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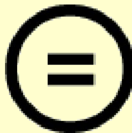

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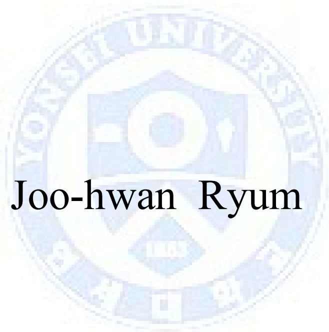
Functional Study of *O*-GlcNAcylation on Cdk4 during G1/S transition



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Functional Study of *O*-GlcNAcylation on Cdk4 during G1/S transition

A Dissertation
Submitted to the Department of Integrated OMICS for Biomedical Science
and the Graduate School of Yonsei University
in partial fulfillment of the
requirements for the degree of
Master of Biomedical Science

Joo-hwan Ryum

June 2015

This certifies that the dissertation
of Joo-hwan Ryum is approved.

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The Graduate School
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June 2015

감사의 글

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Abbreviation

O-GlcNAc : *O*-linked β-*N*-acetylglucosamine

UDP-GlcNAc : uridine 5'-diphospho-*N*-actylglucosamine

OGT : *O*-GlcNAc transferase

OGA : *O*-GlcNAcase

HBP : hexosamine biosynthetic pathway

Cdk4 : cyclin-dependent kinase 4

sWGA : succinylated wheat germ agglutinin

shRNA : small hairpin RNA

CTL : control

Abstract

O-GlcNAcylation is a post-translational modification which occurs on proteins located in the cytosol and nucleus of cells. This modification regulates the expression and functions of proteins, thereby participating in various cellular signalings and cell mechanisms. UDP-GlcNAc, the *O*-GlcNAc donor of the *O*-GlcNAcylation is synthesized from glucose via hexosamine biosynthetic pathway. Production of UDP-GlcNAc is greatly reliant on the cellular glucose condition. It is also known that *O*-GlcNAcylation is involved in numerous diseases such as cancer, diabetes, and Alzheimer's disease.

Recent scientific reports showed that cancer cells are in an elevated state of *O*-GlcNAcylation when compared with normal cells and furthermore, it was also shown that *O*-GlcNAcylation is involved in cell cycle regulation. From this we hypothesized that higher *O*-GlcNAcylation in cancer cells promotes cell cycle transition. In order to verify this hypothesis, we generated an OGT knock-down stable cell line using lung cancer cell line A549. Through FACS analysis, we observed a relatively delayed G1/S cell cycle transition in these cells. Using Western blot, we determined that hypophosphorylation of pRB was the cause of the delayed G1/S cycle transition.

To gain a better understanding of the relationship between the

decrease in *O*-GlcNAcylation and pRB hypophosphorylation, we examined whether cyclin-dependent kinase 4 (Cdk4), a protein crucially involved in phosphorylation of pRB, is *O*-GlcNAcylated. By Western blot, we detected the presence of *O*-GlcNAcylation on overexpressed Cdk4 and observed an increase in detected *O*-GlcNAcylation when OGT was also overexpressed. Furthermore, using protein *O*-GlcNAc moiety binding sWGA precipitation, we observed *O*-GlcNAcylation on Cdk4 regulation during G1/S cell cycle transition.

To determine the functions of *O*-GlcNAcylation on Cdk4, we focused on Cdk4 and cyclin D binding affinity which is critical to the G1/S transition. By coimmunoprecipitation of overexpressed Cdk4 and cyclin D, we observed increased binding between Cdk4 and cyclin D1, D2 under an OGT overexpressed condition.

In this research we aim to identify that G1/S cell cycle transition is regulated by the *O*-GlcNAcylation on Cdk4, which increases the binding between Cdk4 and cyclin D. Such relationship serve to show that increased global *O*-GlcNAcylation of cancer cells contribute to cancer cell proliferation.

Chapter 1. Introduction

O-GlcNAcylation is a single sugar modification occurring on serine and threonine of proteins. It is also a post-translational modification occurring on proteins in the cytosol and nucleus of a cell (Fig. 1). The *O*-GlcNAc is attached by *O*-GlcNAc transferase (OGT) and removed by *O*-GlcNAcase (OGA)[1]. UDP-GlcNAc, a substrate for *O*-GlcNAcylation, is synthesized through the hexosamine biosynthetic pathway (HBP). For this reason *O*-GlcNAcylation is regarded as a nutrient sensor indicating cellular nutrient conditions [2,3]. *O*-GlcNAcylation can regulate a multitude of intercellular protein functions, either individually or in interplay with *O*-phosphorylation [1,4], and is linked to diseases such as cancer, diabetes and Alzheimer's disease [4,5].

