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DYSREGULATION OF O-LINKED β-N-ACETYLGLUCOSAMINE (O-GLCNAC) CYCLING SUPPORTS TUMORIGENICITY OF CANCERS OF THE FEMALE REPRODUCTIVE TRACT

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BY

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DISSERTATION

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Original approval signatures are on file with the University of New Hampshire Graduate School.

TABLE OF CONTENTS

DEDICATION

John Lennon said, “A dream you dream alone is only a dream. A dream you dream together is reality.” It is with this sentiment that I dedicate this work to my family. To my husband, Justin, who has always viewed my dreams of becoming a scientist as a team effort. Thank you for never doubting my dedication or abilities, even when I doubted myself, and for taking a leap of faith with me to start a new life, far from what we knew. To our daughter, Fiona, for filling my evenings and weekends with joy and laughter; and for renewing my love for science through your endless wonder and excitement for the world around you. I must thank my parents, Mike and Kathy, for showing us what a loving and supportive marriage can be. And a special thank you to my mother, for being my earliest role model of a strong and independent women. Thank you to my sister, Jamie, who can cheer up even the most distraught graduate student with a light-hearted moment. And to all of my family and friends, old and new, who have followed me through this winding and difficult journey, you have my deepest appreciations for the many contributions you have made to my success in my quest for knowledge. A quote from Albert Schweitzer comes to mind, “Success is not the key to happiness. Happiness is the key to success. If you love what you are doing, you will be successful.” Thank you all for making me incredibly happy and enabling me to do what I love.

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A special thank you to, Dr. David H. Townson, for taking a chance on a new graduate student with limited laboratory experience. I am grateful for your patient mentorship and support over the past several years. I must also acknowledge the many undergraduate and graduate students with whom I have learned from and developed lasting relationships with. During my graduate studies I had the unique experience of studying at two Universities, both of which quickly felt like home. Kendall and Terrill Halls have both been wonderful, fun, and supportive places to learn and grow.

Finally, I must acknowledge my dissertation committee, who from near and far richly contributed to my education and growth as a scientist. Dr. Vern Reinhold once wrote to me, "In your research be aggressive, make mistakes and follow crazy thoughts." I hope you will see this spirit in my work, past, present, and future.

LIST OF FIGURES AND TABLES

INTRODUCTION

I had always intended on attending Veterinary School, but when my mother was diagnosed with an aggressive form of Breast Cancer in 2008 my eyes were opened to a career path I had not considered. In August of 2015, I enrolled in the Biochemistry Graduate Program at the University of New Hampshire with the personal goal of participating in cancer research. When I arrived on campus I began exploring the various research opportunities. At this time, I became familiar with the work of Research Professor, Dr. Vernon Reinhold who introduced me to the field of Glycomics. My previous experience with glycomics had been a single brief lecture in my undergraduate Biochemistry course. I was immediately intrigued by the vast and critical influence glycosylation could have on cell function and human health and surprised by the lack of attention these post-translational modifications received.

When I began my second rotation in the laboratory of Reproductive Physiologist, Dr. David Townson, I was presented with previous Masters Student, Brian Sullivan's, research project investigating the functional role of cytokeratin intermediate filaments 8/18 (K8/18), which had been previously noted to be up-regulated in more aggressive cervical cancers. Through Brian's work, the Townson group discovered that within the cervical cancer cell line, HeLa cells, two cellular phenotypes could be identified; those expressing K8/18 and those lacking K8/18 expression. The two phenotypes were separated by serial dilution in order to analyze various parameters of cell survival and immune system evasion. The study supported the hypothesis that reduced K8/18 expression increased cellular susceptibility to immune mediated death, specifically by the cytokines Fas Ligand (FasL) and Tumor Necrosis Factor α (TNF-α).

This work suggested that K8/18 filaments provided protection against immune mediated death, subsequently allowing for the progression of neoplastic disease.

This work led me to ask the question, what is the mechanism that provides this protection? Having just completed my rotation in the UNH Glycomics Center, I went to the literature to determine if, and how, K8/18 filaments are glycosylated. I soon discovered Keratin 18 filaments are highly phosphorylated and glycosylated. However, this protein is not glycosylated in the traditional branched chain formation but is O-linked β-N-acetylglucosaminylated (O-GlcNAcylated). This discovery led me down a winding and fascinating path into a relatively new and exciting area of science. I initially used Brian's work as a jumping off point and began investigating the role of O-GlcNAcylation in cervical cancer disease progression and metastasis. This led to my first publication, "O-GlcNAcylation enhances the tumorigenic properties of cervical cancer cells *in vitro*.(Jaskiewicz, Hermawan, Parisi, & Townson, 2017)" (Chapters 2 and 3).

Shortly after completing this work I was presented with the opportunity to move to the University of Vermont (UVM) with Dr. Townson and the laboratory. I eagerly accepted the offer and prepared to move. When we arrived at UVM, I spent several months working with Dr. Townson to establish the new laboratory and explore new opportunities at the University. Perhaps one of the most exciting components of this transition was the close proximity of the main campus to the University of Vermont College of Medicine. This opened up a number of resources that have furthered my research and training.

It was at this time that I contacted Dr. Elizabeth McGee, an OB/GYN research physician, for guidance with continuing my research. After consulting with Dr. McGee, it was decided that while the results of my first study were interesting and warranted further investigation, the clinical relevance of studying O-GlcNAcylation in cervical cancer was not clear. However, O-GlcNAcylation's connection to diabetes encouraged us to transition to an endometrial cancer cell model. Women with Type II Diabetes have a 2-3 fold increased risk of developing endometrial cancer, and mRNA analysis of endometrial tumors showed an increase in the O-GlcNAc cycling enzyme mRNA in the leading edge of tumors. This led to my second manuscript, "Hyper-O-GlcNAcylation promotes Epithelial-Mesenchymal Transition in endometrial cancer cells," which is currently in revision for the journal, *Oncotarget*. (Chapter 4). Finally, my interest extended from disease progression to possible treatment interference, and in my final manuscript I focused on the impact hyper-O-GlcNAcylation may have on the efficacy of progesterone treatment to reduce invasion and cell proliferation in endometrial cancer (Chapter 5).

Throughout my graduate student career, I was incredibly fortunate to be afforded the independence to build a research project that both supported my interest in women's health and facilitated an interdisciplinary research education. I hope that you will recognize the unique graduate experience that Dr. Townson supported and guided me through in the proceeding chapters. As my graduate student career draws to an end, I look forward to continuing to explore the fields of women's reproductive health and glycobiology as a lifelong learner.

