이학석사 학위논문

GATA4의 *O*-GlcNAcylation에 의한 골관절염 연골의 노화 분비표현형 조절

Senescence-induced *O*-GlcNAcylation of GATA4 governs senescence-associated secretory phenotype in osteoarthritic cartilage

2019 년 2 월

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2018 년 **12** 월

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신 중 권

신중권의 석사 학위논문을 인준함

2018 년 **12** 월

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Abstract

Senescence-induced *O*-GlcNAcylation of GATA4 governs senescence-associated secretory phenotype in osteoarthritic cartilage

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Osteoarthritis (OA) is one of the most prevalent joint disease that causes the degeneration of articular cartilage. Among various risk factors, age is the most prominent risk factor for development and progression of OA. During OA development, senescence of chondrocytes promotes the secretion of the senescence-associated secretory phenotype (SASP) contributing to matrix catabolism in cartilage. *O*-linked N-acetylglucosamine (*O*-GlcNAc) modification regulates various signaling pathways in response to cellular stresses and affects the pathogenesis of various age-related diseases. Here, I show that GATA4, a key regulator of SASP, is directly

O-GlcNAcylated, and *O*-GlcNAcylation of GATA4 regulates the stability of GATA4 protein. I also found that the expression of SASP factors could be regulated by the modulation of *O*-GlcNAcylation status in senescent chondrocytes. These results indicate that the modulation of *O*-GlcNAcylation status of GATA4 effectively controls the expression of SASP factors in OA cartilage. Thus, I conclude that targeting *O*-GlcNAc status of GATA4 may serve as a potential therapeutic strategy for OA.

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keywords : Osteoarthrits, Senescecnce, *O*-GlcNAcylation, GATA4, Senescence-associated secretory phenotypes

Student Number : 2016-20389

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INTRODUCTION

Osteoarthritis and chondrocyte senescence

Osteoarthritis (OA), the most prevalent joint disease, is characterized by degeneration of articular cartilage. The pathogenesis of OA involves various risk factors such as physical injury, obesity, genetic factors, and abnormal joint alignment (Price et al., 2002). However, increasing age is the most prominent risk factor for development and progression of OA (Loeser et al., 2016). According to the recent studies, cellular responses such as cellular stress or damage response in chondrocytes contribute to cellular senescence leading to age-related OA (Green et al., 2006; Loeser, 2009). The cellular senescence of chondrocytes promotes the secretion of the senescence-associated secretory phenotype (SASP), including growth factors, chemokines, cytokines, and matrix-degrading proteases (Brandl et al., 2011; McCulloch et al., 2017). Of note, recent studies suggest that removal of senescent chondrocytes in OA cartilage attenuates the progression of OA (Jeon et al., 2017; Zhang et al., 2016). The results of these studies suggest that targeting senescent chondrocytes, active producer of SASP in cartilage, might be an effective therapeutic strategy for treatment of OA.

GATA4 is a key regulator of SASP factors

Cellular senescence is an irreversible cell cycle arrest triggered by various cellular stresses that induce the activation of DNA damage

response. Senescence-inducing signals are usually controlled by either p53 or $p16^{INK4A}$/Rb tumor suppressor pathways (Beausejour et al., 2003). Recently, the transcription factor GATA4 has been identified as a core regulator of senescence and SASP secretion (Kang et al., 2015). Under senescence-inducing conditions, the GATA4 pathway is activated. GATA4 protein sstimulates senescence by upregulating the expression of SASP through inducing TRAF3IP2 (tumor necrosis factor receptor - associated factor interacting protein 2) and IL1A (interleukin 1A), and corresponding activation of NF-κB. Upon senescence condition, GATA4 is stabilized by evading of selective autophagy mediated by p62 adaptor protein, and thus is able to regulate the SASP pathway (Kang et al., 2015). However, how GATA4 protein can evade from selective autophagy pathway and stabilize under senescence condition is not fully understood.

O-GlcNAcylation and aging

The post-translational modification (PTM) of proteins allow cells to respond immediately to intrinsic and extrinsic signal through dynamic regulation of protein function. *O*-linked N-acetylglucosamine (*O*-GlcNAc) modification is one of the PTMs that can regulate fundamental cellular processes (Hart et al., 2011). *O*-GlcNAcylation is a non-canonical glycosylation that attachs uridine diphosphate GlcNAc (UDP-GlcNAc) to Ser and Thr residues of proteins. Thousands of proteins including transcription factors, cytoskeletal proteins, cell cycle regulators, phosphatases, and kinases are known

to be *O*-GlcNAcylated. These dynamic *O*-GlcNAcylation can control the cellular pathways in response to cellular stresses (Bond and Hanover, 2013; Slawson and Hart, 2011).

The alteration of protein *O*-GlcNAc modification has been known to have an effect on the pathogenesis in various degenerative and age-related diseases such as diabetes, and neurodegenerative diseases (Chatham et al., 2008; Hart et al., 2011; Zachara, 2012). In addition to these reports, it has been recently documented that *O*-GlcNAc-modified proteins are increased in human OA chondrocytes (Tardio et al., 2014). However, dynamic changes of *O*-GlcNAc state affect OA pathgenesis is not fully understood.

Here, I suggest that the modulation of *O*-GlcNAcylation status of GATA4 can regulate its stability and serve as a potential SASP-targeting therapeutic strategy for OA without removal of senescent cells.

MATERIALS AND METHODS

Experimental OA in mice.

All animal experiments were approved by the Seoul National University Institutional Animal Care and Use Committee (IACUC No. SNU-180109-1). The design, analysis, and reporting of animal experiments followed the ARRIVE guidelines (Animals in Research: Reporting of In Vivo Experiments). Mice were housed in a specific-pathogen-free animal facility at Seoul National University. The animals were kept under constant temperature and humidity and controlled 12 h light-dark cycles. Mice were fed a standard laboratory chow and water ad libitum. Post-traumatic OA was induced by DMM surgery in 12-week - old mice (Glasson et al., 2007); sham-operated mice were used as controls. Mice were sacrificed at 10 weeks after DMM. The extent of cartilage destruction in knee joints was analyzed by safranin O staining and scoring using the OARSI grading system (Glasson et al., 2010; Pritzker et al., 2007).

Collection of human tissue samples.

Human OA cartilage specimens were sourced from 10 female OA patients (67- to 77-year - old) undergoing total knee replacement at SNU Boramae Medical Center. The Institutional Review Board (IRB) of SNU Boramae Medical Center (IRB No. 26-2016-143 and IRB No. 30-2017-48) approved the collection of materials and the IRB of Seoul National University (IRB No. E1612/003-005 and IRB No.

