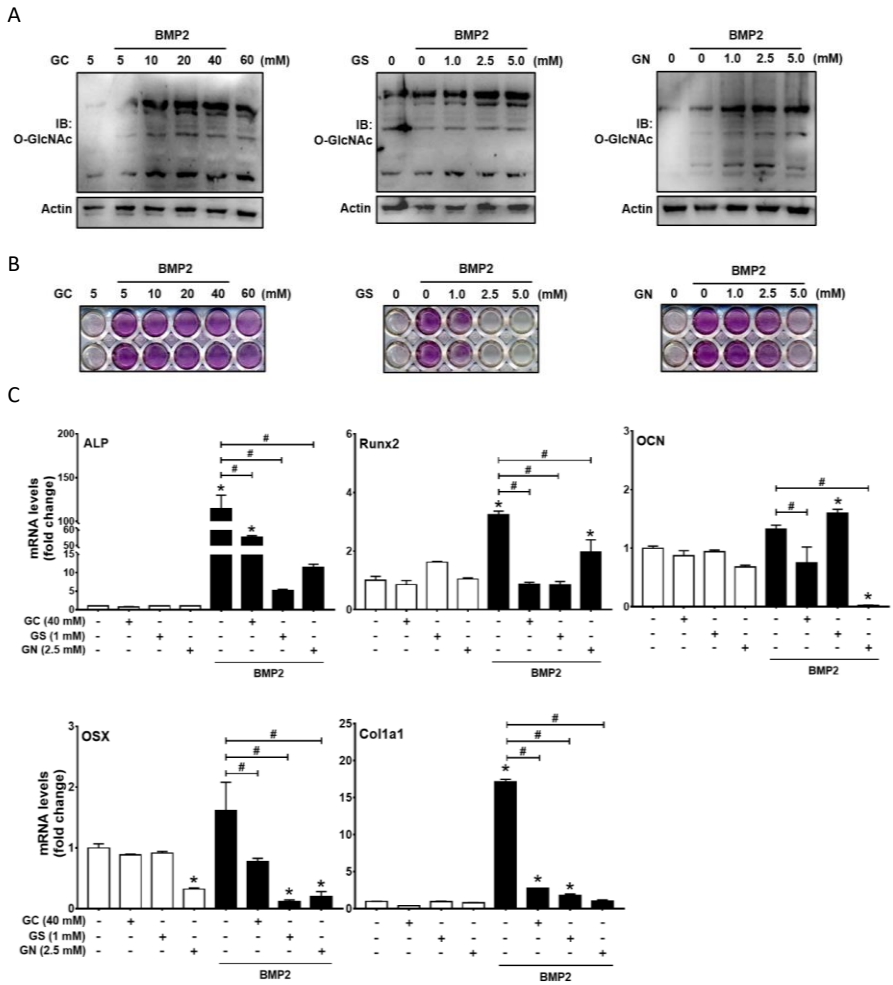

Fig. 1. High glucose (GC), glucosamine (GS), and N-acetylglucosamine (GN) concentrations inhibited BMP2-induced osteogenic differentiation in C2C12 cells.

(A) C2C12 cells were incubated for 6 h in the presence or absence of rhBMP2 (50 ng/mL) and GC, GS, or GN at the indicated concentrations, and Western blot analysis was performed to examine the levels of global protein O-GlcNAcylation; (B, C)

C2C12 cells were incubated in the presence of the indicated reagents for 48 h, and osteogenic differentiation was examined by alkaline phosphatase (ALP) staining (B) and quantitative RT-PCR of osteogenic marker genes (C). Quantitative data are presented as mean ± SD of triplicates. * $p < 0.05$ compared with the non-treatment control; # $p < 0.05$ for the indicated pairs. OSX, osterix; OCN, osteocalcin.

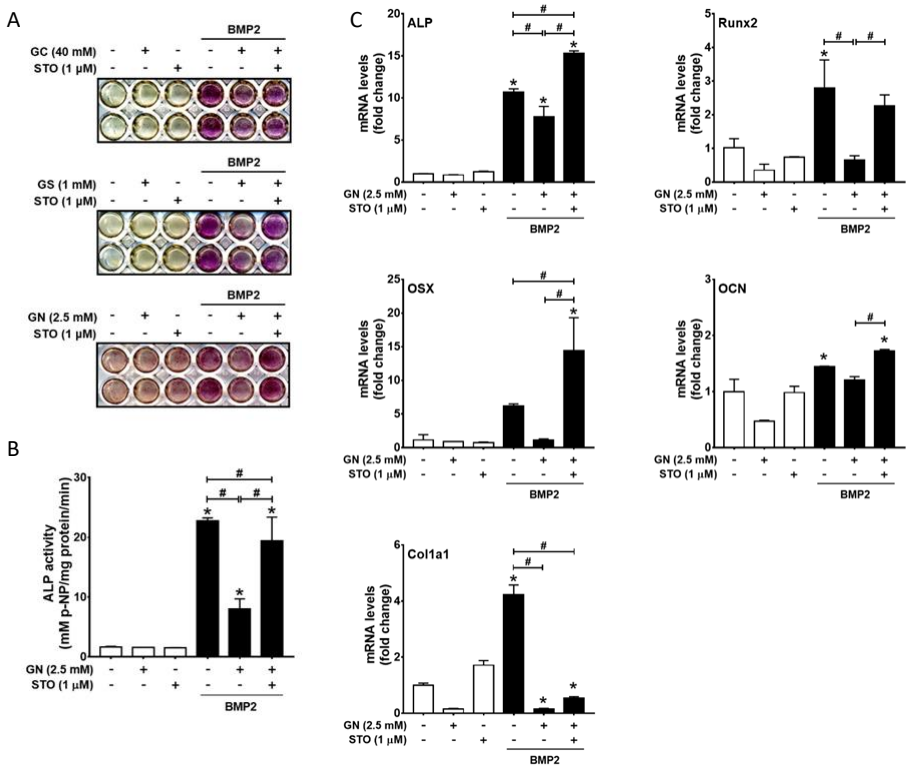

Fig. 2. OGT activity inhibition by STO45849 (STO), an OGT inhibitor, rescued BMP-induced ALP activity and osteogenic marker gene under excessive O-GlcNAcylayion-inducing conditions.

C2C12 cells were incubated for 48 h in the presence or absence of the indicated reagents, and osteogenic differentiation was examined by ALP staining (A); ALP activity assays (B); and qRT-PCR analysis of osteoblast differentiation marker genes (C). * $p < 0.05$ compared with the non-treatment control; # $p < 0.05$ for the indicated pairs.

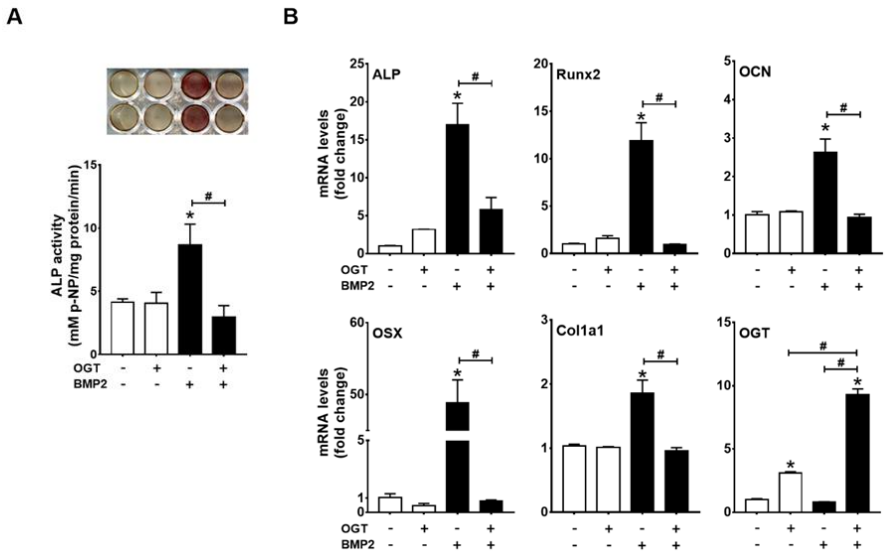

Fig. 3. OGT overexpression inhibited osteogenic differentiation of C2C12 cells.