ABSTRACT

DYSREGULATION OF O-LINKED β-N-ACETYLGLUCOSAMINE (O-GLCNAC) CYCLING SUPPORTS TUMORIGENICITY OF CANCERS OF THE FEMALE REPRODUCTIVE TRACT

by

Nicole Morin Jaskiewicz

University of New Hampshire, September, 2018

Hyper-O-GlcNAcylation of proteins is a subsequent artifact of metabolic disorder and is indicative of many cancers, including cancers of the female reproductive tract. While the incidence of most cancer types has been declining in the U.S., endometrial and cervical cancer remain among the most common cancers diagnosed in women. Diabetic women have a 2-3 fold increased risk of developing endometrial cancer, and tend to have more aggressive cases of cervical cancer, however, the molecular aspects of these risks are not fully understood. This study investigated the alteration of cellular O-GlcNAcylation of proteins as the potential mechanistic connection between diabetes and tumorigenicity in cancers of the female reproductive tract. The cervical cancer cell line (SiHa) and the endometrial cancer cell line (Ishikawa) were utilized to study the effect of dysregulation of O-GlcNAcylation on the proliferation, migration, invasion, and related molecular mechanisms. In cervical cancer, O-GlcNAcylation was found to be an important regulator of tumorigenicity. Overall, inhibition of O-GlcNAcylation (via the inhibitor, OSMI-1) in SiHa cells impaired cell proliferation ($p<0.01$) and invasion ($p<0.01$) yet did not affect cell cycle progression. These effects occurred

concomitantly with an alteration of cellular morphology, principally the disruption/decline of K8/18 and β-actin filament expression. The results suggest O-GlcNAcylation regulates several aspects of tumorigenesis in cervical cancer cells, and cytoskeletal proteins are among the targets. Similarly, in endometrial cancer cells, hyper-O-GlcNAcylation (via 1μM Thiamet-G/ThmG or 25mM Glucose) enhanced the expression of EMT-associated genes (WNT5B and FOXC2), and the E-Cadherin suppressor, Snail. Reorganization of actin filaments into stress filaments, consistent with EMT, was also noted in ThmG-treated cells. Interestingly, Hypo-O-GlcNAcylation (via 50 μM OSMI-1) also upregulated WNT5B, inferring that any disruption to O-GlcNAc cycling impacts EMT. However, Hypo-O-GlcNAcylation reduced cellular proliferation/migration and the expression of the pro-EMT genes (AHNAK, TGFB2, FGFBP1, CALD1, TFPI2). Finally, Ishikawa cells were used to investigate the effect hyper-O-GlcNAcylation on the efficacy of progesterone (P4) in therapy for endometrial cancer proliferation and invasion. Ishikawa cells were exposed to ThmG, to induce hyper-O-GlcNAcylation, and 100nM P4. P4 alone, significantly decreased cell proliferation, however, the addition of ThmG, and subsequent hyper-O-GlcNAcylation, negated this affect, returning the cells to control level proliferation ($p<0.05$). A similar pattern was noted in Matrigel invasion assays, where Hyper-O-GlcNAcylation augmented invasion compared to P4 treatment alone, both with and without progesterone treatment ($p<0.05$). Progesterone treatment has been shown to induce the expression of p21 and p27, reducing cell growth. In this study, P4 maintained p21 expression and increased p27 expression, however, ThmG decreased p27 expression and the expression of endogenous progesterone receptor B (PR B) despite P4 treatment. These results suggest that hyper-O-GlcNAcylation, common in obese and diabetic patients, may promote

tumorigenicity in female cancers and could impair the efficacy of progesterone treatment. O-GlcNAcylation has the potential to serve as a biomarker for early diagnosis and could predict treatment success. O-GlcNAc cycling enzyme inhibitors could prove to be useful tools for providers when treating cancer patients with metabolic disorders.

CHAPTER 1

LITERATURE REVIEW AND RESEARCH PROPOSAL

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Glycobiology

There are four major classes of macromolecules that come together to support all living organisms; nucleic acids, protein, lipids, and carbohydrates. While nucleic acids (transcription) and proteins (translation) have been the focus of substantial biological research, referred to as the Central Dogma of Biology, carbohydrates have long been down played as a mere means for energy storage. Despite the discovery of protein bound glycans in the 19th Century, the importance of glyco-proteins and glyco-lipids has only recently been realized by the scientific community (Schachter, 2001). While carbohydrates do serve the purpose of energy storage in the form of glucose and glycogen, protein glycosylation is considered one of the major forms of protein post-translational modification, massively contributing to the vast diversity of the proteome (Love et al., 2010).

Glycans, which are branched chain polysaccharides, can be bound to proteins by asparagine-linkage (N-linked) or serine/threonine linkage (O-linked). These branched chains are comprised of 6 different sugar moieties: mannose, GlcNAc, Neu5Gc, Galactose, Sialic Acid, and Fucose. N-linked and O-linked glycosylation are major structural components of both cell surface and secreted proteins, and significantly influence their structure and function (Varki et al., 2009). The biological significance of these structures is highlighted by debilitating glycan associated diseases (Freeze & Schachter, 2009; Varki et al., 2009). A complete lack of N-glycosylation is fatal, but several genetic defects resulting in partial N-glycan pathway dysfunction result in extensive disability including severe mental retardation, epilepsy, and

failure to thrive (Hudson H. Freeze, n.d.). In recent years, it has become clear that several forms of Muscular Dystrophy stem from under-glycosylation by O-mannose (Kanagawa & Toda, 2006). While our biological understanding of glycosylation has flourished in the past decades, there is still much to be learned about a unique form of O-linked glycosylation, β-N-acetylglucosamine (O-GlcNAc).

The history and biochemistry of O-GlcNAcylation

O-GlcNAcylation was first described by Hart, et al. in 1984. Originally investigating the presence of surface glycans with terminal GlcNAc residues, Hart et al. discovered evidence suggesting the "-presence of O-glycosidicly linked monosaccharides on many lymphocyte cell surface proteins (Torres & Hart, 1984). Additionally, they discovered a majority of these moieties appeared to be intracellular, thus a novel single moiety form of glycosylation was discovered. In the 34 years since this ground breaking publication, more than 1,400 peer reviewed papers discussing O-GlcNAc have been produced (Lefebvre & Issad, 2015), and while many of its important mechanisms have been determined, O-GlcNAcylation and its relationship to disease is still largely an enigma.

While we know substantially less about O-GlcNAcylation compared to some of its branched counterparts, it is surprising to note that O-GlcNAcylation is both ubiquitous and abundant in eukaryotic cells. To date, more than 600 O-GlcNAcylated proteins have been identified, and more are discovered each day. O-GlcNAcylation is a unique form of glycosylation in many ways. While glycosylation is generally an extra-cellular protein modification, O-GlcNAcylation occurs in nearly all intracellular compartments. It is found mostly within the nuclear and cytoplasmic compartments. Beyond its general abundance, O-

GlcNAcylation is remarkable for its highly dynamic nature, akin to phosphorylation as a post-translational modification (Wells et al., 2002). In contrast to phosphorylation, O-GlcNAc modification is regulated by just two highly-conserved enzymes. β-N-acetylglucosamine transferase (O-GlcNAc Transferase, OGT) is one of these enzymes and is responsible for adding the free form substrate UDP-O-GlcNAc to serine and threonine residues on proteins by beta-glycosidic linkage. Conversely, β-N-acetylglucosaminase (O-GlcNAcase, OGA) removes the modification from proteins (F. V Rao et al., 2006).

