Functional Study of *O*-GlcNAc Modification on Extracellular Signal-Regulated Kinase 2

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Functional Study of *O*-GlcNAc Modification on Extracellular Signal-Regulated Kinase 2

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This certifies that the master's thesis
of Ji Young Yoon is approved.

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감사의 글

아마도 당 생물학 연구실의 일원으로서는 마지막 실험이 될 Cell counting을 마치고 컴퓨터 앞에 앉아 글을 씁니다. 오늘로 2015년도 이틀이 남았습니다, 한 해의 끝에 서서 지난 4년을 되돌아보니 설레면서도 동시에 마음이 복잡합니다. 조금은 막연하고, 조금은 큰 꿈을 가지고 실험실에 처음 발을 딛게 된 지 4년, 그 꿈의 한 부분을 예쁘게 포장해서 세상에 내놓을 수 있게 되기까지 지도하고 도와주신 교수님과 곁에서 응원과 격려를 주셨던 분들께 서투르지만 감사의 말씀을 드리고 싶습니다.

먼저 저에게 *O*-GlcNAc의 세계를 알려주시고 연구자로서의 지금의 저를 있게 해주신 조진원 교수님께 진심으로 감사의 마음을 전하고 싶습니다. 3년간 연구를 수행하면서 교수님의 끝없는 배려와 실험에서 막힐 때마다 해주신 조언, 지도 덕분에 무사히 연구를, 그리고 학업을 마칠 수 있었습니다. 끝까지 믿어주신 덕분에 좋은 결과로 학업을 마무리할 수 있어 감사드립니다. 또한, 바쁘신 와중에 논문 심사에 응해 주시고, 실험에 대해 아낌없이 조언해 주시고 논문이 아름다운 형태로 빛을 볼 수 있게끔 도와주신 이태호 교수님, 김지현 교수님께 감사를 드립니다.

4년간 가장 가까운 곳에서 지낸 당 생물학 연구실 식구들에게도 감사의 인사를 하고 싶습니다. 처음 실험실 생활을 시작할 때 공부도 실험도 많이 도와주시고 가이드 해주신 한별오빠, 선택한 진로를 격려해주시고 졸업하는 데 있어 끝없는 지원을 주신 현규오빠, 조교님으로 만나 의지 되는 실험실 언니가 되어주신 인숙언니, 마지막까지 도와주셔서 감사한 연정언니, 입학하면 잘 챙겨주겠다고 한 약속을 지켜주신 은아언니, 자주 뵙지는 못했지만 중국에서 맥주를 나눠주신 양신언니, 그리고 졸업을 목전에 두고 한창 바쁠 동기 진원오빠, 의욕 만만하고 열심히 하는 게 보여 걱정이 안 되는 든든한 정화까지, 지면이 부족 할 정도로 많이 받은 배려와 도움 그리고 상냥함에 감사를 전하고 앞으로의 일에서 행복과 축복이 따르길 바랍니다. 또한, 함께 실험실 생활을 한 학부생 친구들, 그리고 그중에서도 PCR 트러블을 한 방에 날려준 미다스의 손 우택이에게 감사를 표합니다.

지금은 졸업하셨지만, 언제나 차근차근히 실험을 가르쳐 주신 호중 오빠, 이전 프로젝트로 고생할 때 많은 조언을 주셨던 수진 언니, 그리고 마지막으

로 실험 그리고 개인적인 고민거리에 기꺼이 귀를 빌려주신 주환 오빠에게 고마운 마음을 전하고 싶습니다.

To friends, who were along with me for the long-long ride through this chapter in my life. 바쁜 와중에도 재미있는 신작 영화가 나오면 언제나 시간을 내준 주은이, 그리고 신기하게도 연이 닿아 학부부터 대학원까지 같이 할 수 있었던 현진이에게도 고맙다는 말과 앞으로의 과학자로서의 무한한 성공을 바랍니다. 아직은 어색하지만 이제는 원오스님이라 불리는 노사, 수다 타임과 기도 덕분에 무사히 졸업할 수 있었습니다. To Bori, Panna and Agi, whose existence alone was so often a comfort during the difficult times, I give my heartfelt thanks.

그리고 서로 걷는 길은 달라도 10년째 good times and the bad times를 함께 해온 마마곰 보슬이, 천사의 보이스 선영이 그리고 CU 유민이와 이 기쁨을 함께하고 싶습니다. 학부 때부터 진로로 고민할 때 많이 신경 써주시고 제게 맞춤 조언과 격려를 아낌없이 주신 Underwood International College 은사님 Helen Lee 교수님께도 졸업의 기쁨을 나누며 감사를 드리고 싶습니다.

사랑하는 부모님에게 아주 고맙고 또 사랑한다고 전하고 싶습니다. 수학 때문에 고전하던 제가 이과로 진학해서 많이 걱정하셨지만 그래도 전폭적인 지지와 격려를 해주셨기 때문에 오늘 이 글을 쓸 수 있게 되었습니다. 언제나 재미있는 일이 생기면 제일 먼저 알려줘서 생활에 활력을 불어넣어 준 사랑하는 지예에게도 고맙다는 말을 전합니다. 또한 귀여운 토미에게도 고마움을. 그리고 언제나 관심을 두고 격려해준 먼 가족분들과 사촌들, 귀염둥이 아기 사촌들에게도 감사드립니다.