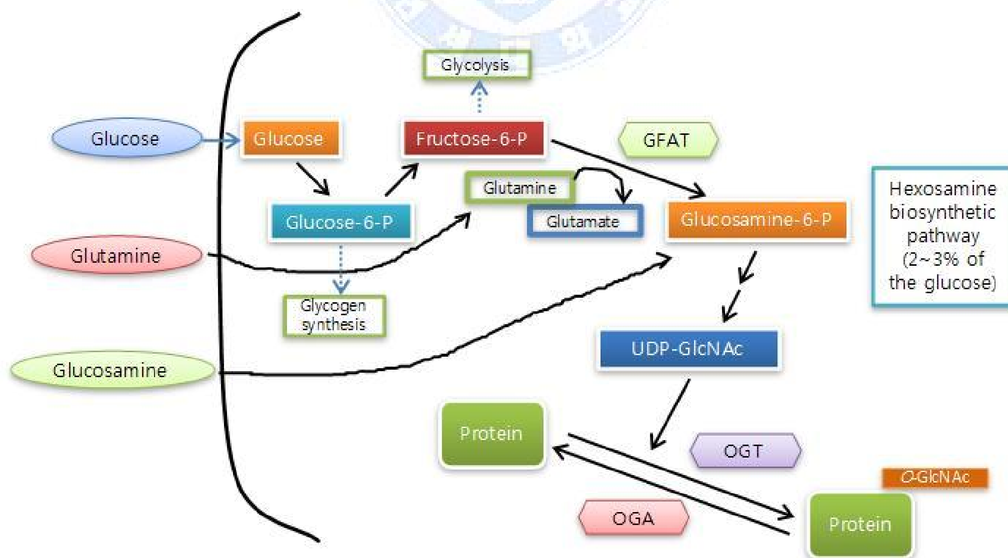


Figure 1. The regulation of *O*-GlcNAcylation

Naturally, the relationship between cancer and *O*-GlcNAcylation has been studied for a long time. A common point in these research was that cancer cells possessed a higher level of global *O*-GlcNAcylation compared to normal cells [6]. This phenomenon has been reported in prostate [7], primary colorectal [8], breast [9], lung and colon cancer [10]. Therefore, we hypothesized that high *O*-GlcNAcylation conditions positively influence cancer cell proliferation.

From here we took the cell cycle into consideration as an influential factor in cancer proliferation. Cell cycle directly regulates cell proliferation of not only cancer cells, but all cells. Further, studies have shown that *O*-GlcNAcylation is involved in the regulation of whole cell cycle [11]. However, the relationship between *O*-GlcNAcylation and G1/S phase as not yet been studied in depth.

Using lung cancer cell line, we discovered that decreasing cancer cell *O*-GlcNAcylation by OGT knock-down resulted in a delay in cell G1/S transition. And by Western blot, we determined that this delay was caused by pRB hypophosphorylation. Furthermore we identified that cyclin-dependent kinase 4 (Cdk4), a crucial protein in G1/S transition, is *O*-GlcNAcylated. This modification is regulated during G1/S cell cycle transition. Lastly upon overexpression of OGT, we observed increased binding of Cdk4 and cyclin D which is crucial to pRB phosphorylation and G1/S cell cycle transition. Such result confirmed the possible functional property of *O*-GlcNAcylation on Cdk4.

Chapter 2. Materials and Methods

2.1 Cell culture

HEK293 (tumorized human embryonic kindney cell), A549 (human lung adenocarcinoma epithelial cell) cell lines were cultured in High Glucose DMEM (Dulbecco's Modified Eagle's Medium, HyClone^{TM}) supplemented with 10% FBS (Fetal Bovine Serum, HyClone^{TM}) and 1% P/S (Penicilline/Streptomycin, HyClone^{TM}) in 5% CO_2 at 37℃.

2.2 DNA plasmids and transfection

cDNA of human full-length Cdk4, cyclin D1, cyclin D2, cyclin D3 were generated by PCR and cloned into the p3XFLAG-CMV^{TM}-7.1 expression vector (Sigma Aldrich). FLAG-Cdk4 was subcloned into the pEXPR-IBA105 One-Strep tag vector (IBA). Untagged OGT was used for OGT overexpression [12]. The sequences of all constructs were checked by DNA sequencing. These DNA plasmids were transfected into cells using polyethylenimine (PEI, Sigma Aldrich).

2.3 Establishment of OGT knock-down stable cell line

For establishment of stable OGT knock-down cells, lentiviral clones expressing shOGT were acquired from Sigma Aldrich. Lentiviruses were produced in 293FT cells with Lipofectamine 2000 (Invitrogen) and infected into A549 cells according to the manufacturer's directions. The

stable clones were selected via puromycin (5μg/ml) treatment (shOGT). Non-target shRNA lentiviral transduction particles (Sigma Aldrich) were used as a negative control (shCTL).

2.4 Synchronization of cell culture

Cell cycle synchronization was achieved by serum deprivation experiments. A549 cells were washed twice with PBS and cultured in serum-free media for 48hr. To release the cell cycle block, cells were grown in the presence of 10% FBS and harvested at the indicated time periods.

2.5 FACS analysis

For flow cytometric analysis of the cell cycle, FACS analysis was performed by staining DNA with propidium iodide (PI). A549 cells treated as described above were washed in PBS and harvested using trypsin. After centrifugation at 3,000rpm for 3min, the cell pellet was washed with PBS and fixed in 70% (v/v) ice-cold ethanol at −20℃ for 3h. Cells were centrifugated, washed with PBS, and then incubated with 50μg/ml PI (Sigma Aldrich) and 20μg/ml RNAse A (Sigma Aldrich) at 37℃ for 30min. After washing with PBS, flow cytometric analysis was performed on a FACScalibur (Becton Dickinson) instrument using the associated CellQuest^{TM} software package (Becton Dickinson). The distribution of cells in the different phases of the cell cycle was analysed by appropriate software (ModFit *LT*; Becton Dickinson).

2.6 Cell lysis, SDS-PAGE, and Western blot

Cells were lysed on ice for 30min in NET buffer [150mM NaCl, 50mM Tris-HCl, 1% NP-40, 1mM EDTA and protease inhibitor cocktail (Roche Appled Science)] and centrifuged at 13,900rpm for 20min. Protein concentration of the supernatant was measured by Bradford Assay using Protein Assay Dye Reagent Concentrate (BIO-RAD). The lysates were boiled in 4x SDS sample buffer. The samples were separated by SDS-PAGE using 10~12% polyacrylamide gel and transferred onto nitrocellulose membranes (GE Healthcare). The membranes were incubated in 5% skin milk for 1hr at room temperature and washed 3 times with TBS-T. After washing, the membranes were incubated in primary antibodies diluted in TBS-T for 16hr at 4℃ and washed 3 times with TBS-T. They were incubated in secondary antibodies diluted in TBS-T for 1hr at room temperature, and washed 3 times with TBS-T. Immunoreactive bands were detected using an ECL system (GE Healthcare, Bio-Science).

2.7 Succinylated wheat germ agglutinin (sWGA) precipitation

For sWGA precipitation, cell lysates were incubated with agarose-sWGA (Vector Lab) at 4℃ overnight. After washing 4 times with lysis buffer, precipitates were eluted in 2x SDS sample buffer.