O-GlcNAc Transferase is heterotrimeric, consisting of two 110 kDa and one 78 kDa sub-units, encoded by the OGT gene, which is located on the X chromosome (Xq13). As mentioned previously, OGT is highly conserved from worms to humans, and complete inhibition of the enzyme is lethal (Shafi et al., n.d.). Three isoforms of the enzyme have been identified: a full length (ncOGT), a short length (sOGT), and a mitochondrial variant (mOGT). OGT is tyrosine phosphorylated, and is O-GlcNAcylated itself, possibly operating as its own regulator of expression (Aquino-Gil, Pierce, Perez-Cervera, Zenteno, & Lefebvre, n.d.). Comparatively, OGA is a hydrolase enzyme encoded for by the Meningioma Expressed Antigen 5 (MGEA5) gene, located on chromosome 10 (10q24.32) (Comtesse, Maldener, & Meese, 2001). There are 3 known isoforms of OGA; a full length (fOGA), a short length (sOGA), and a variant of OGA (vOGA) (Alonso, Schimpl, & van Aalten, 2014).

Current tools and techniques for studying O-GlcNAcylation

O-GlcNAcylation is described by glycobiologist, John Hanover, as the dark matter of the glycobiology field (Bond & Hanover, 2015). For nearly 60 years, O-GlcNAcylation has influenced cellular functions without first being identified. This modification evaded scientific

discovery for so long because of its small size and labile nature (Greis & Hart, 1998). For instance, cells have abundant, intrinsic hydrolases, which can remove O-GlcNAc during protein purification. Further, unlike charged post-translational modifications or PTMs, such as phosphorylation, O-GlcNAcylation has no charge and thus does not influence the migration pattern of a protein during gel electrophoresis. Additionally, the addition of an O-GlcNAc molecule (+203), typically does not impact the apparent molecular mass of a protein, unless there are many clustered O-GlcNAc modifications. These characteristics also make detection by mass-spectrometry difficult, as O-GlcNAc modifications tend to be lost during ionization; the free O-GlcNAc is then overshadowed by abundant peptides (Varki et al., 2009; Zhongping Tan and Lai-Xi Wang, 2017; Trinidad, et al., 2012).

While O-GlcNAcylation presents many structural and analytical challenges, several techniques have been developed to overcome these. For example, the development of a monoclonal antibody for O-GlcNAc modified proteins (CTD 110.6) facilitated the efficient detection and purification of O-GlcNAcylated proteins (Whelan & Hart, 2006). There are also several tools for the manipulation of O-GlcNAcylation, such as small molecule inhibitors of OGA and OGT, cell culture supplementation of glutamine and glucose, small interfering (siRNA) or short hairpin RNA (shRNA), and now CRISPR/Cas9 (Banerjee, Hart, & Cho, 2013; Esko & Bertozzi, 2009; E. J. Kim, Bond, Love, & Hanover, 2014).

Pharmacological inhibitors of OGA exist, N-Acetylglucosaminono-1,5-lactone O-(phenylcarbamoyl)oxime (PUGNAc) (Horsch, Hoesch, Vasella, & Rast, 1991), and Thiamet-G (Yuzwa et al., 2012) are those most notable. The OGA inhibitors PUGNAc has been widely used to induce a state of hyper-O-GlcNAcylation in a variety of cell types and cell culture systems (Ali Mehdy, Willy Morelle, Claire Rosnoblet, Dominique Legrand, Tony Lefebvre,

2011; Seung Yoon Park, Ryu, & Lee, 2005; Vosseller, Wells, Lane, & Hart, 2002). While both OGA inhibitors are effective, Thiamet-G has been identified as the more specific inhibitor as it has higher Ki value for OGA than PUGNAc, and is a more stable bioactive inhibitor of OGA (Banerjee et al., 2013; Yuzwa et al., 2008).

On the other hand, the development of reliable OGT inhibitors has been extremely difficult. For instance the drug Alloxan, often used to induce diabetes in mice (Moley, Pellicer, & Decherney, n.d.), also has OGT inhibitory effects (Konrad et al., 2002). However, Alloxan was known to be cytotoxic and has many off target effects beyond the inhibition of OGT, such as the "formation of the reactive oxygen species (ROS) superoxide anions (O_2-), hydroxyl radical ($OH\cdot$) and hydrogen peroxide (H_2O_2), causing cell damage through a number of complex interactions probably involving several different cellular structures" (Zhang, Gao, & Brunk, 1992), and for this reason is not considered a suitable OGT inhibitor. After this, Ac4-5S-GlcNAc and BADGP, mimics of the OGT donor substrate UDP-GlcNAc were used, however they caused indirect inhibition of other glycotransferases, impacting cell surface glycan expression (Liu et al., 2017). In the interim, the chemotherapeutic drug, 6-Diazo-5-oxo-L-norleucine (DON) (Henry W. Dion, Salvatore A. Fusari, Zbigniew L. Jakubowski & Bartz, 1956) has some indirect potential. This drug suppresses the presence of UDP-GlcNAc by inhibiting the enzyme glutamine: fructose-6-phosphate-amido- transferase-1 (GFAT-1). The inhibition of GFAT-1, in turn, limits the catalytic ability of the Hexosamine Biosynthesis Pathway (HBP), of which the end product is the OGT substrate, UDP-GlcNAc (Marshall, Bacote, & Traxinger, 1991). More recently, the Walker laboratory at Harvard developed a small molecule inhibitor of OGT, OSMI-1, which effectively inhibits O-GlcNAcylation without impacting transmembrane protein glycosylation (Ortiz-Meoz et al., 2015). Around this same time, similar inhibitors were also

introduced to the market in the form of various small molecule and bi-substrate inhibitors (E. J. Kim et al., 2014; Trapannone, Rafie, & Van Aalten, 2016).

While pharmacological manipulation is an important tool for preliminary research, these small molecule inhibitors can have unintended consequences. Thus, molecular tools have become critical for advancing our knowledge. This includes the use of small interfering RNAs (siRNAs) and short hairpin RNAs (shRNA) to disrupt transcriptional/translational events (D. D. Rao, Vorhies, Senzer, & Nemunaitis, 2009), or for specific site knockout, point mutations (Butkinaree et al., 2008). Several studies have been published utilizing point mutation technology to eliminate an O-GlcNAc binding site of interest. In this technique, minor point mutations are made at the DNA or RNA level to result in the substitution of a neutral amino acid residue (typically alanine (Ala)) for a serine or threonine glycosylation site. In example, when O-GlcNAcylated serine 30, 31, and 49 of K8/18 filaments are replaced by alanine residues in transgenic mice, the non-glycosylated mutant mice are more susceptible to liver and pancreatic injury/apoptosis compared to wild-type mice. The loss of O-GlcNAc resulted in inactivation and reduced phosphorylation of Akt1 and Protein Kinase Cθ, suggesting that O-GlcNAcylation supports the phosphorylation of cell-survival kinases (N.-O. Ku, Toivola, Strnad, & Omary, 2010).