C2C12 cells were transfected with OGT expression plasmids and incubated in the presence or absence of BMP2 for 48 h. Subsequently, ALP staining and ALP activity assays (A) as well as qRT-PCR analysis (B) were performed to examine osteogenic differentiation. * $p < 0.05$ compared with the non-treatment control; # $p < 0.05$ for the indicated pairs.

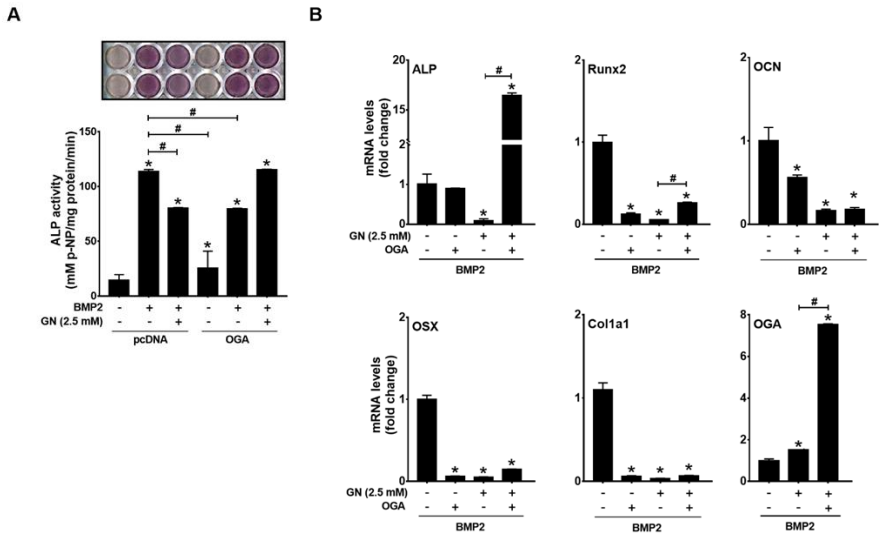

Fig. 4. OGA overexpression rescued BMP2-induced osteogenic differentiation under excessive O-GlcNAcylation conditions.

C2C12 cells were transfected with OGA expression plasmids and osteogenic differentiation was induced by BMP2 in the presence or absence of N-acetylglucosamine (GN). Subsequently, ALP staining and ALP activity assays (A) or qRT-PCR analysis (B) were performed. * $p < 0.05$ compared with the non-treatment control; # $p < 0.05$ for the indicated pairs.

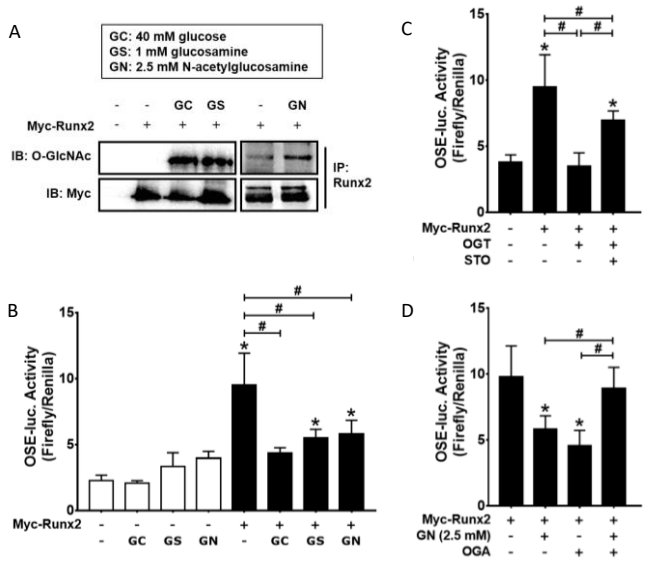

Fig. 5. Excessive O-GlcNAcylation inducers suppressed Runx2 transcriptional activity, which was restored by OGT inhibitor (STO) addition or OGA overexpression

(A) C2C12 cells were transiently transfected with Myc-Runx2 expression plasmids and incubated under the indicated conditions for 24 h. Subsequently, immunoprecipitation with Runx2 antibody was performed, followed by immunoblotting with O-GlcNAc or Myc antibodies. High glucose (GC), glucosamine (GS), and N-acetylglucosamine (GN) enhanced Runx2 O-GlcNAcylation; (B–D) C2C12 cells were transiently transfected with the indicated expression plasmids and OSE-luc (a Runx2 reporter plasmid) and incubated for 24 h in the presence or absence of O-GlcNAcylation inducers and STO. Luciferase activity was then measured. * $p < 0.05$ compared with the non-treatment control; # $p < 0.05$ for the indicated pairs.

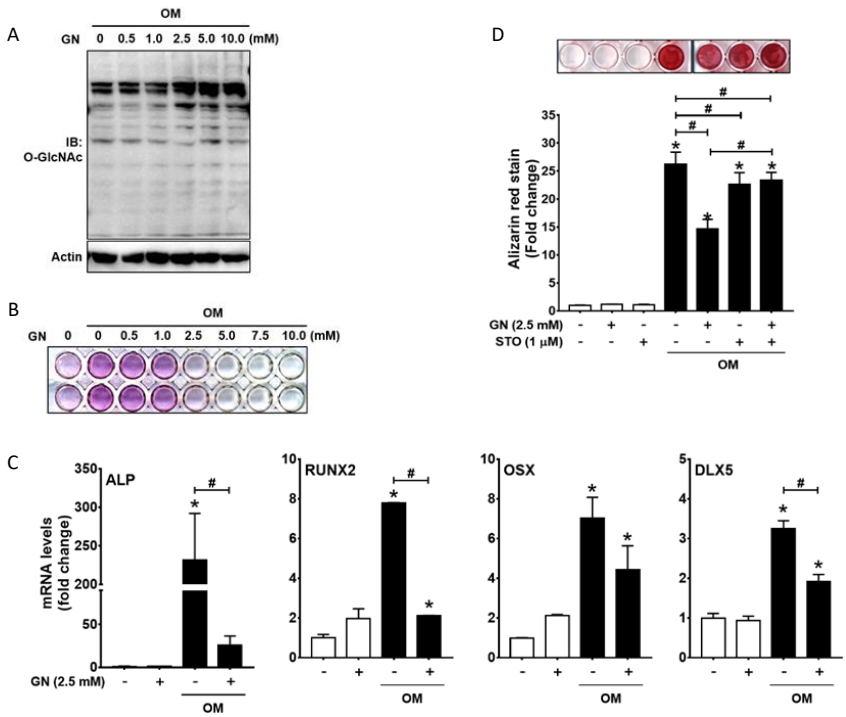

Fig. 6. N-acetylglucosamine inhibits osteogenic differentiation and matrix mineralization in human periodontal ligament (hPDL) cells.

(A) hPDL cells were incubated for 6 h in the presence of N-acetylglucosamine (GN) at the indicated concentrations, and Western blot analysis was performed to examine the levels of protein O-GlcNAcylation; (B,C) Cells were incubated for seven days and osteogenic differentiation was examined by ALP staining (B) and qRT-PCR of osteogenic marker genes (C); (D) hPDL cells were incubated for 21 days in the presence or absence of the indicated reagents, and Alizarin red S staining and quantification were performed. * $p < 0.05$ compared with the non-treatment control; # $p < 0.05$ for the indicated pairs. OM, osteogenic medium.