글재주가 부족하여 마음을 전하는데 충분한지 모르겠습니다만, 글자 사이에 담긴 진심이 전해졌기를 바랍니다. 이렇게 올해로 대학원생 연구자로서의 가운을 벗고 새로운 인생의 장이 펼쳐지길 기대해봅니다. 힘들었던 만큼 좋아했기 때문에 보람찬 4년이었고, 앞으로도 생물학을 계속 접할 수 있는 길을 걸을 수 있었으면 좋겠다고 생각하며 이만 줄이겠습니다.

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Abbreviations

CRS/CD: cytosolic retention sequence/common docking domain

ERK2: extracellular signal-regulated kinase 2

GFAT: Glutamine:fructose-6-phosphate amidotransferase

HBP: hexosamine biosynthetic pathway

IP: immunoprecipitation

MAPK: mitogen-activated protein kinase

MEK1: mapk/erk kinase 1

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

OGA: *O*-GlcNAcase

OGT: *O*-GlcNAc transferase

sWGA: succinylated wheat germ agglutinin

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

Abstract

O-GlcNAcylation is a post-translational modification on nuclear and cytoplasmic proteins, attached to and removed from serine or threonine residues by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA). *O*-GlcNAcylation is known to affect various cellular mechanisms, one of which is cell proliferation. The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway is involved in cell proliferation, and activation of this pathway if known to increase OGT expression and *O*-GlcNAcylation.

This study aims to identify presence of *O*-GlcNAcylation in the MAPK pathway, specifically on ERK2, and determine the function of ERK2 *O*-GlcNAcylation in the pathway. Here we discovered that OGA inhibition increased nuclear localization of ERK2, indicating a relevance between the MAPK pathway and *O*-GlcNAcylation. Through immunoprecipitation and lectin precipitation of ERK2, we identified *O*-GlcNAcylation on both endogenous and exogenous ERK2. Co-immunoprecipitation results of MEK1 and ERK2 showed that OGT induced *O*-GlcNAcylation change corresponded to an increase in ERK2-MEK1 interaction. Examination of subcellular fractionation results showed that upon OGT overexpression, ERK2 nuclear localization was decreased.

In conclusion, we have observed that ERK2 is *O*-GlcNAcylated, and that this modification increased the interaction between ERK2 and MEK1, leading to decreased nuclear localization of ERK2 in HEK293 cells.

Keywords: *O*-GlcNAc, OGT, ThiaMet-G, MAPK pathway, cell proliferation, ERK2, nuclear localization

Chapter 1. Introduction

A crucial modulator in a multitude of cellular mechanisms, *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) modification – also known as *O*-GlcNAcylation – is a post-translational single sugar modification occurring on serine and threonine residues of nuclear and cytoplasmic proteins[1].

O-GlcNAc is dynamically attached to and removed from proteins in response to cellular cues, through the functions of *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA)[1]. 2-3% of glucose entering the cell is processed by the hexosamine biosynthetic pathway (HBP) to produce uridine 5'-diphospho-*N*-acetylglucosamine (UDP-GlcNAc). This UDP-GlcNAc is used as substrate by OGT to modify proteins, resulting in *O*-GlcNAcylation on proteins[2,3,5]. Since *O*-GlcNAc modification can occur on serine and threonine residues, it can reciprocally interplay with phosphorylation. The *O*-GlcNAcylation may occur on the same site as a phosphorylation or an adjacent site to a phosphorylation[1,4]. Through this reciprocal relationship the two modifications can either positively or negatively affect one another's modification and function[4].

Since UDP-GlcNAc, a substrate for *O*-GlcNAcylation is composed of many molecules related to energy, *O*-GlcNAc is thought to function as a nutrient sensor[3,5,6]. It is also implicated in a variety of cellular mechanisms, one of which is cell proliferation[7]. In idiopathic pulmonary arterial hypertension (IPAH) disease cells which exhibit increased cell proliferation, the activity of HBP was also found to be augmented. Down regulation

of OGT through siRNA knockdown in these cells resulted in decreased smooth muscle cell proliferation[8]. In a different research it was reported that cells lacking the HBP rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) showed not only decreased *O*-GlcNAc but also decreased cell proliferation[9]. Thus we sought to understand the impact of *O*-GlcNAcylation on the pathways regulating cell proliferation.

In order to study the relevance of *O*-GlcNAcylation to cell proliferation, the MAPK/ERK signaling pathway was chosen. The MAPK-ERK pathway is a cellular signaling pathway consisting of a kinase cascade that transmits signals initiated by extracellular stimuli (ex. growth factor, cytokines) to their intracellular targets. The pathway is most prominently known to be involved in cell proliferation[7].

Three kinases Raf, MEK1/2 and ERK1/2 relay signals received from membrane receptors (ex. receptor-linked tyrosine kinases) by phosphorylating their downstream kinases. The kinase cascade ends with the phosphorylation of ERK1/2 which is then activated[10]. The activated ERK1/2 goes on to phosphorylate its cytosolic targets, or localizes to the nucleus to phosphorylate its nuclear targets such as transcription factors (ex. c-Fos), enabling production of proteins which prepare the cell for proliferation[10,11].

The kinases ERK1 and 2 possess 84% sequence identity, with the two isoforms distributed differently across tissues[12]. However, it has been studied that while ERK1 knock out in mice was not detrimental to growth, ERK2 knock out led to an early embryonic death[13,14,15]. These researches have supported the claim that ERK2 was capable of substituting for ERK1. Therefore we chose ERK2 as our matter of study.

Human ERK2 is activated upon phosphorylation at theronine 185 and tyrosine 187 by MEK. Following activation, threonine 190 is phosphorylated by autocatalysis[16,17]. The phosphorylated ERK2 can then be localized to the nucleus to carry on phosphorylation of its substrates[18].