Cellular effects of O-GlcNAcylation

Because O-GlcNAc and O-phosphate both modify threonine and serine residues, they frequently compete with each other for binding. In fact, in many instances, O-GlcNAcylation inhibits phosphorylation (Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011). One mechanism to explain this phenomenon is the observation that OGT forms complexes with some phosphatases

(Chen et al., 2008; Wells, Kreppel, Comer, Wadzinski, & Hart, 2004), which presumably activates them and then cleaves phosphates from the targeted proteins. Inhibition can also be attained through direct competition of shared binding sites or steric hindrance in adjacent residues (Z. Wang et al., 2010). Additionally, instances have been noted where the presence of an O-GlcNAc moiety on one residue of a protein can impair phosphorylation at a distant site (Comer & Hart, 2000). One example of this is the cytoskeletal intermediate filament component keratin 18 (Tao et al., 2006), however the mechanism of inhibition is still unclear.

O-GlcNAcylation has a diverse set of cellular functions in that it can control protein stability (Sang Yoon Park et al., 2010; Srikanth, Vaidya, & Kalraiya, 2010a; Yuzwa et al., 2012), influence enzymatic activity, and affect protein-protein interactions, including transcription/translation (Hardivillé & Hart, 2014; Tarik Issad & Kuo, 2008), cell structure (Rotty, Hart, & Coulombe, 2010), cell cycle regulation (Jiang et al., 2016; Phie Tan, Duncan, & Slawson, n.d.) and perhaps most notably, metabolism (Jóźwiak, Forma, Bryś, & Krześlak, 2014a). The novelty of O-GlcNAcylation as a sentinel to cellular metabolism is the origin of its nucleotide sugar substrate, UDP-GlcNAc. UDP-GlcNAc is the end-product of the Hexosamine Biosynthesis Pathway (HBP). Approximately 2-5% of all glucose that enters the cell is shuttled into the HBP by the addition of glutamine to Frustose-6-Phosphate. Further, the fatty acid, Acetyl-CoA and the nucleotide, Uridine Tri-Phosphate (UTP) pathways converge to be incorporated into UDP-GlcNAc (Frank, 2012). Thus, the direct link of the HBP to glycolysis and the amino acid, fatty acid, and nucleotide metabolic pathways make O-GlcNAcylation the ideal nutrient-sensing modification. As such, the occurrence of hyper-O-GlcNAcylation is associated with the development of insulin resistance (IR) (Vosseller et al., 2002) and is a characteristic of Type 2 Diabetes (T2D) and many of its comorbidities (J. Ma & Hart, 2013). Today, many

scientists consider the development of IR as an adaptive mechanism to protect cells when nutrients, such as glucose and lipids, are too abundant.

In light of the above observations, it is hypothesized that a high prevalence of O-GlcNAcylation in cells provides the trigger for an IR response. Indeed, experimental overexpression of OGT, which in turn leads to hyper-O-GlcNAcylation, triggers IR in mice (Yang et al., 2008). Moreover, many of the effects of O-GlcNAc on intracellular signaling are attributed to the large number of transcription factors that are O-GlcNAcylated, which ultimately influence gene expression (Tarik Issad & Kuo, 2008).

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia. Type 1 Diabetes (T1D) stems from the failure of the pancreas to produce sufficient insulin, the peptide hormone that promotes the absorption of glucose in the cell. Type 2 Diabetes (T2D) is the failure of cells to respond properly to the insulin that is being produced, known as insulin resistance (IR), which in addition to hyperglycemia can cause hyperinsulinemia. Although the mechanisms which lead to development of T1D remain unclear, several risk factors linked to the onset of T2D have been identified. These include the modifiable risk factors of overweight, unhealthy diet, and physical inactivity. These same risk factors also lead to an increased flux of glucose into the HBP, prompting the global hyper-O-GlcNAcylation of proteins (C. Slawson, Copeland, & Hart, 2010). For instance, a recent study showed that consumption of a Western diet (high in saturated fats and carbohydrates) led to increased O-GlcNAcylation in rats (Medford, Chatham, & Marsh, 2012). Increases in O-GlcNAcylation (hyper-O-GlcNAcylation) are directly linked to insulin resistance and hyperglycemia-induced glucose toxicity (McClain, 2002). Moreover, there are several proposed mechanisms for the link between T2D and O-GlcNAcylation. The current working model proposes O-GlcNAc to be a

nutrient sensing “master switch” that attenuates cellular responses to extracellular stimuli, such as the metabolic state. In this case, excess glucose within the cell results in an accumulation of fructose-6-phosphate, which then is shunted to the HBP, causing increased O-GlcNAcylation. The effect establishes a negative feedback loop through the rate limiting enzyme GFAT, which then reduces the rate of glucose transport in response to insulin stimulation (Ruan, Singh, Li, Wu, & Yang, 2013).

O-GlcNAcylation and Cancer

Obesity, diabetes, and excess nutrient intake increase one’s risk for developing several types of cancer (Calle & Kaaks, 2004; Zelenko & Gallagher, 2014). For example, metabolic disorders are linked to an increased risk of cancers of the digestive and women’s reproductive tracts including pancreas (F. Wang, Herrington, Larsson, & Permert, 2003), liver (Adami et al., 1996), breast (Larsson, Mantzoros, & Wolk, 2007), colorectal (Meyerhardt et al., 2003), urinary tract (Xu, Huo, Chen, & Yu, 2017), and female reproductive organs (Byrne et al., 2014; Cantrell et al., 2010; Shoff & Newcomb, 1998). As such, hyper-O-GlcNAcylation is a frequent harbinger of these cancers (Z. Ma & Vosseller, 2013). The two most critical nutrients to cell proliferation and growth in cancer are glucose and glutamine, a phenomenon often described as the Warburg effect (Tekade & Sun, 2017). In the 1960’s Otto Warburg discovered that cancer cells, in comparison to their normal counterparts, more heavily depended upon anaerobic metabolism, rather tradition aerobic metabolism, for proliferation and growth even when oxygen is present. In the average cell, glucose is metabolized to pyruvate, and then enters the Kreb Cycle in the mitochondria, where the majority of the energy for the cell is generated through oxidative metabolism. In cancer cells, conversely, glucose is still metabolized to pyruvate, but instead of undergoing oxidative metabolism, it is converted to lactic acid and secreted by the cell. This less

efficient form of metabolism is nevertheless viewed as a resourceful mechanism to enable the rapidly proliferating cancer cells to meet ongoing demands for anabolism of macromolecules (Heiden et al., 2009). In this context, the shift in metabolism of cancer cells has become a focal point for the development of new chemotherapeutic drugs.

The nutrient sensing characteristics of OGT link the HBP to oncogenic signaling and subsequent regulation of glucose and lipid metabolism. Additionally, metabolic reprogramming in cancer cells is connected to epigenetic changes, including effects of O-GlcNAcylation on histone modification and regulation of transcription factors (Singh, Zhang, Wu, & Yang, 2015). For example, Hypoxia-Induced Factor (HIF-1) mediates the cell's response to reduced O_2 availability. Under hypoxic conditions, HIF-1 binds to the hypoxia response element of genes encoding for glucose transporters (GLUT 1 and 3) and most glycolytic enzymes activating their transcription (J. Kim, Tchernyshyov, Semenza, & Dang, 2006). O-GlcNAcylation stabilizes HIF-1 and results in metabolic reprogramming in cancer cells, and high levels of HIF-1 are associated with elevated levels of OGT (Ferrer et al., 2014).

