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Inhibition of protein phosphatase 2A by okadaic acid induces translocation of nucleocytoplasmic *O*-GlcNAc transferase

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ABSTRACT

Post-translational modification (PTM) is crucial for many biological events, such as the modulation of bone metabolism. Phosphorylation and O-GlcNAcylation are two examples of PTMs that can occur at the same site in the protein: serine and threonine residues. This phenomenon may cause crosstalk and possible interactions between the molecules involved. Protein phosphatase 2 A (PP2A) is widely expressed throughout the body and plays a major role in dephosphorylation. At the same location where PP2A acts, O-GlcNAc transferase (OGT) can introduce uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) molecules and mediates O-GlcNAc modifications. To examine the effects of PP2A inhibition on OGT localization and expression, osteoblastic MC3T3-E1 cells were treated with Okadaic Acid (OA), a potent PP2A inhibitor. In the control cells, OGT was strictly localized in the nucleus. However, OGT was observed diffusely in the cytoplasm of the OA-treated cells. This change in localization from the nucleus to the cytoplasm resulted from an increase in mitochondrial OGT expression and translocation of the nucleocytoplasmic isoform. Furthermore, knockdown of PP2A catalytic subunit α isoform (PP2A Cα) significantly affected OGT expression ($p < 0.05$), and there was a correlation between PP2A Cα and OGT expression ($r = 0.93$). These results suggested a possible interaction between PP2A and OGT, which strengthens the notion of an interaction between phosphorylation and O-GlcNAcylation.

1. Introduction

Post-translational modification (PTM) is a change in a protein that occurs after translation and can affect protein activity, function, or interactions with another protein [1,2]. PTM is important for the regulation of various biological events, including bone metabolism [3], disease progression [4,5], and organogenesis, such as tooth development [6].

Among many PTMs, phosphorylation and *O*-GlcNAcylation are unique because they can occupy the same amino acids in target proteins and provide them with quite different outcome [7]. For example, *O*-GlcNAcylation of β-catenin at Thr41 is known to translocate β-catenin to the nucleus and stimulates osteoblast differentiation [4,8]. In contrast, phosphorylation of β-catenin at Thr41 induces β-catenin degradation [9].

O-GlcNAcylation cannot be performed at the same site that has been phosphorylated by kinases and vice versa. Therefore, for *O*-GlcNAcylation to occur, removal of phosphate group by protein phosphatases at that site is necessary. Based on these observations, there is thought to be a close correlation between dephosphorylation and *O*-GlcNAcylation.This small but important switching mechanism has enormous potential in regulating biological mechanisms.

PP2A is a ubiquitously expressed phosphatase involved in the dephosphorylation of various proteins in mammalian cells. PP2A comprises catalytic, structural, and regulatory subunits. The catalytic subunit (also known as the PP2A C subunit) is encoded by the PPP2CA gene and exists as two isoforms: alpha and beta [10,11]. The alpha isoform of the C subunit (PP2A Cα) is the most common isoform found in most tissues [12].

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OGT is the only protein responsible for *O*-GlcNAcylation by introducing UDP-GlcNAc into target proteins [13]. OGT is encoded by the OGT gene and exists as multiple isoforms that are produced through alternative splicing of the OGT gene. The three OGT isoforms are nucleocytoplasmic OGT (ncOGT), mitochondrial OGT (mOGT), and short OGT (sOGT). ncOGT and sOGT are mainly localized in the nucleus and mOGT in the mitochondria [13–15].

Given the necessity of dephosphorylation before *O*-GlcNAcylation by OGT, PP2A activity is thought to be involved in the function of OGT as well as its target proteins. However, there is no information regarding the relationship between PP2A and OGT. Therefore, in this study, we examined the effect of PP2A inhibition on the localization and expression of OGT.

2. Materials and methods

2.1. Cell culture

Pre-osteoblastic cell line MC3T3-E1 was purchased from Riken BRC Cell Bank (Tsukuba, Japan) and cultured at 37 °C incubator with 5% CO_2. Cells were maintained in Minimum Essential Medium α (MEMα) (Gibco/Life Technologies Corporation, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (Gibco/Life Technologies Limited, Paisley, Scotland, UK), and 1% penicillin-streptomycin mixed solution (Nacalai Tesque, Kyoto, Japan). For immunostaining experiment, cells were cultured in 6 cm dish (Thermo Fisher Scientific, Rochester, NY, USA) with coverslips (Matsunami, Kishiwada, Osaka, Japan).