E1803/003-009) approved the use of these materials. Full written informed consent was provided by all participants before the total knee replacement arthroplasty operative procedure.

Cell culture.

For primary culture of mouse articular chondrocytes, cells were isolated from femoral condyles and tibial plateaus of 5-day - old ICR mice as previously described (Gosset et al., 2008). Mouse chondrocytes were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. After 3 days, the cells were treated as indicated in each experiment. Cultured cells were maintained in a humidified 37°C, 5% CO2, and 3% O2 atmosphere. SW1353 cell line was maintained in DMEM supplemented with 10% FBS and 1% antibiotics. For primary culture of human articular chondrocytes, the cells were isolated from femoral condyles and tibial plateaus of human cartilage specimens and cultured as previously described (Goldring et al., 2005). Human articular chondrocytes were maintained in DMEM/F-12 supplemented with 10% FBS, 1% antimycotics, and 1% antibiotics. Cultured cells were maintained in a humidified 37°C, 5% CO2, and 3% O2 atmosphere. Human and mouse chondrocytes were treated with hyaluronidase type I-S (4 units/ml) for 4 h prior to transfection under serum-free conditions. Transfection was performed using METAFECTENE® PRO (Biontex) according to the manufacturer's instructions. For siRNA transfection in mouse chondrocytes or SW1353 cells, 50 nM of siRNA was transfected into the cells.

siRNAs used in the study were purchased from Bioneer and are listed in Supplemetary Table 1. For cell line authentication, SW1353 cell line has been tested for short-tandem repeat profiling by the Korean Cell Line Bank.

Histology and immunohistochemistry.

Human OA cartilage specimens were cryo-embedded in OCT compound and sectioned (5 μm thickness). OA-affected cartilages were acquired from the medial side of the tibial plateau, while relatively undamaged cartilage regions from the lateral side of the tibial plateau were used as controls. Cryosections were air-dried for 20 min and fixed in pre-chilled acetone for 10 min prior to histological analysis. Human cartilage sections were stained with Alcian blue staining for OARSI grading. Knee joint tissues collected from DMM-induced OA and aged mice were fixed with 4% paraformaldehyde (PFA), decalcified in 0.5 M EDTA (pH 7.4), and processed by dehydration in increasing concentrations of ethanol and incubation in xylene. After sample processing, these samples were embedded in paraffin and sectioned (5 μm thickness). For histological staining, the sections were deparaffinized in xylene, hydrated with decreasing concentrations of ethanol, and stained with safranin O, immunohistochemistry, or in situ hybridization. Comprehensive histological assessment of joint tissues was conducted by two orthopedic pathologists at SNU Boramae Medical Center who have extensive experience in evaluating human and mouse OA. Sections were reviewed by two additional pathologists and approximately 90%

complete agreement was achieved for the OARSI grade. The observers were blinded to the treatment of the mice and section images were randomized to prevent observer bias. Cartilage destruction was identified by safranin O staining and was scored using the OARSI grading system (Glasson et al., 2010). Primary antibodies used for immunohistochemistry were as follows: $p16^{INK4a}$ (Proteintech, 10883-1-AP; Abcam, ab54210), MMP3 (Abcam, ab52915), MMP13 (Abcam, ab51072), GATA4 (Santa Cruz, sc-25310), and *O*-GlcNAc [RL2] (Abcam, ab2739).

Immunofluorescence.

Primary cultures of human or mouse chondrocytes were fixed in 50% methanol and 10% acetic acid solution, washed three times with PBS, and blocked with blocking solution (0.1% bovine serum albumin and 10% FBS in PBS) for 1 h. The cells were incubated in primary antibodies against $p16^{INK4a}$ (Proteintech, 10883-1-AP), and γ-H2AX (Santa Cruz, sc-517348). The cells were rinsed three times with PBS and incubated with secondary antibodies as follows: rabbit anti-mouse IgG+IgM (H&L) conjugated with Alexa Fluor 488 (Jackson ImmunoResearch, 315-485-044) and donkey anti-rabbit IgG (H&L) conjugated with Alexa Fluor 488 (Life Technologies, A-21206). The nuclei were stained with DAPI.

Static weight bearing.

Incapacitance measurements were performed using the Incapacitance Meter for mice/rat (IITC Life Science, 600MR). Mice were trained to

walk into and remain in the chamber at least three times before measurement. The adaptation was performed until mice remained still and did not lean on either side of the chamber. Before measurement, each hindlimb of mice was positioned on each recording pad. The weight placed on each recording pad was measured over 1 s for at least three separate measurements. Data were represented as a percentage of the DMM-operated ipsilateral limb versus non-surgical contralateral limb. The observers were blinded to the treatment or procedure of the mice.

RT-PCR and quantitative RT-PCR (qRT-PCR).

Total RNA was extracted using the TRI reagent (Molecular Research Center, Inc.) and reverse-transcribed using EasyScript Reverse Transcriptase (TransGen Biotech). cDNA was amplified by RT-PCR or qRT-PCR. The PCR primers are listed in Supplementary Table 2. To quantitatively analyze mRNA transcript levels, qRT-PCR was performed using SYBR TOPreal qPCR 2× premix (Enzynomics) on StepOnePlus Real-Time PCR System (Applied Biosystems). For qRT-PCR-based quantification data comparing the effect of various treatments, I normalized measurements from treatment groups to those from control groups for each biologically independent trial. For cell-based experiments, each set of primary cultured chondrocytes was considered a biologically independent trial. I then averaged normalized values from multiple biologically independent trials.

Plasmids.

For reporter gene assay of GATA promoter, promoter regions of consensus sequence of GATA protein family were extracted from genomic DNA of mouse chondrocytes and cloned into pGL3-basic via XhoI and HindIII sites. pInducer20 hGATA4 vector was received from Prof. Chanhee Kang's lab. pCMV sport6 hGATA4 vector was cloned by previously described.

SA-β-Gal staining.

I performed SA-β-Gal staining as previously described method (Debacq-Chainiaux et al., 2009). Briefly, the cells were washed twice with PBS and fixed with 2% PFA and 0.2% glutaraldehyde for 5 min. Fixed cells were washed and incubated at 37°C for 12 to 16 h in SA-β-Gal staining solution. After incubation, the cells were washed with PBS twice and methanol, followed by air drying. Dried cells were imaged using Eclipse Ni-U microscope (Nikon). Total cells and SA-β-Gal - positive cells were counted in three random fields per culture dish.