The relationship between the MAPK pathway and *O*-GlcNAc has not yet been studied in depth. One recent research showed that activation of the MAPK pathway through drugs led to an increase in OGT expression and consequently to an increase in *O*-GlcNAcylation[19]. We were curious as to whether a reverse relationship existed, where an increase in *O*-GlcNAcylation would exercise an effect on the MAPK pathway.

In this study we found that ERK2, the last kinase of the MAPK pathway, is *O*-GlcNAcylated. Under the conditions of increased ERK2 *O*-GlcNAcylation, interaction between ERK2 and MEK1 was found to be increased. From our experimental results we hypothesize that this interaction, enhanced by increased *O*-GlcNAc, leads to a decrease in nuclear localized ERK2

Chapter 2. Materials and Methods

2.1 Cell culture

HEK293 cells were cultured in 25mM Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) supplemented with 10% foetal bovine serum at 37°C in 5% CO_2. Cells were cultured every 48 hours, seeded at 1:5 dilutions.

2.2 Cell counting

HEK293 cells were seeded in 6-well plates, $10x10^4$ cells per well. After treatment the cells were incubated with 0.25% Trypsin-EDTA (Gibco) for 5 minutes and then harvested. The cells were re-suspended in 25mM Dulbecco's modified Eagle's medium. 1:1 ratio diluted cell solution was prepared by mixing with 0.4% Trypan blue stain (Gibco) and incubating for 5 minutes. The stained cells were then transferred to Neubauer improved chamber (Marienfeld) and viable cells were counted under OLYMPUS CK2-TRC microscope. The number of cells in 0.1ul of cell solution was obtained by counting four large squares, then dividing the sum of the four cells by 4, and multiplying by dilution factor 2. The number of cell per ml was obtained by multiplying the cell number from previous calculation with 10^4.

2.3 DNA plasmids and transfection

DNA plasmids used in the experiments are the following. FLAG-ERK2 and FLAG-

OGT were cloned into p3xFLAG-CMV™-7.1 Expression Vector (Sigma-Aldrich, St Louis, MO). Untagged pCMV-OGT was used in overexpression. MEK1 was cloned into pRK5 in frame with an N-terminal Myc epitope. For transfection polyethylenimine (Sigma-Aldrich, St Louis, MO) was used, at ratio of 3µl 1x polyethylenimine for transfection of 10µg DNA plasmid.

2.4 Cell lysis, cell fractionation

Cells were lysed using NET buffer (150 mM NaCl, 1% Nonidet P-40 [NP-40], 50 mM Tris-HCl and 1 mM EDTA, pH 8.0), supplemented with Protease Inhibitor Cocktail (Roche, Mannheim, Germany), 1mM DTT, 5mM NaF and 1mM Na_3VO_4, and incubated on ice for 30 minutes with vortexing every 5 minutes.

Subcellular fractionation was carried out using hypotonic Buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM DTT) and extraction Buffer C(20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 10% glycerol). Both buffers were supplemented with Protease Inhibitor Cocktail (Roche, Mannheim, Germany), 1mM DTT, 5mM NaF and 1mM Na_3VO_4. HEK293 cells were rinsed with PBS, then suspended in Buffer A. After 15 minute incubation in ice, the cells were vortexed with 0.5% NP-40. The extracts were centrifuged at 4000rpm, 4 min., 4°C. The cytoplasm fraction supernatant was placed in a new tube. The pellets were suspended in Buffer C and incubated on ice for 30 minutes with vortex every 5 minutes, then centrifuged at 15000rpm, 10 min., 4°C to obtain supernatant containing the nuclear

fraction.

Protein concentrations of the lysates were calculated by Bio-Rad protein assay (Hercules, CA). The lysates were mixed with 4xSDS sample buffer, and subjected to western blot.

2.5 Succinylated wheat germ agglutinin (sWGA) precipitation

For sWGA precipitation, 2~4mg of NET buffer cell lysates were incubated with PBS-washed agarose-sWGA (Vector Lab) for 6 hours at 4°C. The sWGA beads were washed 3 times with NET buffer (supplemented with protease inhibitor cocktail). Precipitates were eluted by addition of 2x SDS sample buffer and boiling.

2.6 Immunoprecipitation

For FLAG immunoprecipitation, 2~4 mg of NET buffer cell lysates were incubated with PBS-washed anti-FLAG M2 affinity Gel (sigma Aldrich) for overnight at 4°C. The anti-FLAG M2 affinity Gel was washed 3 times with NET buffer (supplemented with protease inhibitor cocktail). Precipitates were eluted by addition of 2x SDS sample buffer and boiling.

2.7 SDS-PAGE, Western blotting

The protein samples obtained from cell lysis, subcellular fractionation, sWGA lectin precipitation and immunoprecipitation were subjected to SDS-PAGE and transferred to

nitrocellulose membranes (Amersham, Piscataway, NJ). The samples were loaded onto 10–12% Bis-Tris NuPAGE gel (NOVEX, San Diego, CA) and separated according to size by electrophoresis and transferred to nitrocellulose membranes (GE Healthcare). The membranes were incubated in 5% skim milk TBS-T for 1 hour in room temperature and washed 3 times for 5 minutes by TBS-T. The membranes were then incubated with respective primary antibodies diluted to specific concentration in 1% skim milk TBS-T either overnight at 4°C or for 8 hours at room temperature. Again the membranes were washed 3 times for 5 minutes by TBS-T to remove excess antibodies. Finally the membranes were incubated with HRP-conjugated secondary antibodies diluted to 1:5000 in TBS-T for 1 hour in room temperature, and washed 3 times for 5 minutes by TBS-T. Immunofluoresence was detected using an ECL system (GE Healthcare, Bio-Science).