2.2. OA treatment

OA (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) was prepared according to the manufacturer's protocol as a stock solution of 100 μM. The stock solution was then diluted to the final working concentration of 5, 10, and 20 nM using the medium used to maintain the cell as mentioned above and then used to incubate the cells for 1 h.

2.3. Immunofluorescence microscopy

Cells were rinsed with phosphate-buffered saline (PBS) (Takara, Kusatsu, Shiga, Japan) and then fixed with 4% paraformaldehyde-PBS (Nacalai Tesque Inc., Kyoto, Japan) for 15 min. Permeabilization was executed using 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 2 min on the ice followed by blocking using 5% bovine serum albumin (Nacalai Tesque). Incubation overnight with primary antibody was conducted at 4 °C. On the next day, samples were washed with PBS and then subjected to secondary antibody incubation at room temperature for 2 h. After another washing step with PBS, samples were incubated with DAPI solution (Molecular Probes, Eugene, OR, USA) for 30 min, washed again with PBS, and then mounted on the glass slide (Matsunami) by using mounting medium (Dako, Carpinteria, CA, USA). The primary and secondary antibodies used are as follows: rabbit anti-OGT mAb (D1D8Q; Cell Signaling Technology, Danvers, MA, USA), anti-ATP5A (15H4C4; Abcam, Cambridge, Danvers, MA, USA), mouse anti-Flag monoclonal antibody (Sigma), Alexa Fluor® 594 goat anti-rabbit IgG (A11037; Invitrogen/Molecular Probes), Alexa Fluor® 488 goat anti-mouse IgG (A-11029; Molecular Probes, Leiden, The Netherlands). Cell imaging was performed using confocal microscope (Zeiss, Germany).

2.4. Cellular fractionation

Cellular fractionation was done following a previously published method [16]. Briefly, the cells were resuspended by buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and were allowed to swell on ice for 15 min. Subsequently, 10% Nonidet NP-40 was added, and the samples were mixed by vortexing vigorously for 10 s. The samples were then centrifuged at a high speed for 5 min. The supernatant was collected as the cytoplasmic sample and the pellet was resuspended in buffer C (20 nM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). The nuclear pellet and buffer C were mixed by vortexing every 5 min for 30 min and subjected to high-speed centrifugation. The supernatants from both cytoplasmic and nuclear samples were then used for western blot.

2.5. Cell transfection

The Flag-ncOGT plasmid (pcDNA3x flag-OGT) was a gift from Professor Mutsuhiro Takekawa (The University of Tokyo). The plasmid was transformed to DH5α *E. coli* Competent Cells (DNA-913F; Toyobo Co., Ltd., Osaka, Japan) and antibiotic selection was accomplished using ampicillin (50 μg/ml, Sigma-Aldrich). siRNAs targeting the mouse PP2A Cα gene (Stealth siRNA) sense: GGAACUCUUUAGAAUUGGUGGUAAA, antisense: UUUACCACCAAUUCUAAAGAGUUCC, and control Stealth RNAi™ siRNA Negative Control Med GC were purchased from Invitrogen. Transfection was carried out using Opti-MEM (Gibco/Invitrogen Corporation, Paisley, Scotland, UK) and PEI max (Polysciences, Inc., Warrington, PA, USA). Stable cell line for shOGT was established using the methods described in the previous study [17].

2.6. Protein collection and western blotting

After OA treatment or after cell transfection, the cells were washed with cold PBS. The collected pellets were resuspended in lysate buffer (1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM EGTA) and sonicated for 20 s. After that, cells were centrifuged for 15 min at high speed and the supernatants were collected. Protein concentration was examined using Bradford assay and then adjusted prior to western blot experiments. Ten micrograms of total protein was separated using 10% SDS-PAGE and then blotted onto PVDF membranes (Merck, Darmstadt, Germany). After blocking with 5% skim milk in TBST for 1 h, the membranes were incubated with primary antibody (1:1000) overnight at 4 °C. The next day, the membranes were washed with TBST and incubated with a secondary antibody (1:1000) at room temperature for 2 h. The bands were visualized by chemiluminescence after the addition of HRP substrate (WBLUF0100; Millipore, Burlington, MA, USA). The densitometric analysis of bands was performed by using ImageJ (National Institutes of Health, Bethesda, MD, USA). The antibodies used are as follows: rabbit *anti*-OGT mAb (D1D8Q; Cell Signaling Technology), rabbit *anti*-PP2A C Subunit (#2038; Cell Signaling Technology), rabbit *anti*-LSD1 (#2184, C69G12; Cell Signaling Technology), mouse *anti*-α-Tubulin mAb (#3873, DM1A; Cell Signaling Technology), mouse *anti*-β-Actin mAb (#3700, 8H10D10; Cell Signaling Technology), anti-rabbit IgG HRP-linked antibody (7074S; Cell Signaling Technology), anti-mouse IgG HRP-linked antibody (7076S; Cell Signaling Technology).