In addition to promoting cell proliferation, O-GlcNAcylation also increases migration and invasion of some types of cancer. For example, O-GlcNAcylation supports a fibroblast-like phenotype in a colorectal cancer cell line, and silencing of OGA affected the expression of genes associated with migration and growth (Chaiyawat, Netsirisawan, Svasti, & Champattanachai, 2014). In the lung cancer cell line A549, transformation of cells to a more invasive phenotype was directly associated with activation of the HBP (Lucena et al., 2016). For cancer cells to metastasize from a primary tumor site, the cells undergo a transformation. This transformation is referred to as the epithelial-mesenchymal transition (EMT) because polarized epithelial cells, bound to the basement membrane, undergo biochemical and morphological transitions to become

more “mesenchymal like” in their phenotype. Mesenchymal cells have an increased ability to migrate and invade other tissues, are more resistant to apoptosis than epithelial cells, and have increased expression of extra cellular matrix (ECM) proteins (Kalluri & Weinberg, 2009). One key feature of EMT is the notable switch of E-Cadherin vs. N-Cadherin protein expression, in which cells reduce the expression of the epithelial adhesion molecule, E-Cadherin, in favor of the expression of the mesenchymal adhesion molecule, N-Cadherin. The reduction in E-Cadherin expression is largely regulated by the transcription factor, Snail1, which is stabilized by O-GlcNAcylation during hyperglycemic conditions (Sang Yoon Park et al., 2010). Additionally, the HBP is closely connected with several other pathways relevant to the EMT process, including Wnt (Wu et al., 2014; Zhou et al., 2016) and NOTCH (Taparra, Tran, & Zachara, 2016).

Importantly, O-GlcNAcylation influences many cancers of the female reproductive system. O-GlcNAcylation and OGT are both regularly elevated in breast and ovarian cancer (Trinca & Hagan, 2017), and both O-GlcNAc cycling enzymes are elevated in the leading edge of invasive endometrial tumors (Krześlak, Wójcik-Krowiranda, Forma, Bieńkiewicz, & Bryś, 2012). In cervical cancer, O-GlcNAcylation has been shown to support tumorigenicity through the HPV genes E6 and E7 (M. Kim et al., 2016). Among these examples, endometrial cancer is the most common cancer of the female reproductive tract. Women with Type 2 Diabetes (T2D) have a 2-3 fold increased risk for developing endometrial cancer compared to a metabolically healthy cohort (Friberg, Orsini, Mantzoros, & Wolk, 2007). This connection provides the motivation for studies of O-GlcNAc in models of endometrial cancer; however, the literature is wanting. Among the first few studies to make a connection between O-GlcNAc and endometrial cancer, in 2012 Krzeslak et al. found that O-GlcNAc cycling enzyme genes (OGT and MGEA5)

are upregulated along the myometrial-invading edge of endometrial tumors, suggesting a role for O-GlcNAcylation in metastasis (Krześlak et al., 2012). This discovery supported earlier work that noted a variation in the extent of O-GlcNAcylation between histological samples of non-pathologic and pathologic endometrium as evaluated immunohistochemically (Sgantzos et al., 2007). In 2016, Zhou et. al., determined that high glucose culture conditions impair Wnt signaling and increase the expression of the mesenchymal marker, β-catenin, in the endometrial cancer cell lines, AN3CA and HEC-1. Additional experiments using the OGA inhibitor, PUGNAc, revealed these outcomes resulted from increases in HBP activity and subsequent O-GlcNAcylation (Zhou et al., 2016). These observations suggest that O-GlcNAcylation plays a role in the development and progression of endometrial cancer.

Unlike breast and endometrial cancer, obesity and diabetes are not considered leading risk factors for cervical cancer. However, the autoimmune disease, T1D evidently does double the risk for cervical cancer (Zendehdel, 2003). The leading cause of cervical cancer is infection by the human papilloma virus (HPV). There are more than 100 types of HPV, however, only a handful of these have been linked to cervical cancer, these are referred to as the high risk (HR) HPV's. Most notably HR-HPV 16 and 18 are responsible for nearly 71% of all cervical cancer cases (Burd, 2003). Interestingly, OGT interacts with the HPV oncogenes E6 and E7 to promote tumorigenesis (M. Kim et al., 2016). Specifically, O-GlcNAcylation, and the expression of OGT and O-GlcNAcylated HCF-1 are all elevated in cervical cancers, which in turn promotes the transcription of E6/E7 oncogenes (M. Kim et al., 2016). A similar study corroborated these observations and also demonstrated that HPV E6 alone induces elevated O-GlcNAcylation (Zeng et al., 2016). While metabolism does not directly influence the risk of cervical cancer, changes in O-GlcNAcylation still have a significant impact on cell function and disease progression.

While insight into the role O-GlcNAcylation in the development and advancement of cancer has been attained in recent years, the field is still relatively new, and many questions remain unanswered, especially as they pertain to women's cancers. The following chapters are dedicated to new discovery of the impact O-GlcNAcylation on the tumorigenic and metastatic potential of cervical and endometrial cancer. As is evident from the most current literature, these areas of cancer biology represent a new frontier that has not been previously explored.

The Potential Link Between Dysregulation of O-linked β-N-acetylglucosamine (O-GlcNAc) Cycling And Tumorigenicity of Cancers of the Female Reproductive Tract

Diabetes Mellitus is a major risk factor for cancer and continues to be a disease becoming more prevalent in today's society. For instance, in 2012 there were 29.1 million Americans diagnosed with DM; with 1.4 million new cases diagnosed each year (Gallagher & LeRoith, 2015). Of these cases, a shocking 208,000 patients were under the age of 20 (McCarthy, 2014). As DM affects more people and a higher percentage of youth, it is important that work continue toward understanding the mechanisms controlling this disease and its associated complications. In particular, DM associated with both cervical and endometrial cancer threatens the fertility of the next generation of reproductive-age individuals (Vrachnis et al., 2016). To date, the most effective, yet least desirable, form of treatment for these cancers is surgery; and while hormonal treatment is a fertility-sparing option (Kesterson & Fanning, 2012; Montz, Bristow, Bovicelli, Tomacruz, & Kurman, 2002), the possibility exists that diabetic complications may impact its effectiveness. Thus, an overarching goal of this study is to determine whether O-GlcNAc status and its manipulations affect the aggressiveness of cervical and endometrial cancers and how

hyper-O-GlcNAcylation may pre-dispose cells to metastasis. Additionally, the question of how high levels of O-GlcNAcylation impact the effectiveness of progesterone treatment will be explored. Collectively, the work will provide insight about the mechanisms of metastasis in cancers of the female reproductive tract, which may in turn inform treatment protocols. Additionally, in the future, O-GlcNAcylation may serve as a biomarker for prognosis and treatment success, particularly in the context of individualized medicine in which coupling of dietary or metabolic treatment with current hormone-based methods may be beneficial to at-risk patients.