Immunoblotting.

HEK293T cells were transfected with pInducer20 hGATA4-Flag tag vector using PEI polymer. Human chondrocytes were treated with doxorubicin. Cells were washed twice with PBS and lysed with RIPA buffer containing protease inhibitor (Sigma Aldrich). Next, 50 μg of cell lysates were fractionated by 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare). The membranes

were blocked with 3% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and incubated with primary antibodies at 4°C for overnight. After removing unbound antibodies by washing three times, the membranes were incubated with secondary antibodies. Immunoreactive protein bands were detected using an ECL substrate (Thermo Scientific) with LuminoGraph II system (ATTO). Antibodies used for immunoblotting were as follows: $p16^{INK4a}$ (Proteintech, 10883-1-AP), HA (Abcam, ab9110), MMP3 (Abcam, ab52915), IL-6 (Santa Cruz, sc-28343, GATA4 (Santa Cruz, sc-25310, Vinculin (Sigma Aldrich, V9131), Actin (Santa Cruz, sc-1615), *O*-GlcNAc [RL2] (Abcam, ab2739), goat anti-rabbit IgG (H&L) conjugated with horseradish peroxidase (HRP) (Jackson ImmunoResearch, 111-035-003), goat anti-mouse IgG+IgM (H&L) conjugated with HRP (Jackson ImmunoResearch, 116-035-044), and donkey anti-goat IgG conjugated with HRP (Santa Cruz, sc-2020).

sWGA pulldown assay.

pInducer20 hGATA4-HA vector was transfected to HEK293T cells for 24 h, and the recombinant GATA4-HA proteins were then collected and purified by anti-HA antibody using previously described. To pull down *O*-GlcNAcylated GATA4, 50 ml sWGA agarose were added to cell lysate and incubated at 4 C for 12 h, and washed five times with NETN lysis buffer. Finally, *O*-GlcNAcylated proteins were eluted by 5 sample buffer containing 250mM Tris-Cl (pH 6.8), 4% bromophenol blue, 2% SDS, 50% glycerol and 10% b-mercaptoethanol at 95 C for 5min for following immunoblotting analysis.

Induction of DNA damage in chondrocytes

Doxorubicin (Adriamycin), a topoisomerase II inhibitor, was purchased from Sigma Aldrich. For doxorubicin-induced DNA damage in mouse chondrocytes, the cells were treated with doxorubicin for 5 days. For IR-induced DNA damage in chondrocytes, the cells were irradiated with gamma rays at a dose rate of 4 Gy/min using GC 3000 Elan irradiator (MDS Nordion). IR was delivered at the indicated doses and cells were analyzed 5 days later or as otherwise indicated.

Genes	Strand	siRNA sequences	Species
siGata4#1	S AS	5′-CGAGGAAAGCGAACUCGAGdTdT-3′ 5′-CUCGAGUUCGCUUUCCUCGdTdT-3′	Mouse
siGata4#2	S AS	5′-GUACAUAGUUCUGAUGAUAdTdT-3′ 5′-UAUCAUCAGAACUAUGUACdTdT-3′	Mouse
siGata4#3	S AS	5′-CUCCAAGACAGCUUUCCAAdTdT-3′ 5′-UUGGAAAGCUGUCUUGGAGdTdT-3′	Mouse
siGATA4 #1	S AS	5′-CAGAUGUUACUGAAUGCUUdTdT-3′ 5′-AAGCAUUCAGUAACAUCUGdTdT-3′	Human
siGATA4 #2	S AS	5′-CACCAAACUCACCUACGAUdTdT-3′ 5′-AUCGUAGGUGAGUUUGGUGdTdT-3′	Human

Supplementary Table S1. List of siRNAs.

Genes	Strand	Primer sequences	Species
MMP3	S AS	5′-CGAGAACGGTGGAACTTTGAC-3′ 5′-GTGATAGAAATCTGTCAGGCTGG-3′	Human
MMP13	S AS	5′-CGTACCCCGATTCAGGTGAT-3′ 5′-TTGAGCAGAAGAGCTGCTACGT-3′	Human
IL6	S AS	5′-AGAGGACAGAGGCGTTTTCA-3′ 5′-CGAATCCACCGACACTCAGGA-3′	Human
GATA4	S AS	5′-CCTCTCCCAGGAACATCAAA-3′ 5′-TGAGGTGCAGATGAGCCATA-3′	Human
HPRT	S AS	5′-AGAGCCAAAGCAGTGAGCG-3′ 5′-GACAGCGAGTGATCTTGCAC-3′	Human
Mmp3	S AS	5′-CACAGCACATGACGGAGGT-3′ 5′-AAGGTTCCCACTGGAGTCTTC-3′	Mouse
Mmp13	S AS	5′-AGGAATGGCAACTGGAGCTT-3′ 5′-TGACGAATGACACCTGTGTTGA-3′	Mouse
Il6	S AS	5′-AGAGACCCAGGATGGCTACTT-3′ 5′-CAGGCATGTACTCCACAGCA-3′	Mouse
Gata4	S AS	5′-CTGGAGACTCTCAGGGTCGAA-3′ 5′-GGCGTTTGGAGTGGTAGAAATCT-3′	Mouse
Hprt	S AS	5′-GATTTGAGGGACAGGGTCGG-3′ 5′-ATGGTTACTGCCTCTGGTGC-3′	Mouse

Supplementary Table S2. List of PCR primers.

Genes	Strand	Primer sequences	Restriction Sites	Species
pCMV sport6 GATA4	S AS	5′-ATATTCTAGAGAGGAGCTTCCATAATGT-3′ 5′-ATATGAATTCATCTCTTCCAATGTCTTT-3′	XbaI EcoRI	Humand

Supplementary Table S3. List of Cloning primers.

RESULTS

Senescence-induced stabilization of GATA4 promotes expression of SASPs in osteoarthritic cartilage

To study whether GATA4 contributes to OA pathogenesis, I first measured the expression level of GATA4 in human OA cartilage. GATA4 protein was highly expressed in OA-affected human cartilage, but was rarely detected in undamaged cartilage. This expression pattern correlated with that of $p16^{INK4A}$, which is known as a biomarker of cellular senescence (Fig. 1A). Together with the expression of GATA4 proteins, the expression of SASP factors, including MMP3, MMP13, and IL-6, was also increased in OA-affected cartilage (Fig. 1B). In a post-traumatic OA mouse model, where the OA phenotype was appeared in the articular cartilage, SASP factors were elevated along with that of $p16^{INK4a}$ (Fig. 2A and B).