2.8 Reagents and antibodies

ThiaMet-G, provided by Dr Kwan Soo Kim (Yonsei University, Seoul, Korea) was used at 50μM concentration, with incubation time of 24 hours. Antibodies were used against ERK2 (D-2, mouse monoclonal, Santa Cruz, Dallas, Texas), FLAG (F-3156, mouse monoclonal, Sigma-Aldrich, St Louis, MO), cMyc (B-14, mouse monoclonal, Santa Cruz, Dallas, Texas), α-tubulin (TU-02, mouse monoclonal, Santa Cruz), lamin A/C (#2032, rabbit polyclonal, Cell Signaling, Beverly, MA) and *O*-GlcNAc detecting antibody CTD110.6 was purchased from Covance (Princeton, NJ). Prosi Prestained Protein Marker (P8500, GenDepot) was used to determine approximate size of western blots.

Chapter 3. Results

3.1 Inhibition of OGA affects MAPK pathway by changing cellular localization of Extracellular signal-regulated kinase 2 (ERK2)

To observe how change in global *O*-GlcNAcylation impacted the MAPK/ERK pathway, HEK293 cells were incubated with OGA inhibitor ThiaMet-G for 18hours at 50μM concentration and lysed to obtain nucleus, cytoplasm and total lysate fractions. The samples were then separated by SDS-PAGE and Western blot was carried out. The amount of ERK2 in each subcellular fraction was evaluated by immunoblotting with ERK2 antibody (Fig.1A). Under increased *O*-GlcNAcylation conditions the ratio of the ERK2 localized to the nucleus was found to increase by 2.89 times compared to the ratio of ERK2 localized to the nucleus in control cells (Fig.1B).

ThiaMet-G treatment also influenced proliferation of HEK293 cells. 24 hours following treatment with ThiaMet-G, the cells were harvested using trypsin and stained with 0.4% Trypan blue. The number of cells were counted using Neubauer improved counting chamber. We found that ThiaMet-G treated HEK293 cells showed greater cell proliferation compared to non-treated cells (Fig.1C).

Through these experiments we showed that OGA inhibition by ThiaMet-G increased nuclear localization of ERK2, as well as enhanced cell proliferation of HEK293. Since localization of ERK2 to the nucleus is a sign of MAPK pathway activity[18], thus we concluded that increased *O*-GlcNAc by inhibition of OGA affected the MAPK pathway.

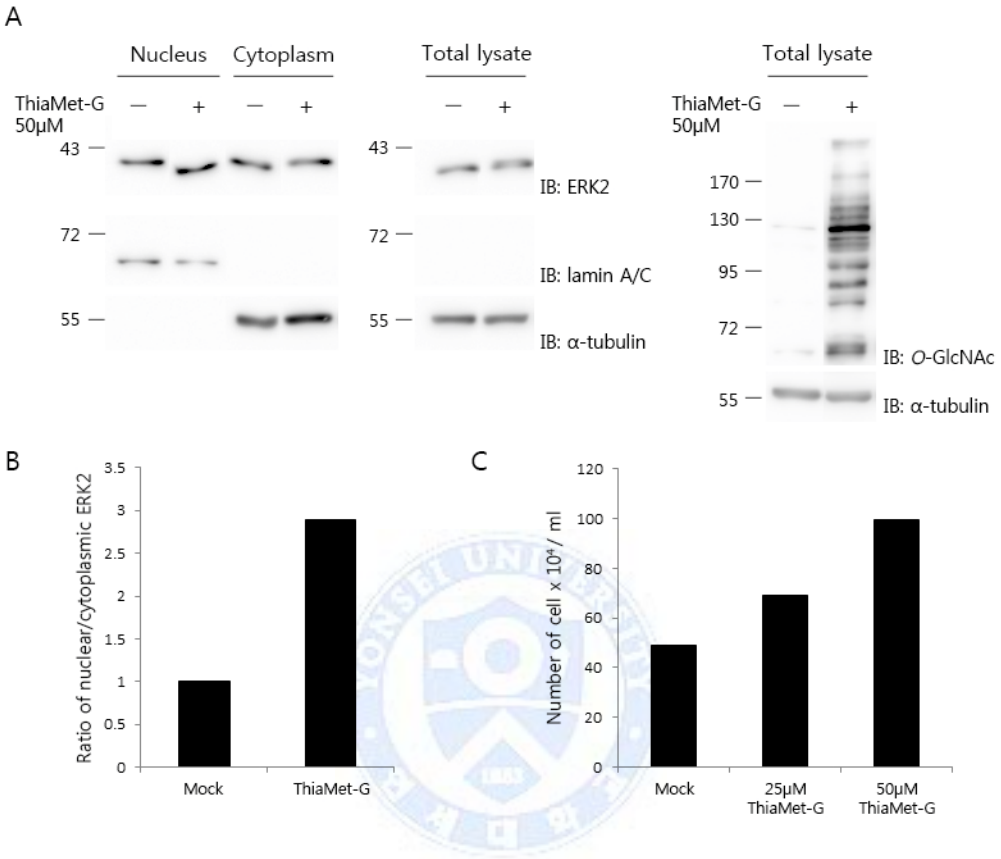

Figure 1. Inhibition of OGA affects MAPK pathway by changing cellular localization of Extracellular signal-regulated kinase 2 (ERK2). (A) HEK293 cells were treated with 50μM ThiaMet-G and incubated for 24 hours. The cells were harvested and subjected to fractionation to obtain nucleus, cytoplasm and total lysate fractions. Western blotting of fractions was done by ERK2, *O*-GlcNAc, lamin A/C, and α-tubulin. (B) The ratio of nuclear/cytoplasmic ERK2 was obtained by quantification and normalization. Nuclear ERK2 was normalized to the amount of lamin A/C, and cytoplasmic ERK2 was normalized to the amount of α-tubulin. The values are an average