Discussion

DM is a common metabolic disorder characterized by hyperglycemia, insulin resistance, and relative insulin deficiency [3]. DM is a complex syndrome with more than one cause and is responsible for numerous complications that affect the entire body. Increasing evidence has demonstrated that DM can cause bone metabolism disorder, leading to decreased bone mineral density and defective bone healing [7-9]. Under hyperglycemic conditions, the unbalanced coupling of bone resorption and formation in remodeling processes promotes bone loss [12].

Previously, it was reported that hyperglycemia increases sclerostin expression by enhancing reactive oxygen species and tumor necrosis factor alpha production in osteoblasts and osteocytes [13], suggesting that increased sclerostin expression contributes to DM-associated osteopenia and accelerated alveolar bone loss in diabetic patients with periodontitis. It has also been demonstrated that high glucose concentrations inhibit osteogenic differentiation of bone marrow mesenchymal stem cells by attenuating the effect of BMP2 [10]. Hyperglycemia is considered a potential contributor to diabetic osteoporosis [9], but the mechanism underlying hyperglycemia-induced bone loss is still unclear.

Previous reports have demonstrated that global protein O-GlcNAcylation increases during the early stages of osteoblast differentiation and correlates with increased Runx2 transcriptional activity and increased osteogenic marker gene expression levels [45-47]. Similarly, in this study it was also observed that BMP2 treatment moderately increased overall O-GlcNAcylated protein levels, which was

clear even after incubation with BMP2 for 30 min. In addition, the inhibition of BMP2-induced protein O-GlcNAcylation by OGA overexpression significantly suppressed BMP2-induced ALP activity and Runx2 transcriptional activity. These results further support the notion that a moderate increase in protein O-GlcNAcylation is required for the progression of osteogenic differentiation.

However, when O-GlcNAcylation was further increased in the presence of BMP2 by treating cells with high glucose, glucosamine, or N-acetylglucosamine or by OGT overexpression, BMP2-induced ALP activity and osteoblast differentiation marker gene expression were clearly suppressed. Furthermore, N-acetylglucosamine treatment attenuated the expression levels of osteogenic differentiation marker genes and matrix mineralization of hPDL cells. These results are consistent with the decreased bone formation phenotype observed in type 2 DM patients. They are, however, in contrast to the results of previous studies, which showed that the upregulation of O-GlcNAcylation by the addition of OGA inhibitors, such as PUGNAc or thiamet G, further increases osteogenic stimuli-induced Runx2 transcriptional activity and matrix mineralization [45, 46]. The reason for this discrepancy is still unclear. Considering the suggestion that global O-GlcNAcylation levels should be maintained within an optimal zone to preserve normal cellular function [36], excessive global O-GlcNAcylation may disrupt the progression of normal osteogenic differentiation in C2C12 cells and hPDL cells.

Runx2 is a transcription factor critical for the progression of osteoblast differentiation. Runx2 is post-translationally modified downstream of a diverse set

of signaling pathways, whose coordinated action controls osteoblast differentiation and bone development [48]. Similar to previous studies [45, 47], it was also confirmed that Runx2 is O-GlcNAcylated and that its O-GlcNAcylation levels are increased by O-GlcNAcylation inducers. However, in contrast to the findings of previous studies, Runx2 transcriptional activity in this study was significantly suppressed by O-GlcNAcylation inducers. Reportedly, NF-κB O-GlcNAcylation exerts stimulatory or inhibitory effects on target gene transcription, depending on the cellular context and/or the type of O-GlcNAcylation-inducing conditions [70-72]. One possible explanation for these discrepancies is that the effects of metabolic treatments (for example, the addition of high glucose, glucosamine, or N-acetylglucosamine) and pharmacological treatments (for example, OGA inhibitors) are not the same: OGA inhibitors increase O-GlcNAcylation levels by breaking the dynamic on/off cycle, whereas metabolic treatments or OGT overexpression elevates O-GlcNAcylation levels by shifting the equilibrium toward modification [43]. Furthermore, other studies have demonstrated that the concentrations of UDP-GlcNAc, the donor for O-GlcNAcylation, may affect OGT substrate selectivity [73, 74], suggesting that the increase in UDP-GlcNAc concentration induced by metabolic treatments may differentially regulate modification sites in Runx2 or the type of modified proteins. Furthermore, it is unclear whether the inhibitory effect of O-GlcNAcylation inducers on Runx2 transcriptional activity results directly from Runx2 O-GlcNAcylation. These are important issues that should be clarified in future studies.

In summary, in this study it was demonstrated that during BMP2-induced osteogenic differentiation, exposing C2C12 cells to excessive O-GlcNAcylation-inducing conditions, including high glucose, glucosamine, N-acetylglucosamine or OGT overexpression, led to an excessive increase in protein O-GlcNAcylation, which subsequently attenuated BMP2-induced osteogenic differentiation. Similarly, N-acetylglucosamine attenuated ALP activity and matrix mineralization in hPDL cells. These results suggest that investigating the role of excessive protein O-GlcNAcylation in bone cells under hyperglycemic conditions is necessary to explain pathological bone loss in diabetic patients in future studies. In this context, the consequences of *in vivo* chronic hyperglycemia on protein O-GlcNAcylation in osteoblasts and the role of excessive O-GlcNAcylation in the development of diabetic bone phenotype should also be evaluated in the near future using osteoblast-specific knockout of OGT and OGA in a mouse model.

IV. PART II

Introduction

DM is one of the most common chronic diseases in nearly all countries, and continues to increase in numbers and significance, as economic development and urbanization lead to changing lifestyles. In 2011 there are 366 million people with diabetes, and this is expected to rise to 552 million by 2030 [1, 2]. Type 2 diabetes mellitus (T2DM) has become an increasing pandemic metabolic disease in the past decade [75]. The disease is associated epidemiologically to impaired bone regeneration, increased risk of bone fracture and various disorders of the skeletal system [76]. DM has also been linked to periodontitis, which is a common chronic inflammatory disease characterized by alveolar bone resorption and supporting tissue destruction around the teeth [15]. Poorer glycemic control leads to both an increased risk for alveolar bone loss and more severe progression over those without T2DM [16, 17].

O-GlcNAcylation, the addition of O-GlcNAc moiety to Ser or Thr, is a sensor of the cell metabolic state. Protein O-GlcNAcylation is reversibly catalyzed by OGT and OGA. Increased O-GlcNAcylation is directly linked to insulin resistance and to hyperglycemia-induced glucose toxicity, two hallmarks of diabetes and diabetic complications [35]. The biology of O-GlcNAc modification is still not well understood and cell-permeable inhibitors of OGT are needed both as research tools and for validating OGT as a therapeutic target.

Wnts are secreted, cysteine-rich glycoproteins involved in controlling cell proliferarion, cell-fate specification, gene expression, and cell survival. Wnts trigger several signaling cascades. Wnt signaling pathways have been the focus of intense research activity in bone biology laboratories because of their importance in skeletal development, bone mass maintenance, and therapeutic potential for regenerative medicine [52]. The canonical, Wnt/β-catenin signaling pathway is one of the critical pathways that regulate cell differentiation, skeletal system development and osteogenesis [77]. Several studies demonstrated that the accumulation of β-catenin activated the Wnt signaling pathway, which enhanced bone formation [49-51]. Previous study has demonstrated that high glucose can target different components of the canonical Wnt pathway to induce β-catenin destabilization in mouse osteoblastic cells [55].

In part I, it was demonstrated that excessive O-GlcNAcylation inhibits BMP2-induced osteogenic differentiation of C2C12 cells via inhibiting transcriptional activity of Runx2. In part II, it has been investigated whether excessive O-GlcNAcylation affects Wnt/β-catenin signaling under osteogenic conditions *in vitro* and OGT inhibitors or Wnt/β-catenin signaling enhancer rescue delayed calvarial defect healing in T2DM mice model.