2.8 Immunoprecipitation

For FLAG immunoprecipitation, cell lysates were incubated with

Ezview™ Red anti-FLAG M2 Affinity Gel (Sigma Aldrich) at 4℃ overnight. After washing 4 times with lysis buffer, precipitates were eluted in 2x SDS sample buffer.

The One-strep tag system is a method which allows the purification and detection of proteins by affinity chromatography. The One-strep tag is a synthetic peptide consisting of 8 amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys). This peptide sequence exhibits intrinsic affinity towards Strep-Tactin, a specifically engineered streptavidin and can be N- or C- terminally fused to recombinant proteins. Streptavidin is a tetrameric protein expressed in *Streptomyces avidinii*. Because of its high affinity for the vitamin h-biotin, streptavidin is commonly used in the fields of molecular biology and biotechnology (Wikipedia).

For One-strep immunoprecipitation, cell lysates were incubated with Strep-Tactin Sepharose (IBA) at 4℃ overnight. After washing 4 times with lysis buffer, precipitates were eluted in 2x SDS sample buffer.

2.9 Antibodies

The following primary antibodies were used: α-*O*-GlcNAc (CTD110.6, MMS-248R, Covance), α-OGT (DM-17, O5264, Sigma Aldrich), α-OGT (TI-17, O6014, Sigma Aldrich), α-Cdk4 (H-22, sc-601, Santa Cruz Biotechnology), α-cyclin D1 (M-20, sc-718, Santa Cruz Biotechnology),

α-phospho-Ser807/811-pRB (8516S, Cell Signaling), α-One-strep (2-1507-001, IBA), α-FLAG (F7425, Sigma Aldrich), α-GAPDH (MAB374, Millipore).

The secondary antibodies conjugated to HRP (Horse radish peroxidase) were purchased from Santa Cruz Biotechnology (α-mouse IgG; sc-2005, α-rabbit IgG; sc-2004, α-mouse IgM; sc-2064).

Chapter 3. Results

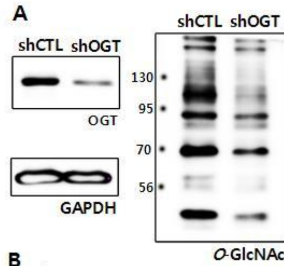
3.1 Decreased OGT level delays G1/S cell cycle transition via pRB hypophosphorylation in human lung cancer cell

To observe how cancer cell G1/S transition is changed when cancer cell *O*-GlcNAcylation level is decreased, we generated an OGT knock-down stable cell line (shOGT) and control cell line (shCTL) from A549 lung cancer cell line (Fig. 2A). We subjected the two cell lines to serum deprivation in order to synchronize cells to the G0/G1 phase. After releasing cells by adding serum to media, the change in cell cycle phase of cells according to time points was analyzed using FACS analysis (Fig. 2B). When the ratio of cells in S phase was plotted against time in graph we found that the increase in number of cells in the S phase was slower for shOGT cells compared to shCTL cells (Fig 2D).

There are several restriction points that are required for a cell to transition from G1 to S phase. Of these, pRB phosphorylation by Cdk4-cyclin D complex is an event which occurs at an early stage [13, 14](Fig. 3). To determine the cause of the G1/S phase transition delay observed previously, we checked how pRB phosphorylation changed according to time using the same two cell lines. In the Western blot experiment using phospho-pRB antibody, shOGT cells exhibited

hypophosphorylated pRB for serine 807 and 811 according to progression of time when compared to shCTL cells (Fig. 4). From these experiments we concluded that global decrease of *O*-GlcNAcylation induced by knock-down of OGT in lung cancer cells delayed G1/S phase transition through pRB hypophosphorylation.

A



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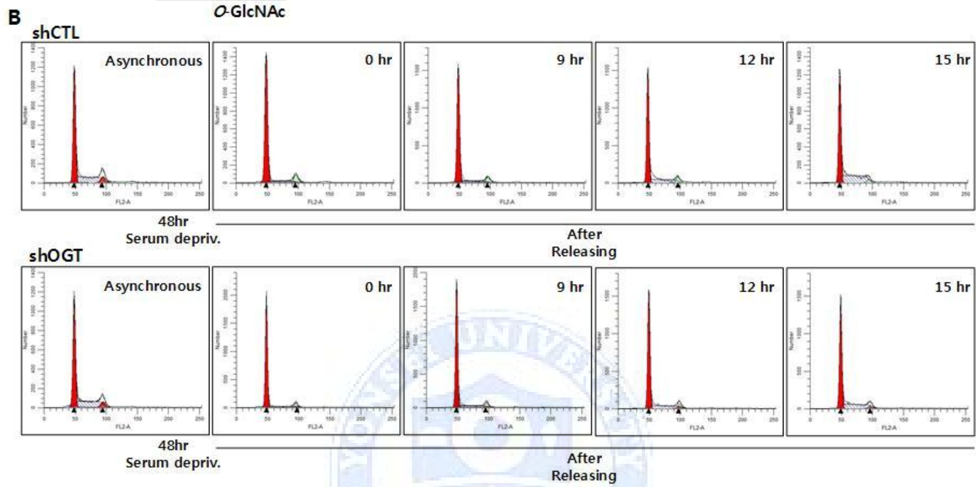

C

1 set	Time	G0/G1	S	G2/M
shCTL	Asynchronous	64.02	29.37	6.6
	0hr	90.03	9.05	0.92
	9hr	88.73	10.76	0.51
	12hr	77.67	22.33	0
	15hr	61.31	38.69	0

1 set	Time	G0/G1	S	G2/M
shOGT	Asynchronous	61.73	31.87	6.4
	0hr	89.18	8.86	1.96
	9hr	85.7	12.78	1.52
	12hr	79.01	18.63	2.35
	15hr	70.03	28.03	1.94

D

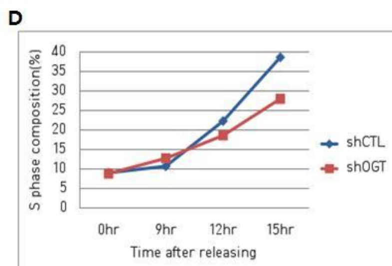

Figure 2. Decreased OGT level delays G1/S cell cycle transition in A549 cells. (A) OGT level was decreased by shRNA in A549 cells (shOGT). Non-targeted shRNA was used as a negative control (shCTL). (B) Cells were synchronized by serum deprivation for 48hr. After releasing by serum addition, cells were harvested at the indicated time points. harvested cells were analyzed by FACS machine. (C) FACS results were digitized by ModFit *LT* software. (D) The cells in S phase were displayed in graph form.