obtained from two separate experiments. (C) HEK 293 cells were treated with 25μM or 50μM ThiaMet-G for 24hours. The cells were then treated with trypsin and counted using 0.4% Trypan blue staining and Neubauer improved counting chamber. The value indicates number of cell per ml.

3.2 ERK2 is *O*-GlcNAcylated under high glucose conditions in HEK293 cells

In the previous experiment, under increased *O*-GlcNAcylation condition, the intracellular distribution of ERK2 was found to be increased in the nucleus. To determine whether ERK2 is *O*-GlcNAcylated, lectin precipitation and immunoprecipitation were carried out on endogenous and exogenous ERK2.

HEK293 cells were transfected with pCMV-OGT or treated with ThiaMet-G. The cells were lysed and the lysate was used in lectin precipitation using *O*-GlcNAc moiety binding sWGA beads. As a result, endogenous ERK2 was precipitated, and the amount of precipitated ERK2 increased under OGT overexpression and ThiaMet-G treatment conditions (Fig.2A). To confirm sWGA binding of ERK2, Flag-ERK2 and pCMV-OGT were co-expressed in HEK293 cells and precipitated with sWGA. Flag-ERK2 was precipitated with sWGA, and the amount of precipitated Flag-ERK2 increased under OGT overexpression condition (Fig 2B).

To confirm presence of *O*-GlcNAcylation on ERK2 using a more direct approach, immunoprecipitation by anti-FLAG M2 affinity Gel was carried out on cells transfected with Flag-tagged ERK2 and pCMV-OGT. Immunoprecipitated Flag-ERK2 was immunoblotted using *O*-GlcNAc antibody CTD 110.6. *O*-GlcNAcylation was detected on Flag-ERK2 and found to be increased under OGT overexpression. (Fig. 2C)

Through these experiments *O*-GlcNAcylation on both endogenous and exogenous ERK2 was observed, and found to be increased under *O*-GlcNAc enhanced conditions.

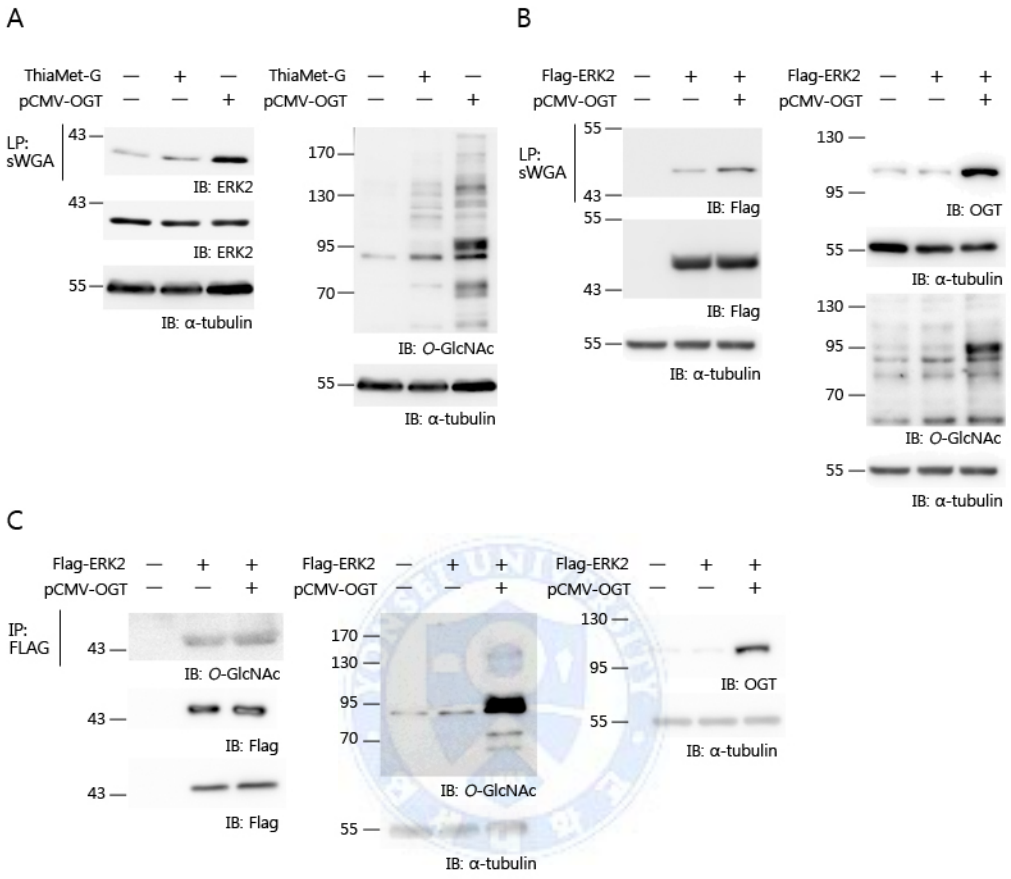

Figure 2. ERK2 is *O*-GlcNAcylated under high glucose conditions in HEK293 cells. (A) HEK293 cells were transfected with pCMV-OGT or treated with 50μM ThiaMet-G. Endogenous ERK2 was precipitated from lysates using sWGA. Western blotting was done by ERK2, *O*-GlcNAc and α-tubulin. (B) Flag-ERK2 was transfected into HEK293 cells together with pCMV-OGT. Each cell lysate was immunoprecipitated with anti-FLAG M2 affinity Gel. *O*-GlcNAc was detected by CTD110.6 antibody. Western blotting was done by Flag, *O*-GlcNAc, OGT and α-tubulin. (C) Flag-ERK2 was transfected into