2.7. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted by using TRIzol reagent (Invitrogen), chloroform, and isopropanol. cDNA was generated using PrimeScript Reverse Transcription Kit (Takara) according to the manufacturer's protocol. Prior to the PCR experiment, samples were prepared and mixed with PCR master mix (Toyobo), RNAse-free dH_2O (Millipore),

and the primers. The primer sequences for each target gene were as follows: *mOgt*-forward, 5′-ATCTTCTTCCCCCTCCCCAAT-3'; *mOgt*-reverse, 5′-TGAATAGCCAGCCAAATCTCC-3'; *Ogt*-forward, 5′-CTGTCACCCTTGACCCAAAT-3'; *Ogt*-reverse, 5′-ACGAAGA-TAAGCTGCGACAG-3'; *Actb*-forward, 5′- GAGAAGATCTGGCACCA-CACC-3'; *Actb*-reverse, 5′-GCATACAGGGACAGCACAGC-3'. The reaction was amplified for 30 cycles with denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 90 s (Takara). The PCR products were inspected using 2% agarose gel (Nacalai Tesque) in 1xTBE, visualized using ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA), and image quantification was conducted using ImageJ (National Institutes of Health).

2.8. Statistical analysis

The statistical analysis was completed by using GraphPad Prism software version 9.0 for Mac (GraphPad Software, San Diego, CA, USA). All results are expressed as means with standard deviation (SD) from at least three different replications. The data were analyzed by *t*-test. Pearson's correlation coefficient was used to evaluate the correlation between OGT and PP2A C subunit level. The p value < 0.05 was considered as significant (*).

3. Results

Okadaic acid (OA) is a widely-used PP2A inhibitor [20,21]. To examine the effect of PP2A inhibition on OGT localization, we treated MC3T3-E1 cells with various concentrations of OA for 1 h. Immunostaining results using an OGT antibody showed that OGT colocalized with DAPI staining of the nucleus in control cells. Following treatment with 10 and 20 nM OA, OGT localization changed from the nucleus to the cytoplasm (Fig. 1A). To confirm the translocation, we did Western blot analysis using the fractionated protein samples (Fig. 1B). In the control sample, the OGT band was only detected in the nuclear fraction (N) but not in the cytoplasmic fraction (C). On the contrary, in the OA-treated samples, OGT could be detected in the cytoplasmic and nuclear fractions. LSD1 and α-tubulin were used to confirm nuclear and cytoplasmic fractionation, respectively.

Next, we aimed to evaluate in which cellular compartment OGT translocated to in the cytoplasm of the OA-treated cells. Using double staining of OGT with ATP5A, a mitochondrial marker, OGT was detected in the mitochondria after OA treatment (Fig. 2A). qPCR results using specific primers for mOGT and total OGT showed that mRNA level of mOGT, but not total OGT, was significantly increased in 10 nM OA-treated MC3T3-E1 cells compared to control cells (Fig. 2B and C).

To examine whether ncOGT translocates from the nucleus to the cytoplasm, we transduced Flag-ncOGT plasmid into MC3T3-E1 cells. In the control cells, exogenous ncOGT was localized in the nucleus. Treatment with OA stimulated ncOGT translocation to the cytoplasm (Fig. 3). Similar to the results of OA treatment, knockdown of PP2A Cα translocated OGT from the nucleus to the cytoplasm, as determined by immunostaining (Fig. 4A). To confirm whether there was any correlation between PP2A and OGT expression levels, we carried out western blot analysis using the samples obtained from the knockdown cells of PP2A or OGT (Fig. 4B and C). Computational analysis revealed a strong positive correlation between the expression of these two proteins (Fig. 4D). We also detected a positive correlation between PP2A and OGT expression in HEK293 cells (Supplementary Data 1).