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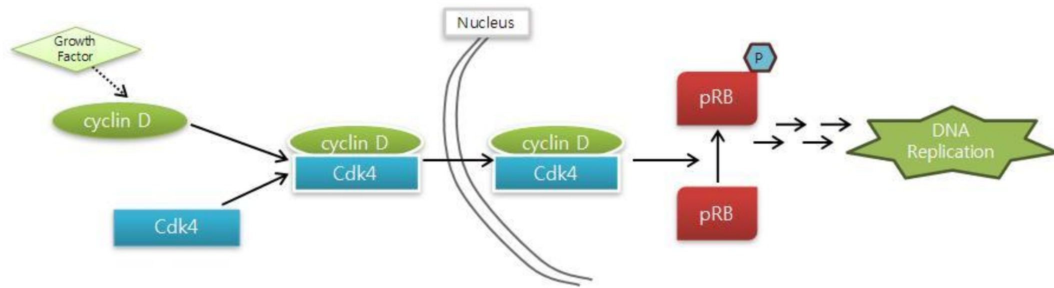


Figure 3. pRB phosphorylation by Cdk4 and cyclin D complex

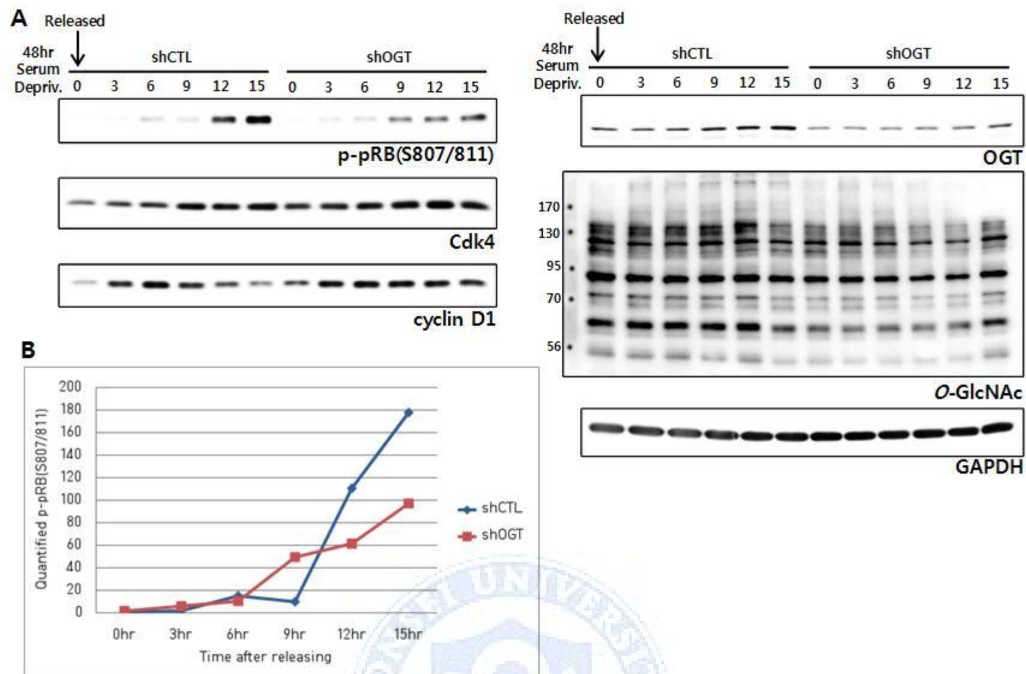


Figure 4. Decreased OGT level causes pRB hypophosphorylation in A549 cells. A comparison was made between shOGT and shCTL. Cells were synchronized by serum deprivation, and released. (A) Harvested cells with indicated time points were analyzed by SDS-PAGE and Western blot with phospho-pRB antibodies (S807/811). (B) Quantified phospho-pRB (S807/811) values are displayed in graph form.

3.2 Cyclin-dependent kinase 4 (Cdk4) is *O*-GlcNAcylated, and this modification is regulated during G1/S cell cycle transition

In order to identify the protein involved in decreased *O*-GlcNAcylation, delayed G1/S phase transition, and hypophosphorylated pRB, we examined whether Cdk4, which is directly involved in pRB phosphorylation [13,15], is *O*-GlcNAcylated. When OGT and FLAG-tagged Cdk4 were overexpressed in HEK293 cells, *O*-GlcNAcylation was detected on Cdk4. In proportion to the increased amount of overexpressed OGT, the amount of detected *O*-GlcNAcylation was also found to increase dose dependently (Fig. 5).

To see whether *O*-GlcNAcylation on Cdk4 is regulated during cell G1/S transition, A549 cells were synchronized and released as described previously. The prepared cells were then used in lectin precipitation with sWGA which binds the *O*-GlcNAc moiety of proteins. The obtained samples were used in Western blot and probed for Cdk4 using the Cdk4 antibody. We discovered that after cell release, the detected Cdk4 blot increased and then decreased over time (Fig. 6). It means *O*-GlcNAcylation on Cdk4 is regulated during G1/S cell cycle transition. Since it is known that the time point when cyclin D1 is maximized correlates with pRB phosphorylation and G1/S transition progression [16]. Our result also indicated the possibility that *O*-GlcNAcylation on Cdk4 could influence cell G1/S transition progress.