Aims and Hypotheses

Aim 1. Determine the effect of global O-GlcNAcylation on tumorigenic potential in cervical cancer (SiHa) cells.

H1: Global O-GlcNAcylation supports proliferation of cervical cancer cells.

H2: Global O-GlcNAcylation augments migration and invasion of cervical cancer cells.

H3: Reduced O-GlcNAcylation increases susceptibility to cell-mediated death and alters the cytoskeleton.

Aim 2. Characterize the relationship between O-GlcNAcylation and Epithelial-Mesenchymal Transition (EMT) in endometrial cancer.

H1: Hyper-O-GlcNAcylation enhances the expression of EMT associated proteins.

H2: Hyper-O-GlcNAcylation stimulates cancer cell proliferation, migration, and invasion.

Aim 3. Determine the impact of Global Hyper-O-GlcNAcylation on the efficacy of progesterone treatment of endometrial cancer.

H1: Hyper-O-GlcNAcylation impairs the anti-proliferative effects of progesterone treatment.

H2: Hyper-O-GlcNAcylation reverses the anti-invasive effects of progesterone treatment.

CHAPTER 2

ALLOXAN INDUCED HYPO-O-GLCNACYLATION IMPAIRS CERVICAL CANCER CELL PROLIFERATION, INVASION, AND CYTOSKELETAL ORGANIZATION

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Introduction

Cervical cancer is among the most common types of cancer world-wide, and is the most readily diagnosed form of cancer for women in developing countries (Vaccarella & Bray, 2015). Infection by human papillomavirus (HPV) is the primary cause of cervical cancer, with approximately 97% of all cervical cancer patients testing positive for HPV DNA (Munoz N, Bosch FX, de Sanjose S, Herrrero R, Castetellaasague X, 2003). Fifteen types of HPV are classified as high-risk (HR-HPV) based upon their prevalence and oncogenic nature (Munoz N, Bosch FX, de Sanjose S, Herrrero R, Castetellaasague X, 2003), with two types (HPV 16 and 18) being closely related and contributing to the majority of all cervical cancer tumors (Crow, 2012). In less-developed nations, cervical cancer continues to be the leading cause of cancer-related death in women (Crow, 2012; Woodman, Collins, & Young, 2007). Prophylactic measures to prevent cervical cancer in these parts of the world present logistical challenges, so there is increasing urgency to gain insight about disease progression and to develop therapeutic methods that will ultimately eliminate the problem.

In many epithelial cancers, there is an increase in the post-translational modification of cellular proteins that includes β-N-acetylglucosaminylation (O-GlcNAcylation) (Fardini, Dehennaut, Lefebvre, & Issad, 2013; Ferrer, Sodi, & Reginato, 2016; Chad Slawson & Hart, 2011). O-GlcNAcylation is a unique form of glycosylation that results in the addition of a single

sugar moiety, β-N-acetylglucosamine (GlcNAc), to proteins at serine and threonine residues. The enzyme, O-GlcNAc Transferase (OGT) adds O-GlcNAc to the proteins via the substrate UDP-GlcNAc, the end product of the Hexosamine Biosynthesis Pathway (HBSP). Conversely, the enzyme O-GlcNAcase (OGA) removes O-GlcNAc from the targeted proteins (Bond & Hanover, 2015). In this manner, the reversible and dynamic nature of O-GlcNAc modification is akin to phosphorylation of cellular proteins rather than simply glycosylation. Because O-GlcNAcylation occurs on serine and threonine residues it is also known to dynamically influence O-phosphorylation (Comer & Hart, 2000). Proteins can be reciprocally modified by O-GlcNAc and O-phosphate (i.e., estrogen receptor β) (Cheng & Hart, 2000), while in other instances O-GlcNAc physiologically competes with phosphorylation via steric hindrance (Ngoh, Facundo, Zafir, & Jones, 2010). Still other proteins, for example many cytokeratin intermediate filaments, exhibit both forms of modifications, but at distant sites (Hart et al., 2011).

The primary cytokeratin heterodimer expressed in most simple epithelial cells is cytokeratin 8/18. The type I monomer, cytokeratin 18 (K18), is O-GlcNAcylated at three serine residues in the head domain, while still highly-phosphorylated in the tail domain (N.-O. Ku et al., 2010; Omary, Ku, Tao, Toivola, & Liao, 2006). The major biological function of cytokeratin is structural, but K18 is also important in many cellular functions including apoptosis, mitosis, cell cycle progression, and cell signaling (Brian T. Sullivan, MS, Jessica A Cherry, MS, Hideo Sakamoto, PhD, Luiz E Henkes, PhD, David H. Townson, PhD, and Bo Rueda, 2010; Pan, Hobbs, & Coulombe, 2013; Yu-Rong Weng et al., 2012). Additionally, K18 is widely-used as a diagnostic tool and biomarker for many epithelial cancers (Fortier, Asselin, & Cadrin, 2013a; Toivola, Boor, Alam, & Strnad, 2015; Yu-Rong Weng et al., 2012), and is known to promote metastasis (Fortier et al., 2013a). Critically, the dynamic equilibrium of K8/18 filament stability

between soluble and filamentous forms is crucial in determining cellular function, and is regulated by both site specific phosphorylation and O-GlcNAcylation (Srikanth, Vaidya, & Kalraiya, 2010b).

Acknowledging that high intracellular concentrations of O-GlcNAc and high levels of O-GlcNAcylation are common among many cancers, the role of this form of glycosylation in disease progression remains poorly understood. There are a variety of endpoints to measure metastatic potential, but the extent to which O-GlcNAcylation impacts these measures in cervical cancer is in its infancy. In the current study we investigated the impact of global O-GlcNAcylation on cervical cancer cells, hypothesizing that it augments metastatic potential, in part by facilitating cytoskeletal re-organization.

Materials and Methods

Cell Culture and Reagents. The human cervical cancer cell line, SiHa (ATCC® HTB35™), derived from the cervical tumor of a HR-HPV 16-infected patient was used (Friedl, Kimura, Osato, & Ito, 1970). At present, there are no reports indicating the state of O-GlcNAcylation or other forms of glycosylation within this cell line. The SiHa cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic purchased from Thermo Scientific (Waltham, MA) and cultured in a humidified 5% CO2 environment at 37°C. Achieving a relative state of hypo-O-GlcNAcylation was accomplished by exposing the cultures to the OGT inhibitor, Alloxan monohydrate (Konrad et al., 2002) (5mM) purchased from Sigma-Aldrich (St. Louis, MO) dissolved in DMSO (Thermo Fisher, Waltham, MA), for a period of 8-24 hours depending upon experimental

endpoints. Additional SiHa cultures were exposed to an equivalent amount of DMSO (1µl/ml) as a negative control.