To investigate the effect of GATA4 on the expression of SASP in human chondrocytes, I examined the effects of GATA4 during the senescence response of human chondrocytes. Doxorubicin, DNA-damaging chemical agent, caused DNA damage and cellular senescence of chondrocytes (Fig. 3A). Under senescence condition induced by doxorubicin, protein levels of GATA4, $p16^{INK4A}$, and SASP factors were markedly increased (Fig. 3B), and mRNA level of SASP factors but not that of *GATA4* was increased in human chondrocytes.

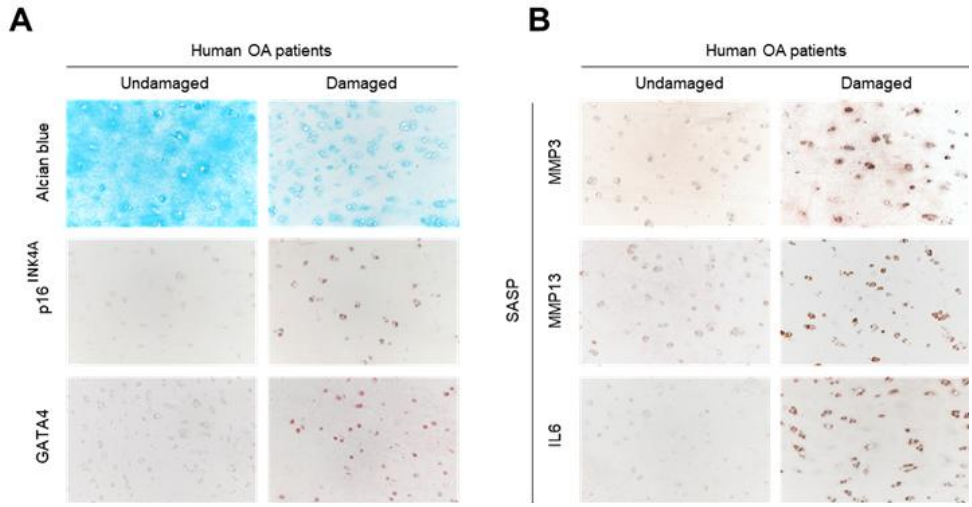

Figure 1. GATA4 is upregulated with the expression of SASPs in human OA cartilage.

(A and B) Cartilage sections from the undamaged or damaged region of human OA cartilage were stained with (A) Alcian blue, immunohistochemistry of $p16^{INK4A}$, GATA4, and (B) SASP factors (MMP3, MMP13, and IL6).

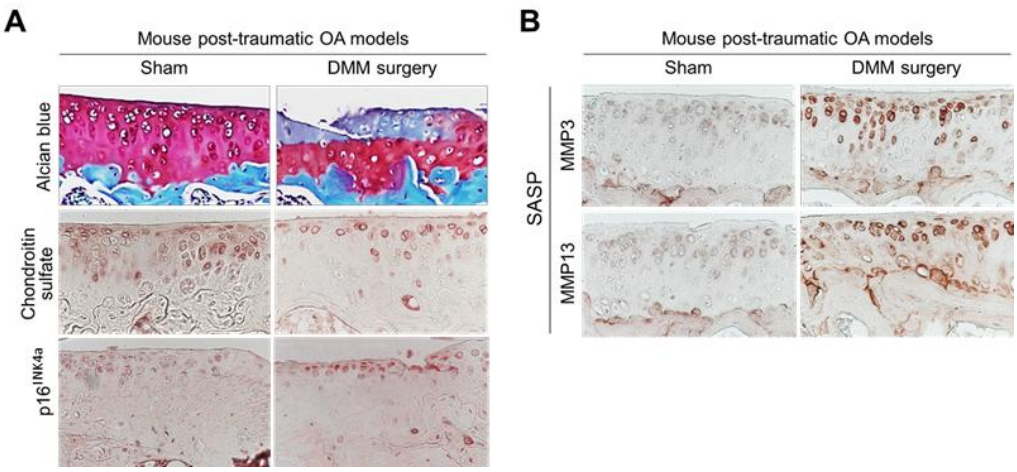

Figure 2. SASP factors were elevated together with cellular senescence in mouse OA cartilage.

(A and B) Cartilage sections from sham- or DMM-operated mouse were stained with (A) safranin O, immunohistochemistry of chondroitin sulfate, $p16^{INK4a}$, and (B) SASP factors (MMP3 and MMP13).

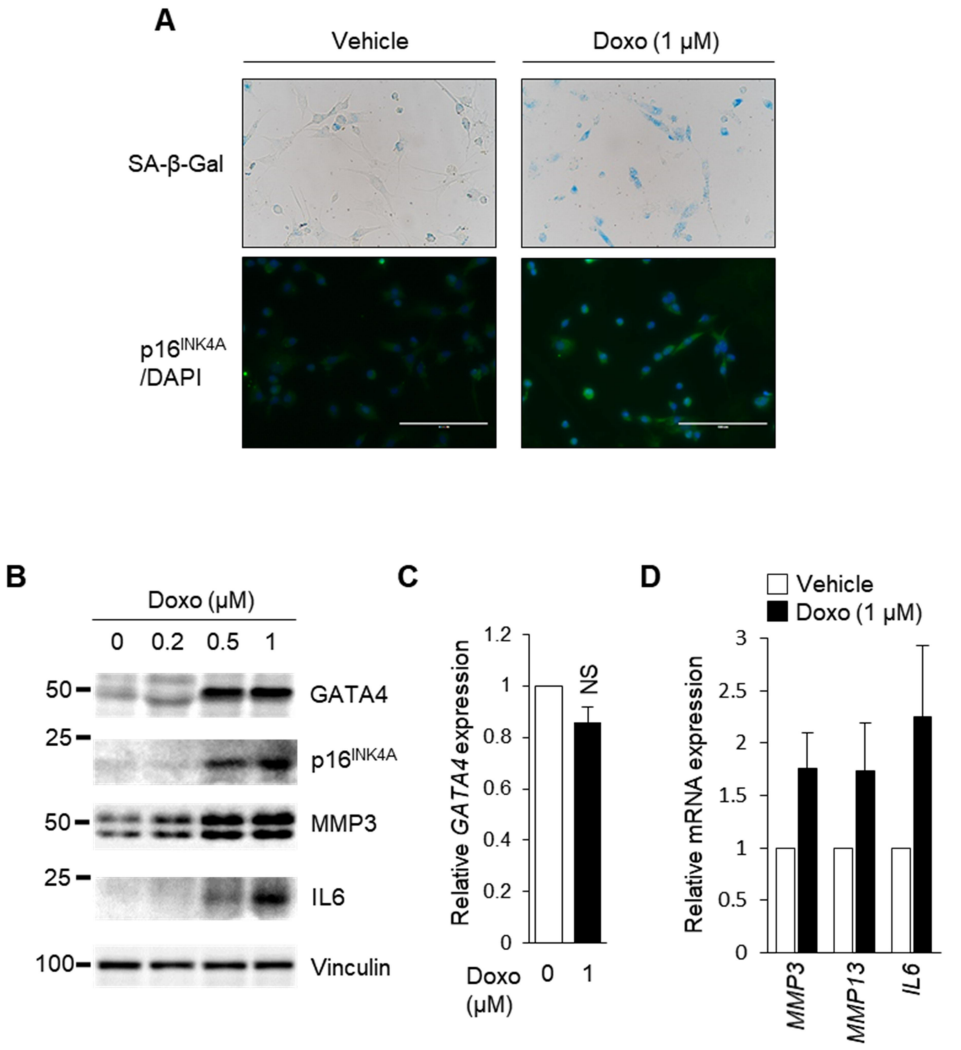
A
Vehicle
Doxo (1 μM)
SA-β-Gal
p16INK4A
/DAPI
B
Doxo (μM)
0 0.2 0.5 1
50
GATA4
25
p16INK4A
50
MMP3
25
IL6
100
Vinculin
C
Relative GATA4 expression
1.2
1
0.8
0.6
0.4
0.2
0
NS
Doxo (μM) 0 1
D
Vehicle
Doxo (1 μM)
Relative mRNA expression
3
2.5
2
1.5
1
0.5
0
MMP3
MMP13
IL6