Results

Excessive O-GlcNAcylation inhibited Osteogenic Differentiation and Matrix Mineralization of hPDL Cells

To confirm whether high dose glucose (GC) and N-acetylglucosamine (GN) exert cytotoxicity in hPDL cells, cell viability assays were performed after an incubation for 48 h, and no significant cytotoxicity was observed in doses used in this study (Fig. 7A). Next, the progression of osteoblast differentiation was assessed after 48 h of incubation under osteogenic conditions. ALP staining demonstrated that minimum concentrations to clearly suppress ALP activity are 80 mM glucose and 2.5 mM N-acetylglucosamine (Fig. 7B). Thus, subsequent experiments were performed using 80 mM glucose or 2.5 mM N-acetylglucosamine. To further examine the effect of high glucose and N-acetylglucosamine on matrix mineralization, hPDL cells were incubated for 21 days, followed by Alizarin Red S staining. High glucose and N-acetylglucosamine significantly suppressed osteogenic stimuli-induced matrix mineralization (Fig. 7C). In addition, qRT-PCR of osteoblast marker genes demonstrated that the expression levels of RUNX2, ALP, osterix, and osteocalcin (OC) genes were increased by osteogenic stimuli, but were significantly suppressed by high glucose and N-acetylglucosamine (Fig. 7D).

High glucose- and N-acetylglucosamine-induced suppression of matrix mineralization and ALP activity was rescued by the addition of ST045849, an OGT inhibitor (Fig. 8). These results indicate that conditions inducing abnormally high

levels of protein O-GlcNAcylation inhibit osteogenic differentiation of hPDL cells, which may be rescued by OGT inhibitors.

Excessive O-GlcNAcylation decreased the levels of β-catenin in hPDL Cells

To investigate whether excessive O-GlcNAcylation inducers inhibit osteogenic differentiation via suppression of Wnt/β-catenin signaling, expression levels of β-catenin protein were examined in hPDL cells under osteogenic conditions. With the initiation of osteogenic differentiation, hPDL cells were exposed to varying concentrations of glucose or N-acetylglucosamine for 6 h. Interestingly, global protein O-GlcNAcylation levels showed inverse correlation with the levels of β-catenin; higher the protein O-GlcNAcylation levels, lower the β-catenin levels (Fig. 9A, 9B). Furthermore, addition of ST045849 into osteogenic medium highly increased β-catenin protein levels in hPDL cells (Fig. 9C). N-acetylglucosamine increased O-GlcNAcylation of β-catenin but decreased β-catenin protein levels, which were suppressed by ST045849 (Fig. 9D). These results indicate that excessive O-GlcNAcylation inducers reduce β-catenin protein levels under osteogenic conditions in an OGT activity-dependent manner.

Excessive O-GlcNAcylation-Inducing Conditions Suppressed Wnt3a-Induced ALP Activity and Osteogenic Marker Gene Expression in ST2 Cells

Osteogenic differentiation of ST2 bone marrow stromal cells has been shown to be efficiently induced by Wnt3a [59]; Wnt3a is more effective than BMP2 in stimulating osteogenic differentiation of ST2 cells, whereas BMP2 is more effective than Wnt3a in C2C12 and MC3T3-E1 cells. Therefore, in this study, Wnt3a-ST2 model system was employed to examine the effect of excessive O-GlcNAcylation on Wnt3a/β-catenin signaling and consequent osteogenic differentiation. *In vitro* cell culture experiments were mostly performed using Wnt3a-condiitoned medium as described in Experimental Techniques. With the initiation of osteogenic differentiation, ST2 cells were exposed to varying N-acetylglucosamine (GN, 1, 2.5, 5.0 or 10.0 mM) concentrations in the presence of Wnt3a for 3 days. ALP staining demonstrated that Wnt3a-induced ALP activity was suppressed by N-acetylglucosamine at 2.5 mM and higher concentrations, which was partially rescued by OGT inhibitor STO45849 or Wnt3a/β-catenin signaling enhancing Noni extract (Fig. 10A, 10B). qRT-PCR of osteoblast marker genes demonstrated that the expression levels of ALP, Runx2, Bsp and β-catenin genes were increased by Wnt3a, which were significantly suppressed by N-acetylglucosamine (Fig. 10C). Similarly, the addition of OGT inhibitors or Noni extract partially rescued Wnt3a-induced mRNA expression levels of ALP, Runx2, Bsp and β-catenin. These results indicated that N-acetylglucosamine downregulates

Wnt3a-induced ALP activity and osteogenic marker gene expression, and OGT activity is required for the inhibitory effect of O-GlcNAcylation inducers on osteogenic differentiation in ST2 cells.

N-acetylglucosamine Suppressed Transcriptional Activity of β-catenin/Tcf/Lef and Destabilized β-catenin Protein

Because N-acetylglucosamine suppressed Wnt3a-induced osteogenic differentiation, it was next examined whether N-acetylglucosamine inhibits transcriptional activity of β-catenin/Tcf/Lef complexes using TOP-Flash/FOP-Flash reporter systems in HEK293 cells (Fig. 11A). Luciferase activity showed the N-acetylglucosamine suppressed Wnt3a-induced TOP-flash activity, which was rescued by the addition of OGT inhibitors, STO45849 and OSMI-1, or Noni extract. Moreover, N-acetylglucosamine increased O-GlcNAcylation of β-catenin but decreased the protein levels of β-catenin in ST2 cells (Fig. 11B). Increase in O-GlcNAcylation of β-catenin by N-acetylglucosamine was reversed by OGT inhibitors, but not by Noni extract (Fig. 11B). However, decrease in β-catenin protein levels by N-acetylglucosamine was reversed by both OGT inhibitors and Noni extract (Fig. 11B).

To further verify the role of N-acetylglucosamine in β-catenin stabilization, ST2 cells were pre-treated with cyclohexamide (CHX, 10 μM), and then incubated in the presence or absence of the indicated reagents for indicated time (Fig. 12A). Western blot analysis was performed to examine β-catenin protein levels. The levels of β-catenin were decreased by N-acetylglucosamine treatment, and the reduction was alleviated by addition of OGT inhibitors or Noni extract (Fig. 12A).

In order to verify whether reduced levels of β-catenin by N-acetylglucosamine is related to enhanced ubiquitination and subsequent proteasomal

degradation, the levels of ubiquitin-bound β-catenin was compared in the presence and absence of N-acetylglucosamine and OGT inhibitors. ST2 cells were transiently transfected with HA-ubiquitin plasmids and then pre-treated with proteosome inhibitor, MG132 (10 μM) and incubated in the indicated reagents for 24 h (Fig. 12B). N-acetylglucosamine increased ubiquitin-bound β-catenin and decreased β-catenin protein levels. were decreased by, while was increased. Both OGT inhibitors and Noni extract recovered the levels of ubiquitin-bound β-catenin and total β-catenin protein levels even in the presence of N-acetylglucosamine (Fig. 12B). These results indicated that N-acetylglucosamine destabilized β-catenin protein by increasing β-catenin ubiquitination in the presence of Wnt3a, thus contribute to attenuate osteogenic differentiation partly via inhibiting Wnt/β-catenin signaling.

High Fat Diet Delayed Bone Repair but OGT Inhibitor or Noni Extract Improved Bone Healing.

Mice given HFD for four-week and a single injection of low dose STZ exhibited increase in body weight, compared to control mice (Fig. 13B). Glucose tolerance tests results demonstrated that compared to control mice, HFD mice showed higher blood glucose levels were maintained before and after an injection of glucose (1 g/kg i.p.), suggesting the development of T2DM (Fig. 13C).