Cell Proliferation Assay. In a set of 3 independent experiments, cells were seeded in flat-sided Thermo Scientific™ Nunc™ Cell Culture Tubes (Waltham, MA) at an initial seeding density of 50k cells/ml of culture medium as described above. Treatment groups included cultures continuously exposed to vehicle (DMSO-Control) or 5mM Alloxan. The conditioned culture medium was exchanged daily, and the cells from one tube for each group were harvested for counting at 24-hour intervals for a total of seven days.

Caspase-Glo 3/7 Assay for Apoptosis. In a set of 3 independent experiments, cells were seeded into 96-well plates (Corning™ Costar™ 96-Well White Clear-Bottom Plates) at a density of 20,000 per well in 100µL of complete media, as described above, for 24 hours. Conditioned medium was then exchanged with fresh medium, now containing 1% FBS, and without/with Alloxan (5mM) for 8hrs. The cells were then pre-treated for one hour with the protein synthesis inhibitor, 30µg/ml Cycloheximide (CHX; Sigma-Aldrich), followed by a 10µl spike of either 10µg/ml TNF-alpha (R&D Systems), 1µg/ml Fas-L antibody (CH11; EMD Millipore), or 10µM Staurosporine (Sigma-Aldrich) for 7.5 hours. Incidence of apoptosis was determined by measuring caspase activity according to the manufacturer's recommendations (Caspase-glo 3/7 Assay; Promega, Madison, WI).

Cell Migration Invasion Assay. Three independent in vitro cell migration-invasion experiments were conducted to measure metastatic potential of the SiHa cells as described in similar experiments (Hu & Verkman, 2006; Kramer et al., 2013; Qian et al., 2012). Briefly, the assays were conducted using Corning® Transwell® polycarbonate membrane cell culture inserts

for 12 well plates (VWR, Radnor, PA) containing a Tissue Culture-treated polycarbonate membrane filter (6.5mm diameter, 8μm pore size). The upper chamber of each well contained the SiHa cell suspension (50K cells/well) in serum-free medium, and the lower chamber contained medium supplemented without/with 10% FBS (chemoattractant). After 6, 12, and 24 hours of incubation at 37°C and 5% CO2, the cells on the top of the membrane (non-invasive) were removed with a cotton swab, and the inserts were fixed and stained with 250μls of crystal violet (Thermo Scientific, Waltham, MA). Invasive cells were photographed (200X magnification) and quantified for 3 random fields (200X magnification) per insert. Relative migration was calculated by dividing the average number of cells/field by the area of the microscope viewing field (0.011cm2) and then multiply this number by the entire area of the Transwell insert (0.3 cm2). Percent invasive was then calculated by dividing this by the number of cells initially seeded in the inserts (50k cells) as described in the manufacturer's protocol.

Migration Wound Healing Assay. To confirm and complement the results of the migration-invasion assay, an in vitro wound healing assay was used to assess metastatic potential of the SiHa cells, as described for other types of metastatic cells in the literature (Hu & Verkman, 2006; Kramer et al., 2013; Moy et al., 2014). In a set of three independent experiments, cells were cultured to confluency in 24 well plates (Thermo Fisher) as described above. The monolayers of cells were then washed twice to remove non-adherent cells, and "wounded" using a small cell scraper (Thermo Fisher, cat# 08-100-241) to induce a linear, 4mm-wide wound. The culture medium was exchanged every 24 hours and Alloxan treatment was maintained throughout the experiment. At 24 hour intervals, the cells were fixed and stained with crystal violet, and photographed. Closure of the wound was calculated as a percentage of the total open area and cell migration rate was calculated as area closed divided by time.

Immunofluorescent Staining. The effect of global O-GlcNAcylation on the stability of K8/18 filaments in SiHa cells was evaluated immunocytochemically. Briefly, SiHa cells were grown on 0.1N HCl-treated coverslips for 24 hours and then treated without/with Alloxan for 8hrs. The cells were washed three times with washing buffer (PBS with 0.1% BSA) and sequentially fixed for 10 minutes using 100% ice-cold methanol for 10 minutes and blocked for 24 hours with 10% normal goat serum in 0.1% BSA and phosphate buffered saline. Cells were then incubated for 24 hours at 4°C in primary antibody (anti-human keratin 18 monoclonal antibody, clone CY90, 1:500 dilution; Sigma, St. Lois, MO). Following primary incubation, the cells were then washed with washing buffer and incubated for one hour at room temperature in detection antibody (rabbit anti-mouse IgG conjugated with Alexa-488, 1:1000 dilution; Life Technologies, St. Lois, MO). The cells were counterstained and mounted to slides using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher), and then imaged using a Zeiss Axiophot microscope (63X).

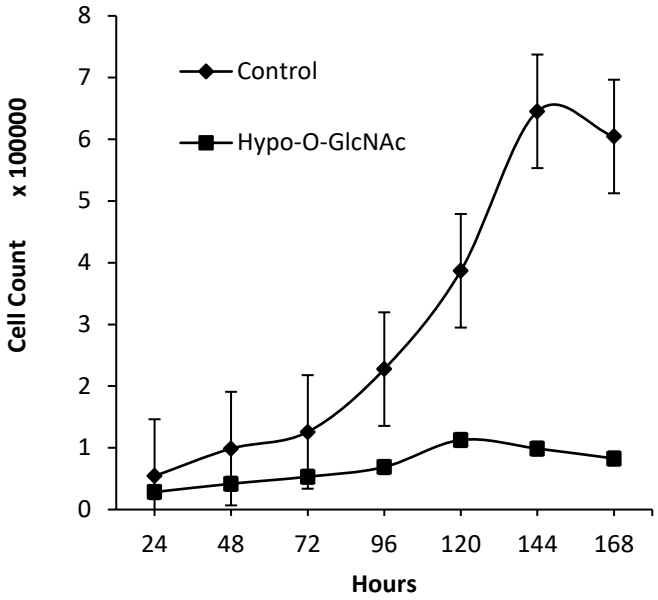

Figure 1: Cell proliferation was impaired by the loss of O-GlcNAcylation (Alloxan) ($p<0.0001$). Total number of generations, multiplication rate, and generation time for Control versus O-GlcNAc-inhibited cells was 2.2 vs. 0.6 generations, 0.4 vs. 0.1 doublings per day, and 2.35 vs. 7.8 days, respectively for 3 independent experiments.