Figure 3. GATA4 is increased with the onset of SASP expression under senescence in human articular chondrocytes.

(A) Representative images of SA-β-Gal staining (top) and immunofluorescence staining of $p16^{INK4A}$ (bottom) of primary cultured human articular chondrocytes 14 days after doxorubicin treatment (1 μM). Scale bar, 50 μm. **(B)** Western blot analysis of GATA4, $p16^{INK4a}$, SASP factors (MMP3 and IL6), and Vinculin in human chondrocytes treated with 1 μM doxorubicin (Doxo). Protein levels of GATA4 and SASP factors were increased in human chondrocytes treated with 1 μM Doxo. **(C and D)** Relative mRNA expression of (C) *GATA4* and (D) SASP factors (*MMP3, MMP13*, and *IL6*) in human chondrocytes treated with 1 μM Doxo (n = 3). mRNA level of SASP factors but not that of *GATA4* was increased in human chondrocytes treated with 1μM Doxo. Data represent means ± SEM.

These results show that the level of GATA4 correlated with that of SASP factors in chondrocytes, as with previous study on fibroblasts (Kang et al., 2015).

Senecence induces O-GlcNAc modification of GATA4, which in turn causes its stabilization and activation.

The change in the level of *O*-GlcNAc modification has been observed in various degenerative and age-related diseases. This alteration in the degree of protein *O*-GlcNAcylation has been described to affect the pathogenesis of diseases (Chatham et al., 2008; Hart et al., 2011; Zachara, 2012). In addition, it has been recently reported that *O*-GlcNAcylated proteins were accumulated in human OA cartilage (L. Tardio et al., 2014). Therefore, I hypothesized that GATA4 can also be *O*-GlcNAcylated along with increased *O*-GlcNAcylated proteins in OA and thus is stabilized by evading from selective autophagy.

I first experimentally confirmed that global *O*-GlcNAcylated proteins are increased in OA-affected cartilage (Fig. 4A). The treatment of doxorubicin also increased the global level of *O*-GlcNAcylated protein together with GATA4 proteins in human chondrocytes (Fig. 3B and Fig. 4B). Consistent with the results in human chondrocyte, *O*-GlcNAcylated proteins were increased in senescent mouse chondrocytes exposed to oxidative stress under prolonged normoxia (Fig. 4, C and D).

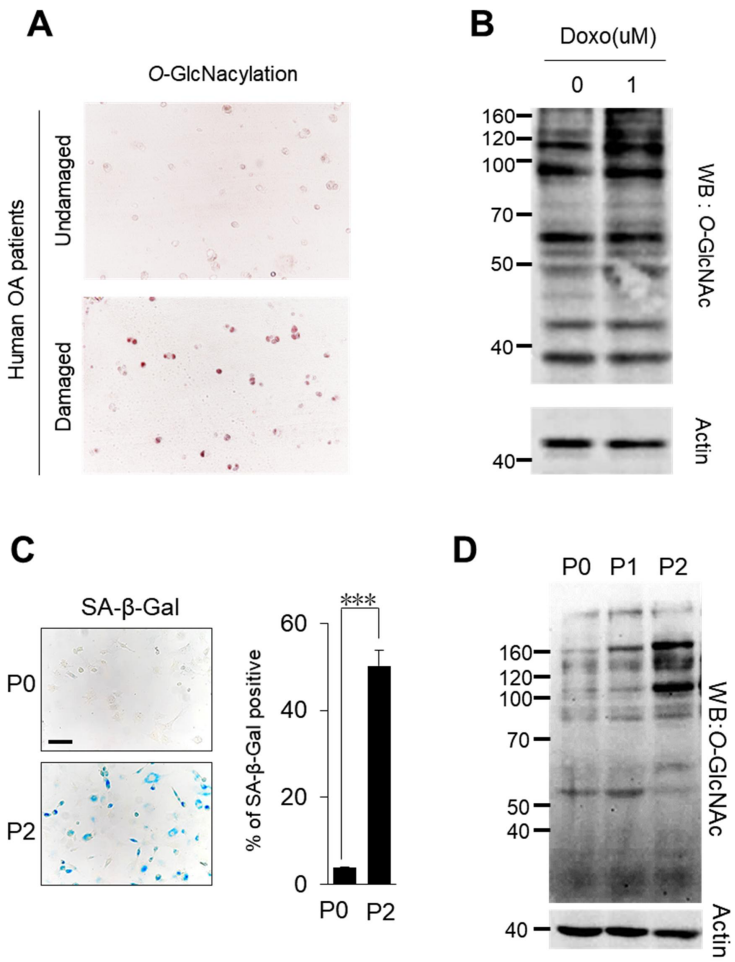
A
O-GlcNacylation
Human OA patients
Undamaged
Damaged
B
Doxo(uM)
0
1
160
120
100
70
50
40
WB : O-GlcNAc
Actin
40
C
SA-β-Gal
P0
P2
% of SA-β-Gal positive
***
60
40
20
0
P0
P2
D
P0
P1
P2
160
120
100
70
50
40
WB:O-GlcNAc
Actin
40

Figure 4. *O*-GlcNAcylated protein is increased in OA-affected cartilage and senescent chondrocytes.