4. Discussion

In this study, we newly found that inhibition of PP2A induced the translocation of OGT from the nucleus to the cytoplasm and a positive correlation between PP2A and OGT expression. Our present results suggest that PP2A activity regulates OGT localization and expression. To our knowledge, this study is the first to report a strong association between PP2A and OGT.

Many studies have indicated the crosstalk between phosphorylation and *O*-GlcNAcylation. However, there is scant information about how the phosphorylated proteins can be *O*-GlcNAcylated, in which the removal of the phosphate group on the target site is needed. This study shows a more detailed relationship between PP2A and OGT than previous reports [7,20].

Based on previous studies, the localization of OGT may be influenced by the presence of its substrate whereas recent reports point out that OGT selects substrates or target proteins through its tetratricopeptide repeat (TPR) [21–23]. However, to date, the mechanism that regulates OGT localization by phosphatases has not been elucidated. The change in OGT localization observed in

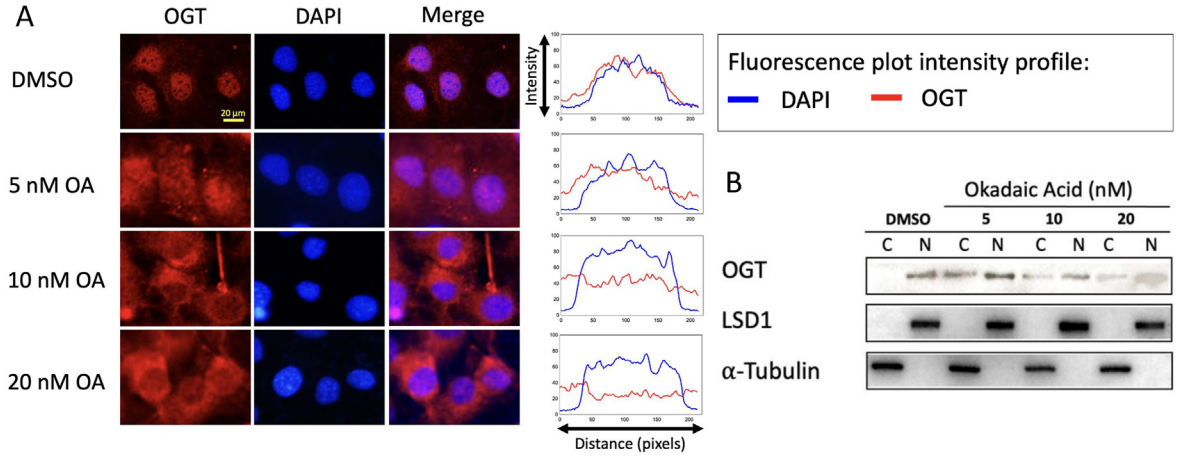

Fig. 1. OA Treatment changed OGT localization from nucleus to cytoplasm. (A) Immunostaining with anti-OGT antibody showed that when MC3T3-E1 cells were treated with OA, OGT translocation occurred from the nucleus to the cytoplasm. It appears that the DAPI and OGT intensity profiles are not at the same peak, which indicates that OGT is not localized in the nucleus. (B) Western blot analysis of the cellular fractionation sample showing that in the DMSO group, most of the OGT was only detected in the nucleus (N) and very little was detected in the cytoplasm (C). On the other hand, under OA treatment the OGT level in the cytoplasm increased. LSD1 and α-tubulin was used to confirm the nuclear and cytoplasmic fractionation respectively.