After the confirmation of T2DM development in HFD mice, 2 mm circular bone defects were created in the parietal bone. The calvarial defects were filled with PBS, OSMI-1, ST045849, or noni extract loaded collagen sponge. Directly after the surgery, *in viv*o microCT images were scanned and follow-up *in vivo* CT scans were made after 2, 5 and 7 weeks to monitor bone structure (Fig. 14A). Compared to normal diet (ND) mice, HFD mice exhibited delayed bone healing and reduced newly formed bone volume and surface although the difference was not statistically significant (Fig. 14A, 14C, 14D). OSMI-1-treated mice showed significantly increased new bone formation, compared to HFD mice (Fig. 14A, 14C, 14D). Moreover, Noni extract-treated mice also showed increased new bone volume and new bone surface (Fig. 14A, 14C, 14D). To further demonstrate the bone healing response in ND and HFD mice, hematoxylin and eosin staining was performed (Fig. 14B). OSMI-1-treated and Noni-treated mice showed narrower fibrous defect tissue than that in HFD mice.

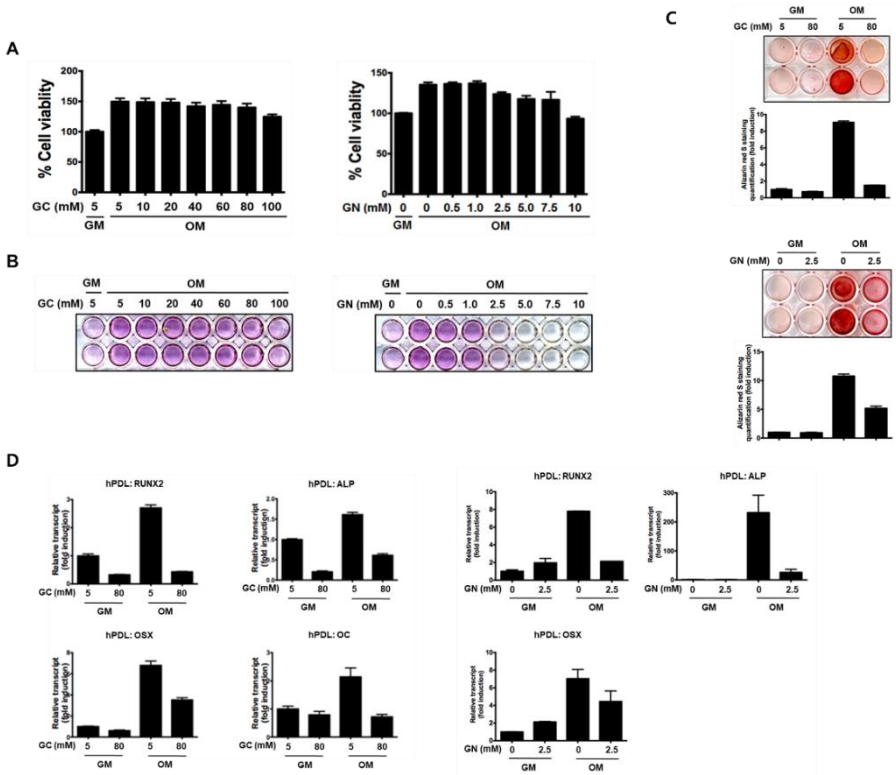

Fig. 7. Glucose and N-acetylglucosamine in high concentration inhibited osteogenic differentiation and matrix mineralization of hPDL cells.

(A) hPDL cells were incubated with glucose (GC) or N-acetylglucosamine (GN) at the indicated concentrations in the presence or absence of the osteogenic medium (OM) and cell viability assay was performed. (B) ALP staining was performed after an incubation for 7 days under the indicated conditions. (C) Alizarin Red S staining was performed after an incubation for 21 days. (D) Expression levels of osteogenic marker genes (RUNX2, ALP, OSX, and OC) were evaluated by qRT-PCR.

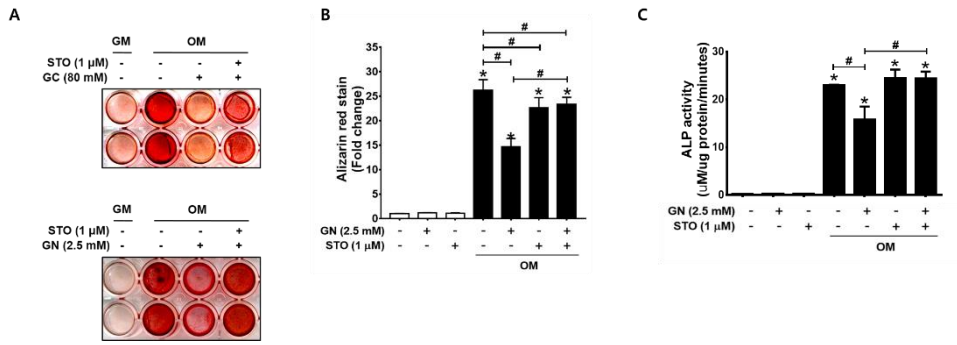

Fig. 8. OGT inhibitor, ST045849 (STO), rescued osteogenic differentiation and matrix mineralization of hPDL fibroblasts in the presence of high glucose or N-acetylglucosamine.

(A, B) hPDL cells were incubated for 21 days in the presence or absence of the indicated reagents, and Alizarin Red S staining and quantification was performed. (C) Cells were incubated for seven days and osteogenic differentiation was examined by ALP assays. * $p < 0.05$ compared with the cells grown in GM without any treatment; # $p < 0.05$ for the indicated pairs.

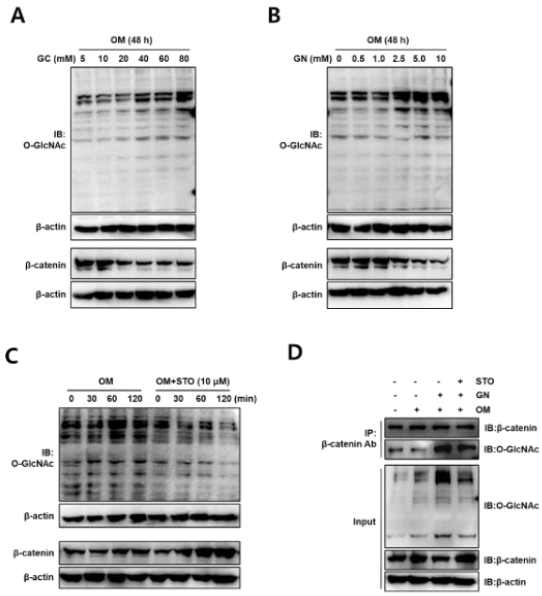

Fig. 9. High dose glucose and N-acetylglucosamine decreased the levels of β-catenin which were rescued by an OGT inhibitor in hPDL cells.

Cells were incubated in the presence or absence of the indicated reagents for the indicated periods, and Western blot analysis (A-C) or immunoprecipitation with β-catenin antibody (D) was performed to examine the levels of β-catenin and O-GlcNAcylation.