HEK293 cells together with pCMV-OGT. Flag-ERK2 was precipitated from lysates using sWGA. ERK2 was detected by Flag antibody. Western blotting was done by Flag, OGT, *O*-GlcNAc and α-tubulin.

3.3 Increased *O*-GlcNAcylation on ERK2 increases interaction between ERK2 and MAPK/ERK Kinase 1 (MEK1)

After determining *O*-GlcNAcylation of ERK2 (Fig.2.), we aimed to determine the function of the modification. ERK2 is known to interact with its upstream kinase MEK1 through its cytosolic retention sequence/common docking domain (CRS/CD), and MEK1 with ERK through its D domain (Lysine3, Lysine 4, Lysine 5)[20,21]. Besides acting as upstream kinase to ERK2, MEK1 has been identified as a cytosolic retention molecule to ERK2[20]. Increase in the amount of MEK1 is known to sequester ERK2 to the cytosol[20].

To observe the effect of increased *O*-GlcNAc on the interaction between ERK2 and MEK1, HEK293 cells were overexpressed with Flag-ERK2, Myc-MEK1 and pCMV-OGT. The transfected cells were then immunoprecipitated using FLAG bead and subjected to Western blotting. The amount of MEK1 interacting with ERK2 was detected using Myc antibody. We found that Immunoprecipitated ERK2 was indeed interacting with MEK1, and that the amount of MEK1 interacting with ERK2 increased upon OGT overexpression (Fig.3).

As a result we determined that increased *O*-GlcNAcylation by OGT expression, which increased *O*-GlcNAcylation on ERK2, enhanced the interaction between ERK2 and MEK1.

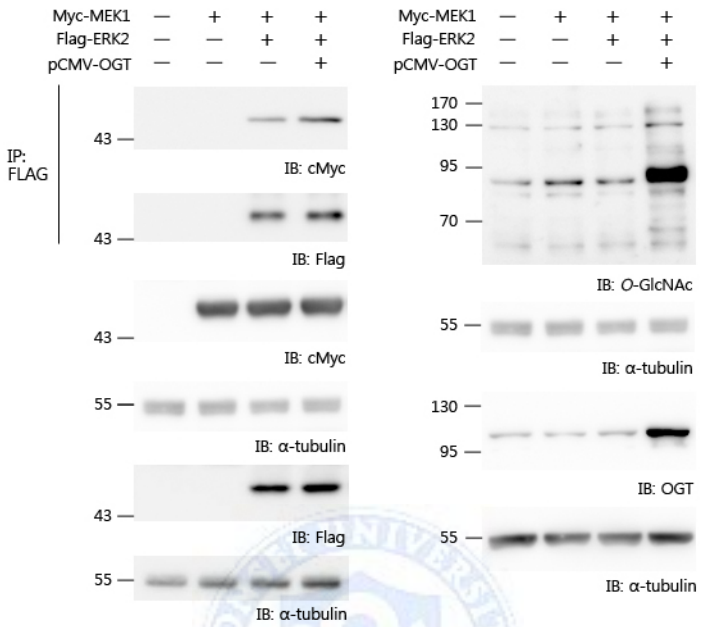

Figure 3. Increased *O*-GlcNAcylation on ERK2 increases interaction between ERK2 and MAPK/ERK Kinase 1 (MEK1). Myc-MEK1 and Flag-ERK2 were overexpressed together with pCMV-OGT in HEK293 cells. Each cell lysate was immunoprecipitated with anti-FLAG M2 affinity Gel. Flag antibody was used to detect ERK2, and cMyc antibody was used to detect MEK1. OGT, *O*-GlcNAc and α-tubulin were immunoblotted using respective antibodies.

3.4 Nuclear localization of ERK2 is decreased under increased *O*-GlcNAc modification conditions

MEK1 plays a role in both promoting and impeding nuclear translocation of ERK2, by means of phosphorylation and molecular interaction[10,20,21]. Because an increase in *O*-GlcNAcylation by OGT overexpression was found to enhance molecular interaction between ERK2 and MEK1 (Fig.3.), we examined whether this change resulted in a change in the cellular distribution of ERK2.

HEK293 cells were each transfected with FLAG-OGT or MEK1. The cells were put through subcellular fractionation to obtain nucleus, cytoplasm, and total lysate fractions. The samples were then Western blotted for endogenous ERK2 using ERK2 antibodies. We observed that the positive control MEK1 overexpression did decrease nuclear localization of ERK2. Nuclear localization of ERK2 was also decreased under OGT overexpression conditions (Fig.4A,B).

From these results we could extrapolate that OGT overexpression condition, which increased the interaction between ERK2 and MEK1, lead to cytosolic retention of ERK2 without increase in total amount of MEK1.