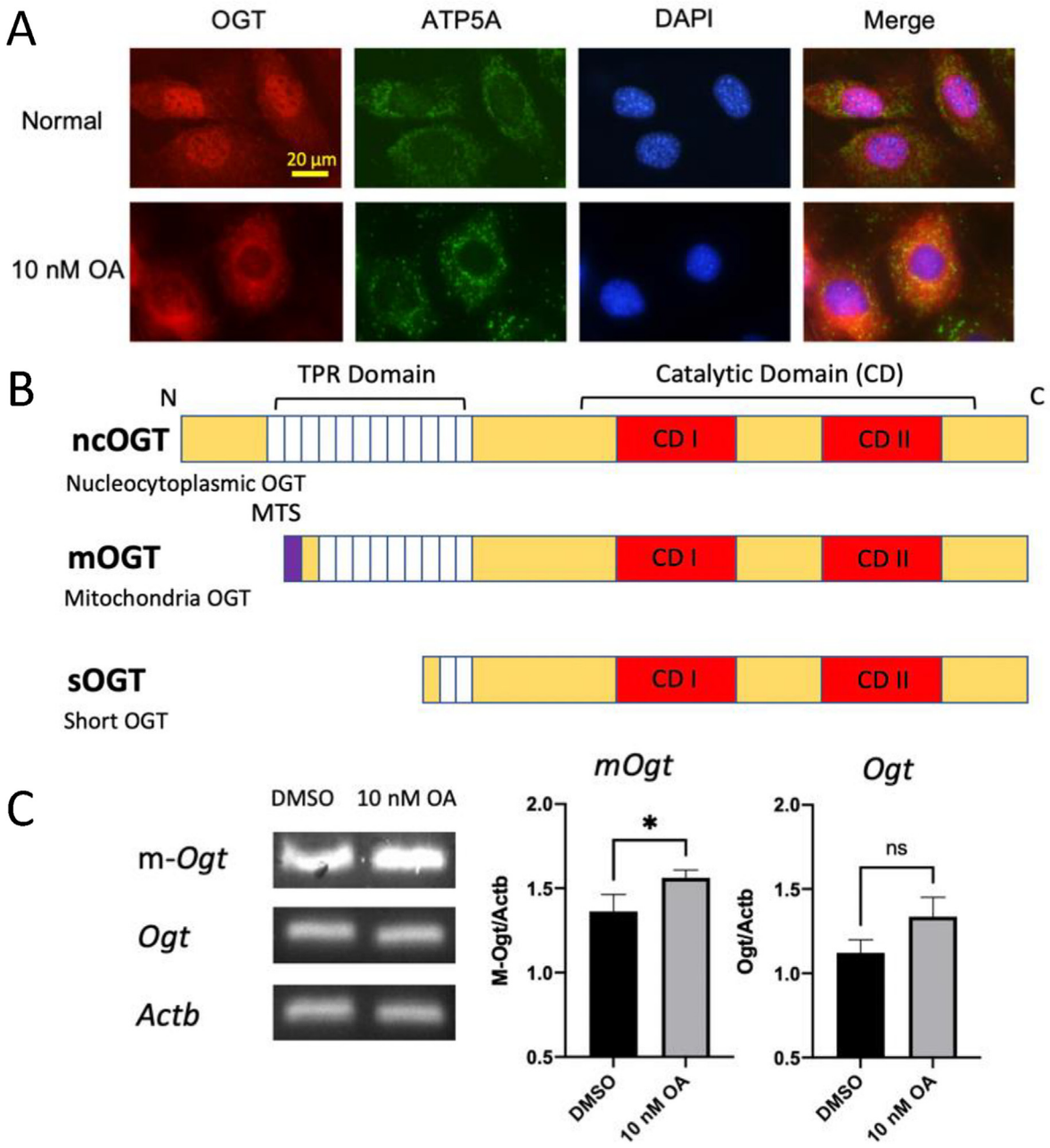

Fig. 2. The localization of OGT in the mitochondria increased after OA treatment. (A) Combination of immunostaining images with anti-OGT and anti-ATP5A shows increased localization of OGT in the mitochondrial area. (B and C) To confirm the expression level of mOGT, a special set of primers was designed to recognize the MTS, which is present only in mOGT and not in other OGT isoforms. mOGT mRNA levels showed a significant increase after treatment with 10 nM OA.

Fig. 1A suggests that PP2A activity is responsible for OGT substrate selectivity or its substrate localization. PP2A is thought to change the substrate selectivity of OGT from mainly nuclear proteins to cytoplasmic proteins or translocates OGT substrates from the nucleus to the cytoplasm.

It has previously been reported that *O*-GlcNAcylation on Ser389 by OGT itself mediates its translocation into the nucleus, allowing importin α5 to recognize the nuclear localization signal (NLS) of OGT [24]. OA treatment has been reported to decrease OGT activity in rat brain extract [25]. Furthermore, our study showed that the suppression of PP2A decreased OGT expression. These findings suggest the possibility that reduced PP2A activity leads to a decrease in *O*-GlcNAcylation on Ser389 of OGT, which prevents nuclear translocation by importin α5. Similar immunostaining results were also obtained in HEK293 cells (Supplementary Data 2).