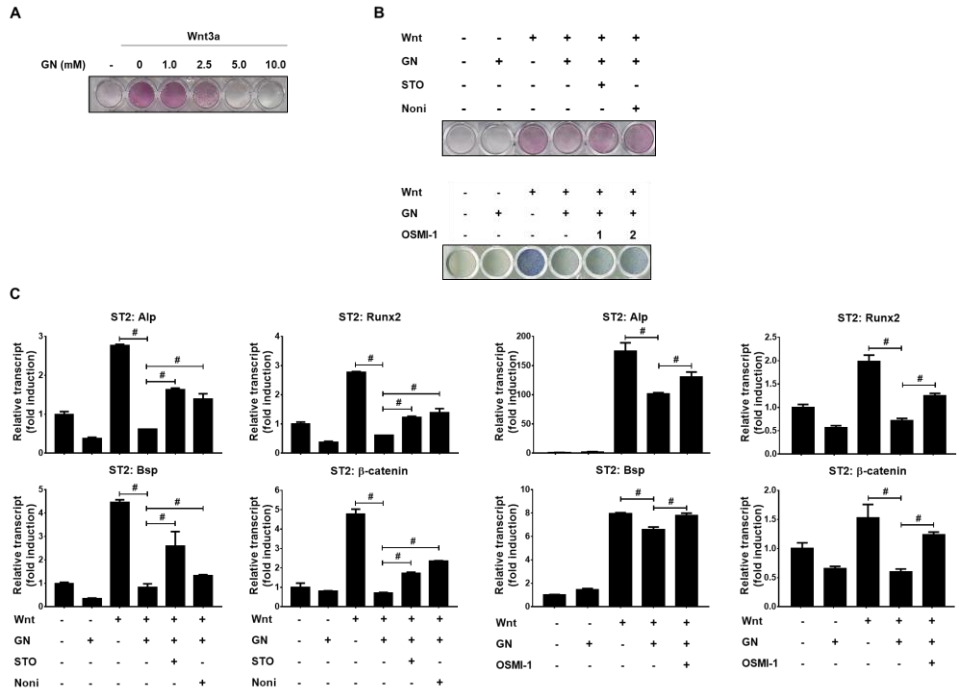

Fig. 10. OGT inhibitors and Noni extracts rescued N-acetylglucosamine-downregulated Wnt3a-induced alkaline phosphatase activity and osteogenic marker gene expression in ST2 cells.

(A, B) ST2 cells were incubated for 3 days in the presence or absence of the indicated reagents, and osteogenic differentiation was examined by ALP staining. (C) ST2 cells were incubated for 48 h in the presence or absence of the indicated reagents, and qRT-PCR analysis was performed to examine osteogenic marker gene expression. # $p < 0.05$ for the indicated pairs.

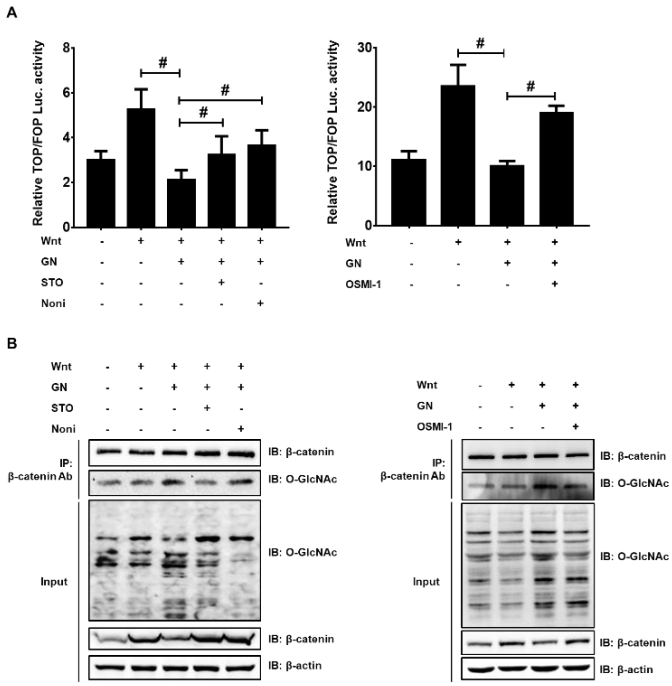

Fig. 11. N-acetylglucosamine suppressed Wnt3a-induced TOP-flash activity and the protein levels of β-catenin, which were rescued by OGT inhibitors and Noni extract.

(A) TOP-Flash/FOP-Flash reporter assays were performed in HEK293 cells after an incubation with the indicated reagents for 24 h. # $p < 0.05$ for the indicated pairs. (B) ST2 cells were incubated for 24 h in the presence or absence of the indicated reagents, and then immunoprecipitation with β-catenin antibody was performed, followed by immunoblotting with β-catenin antibody or O-GlcNAc antibody.

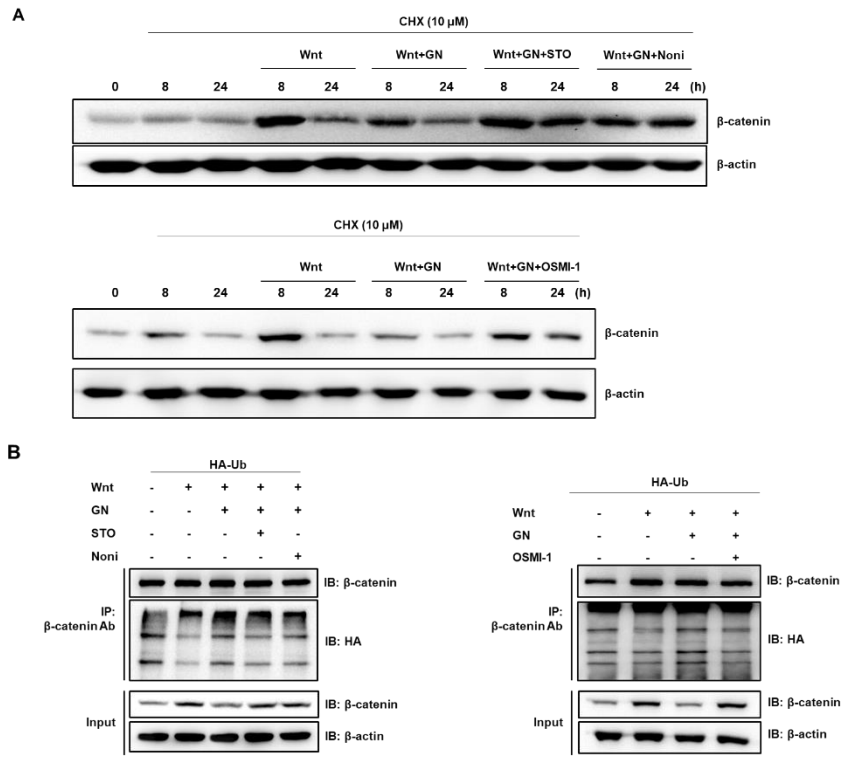

Fig. 12. N-acetylglucosamine destabilized β-catenin protein by increasing β-catenin ubiquitination in the presence of Wnt3a.

(A) ST2 cells were pre-treated with cycloheximide (CHX), and then incubated in the presence or absence of the indicated reagents for the indicated time. Western blot analysis was performed to examine the levels of protein β-catenin. (B) Cells were transfected with plasmids encoding HA-tagged ubiquitin and incubated for 24 h in the presence of the indicated reagents and proteasome inhibitor MG132 (10 μM), followed by immunoprecipitation and immunoblotting.