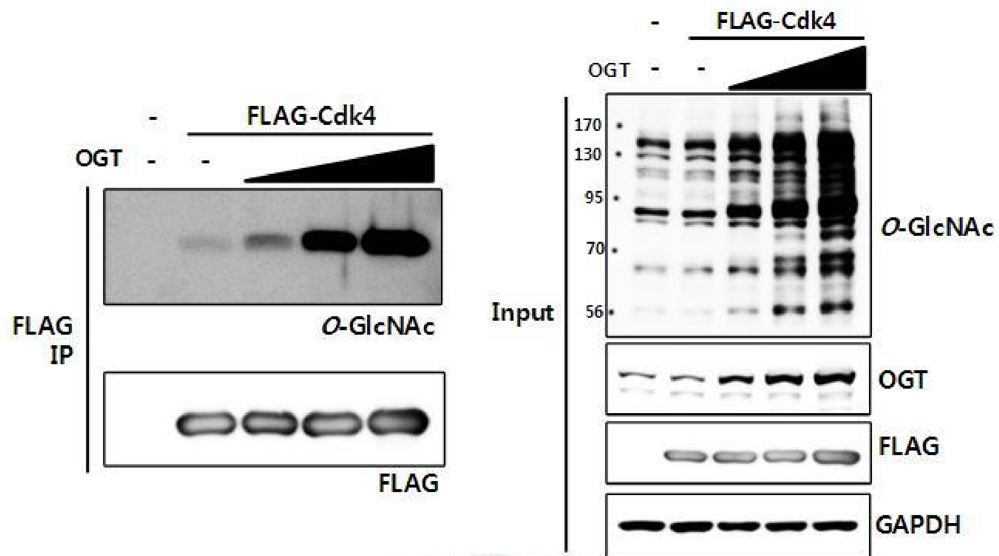


Figure 5. Cdk4 is *O*-GlcNAcylated. FLAG-tagged Cdk4 (FLAG-Cdk4) was overexpressed in HEK293 cells together with untagged OGT (3, 6, 10μg). After immunoprecipitation with FLAG affinity gel, *O*-GlcNAcylated FLAG-Cdk4 was immunoblotted.

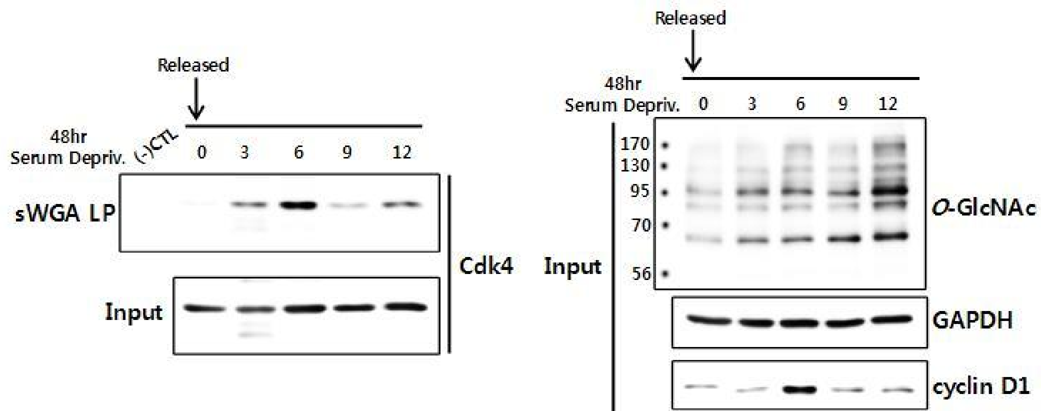


Figure 6. *O*–GlcNAcylation on Cdk4 is regulated during G1/S cell cycle transition. A549 cells were synchronized by serum deprivation, released, and harvested cells at indicated time points. Endogenous Cdk4 was precipitated with sWGA. Cdk4 precipitated with sWGA, total Cdk4, GAPDH, and cyclin D1 were immunoblotted.

3.3 OGT overexpression enhances protein-protein interaction between Cdk4 and cyclin D

Based upon the results in Fig. 6, it was shown that the level of *O*-GlcNAcylation on Cdk4 and the amount of cyclin D was maximized at the same point of time. Thus we hypothesized that *O*-GlcNAcylation on Cdk4 may affect the binding between Cdk4 and cyclin D. To confirm this, HEK293 cells were transfected with One-strep-tagged Cdk4 and FLAG-tagged cyclin D1 together. Using the transfected cells we carried out immunoprecipitation using One-strep affinity gel. The amount of cyclin D1 bound to Cdk4 was detected in Western blot using FLAG antibody. As a result we found that under OGT overexpressed condition, more cyclin D1 was detected compared to when OGT was not overexpressed (Fig. 7). Since there are three kinds of cyclin D (cyclin D1, D2 and D3), the same immunoprecipitation experiment was carried out for cyclin D2 and D3. In case of cyclin D2, OGT overexpression resulted in increased cyclin D2 detection as in the case of cyclin D1 (Fig. 8). However in the case of cyclin D3, OGT overexpression did not result in a change of amount of detected cyclin D3 (Fig. 9). Therefore we concluded that OGT overexpression, which increases *O*-GlcNAcylation on proteins including Cdk4, promotes binding between Cdk4 and cyclin D1, D2.