Besides the mechanism mediated by *O*-GlcNAcylation on Ser389 of OGT and NLS recognition by importin α5, the observation of OGT in the cytoplasm of OA-treated cells raises two new hypotheses: first, the expression of mOGT as OGT isoform that mainly expressed in the cytoplasm is increased, or second, ncOGT that normally resides in the nucleus translocates to the cytoplasm. An increase in the mitochondrial-localized OGT was observed in OA-treated cells in Fig. 2A and a significant increase in mOGT mRNA expression was detected by PCR using mOGT-specific primers (Fig. 2C). The mOGT isoform is produced by alternative splicing of a single *Ogt* gene [14]. PP2A is reportedly a key regulator of alternative splicing [26–28].

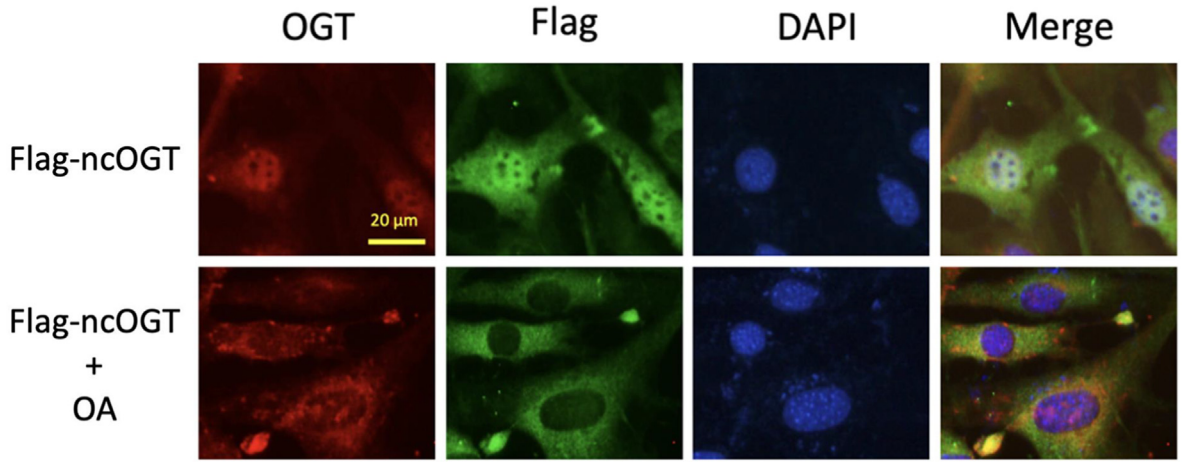

Fig. 3. ncOGT isoform translocated from the nucleus to the cytoplasm after OA treatment. Immunostaining with anti-Flag on MC3T3-E1 cells previously transfected with the plasmid pcDNA3x Flag-ncOGT showed that after treatment with 10 nM OA, exogenous OGT was dislocated into the cytoplasm.