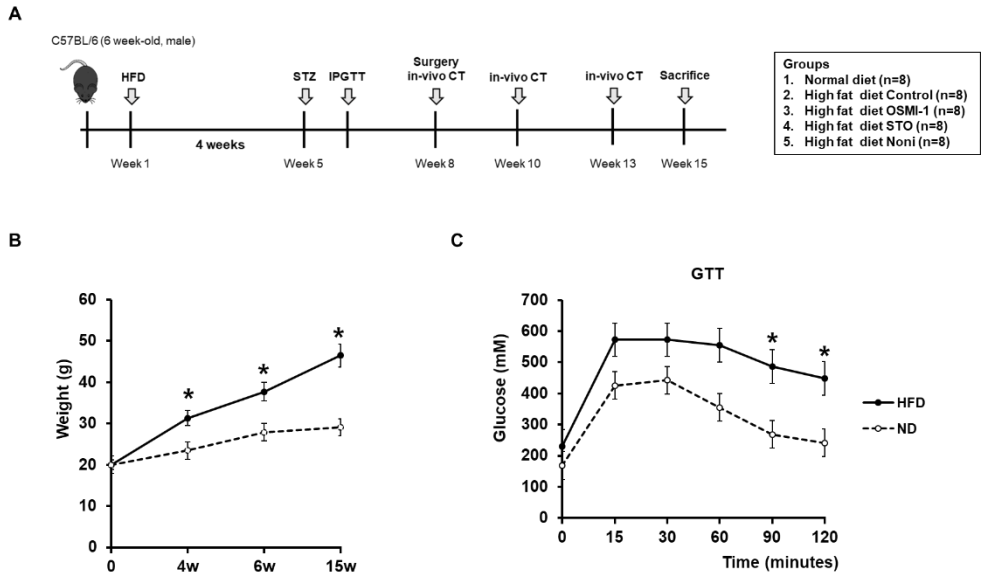

Fig. 13. HFD/STZ type 2 diabetic mice model.

(A) Scheme of animal experiment. (B) Body weights were measured at 4, 6, and 15 weeks. (C) Glucose tolerance tests were performed after STZ injections. Data are means ± SD for n=6 to 8 independent groups. * $p < 0.05$ compared with the ND, Normal diet group.

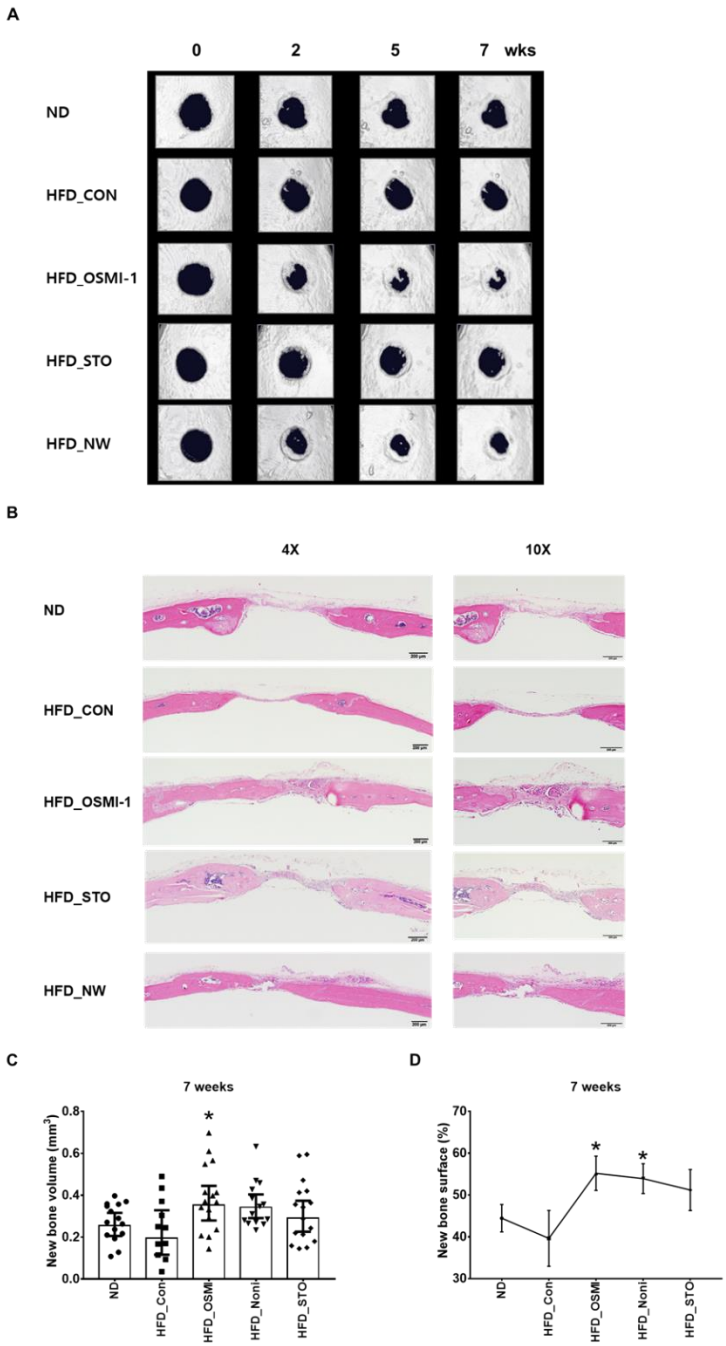

Fig. 14. High fat diet delayed bone repair but OGT inhibitor or Noni extract improved bone healing.

(A) Representative images of *in vivo* microCT obtained at postoperative weeks 0, 2, 5, and 7. (B) Hematoxylin and eosin staining was performed at 7-weeks post-surgery. Slides were examined by light microscopy. (C) New bone volume and new bone surface were analyzed using CTan from microCT data of 7-week post-surgery. Data are means ± SEM for n=6 to 8 mice per group. * $p < 0.05$ compared with the HFD_Con, High fat diet Control group.

Discussion

Hyperglycemia drives excessive chronic O-GlcNAcylation of proteins in diabetic patients, can affect various cellular functions, including the modulation of osteogenic differentiation. T2DM contributes to impaired bone regeneration, increases the risk of bone fracture and various disorders of the skeletal system [76]. Moreover, enhanced formation of advanced glycation end products in the bone matrix contributes to reduced bone strength and increased fracture risk in T2DM [5]. In part I, it has observed that BMP2 treatment moderately increased overall O-GlcNAcylated protein levels and that OGA overexpression significantly suppressed BMP2-induced ALP activity and Runx2 transcriptional activity in C2C12 cells. However, when O-GlcNAcylation was further increased in the presence of BMP2 by treating cells with high glucose, glucosamine, or N-acetylglucosamine or by OGT overexpression, BMP2-induced ALP activity and osteoblast differentiation marker gene expression were clearly suppressed. Similar to these results, N-acetylglucosamine treatment attenuated the expression levels of osteogenic differentiation marker genes and matrix mineralization of hPDL cells. These results seem to be consistent with the decreased bone formation phenotype observed in T2DM patients.

Wnt/β-catenin signaling pathway is one of the key regulators of cellular differentiation, skeletal system development and osteogenesis. The accumulation of β-catenin is induced by canonical Wnt ligands, which enhanced bone formation [49-52, 77]. A previous study has demonstrated that high glucose inhibits Wnt/β-catenin

pathway mainly via inducing β-catenin destabilization in mouse osteoblastic cells and that high glucose-induced β-catenin destabilization seems to be partly dependent on p38 MAPK at downstream of GSK-3β [55]. However, the mechanism of p38 MAPK-induced β-catenin destabilization remains unclear. Similar to the results from this report, glucose of high concentrations clearly decreased β-catenin protein levels in hPDL cells. Furthermore, N-acetylglucosamine also decreased β-catenin protein levels, while OGT inhibitor STO045849 increased β-catenin protein levels, suggesting that there was an inverse correlation between β-catenin protein levels and general protein O-GlcNAcylation levels. Although β-catenin itself is O-GlcNAcylated, it is not clear whether β-catenin O-GlcNAcylation is required for destabilization of β-catenin under osteogenic conditions. Previously, opposite results have been reported that upregulation of O-GlcNAc increased β-catenin protein expression levels, protein stability, and nuclear accumulation in the liver cancer cells [78] (Gao et al., 2019) and that O-GlcNAcylation stabilizes β-catenin via direct competition with phosphorylation at Thr41 in cancer tissues [30] (Olivier-Van Stichelen et al., 2014). The reason for the discrepancies in the effect of O-GlcNAcylation inducing conditions on the protein stability and levels of β-catenin is not clear. However, from these controversies, it is suggested that the effect of enhanced O-GlcNAcylation conditions on β-catenin protein in osteoblasts seem to be different from that in cancer cells and that β-catenin destabilizing effect of O-GlcNAcylation conditions in osteoblasts does not seem to be the results of direct O-GlcNAcylation of β-catenin.