(A) Cartilage sections from the undamaged or damaged region of human OA cartilage were stained with immunohistochemistry of *O*-GlcNAcylation. **(B)** Western blot analysis of *O*-GlcNAcylated proteins and Actin in human chondrocytes treated with 1 μM doxorubicin (Doxo). **(C)** Mouse chondrocytes were exposed to normoxia at passage 0 (P0) and underwent two additional passages under this condition (P2). SA-β-Gal staining and quantification of SA-β-Gal positivity in P0 and P2 chondrocytes (n = 5). Scale bar, 100 μm. **(D)** Western blot analysis of *O*-GlcNAcylated proteins and Actin in P0, P1 and P2 mouse chondrocytes. Data represent means ± SEM. ***$P < 0.001$. Student's t test in (C).

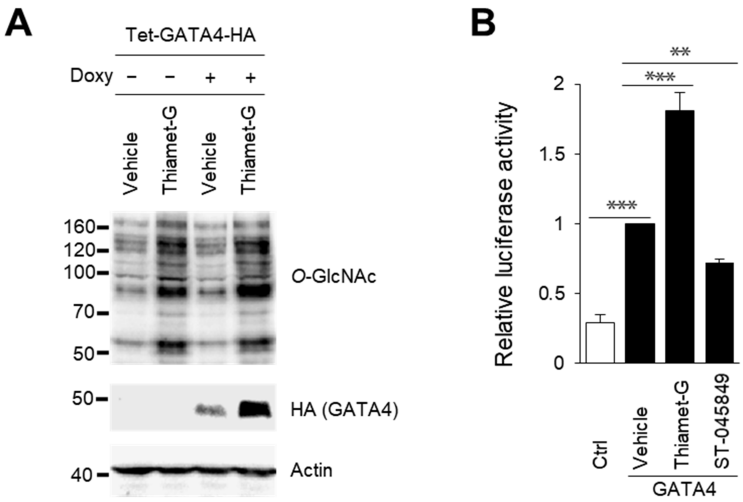

Figure 5. GATA4 levels are affected by *O*-GlcNAcylation status.

(A) Western blot analysis of *O*-GlcNAcylated protein GATA4 and Actin in HEK293T cells overexpressing GATA4-HA and treated with vehicle or 10 μM Thiamet-G. **(B)** Relative luciferase activity of GATA reporter in HEK293T overexpressing GATA4 and treated with vehicle, 10 μM Thiamet-G, or 1 μM ST-045849 (n = 8). Data represent means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. ANOVA followed by post hoc test in (B).

To explore our hypothesis, I tested whether GATA4 proteins can be *O*-GlcNAcylated. Under the treatment of thiamet-G, commonly used as an OGA inhibitor, the increase of *O*-GlcNAc modification state also afftected the GATA4 protein levels (Fig 5A). Additionally, I observed that the modulation of global *O*-GlcNAcylation state using OGA inhibitor and OGT inhibitor could regulate the level of GATA reporter activity (Fig. 5B). These results indicated that GATA4 protein levels could be regulated by its *O*-GlcNAcylation state. To investigate whether GATA4 proteins are *O*-GlcNAcylated, I performed a pull down assay using succinylated wheat germ agglutinin (sWGA), which has high binding affinity for *O*-GlcNAc, in HEK293T cells expressing GATA4-HA tag fusion protein. The result showed that GATA4 proteins were pulled down with sWGA, suggesting that GATA4 may be *O*-GlcNAcylated (Fig. 6A). I further confirmed that GATA4 is directly *O*-GlcNAcylated through immunoprecipitation of GATA4 and *O*-GlcNAc in HEK293T cells expressing GATA4-HA tag fusion protein. GATA4 protein was immunoprecipitated with anti-HA antibody and detected by anti-*O*-GlcNAc antibody, suggesting that GATA4 can be directly modified by *O*-GlcNAc modification (Fig. 6B). Next, to identify *O*-GlcNAcylation site of GATA4, I performed electron-transfer dissociation (ETD) tandem mass spectrometry (MS/MS) analysis. Our MS results showed that Ser 212, and Ser 406 are the candidate *O*-GlcNAcylation site of GATA4 (Fig. 6, C and D).