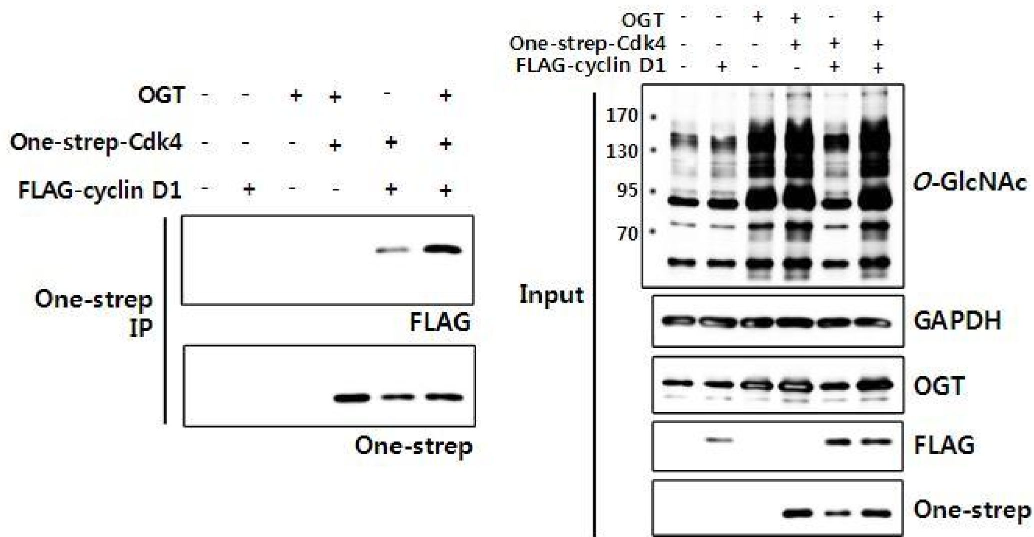


Figure 7. OGT overexpression enhances protein binding between Cdk4 and cyclin D1. One-strep-tagged Cdk4 (One-strep-Cdk4) and FLAG-tagged cyclin D1 (FLAG-cyclin D1) were overexpressed in HEK293 with or without OGT overexpression. After immunoprecipitation with One-strep affinity gel, precipitated cyclin D1 and Cdk4 were immunoblotted.

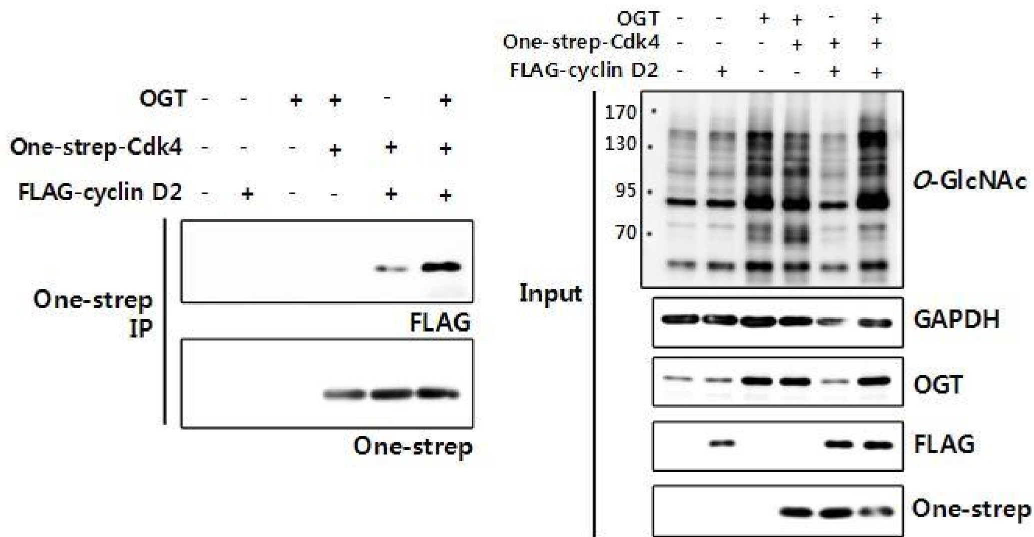


Figure 8. OGT overexpression enhances protein binding between Cdk4 and cyclin D2. One-strep-tagged Cdk4 (One-strep-Cdk4) and FLAG-tagged cyclin D2 (FLAG-cyclin D2) were overexpressed in HEK293 with or without OGT overexpression. After immunoprecipitation with One-strep affinity gel, precipitated cyclin D2 and Cdk4 were immunoblotted.

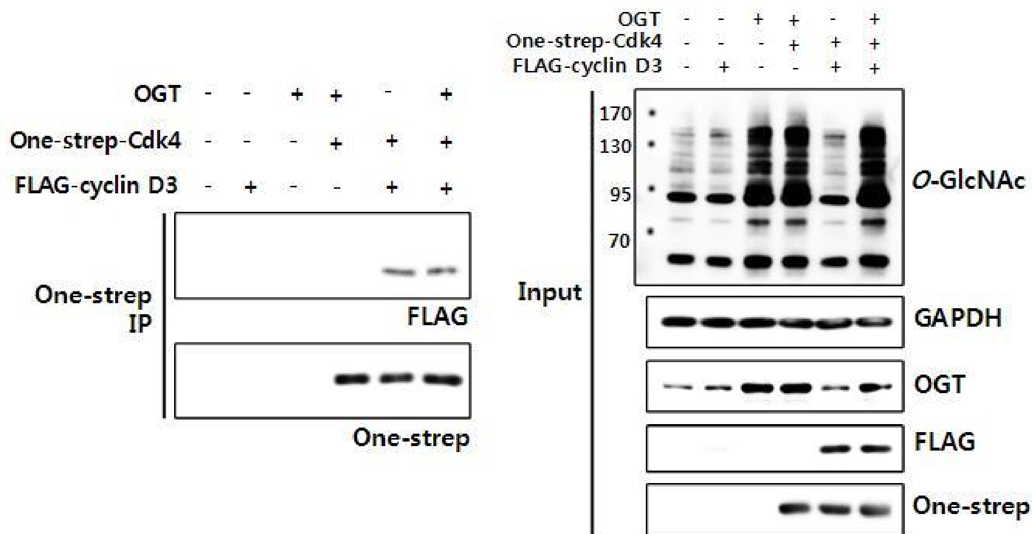


Figure 9. OGT overexpression has no effect on protein binding between Cdk4 and cyclin D3. One-strep-tagged Cdk4 (One-strep-Cdk4) and FLAG-tagged cyclin D3 (FLAG-cyclin D3) were overexpressed in HEK293 with or without OGT overexpression. After immunoprecipitation with One-strep affinity gel, precipitated cyclin D3 and Cdk4 were immunoblotted.