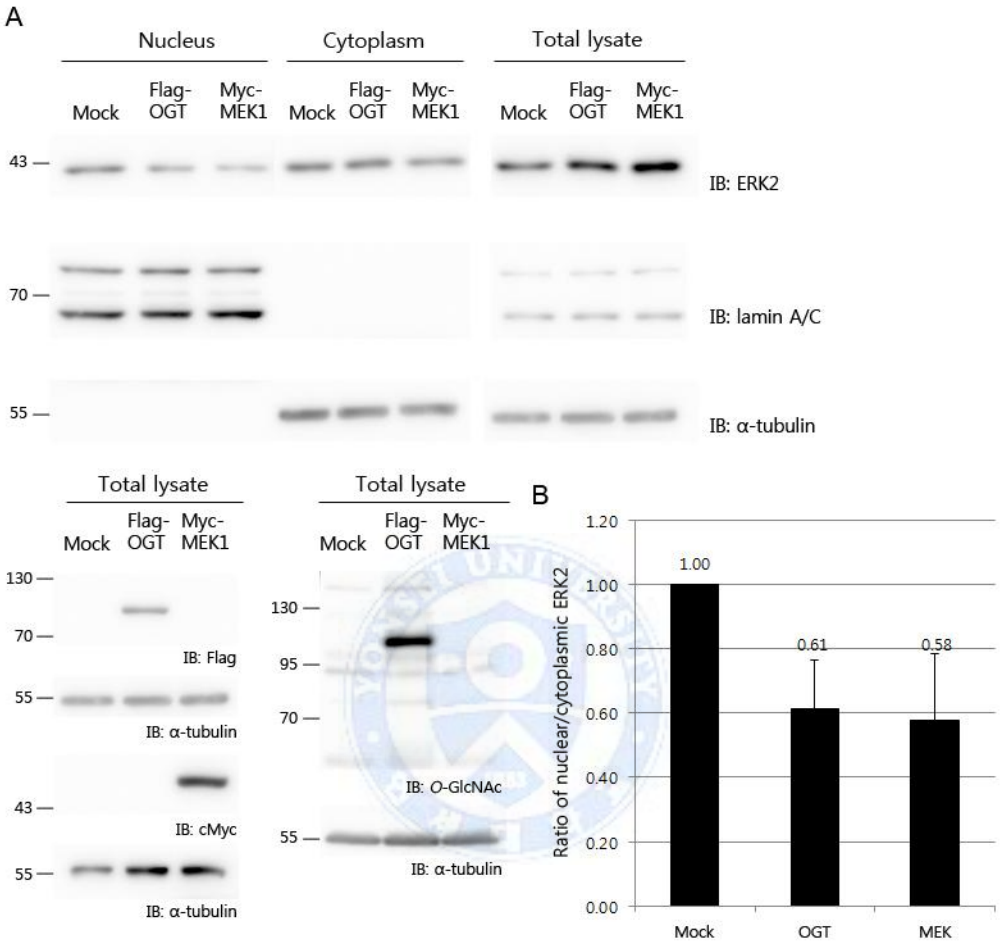

Figure 4. Nuclear localization of ERK2 is decreased under increased *O*-GlcNAc modification conditions. (A) HEK293 cells were transfected with vehicle (Mock), FLAG-OGT and Myc-MEK1 each. Cells were subjected to subcellular fractionation to obtain nucleus, cytoplasm and total lysate cell fractions. Western blotting of subcellular fractions was done by ERK2, lamin A/C, α-tubulin, Flag, cMyc and *O*-GlcNAc antibodies. (B) The ratio of nuclear/cytoplasmic ERK2 was obtained by quantification

and normalization. Nuclear ERK2 was normalized to the amount of lamin A/C, and cytoplasmic ERK2 was normalized to the amount of α-tubulin. The values are an average obtained from two separate experiments.

Chapter 4. Discussion

The prospect of a reciprocal relationship existing between *O*-GlcNAc and the MAPK kinase pathway was highly probable, considering the function of *O*-GlcNAcylation as glucose sensitive nutrient sensor[3,5,6]. However, the effect of changed *O*-GlcNAc on the MAPK pathways has not yet been studied in detail, thus this research aimed to ascertain whether an association existed between the two.

In this study, we demonstrated that the MAPK pathway is influenced by OGA inhibitor ThiaMet-G-induced change in *O*-GlcNAcylation, in the form of increased nuclear localization of ERK2. Since ERK2's cellular distribution was affected by ThiaMet-G, we examined whether ERK2 was *O*-GlcNAcylated. ERK2 *O*-GlcNAcylation was detected and this modification was successfully linked to a change in ERK2 function. Under ERK2 *O*-GlcNAcylation conditions the interaction between ERK2 and MEK1 was found to be enhanced. Furthermore, since MEK1 could function as cytosolic retention molecule to ERK2[20], we hypothesized that *O*-GlcNAc enhanced ERK2-MEK1 interaction may retain ERK2 in the cytosol. The Western blot of cellular fractionation confirmed our hypothesis, with more ERK2 retained in the cytosolic fractions under OGT overexpression conditions.

Combining the results of this research we came to a tentative conclusion that the MAPK pathway was influenced by change in *O*-GlcNAcylation, and that the pathway's final effector kinase, ERK2 was modified with *O*-GlcNAc. The *O*-GlcNAcylation on

ERK2 enhanced ERK2-MEK1 interaction, possibly leading to decreased nuclear localization of ERK2.