Although the regulatory mechanisms how O-GlcNAcylation conditions destabilize β-catenin remain unclear, N-acetylglucosamine significantly downregulated Wnt3a-induced alkaline phosphatase activity and osteogenic marker gene expression, which were rescued by addition of OGT inhibitors. These results indicate that excessive protein O-GlcNAcylation conditions attenuate osteogenic differentiation partly via inhibiting Wnt/β-catenin signaling.

To evaluate the effect of OGT inhibitor on bone regeneration in diabetic bone tissue *in vivo*, combination of HFD and low dose STZ injection was employed to induce T2DM in mice. The researchers attempted to combine HFD feeding with subsequent injection of low dose STZ to set up a stable and reliable T2DM mice model; young adult mice fed a HFD elicit diet-induced obesity and insulin resistance and then single or multiple injections with low-dose STZ (30-40 mg/kg i.p.) elicit partial loss of β cells, which results in hypoisulinaemia and hyperglycemia [79-82]. The results of the studies using this T2DM animal model indicated that the changed levels of blood glucose, total cholesterol, triglyceride, insulin sensitivity and glucose tolerance mimicked the natural history and metabolic characteristics of T2DM in humans [83, 84].

In this study, bone regeneration was examined in the calvarial defects. The size of defect is also a relevant parameter to determine bone wound healing speed besides T2DM. By classical definition, a critical size defect is the smallest size tissue defect that will not completely heal over the natural life time of animals [85, 86]. It has been reported that defects in mice that were smaller than critical-size defects

(1.8-mm diameter) were shown to heal a maximum of 50 percent 1 year postoperatively [87] and that none of 1.8 mm diameter defects healed completely during 28 days of observation [88-90]. Moreover, defects of 2 mm and larger that have been implanted with apatite-coated PLGA without cells did not heal during the 12-week time period, nor did defects of corresponding sizes without implanted scaffolds [91]. These reports indicate that 2 mm defects are critical-size or nonhealing defects. Therefore, 2 mm defect was prepared in calvarial bones of mice in this study.

In vivo results were consistent with the delayed bone regeneration observed in T2DM mice. Furthermore, OSMI-1, ST045849 and Noni extract treated mice showed more newly formed bone compare to PBS treated mice at 2-week post-surgery (data not shown). Although statistically significant difference in newly formed bone volume was only observed in OSMI-1-treated mice at 7-week post-surgery, Noni-treated mice also showed higher levels of new bone formation.

V. CONCLUSION

In the present study, it has demonstrated that during BMP2-induced osteogenic differentiation, exposing C2C12 cells to excessive O-GlcNAcylation-inducing conditions, including high glucose, glucosamine, N-acetylglucosamine or OGT overexpression, led to an excessive increase in protein O-GlcNAcylation, which subsequently attenuated BMP2-induced osteogenic differentiation. Similarly, N-acetylglucosamine attenuated ALP activity and matrix mineralization in hPDL cells. Also, excessive O-GlcNAcylation decreased the levels of β-catenin which were rescued by OGT inhibitor in hPDL cells. In addition, N-acetylglucosamine downregulated Wnt3a-induced ALP activity and osteogenic marker gene expression in ST2 cells and destabilized β-catenin protein by increasing β-catenin ubiquitination. These inhibitory effects of N-acetylglucosamine on Wnt3a/β-catenin signaling were rescued by addition of OGT inhibitors or Noni extract. In animal study, OGT inhibitors and Noni extract treatment showed an effective alternative bone regeneration on T2DM mice. These results suggest that excessive O-GlcNAcylation of proteins contribute to delayed bone healing in T2DM and that OGT inhibitors or Wnt/β-catenin signal enhancers such as Noni extract may have a value as a pharmacologic candidate to enhance bone wound healing in diabetic patients.

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국문 초록

오글루넥당화 조절이 골모세포 분화에 미치는 영향

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구 한 나

당뇨병은 대표적인 대사성 만성질환으로 고혈당으로 인한 여러 증상들이 나타나며 여러 합병증을 유발하기도 한다. 고혈당 상태는 여러 세포 기능에 영향을 주게 되고 골모세포 분화에도 영향을 준다고 알려져 있으며, 당뇨환자 조직세포에서 오글루넥당화 수준이 증가하는 것으로 알려져 있다. 본 연구에서는 과도하게 증가된 오글루넥당화 수준이 골모세포 분화에 미치는 영향을 알아보고자 하였다. 생쥐 근육모 세포주(C2C12 cells)에 Bone morphogenetic protein 2 (BMP2)를 처리하여 골모세포 분화를 유도하였을 때, 고혈당 유사 배양 조건에서 오글루넥당화가 증가하였고, 골모세포 분화 마커(Runx2, Osterix,

alkaline phosphatase)의 발현이 감소하였다. 여기에 OGT 저해제(ST045849)를 추가하면 골모세포 분화 억제효과가 부분적으로 상쇄되었다. 또한 O-GlcNAc transferase (OGT) 과발현을 유도하여 오글루넥을 증가시키는 조건에서도 골모세포 분화가 억제되고, β-N-acetylglucosaminidase (OGA) 과발현을 유도하면 고혈당 유사 배양 조건에 의한 골모세포 분화 억제 효과가 부분적으로 상쇄됨을 관찰하였다. 사람치주인대세포(hPDL cells)에 오글루넥당화 조건을 유도하면 골모세포 분화가 저해되고, 이는 OGT 저해제에 의해 상쇄되었다. 고혈당 유사 배양 조건은 β-catenin 단백질 양을 감소시키며, OGT 저해제는 β-catenin 단백질 양을 증가시켰다. 과도한 오글루넥당화 조건에서는 β-catenin 전사활성이 저해되었고, OGT 저해제를 추가하면 억제효과가 상쇄되었다. 생쥐 중간엽세포주인 ST2 세포에 Wnt3a로 골모세포 분화를 유도하고 과도한 오글루넥당화 조건에서 배양하면 β-catenin 단백질 수준의 감소, 전사활성 감소, 안정화 감소와 더불어 골모세포 분화 억제가 나타났으며, 이는 오글루넥당화 저해제나 천연추출물 노니 잎 추출물에 의해 회복되는 것을 관찰하였다. 선행 연구에서 노니 잎 추출물은 Wnt/β-catenin 신호를 통해 골모세포 분화를 촉진하는 것을 확인하였다. 오글루넥당화 저해제와 노니 잎 추출물이 제2형 당뇨 동물 모델의 골 재생에 미치는

영향을 확인하기 위해 생쥐의 두개골에 결손부를 형성하고, 콜라겐 스폰지에 약물을 흡수시켜 채운 후 in vivo microCT를 통해 7주 동안 골재생 양상을 확인하였다. 오글루넥당화 저해제인 OSMI-1과 노니 처리 군에서 대조군에 비해 골재생이 증가하였고, 특히 OSMI-1 처리군에서 가장 많은 골 생성양을 관찰할 수 있었다. 이상의 결과는 고혈당으로 인해 유도된 과도한 오글루넥당화가 골모세포 분화를 저해하며 그 과정에서 Runx2 와 β-catenin의 전사활성 감소가 관여하고, 오글루넥당화 저해제나 노니 잎 추출물을 당뇨환자의 국소적 골재생 촉진을 위한 약물후보로 고려해볼 가능성이 있음을 시사하였다.

주요어: 오글루넥당화, 골모세포 분화, Runx2 전사인자, Wnt/β-catenin 신호, 제2형 당뇨

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