Chapter 4. Discussion

Considering that *O*-GlcNAcylation is a cellular nutrient sensor [17,18], it is natural to come to the conclusion that it will affect cell proliferation and its crucial cell cycle regulation. This is supported by studies on the function of *O*-GlcNAcylation in relation to the cell cycle [11]. Furthermore, as cancer cells commonly exhibit elevated global *O*-GlcNAcylation [6-10], we can speculate that *O*-GlcNAcylation has a cell proliferation promoting function. This study too shows that *O*-GlcNAcylation is actively involved in cell cycle regulation.

There were several limitations throughout this research. First, although it was confirmed that pRB was hypophosphorylated and G1/S transition was delayed by OGT knock-down, following the release of synchronized cells initially showed more S phase cells in the OGT knock-down cells than in control cells. In addition, pRB phosphorylation also appeared earlier in OGT knockdown cells. This phenomenon requires further explanation through follow up studies.

Second, in sWGA precipitation, *O*-GlcNAcylated proteins and other proteins interacting with the modified proteins can both be precipitated. Thus we cannot simply consider all bands detected in Western blot as *O*-GlcNAcylated proteins. From Fig. 6, we can just conclude that *O*-GlcNAcylation on Cdk4 or *O*-GlcNAcylated binding partners of Cdk4

are regulated during G1/S phase transition. To get more restricted conclusion, further experiments such as Cdk4 immunoprecipitation are needed.

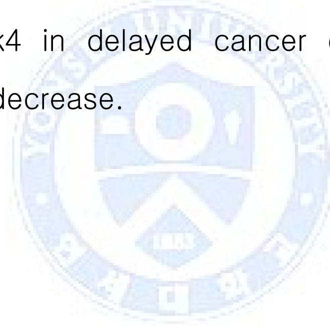
Third, though the binding of Cdk4 and cyclin D is increased upon OGT overexpression, it does not mean that increased binding with cyclin D is due to the increase of *O*-GlcNAcylation on Cdk4. This is because OGT overexpression does not increase only *O*-GlcNAcylation on Cdk4, but also the *O*-GlcNAcylation of other proteins. Thus this results must be further proved by *in vitro* binding assays such as GST pull-down assay.

A possible explanation regarding the lack of change in the binding of Cdk4 and cyclin D3 may be because of the difference in structure between cyclin D3 and the other two proteins (cyclin D1, D2). It is known that while the cyclin D family are homologous proteins with the same function, they are not spliced variants but proteins originating from separate genes [19]. Two recent publications describing the binding structures of Cdk4-cyclin D1 [20] and Cdk4-cyclin D3 [21] also give weight to this hypothesis.

Regardless of these limitations this research provides a novel understanding of the relationship between cancer and *O*-GlcNAcylation, and the connection between *O*-GlcNAcylation and G1/S phase transition with Cdk4 as intermediate. To further this study there is a need to

identify the *O*-GlcNAcylated sites of Cdk4 using mass spectrometry and point mutation. Following that, Cdk4-cyclin D binding experiments using the *O*-GlcNAcylated sites mutated Cdk4, make it more reliable that *O*-GlcNAcylation on Cdk4 acts directly upon the binding between Cdk4 and cyclin D.

Moreover, by generating wild type Cdk4 or *O*-GlcNAcylated sites mutated Cdk4 expressing cell lines from A549, it will be possible to observe delays in G1/S phase transition and pRB phosphorylation in those mutant expressing cell lines. Thus we can unveil the importance of *O*-GlcNAcylation on Cdk4 in delayed cancer cell G1/S transition upon global *O*-GlcNAcylation decrease.



Summary

1. When OGT level is decreased, G1/S cell cycle transition is delayed.

2. Decreased OGT level causes pRB hypophosphorylation.

3. Cdk4 protein is *O*-GlcNAcylated, and this *O*-GlcNAcylation is up-regulated during G1/S cell cycle transition.

4. OGT overexpression enhances protein binding between Cdk4 and cyclin D1, D2.

References

1. Hart, G. W., M. P. Housley and C. Slawson (2007). "Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins." Nature 446(7139): 1017-1022.

2. Bond, M. R. and J. A. Hanover (2015). "A little sugar goes a long way: the cell biology of O-GlcNAc." J Cell Biol 208(7): 869-880.

3. Love, D. C. and J. A. Hanover (2005). "The hexosamine signaling pathway: deciphering the "O-GlcNAc code"." Sci STKE 2005(312): re13.

4. Hart, G. W., C. Slawson, G. Ramirez-Correa and O. Lagerlof (2011). "Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease." Annu Rev Biochem 80: 825-858.

5. Yuzwa, S. A. and D. J. Vocadlo (2014). "O-GlcNAc and neurodegeneration: biochemical mechanisms and potential roles in Alzheimer's disease and beyond." Chem Soc Rev 43(19): 6839-6858.

6. Ma, Z. and K. Vosseller (2014). "Cancer metabolism and elevated O-GlcNAc in oncogenic signaling." J Biol Chem 289(50): 34457-34465.

7. Kamigaito, T., T. Okaneya, M. Kawakubo, H. Shimojo, O. Nishizawa and J. Nakayama (2014). "Overexpression of O-GlcNAc by prostate cancer cells is significantly associated with poor prognosis of patients." Prostate Cancer Prostatic Dis 17(1): 18-22.

8. Phueaouan, T., P. Chaiyawat, P. Netsirisawan, D. Chokchaichamnankit, P. Punyarit, C. Srisomsap, J. Svasti and V.

Champattanachai (2013). "Aberrant O-GlcNAc-modified proteins expressed in primary colorectal cancer." Oncol Rep 30(6): 2929-2936.

9. Caldwell, S. A., S. R. Jackson, K. S. Shahriari, T. P. Lynch, G. Sethi, S. Walker, K. Vosseller and M. J. Reginato (2010). "Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1." Oncogene 29(19): 2831-2842.

10. Mi, W., Y. Gu, C. Han, H. Liu, Q. Fan, X. Zhang, Q. Cong and W. Yu (2011). "O-GlcNAcylation is a novel regulator of lung and colon cancer malignancy." Biochim Biophys Acta 1812(4): 514-519.