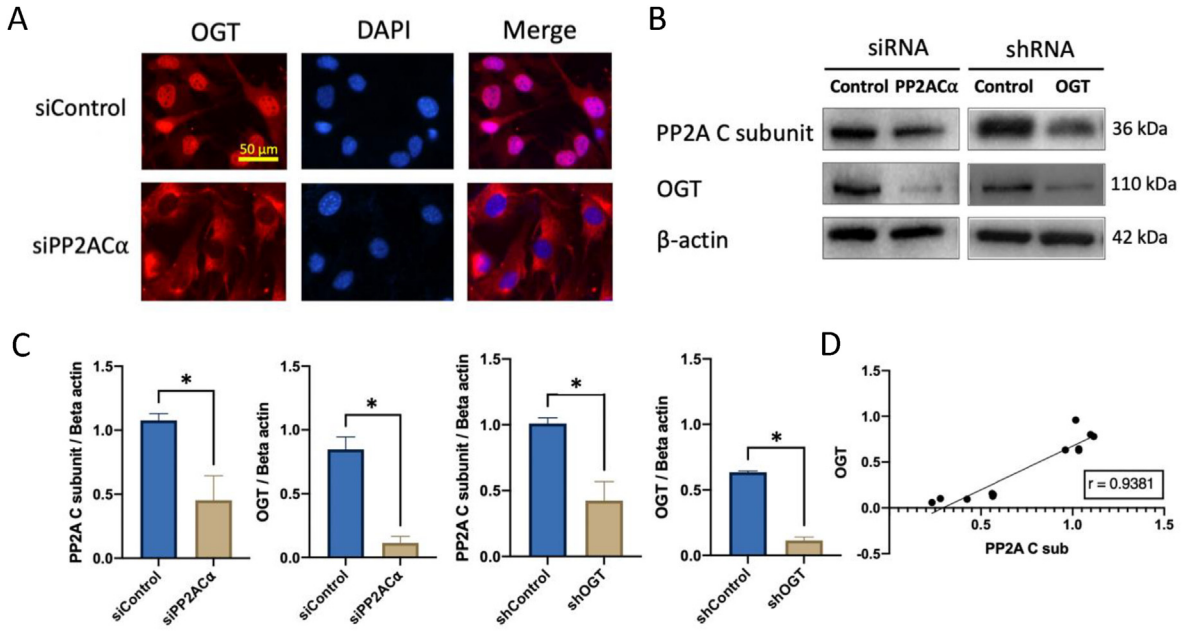

Fig. 4. Knockdown of PP2A Cα alters the localization of OGT and decreases their expression. (A) The OGT was displaced from the nucleus to the cytoplasm in MC3T3-E1 cells treated with transient knockdown of PP2A Cα. Immunostaining with anti-OGT showed localization of OGT in the cytoplasmic area of the cell, outside the nucleus. (B) Western blot results of siPP2A Cα MC3T3-E1 cells showed a decrease in OGT levels. When OGT was knocked down with shRNA hairpin, the level of the PP2A C subunit was also decreased. (C) Quantification of western blot analysis from Fig. 2B. (D) Pearson's correlation coefficient showed a strong positive correlation between OGT and PP2A C subunit levels.

Our data imply that PP2A activity is involved in the alternative splicing pattern of OGT. Moreover, it was reported that mOGT plays a role in the regulation of apoptosis [29]. OA at high concentrations is known to cause apoptosis in osteoblastic cells [19] so the increase in mOGT expression by OA treatment might be related to apoptosis. Little information can be obtained about mOGT, therefore, the present study opens up opportunities for further research.

When we carefully examined t the immunostaining data (Fig. 2A), we can see that in the cytoplasmic area, a prominent level of OGT was not localized to the mitochondria. This suggests the possibility of the second hypothesis proposed above. Given that ncOGT is the most common OGT isoform and is responsible for *O*-GlcNAc levels globally [30], we suspected that this location shift occurred in the ncOGT isoform. The results of immunostaining with anti-Flag antibody showed that OA treatment induced the translocation of ncOGT from the nucleus to the cytoplasm (Fig. 3). This result indicated that PP2A activity controls ncOGT localization.

In this study, knockdown of PP2A Cα caused a decrease in OGT levels, and the knockdown of OGT lowered PP2A C subunit levels. These findings hint a correlation between PP2A Cα and OGT expression level. In addition, a mass spectrometry result and our preliminary data revealed the possibility of PP2A being *O*-GlcNAcylated [31]. Although there is still no report that OGT can be dephosphorylated by PP2A, these findings suggest that they directly or indirectly modify each other through *O*-GlcNAcylation and dephosphorylation. A previous study reported the active complex formed by OGT and PP1 [25]. Another study also signifies the presence of a complex consisting of OGT, OGA, together with Aurora kinase B and protein phosphatase PP1 [32] which further emphasizes the “yin-yang” theory regarding phosphorylation and

O-GlcNAcylation. There is a possibility that such a phenomenon also occurs in OGT and PP2A. Further study to clarify the interaction between PP2A and OGT would be useful to understand the precise regulatory mechanism between phosphorylation and *O*-GlcNAcylation.

5. Conclusion

Taken together, our present study showed that inhibition of PP2A changed the OGT localization through an increase in mOGT isoform expression and the translocation of ncOGT. PP2A and OGT expression level were also significantly correlated. These results indicate that PP2A activity plays an important role in OGT localization and expression to control the balance between phosphorylation and *O*-GlcNAcylation. Further studies are needed to elucidate the interaction between PP2A and OGT. However, the present study sheds some light on the complex relationship between phosphorylation and *O*-GlcNAcylation.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.01.033.

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