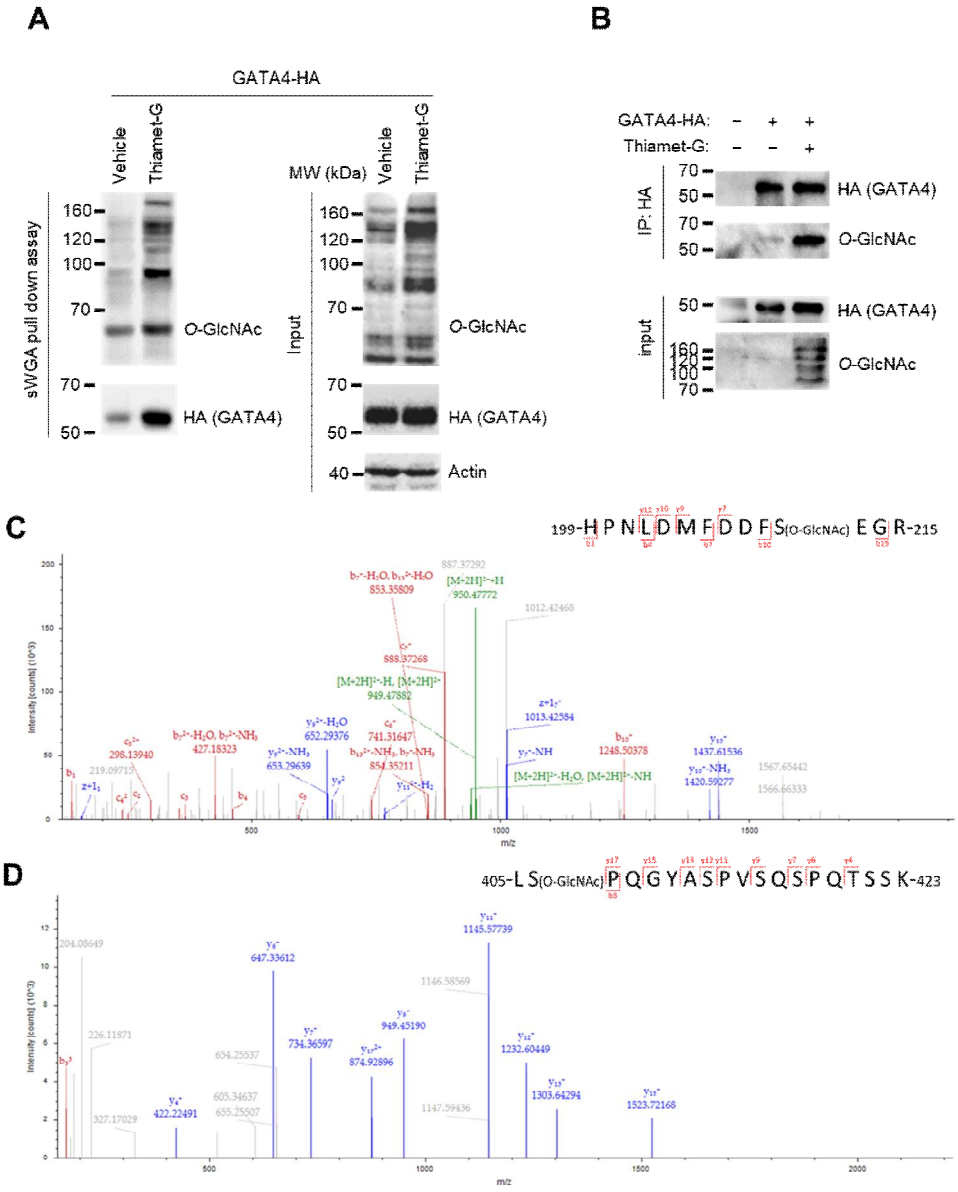
A
GATA4-HA
Vehicle
Thiamet-G
sWGA pull down assay
O-GlcNAc
HA (GATA4)
MW (kDa)
Input
Actin
B
GATA4-HA:
Thiamet-G:
IP: HA
input
C
199-HPNLDMFDDFS(O-GlcNAc)EGR-215
Intensity (counts) (10^3)
m/z
D
405-LS(O-GlcNAc)PQGYASPVSQSPQTSSK-423

Figure 6. GATA4 is *O*-GlcNAcylated at Ser 212, and Ser 406.

(A) sWGA pull down assay of GATA4 in HEK293T overexpressing GATA4 and treated with vehicle or 10μM Thiamet-G. **(B)** GATA4 were immunoprecipitaed with anti-HA antibody in HEK293T cells overexpressing GATA4-HA and treated with vehicle or 10 μM Thiamet-G. GATA4 is detected by anti-*O*-GlcNAc antibody. **(C** and **D)** ETD-MS-based detection of the *O*-GlcNAcylation of GATA4 (Ser 212 (C), and Ser 406 (D)).

Finally, I investigated the effect on OA progression by regulating the stabilization mechanism of GATA4. I found that the mRNA expression of SASP factors including *Mmp3*, *Mmp13*, and *Il6* were increased under treatment of OGA inhibitor in senescent mouse chondrocytes (Fig. 7A). In contrary, the mRNA expression of SASP factors were decreased in senescent mouse chondrocyte under treatment of OGT inhibitor (Fig. 7B). These results suggest that the regulation of GATA4 *O*-GlcNAcylation status could regulate the expression of SASP factors, which are critical mediators of cartilage degeneration.

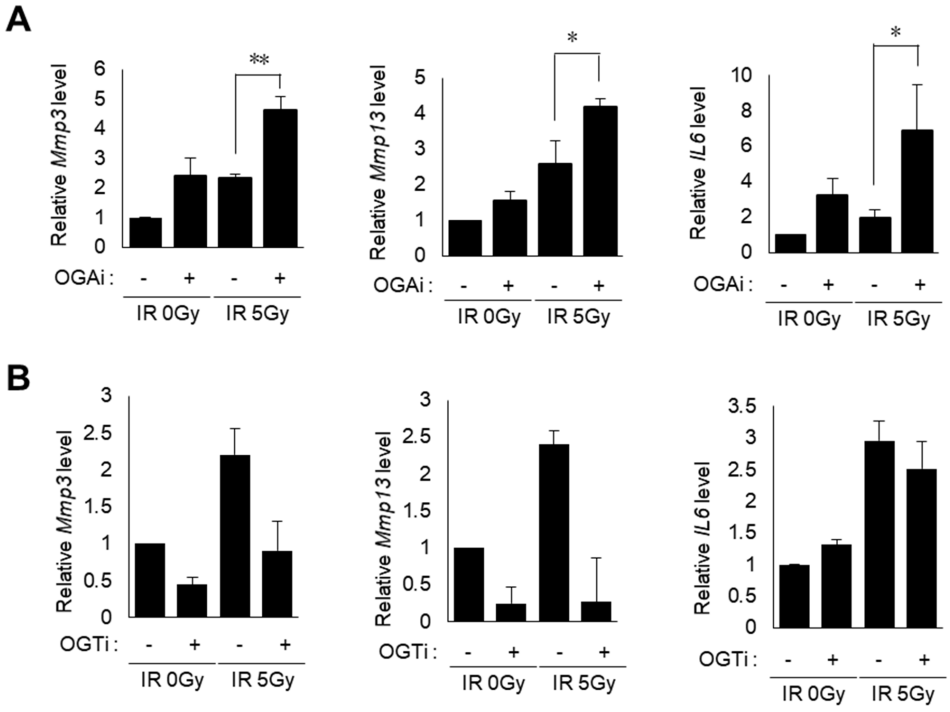

Figure 7. Regulation of O-GlcNAcylation level can control the expression of SASP factors in mouse chondrocytes.

(A) Relative expression of SASP factors (*Mmp3*, *Mmp13*, and *Il6*) in mouse chondrocytes treated with vehicle or 10 μM Thiamet-G after 5 Gy IR radiation (n = 5). **(B)** Relative expression of SASP factors (*Mmp3*, *Mmp13*, and *Il6*) in mouse chondrocytes treated with vehicle or 1 μM ST-045849 after 5 Gy IR irradiation (n = 3). Data represent means ± SEM. $*P < 0.05$, $**P < 0.01$. ANOVA followed by post hoc test in (A).