11. Fardini, Y., V. Dehennaut, T. Lefebvre and T. Issad (2013). "O-GlcNAcylation: A New Cancer Hallmark?" Front Endocrinol (Lausanne) 4: 99.

12. Ji, S., J. G. Kang, S. Y. Park, J. Lee, Y. J. Oh and J. W. Cho (2011). "O-GlcNAcylation of tubulin inhibits its polymerization." Amino Acids 40(3): 809-818.

13. Tashiro, E., A. Tsuchiya and M. Imoto (2007). "Functions of cyclin D1 as an oncogene and regulation of cyclin D1 expression." Cancer Sci 98(5): 629-635.

14. Bockstaele, L., K. Coulonval, H. Kooken, S. Paternot and P. P. Roger (2006). "Regulation of CDK4." Cell Div 1: 25.

15. Paternot, S., L. Bockstaele, X. Bisteau, H. Kooken, K. Coulonval and P. P. Roger (2010). "Rb inactivation in cell cycle and cancer: the puzzle of highly regulated activating phosphorylation of CDK4 versus constitutively active CDK-activating kinase." Cell Cycle 9(4): 689-699.

16. Schachter, M. M., K. A. Merrick, S. Larochelle, A. Hirschi, C. Zhang, K. M. Shokat, S. M. Rubin and R. P. Fisher (2013). "A Cdk7-Cdk4 T-loop phosphorylation cascade promotes G1 progression." Mol Cell 50(2): 250-260.

17. Harwood, K. R. and J. A. Hanover (2014). "Nutrient-driven O-GlcNAc cycling - think globally but act locally." J Cell Sci 127(Pt 9): 1857-1867.

18. Bullen, J. W., J. L. Balsbaugh, D. Chanda, J. Shabanowitz, D. F. Hunt, D. Neumann and G. W. Hart (2014). "Cross-talk between two essential nutrient-sensitive enzymes: O-GlcNAc transferase (OGT) and AMP-activated protein kinase (AMPK)." J Biol Chem 289(15): 10592-10606.

19. Inaba, T., H. Matsushime, M. Valentine, M. F. Roussel, C. J. Sherr and A. T. Look (1992). "Genomic organization, chromosomal localization, and independent expression of human cyclin D genes." Genomics 13(3): 565-574.

20. Day, P. J., A. Cleasby, I. J. Tickle, M. O'Reilly, J. E. Coyle, F. P. Holding, R. L. McMenamin, J. Yon, R. Chopra, C. Lengauer and H. Jhoti (2009). "Crystal structure of human CDK4 in complex with a D-type cyclin." Proc Natl Acad Sci USA 106(11): 4166-4170.

21. Takaki, T., A. Echalier, N. R. Brown, T. Hunt, J. A. Endicott and M. E. Noble (2009). "The structure of CDK4/cyclin D3 has implications for models of CDK activation." Proc Natl Acad Sci USA 106(11): 4171-4176.

국문 요약

O-GlcNAcylation은 핵과 세포질 내에서 단백질에 일어나는 번역 후 수식화 중 하나이다. 이 수식화는 단백질의 발현과 기능을 조절하여, 세포 내 다양한 대사와 신호 전달에 관여하고 있다. 이 수식화의 *O*-GlcNAc 주개인 UDP-GlcNAc은 포도당으로부터 hexosamine biosynthetic pathway (HBP)를 통해 합성되며, 그 합성은 세포의 포도당 농도 환경에 크게 영향을 받는다. 또한, *O*-GlcNAcylation은 암, 당뇨, 알츠하이머 병 등 다양한 질병들에도 관여하고 있음이 알려져 있다.

암세포가 정상세포에 비해 높은 *O*-GlcNAcylation 상태를 가지며, *O*-GlcNAcylation이 세포 주기 조절에 관여하고 있다는 여러 보고에 따라, 우리는 암세포의 높은 *O*-GlcNAcylation이 세포 주기 전환을 촉진한다는 가설을 세웠다. 그것을 확인하기 위해, 폐암 세포주인 A549를 가지고 OGT가 감소된 안정 세포주를 만들었다. FACS 분석를 통해 이 세포들의 G1/S 세포 주기 전환이 상대적으로 느려진 것을 확인하였다. 그리고 Western blot을 통해 이러한 G1/S기 전환 저해가 pRB 단백질의 인산화 감소로 인한 것임을 확인하였다.

O-GlcNAcylation 감소와 pRB의 인산화 감소 간의 연관성을 더 깊이 이해하기 위해, pRB의 인산화에 관련된 단백질인 cyclin-dependent kinase 4 (Cdk4)가 *O*-GlcNAcylation 되는지 확인해보았다. Western blot을 통해, 과발현된 Cdk4에서 *O*-GlcNAcylation이 탐지되는 것을 확인하였고, 그것이 OGT를 과발현시켰을 때 더 증가하는 것을 확인하였다. 또한, 단백질의 *O*-GlcNAc

부분와 결합하는 sWGA 렉틴 침강법을 이용해, 세포의 G1/S기 전환동안 Cdk4의 *O*-GlcNAcylation이 조절되는 것도 확인하였다.

이러한 Cdk4의 *O*-GlcNAcylation이 가지는 기능을 밝히기 위해, G1/S기 전환에 있어서 핵심적인 역할을 하는 Cdk4와 cyclin D의 결합에 초점을 맞추었다. 과발현된 Cdk4와 cyclin D의 공동 면역침강법을 통해, OGT를 과발현 시켰을 때 Cdk4와 cyclin D1, D2 간의 결합이 증가하는 것을 확인하였다. 이번 연구를 통해, 우리는 G1/S 세포 주기 전환이 Cdk4의 *O*-GlcNAcylation에 의해 조절되며, 이 수식화가 Cdk4와 cyclin D의 결합을 증가시킨다는 것을 밝힘으로써, 증가된 암세포의 *O*-GlcNAcylation이 암세포의 증식에 기여하는 기작 중 하나를 제안하였다.

Keywords : O-GlcNAc, cancer cell, cell cycle, OGT, G1/S transition, pRB phosphorylation, Cdk4, cyclin D