During the research we faced several complications. The first was the conflicting subcellular fractionation result of OGA inhibitor ThiaMet-G and OGT overexpression conditions. While both ThiaMet-G and OGT overexpression resulted in increased global *O*-GlcNAc, the effects of the two conditions on the nuclear-cytosolic localization of ERK2 were markedly different. ERK2 was dominantly localized to the nucleus under ThiaMet-G treatment (Fig.1A), whereas it was dominantly retained in the cytosol under OGT overexpression conditions (Fig.3A). We hypothesized that although both ThiaMet-G treatment and OGT overexpression resulted in increased global *O*-GlcNAcylation, because the two conditions employ different mechanisms to achieve the result, ERK2 localization was affected differently. ThiaMet-G enhances global *O*-GlcNAcylation by inhibiting OGA from removing existing *O*-GlcNAcylation, whereas OGT overexpression increases total OGT proteins allowing *O*-GlcNAcylation of yet unmodified proteins. Our hypothesis is that the *O*-GlcNAcylation on ERK2 which enhanced interaction with MEK1 and subsequently influenced cytosolic retention is newly gained from OGT overexpression, but not from ThiaMet-G treatment. In order to confirm this hypothesis, identification of ERK2 *O*-GlcNAcylation by Mass spectrometry is required. By comparing ERK2 under ThiaMet-G treatment and OGT overexpression conditions, it may be possible to determine whether ERK is differently modified under the two circumstances, which would shed light on the differing subcellular fractionation result of

this research.

The second limitation of this study was the effectiveness of coimmunoprecipitation as a method for studying interaction between two proteins, in our case ERK2 and MEK1. While we were successful in detecting MEK1 on immunoprecipitated ERK2, we could not achieve the reverse. Immunoprecipitation of MEK1 using A/G beads and Myc antibody was successful, but due to an excess of background we could not detect the presence of ERK2.

Third, while interaction between ERK2 and MEK1 increased under OGT overexpression (Fig.3), we cannot conclude that the *O*-GlcNAcylation on ERK2 alone functioned in enhancing ERK2-MEK1 interaction. To be certain that the increase in ERK2 *O*-GlcNAcylation was crucial to enhanced ERK2-MEK1 interaction, an *in vitro* binding assay (ex. GST pull-down) is required to eliminate all other cellular variables.

Though limitations existed, the research fulfilled its aim of establishing a connection between *O*-GlcNAcylation and the MAPK pathway, through its study focused on ERK2. Our findings suggest that an influencing relationship exists between *O*-GlcNAcylation and the MAPK pathway, which through the regulation of ERK2 cellular localization, may possibly be further implicated in influencing cell proliferation.

Chapter 5. Summary

1. When global *O*-GlcNAcylation is increased by OGA inhibition, ERK2 is dominantly localized in the nucleus.

2. ERK2 is *O*-GlcNAcylated in HEK293, and *O*-GlcNAcylation increases under OGT overexpression.

3. When *O*-GlcNAcylation on ERK2 is increased, interaction between ERK2 and MEK1 is enhanced.

4. When global *O*-GlcNAcylation is increased by OGT overexpression, ERK2 is decreased in the nucleus.

Chapter 6. References

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국문 요약

Extracellular Signal-Regulated Kinase 2의 *O*-GlcNAc 수식화 기능 연구

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O-GlcNAcylation은 세포질과 핵에 존재하는 단백질들에 일어나는 post-translational 수식화의 한 종류이다. 이 수식화는 *O*-GlcNAc transferase (OGT)와 *O*-GlcNAcase (OGA) 단백질들의 작용을 통해 serine 또는 threonine 아미노산에 붙여지고 또한 제거된다. *O*-GlcNAcylation은 다양한 세포 내 대사와 신호전달에 관여하고 있고, 이 중 하나가 세포 증식이다. Mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) 신호전달체계는 세포 증식에 있어 중요한 역할을 하고, 이 신호전달 체계의 활성화는 OGT 발현 증가 및 *O*-GlcNAcylation 증가로 이어지는 것으로 밝혀져 있다.

본 연구는 MAPK 신호전달 체계에 속한 ERK2 단백질의 *O*-GlcNAcylation 여부를 규명하려 하였고, 또한 ERK2 단백질의 *O*-GlcNAcylation이 MAPK 신호전달 체계에서 어떠한 역할을 하는지 알아보자고 하였다. 우리는 OGA 저해제로 인해 ERK2가 핵에 집중되는 것을 확인하여 *O*-GlcNAcylation과 MAPK 신호전달 체계 사이에 연관성을 찾았다. Immunoprecipitation과 lectin precipitation을 통해 exogenous, 그리고 endogenous한 ERK2에 *O*-GlcNAcylation이 존재하는 것을 확인하였다. ERK2와 MEK1을 co-immunoprecipitation한 결과, OGT 과발현으로 인한 ERK2 *O*-GlcNAcylation 증가와 함께 ERK2-MEK1 사이의 상호작용이 증가함을 볼 수 있었다. 핵-세포질 분리 실험을 통해 OGT 과발현 조건에서 ERK2는 핵에서 줄어 드는 것을 확인하였다.

이 실험을 통해 우리는 ERK2가 *O*-GlcNAcylation 되어 있고, 또한 이 수식화증가로 인해 ERK2-MEK1 사이의 상호 작용이 증가하게 되어 궁극적으로는 HEK293 세포의 핵에서 ERK2가 줄어드는 것을 발견하였다.

Keywords: *O*-GlcNAc, OGT, ThiaMet-G, MAPK pathway, cell proliferation, ERK2, nuclear localization