DISCUSSION

OA is one of the most common age-related disease characterized by the degeneration of articular cartilage. During OA progression, chondrocytes within articular cartilage have many senescence features such as an increase in senescence marker expression and the secretion of senescence-associated inflammatory cytokines (Zhang et al., 2016; Jeon et al., 2017). Chondrocyte senescence is considered to promote OA by inducing the imbalance of cartilage matrix metabolism (Childs et al., 2015; Loeser et al., 2016). Thereby, therapeutic strategies for the removal of senescent cells in variety of age-related diseases have emerged, and this senolytic approaches have also been studied in OA research field (Chang et al., 2016; Jeon et al., 2017). However, the development of therapeutic strategy based on the specific regulation of the production of SASP factors, a key mediators which induce the imbalance of cartilage matrix metabolism under senescence, remains elusive.

GATA4 is a key regulator in senescence regulatory pathway to activate the SASP. Senescence signal-induced GATA4 regulates SASP factors via activation of NF-kB pathway. Previously, GATA4 is known to be stabilized through avoiding selective autophagy (Kang et al., 2015). Our study investigates the mechanism of GATA4 stabilization, and shows that *O*-GlcNAcylation of GATA4 might contribute to evasion of selective autophagy by interfering with the

interaction of GATA4 and p62 proteins. Therefore, modulation of *O*-GlcNAcylation status of GATA4 could effectively control the SASP factors in OA cartilage.

This study suggests a possible therapeutic strategy for OA treatment by controlling SASP expression via modulation of GATA4 stability. Recently, senolytic therapy has emerged as a potential disease-modifying therapy for OA. Previous study of senolytic approach suggested that targeting senescent chondrocytes slows the progression of OA (Jeon et al., 2017). However, there are several critical challenges to be resolved. One of the important challenges of senolytic therapy is to minimize off-target effects on non-senescent cells while specifically removing senescent cells (Childs et al., 2015; Demaria et al., 2014). Our approach targeting SASP pathway may be used as an alternative therapeutic strategy without the removal of senescent cell and off-target effects of senolysis.

In further studies to modulate *O*-GlcNAcylation status of GATA4, it is necessary to confirm which of amino acids can be directly modified by O-GlcNAcylation among S212, and S406 amino acids of GATA4, through mutagenesis-based study involving immunoprecipitation assay. The identification of the *O*-GlcNAcylation site of GATA4 would enable specific control of GATA4 *O*-GlcNAcylation which affects its stability and may serve as more effective therapeutic approach for controlling SASP pathway.

In conclusion, this study reveals that *O*-GlcNAc modification of GATA4 might regulate the stability of GATA4, and the modulation of its modificstion status can control the SASP factors in OA cartilage (Fig. 8). Therefore, targeting *O*-GlcNAc status of GATA4 may serve as a potential therapeutic strategy for OA without removal of senescent cells by regulating SASP factors.

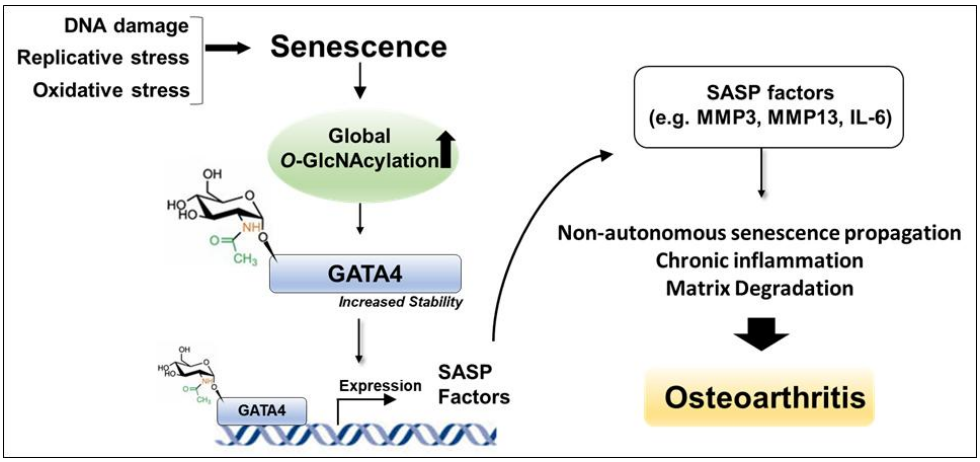

Figure 8. Schematic representation of senescence-induced signaling pathway mediated by *O*-GlcNAcylation of GATA4 in OA development.

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국문초록

GATA4의 *O*-GlcNAcylation에 의한 골관절염 연골의 노화 분비표현형 조절

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퇴행성 관절염은 가장 흔한 관절 질병 중 하나로 관절 연골의 퇴행을 유발한다. 다양한 위험인자들 중에서, 퇴행성 관절염의 발병과 진행에 있어 가장 중요한 위험인자는 노화이다. 퇴행성 관절염이 진행되는 동안 연골세포의 노화는 연골 조직 내 기질 대사의 불균형을 초래할 수 있는 노화 연관 분비표현형 (SASP)의 분비를 촉진한다. 전사 후 변형 중의 하나인 *O*-GlcNAc 수식화는 다양한 세포내 자극에 반응하여 세포 내 신호전달 기전을 조절한다. 이러한 *O*-GlcNAc 수식화는 다양한 노인성 질병에서 병인기전에 영향을 미친다. 이 연구에서 나는 SASP를 조절하는 핵심인자인 GATA4가 *O*-GlcNAc 수식화가 됨을 확인하였고, GATA4의 *O*-GlcNAc 수식화는 GATA4 단백질의 안정성을 조절할 수 있음을 보여주었다. 또한, 노화연골세포 내에서 전반적인 *O*-GlcNAc 수식화 수준을 조절하였을 때, SASP 인자들의 발현이 조절되는 것을 확인하였다.

이는 GATA4의 *O*-GlcNAc 수식화 상태의 조절을 통해 퇴행성 관절 연골에서 SASP 인자들의 발현을 효과적으로 조절할 수 있다는 것을 간접적으로 보여준다. 따라서, 나는 GATA4의 *O*-GlcNAc 수식화 조절을 목표로 하는 관절염 치료 전략이 노화세포의 제거 없이 SASP 인자를 조절하여 퇴행성 관절염을 완화시키는 치료 전략으로서의 가능성을 제시한다.

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주요어 : 퇴행성관절염, 세포노화, 세포노화 연관 분비표현형, *O*-GlcNAc 수식화, GATA4

학 번 : 2016-20389