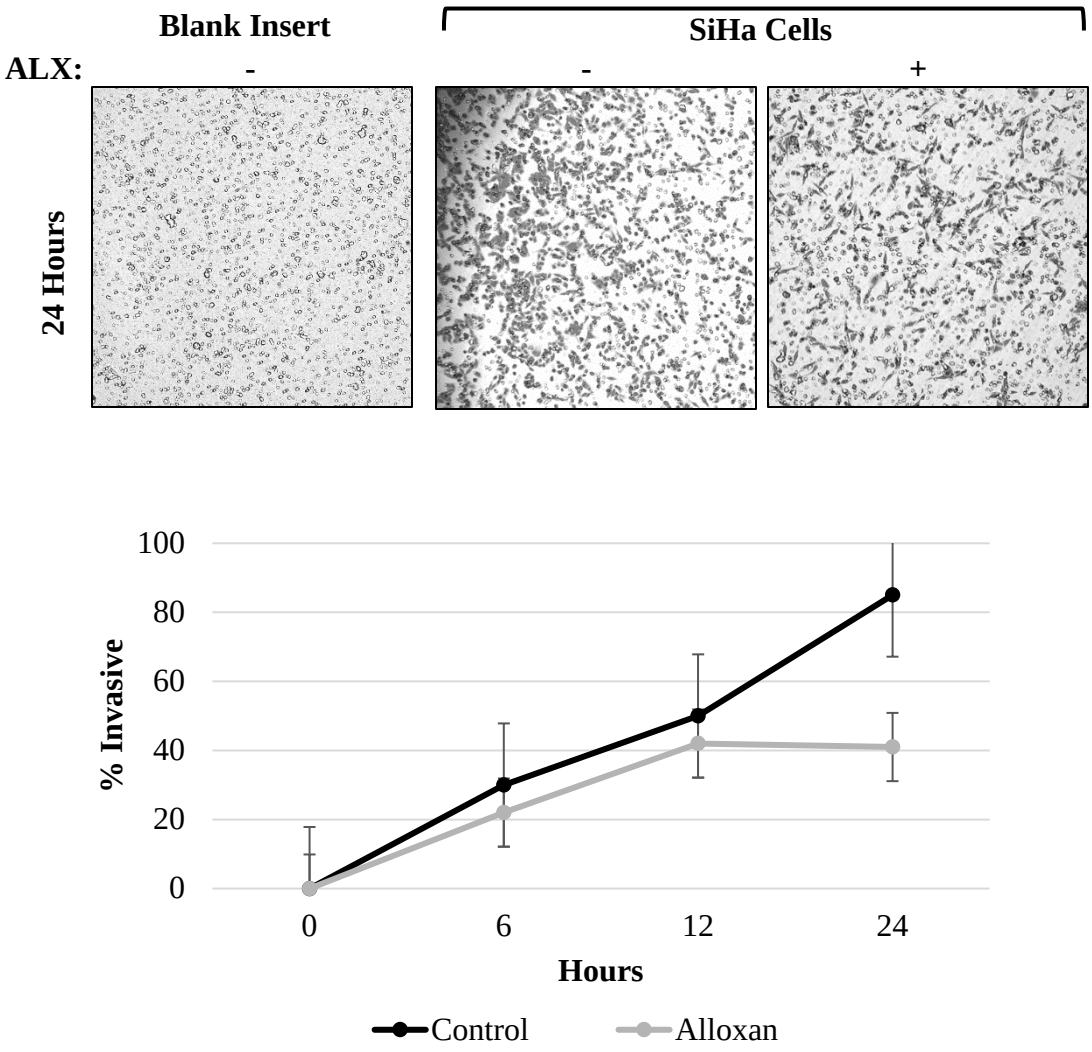

Figure 2: [A] Representative images of cell migration from three independent migration invasion experiments. [B] Percent migration was calculated by dividing the invasive population (determined by counting 3 random fields) by the number of cells seeded. Invasion was impaired at 24 hours following hypo-O-GlcNAcylation (Alloxan; $P<0.05$).

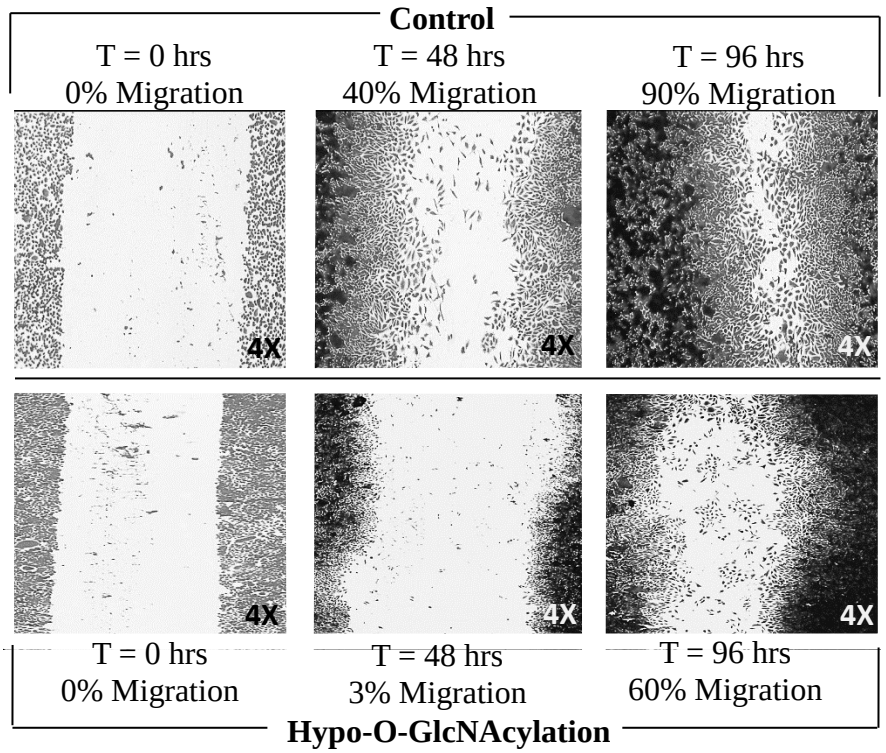

Figure 3: Representative images of cell migration from three independent wound healing assays. Inhibition of O-GlcNAcylation reduced the migration rate ($P<0.001$), and thus the wound healing potential of the SiHa cells. The migrated surface area and migration rate for control vs. O-GlcNAcylation inhibited (Alloxan-treated) cells was 5.5 vs. 4.1 mm2, and 5.5 vs. 4.1μm/hour respectively. Full wound closure was achieved in control cultures by 120 hours, whereas O-GlcNAcylation inhibited cultures did not reach 100% confluency until 168 hours.

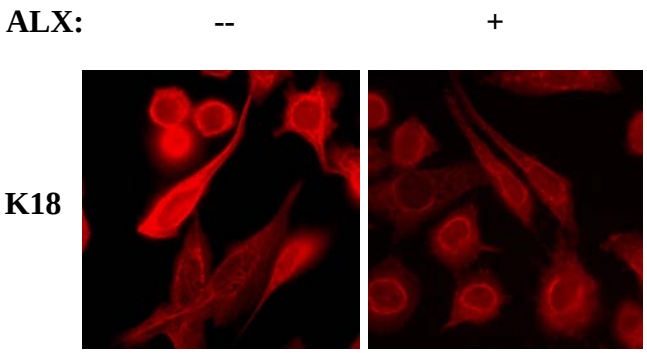

Figure 4: Immunofluorescent staining of the keratin 8/18 filament network suggests a reduction in K18 expression in Hypo-O-GlcNAcylated cells. Keratin 18 also appears to be more granular (soluble) in treated cells compared to the filamentous staining seen in control cells.

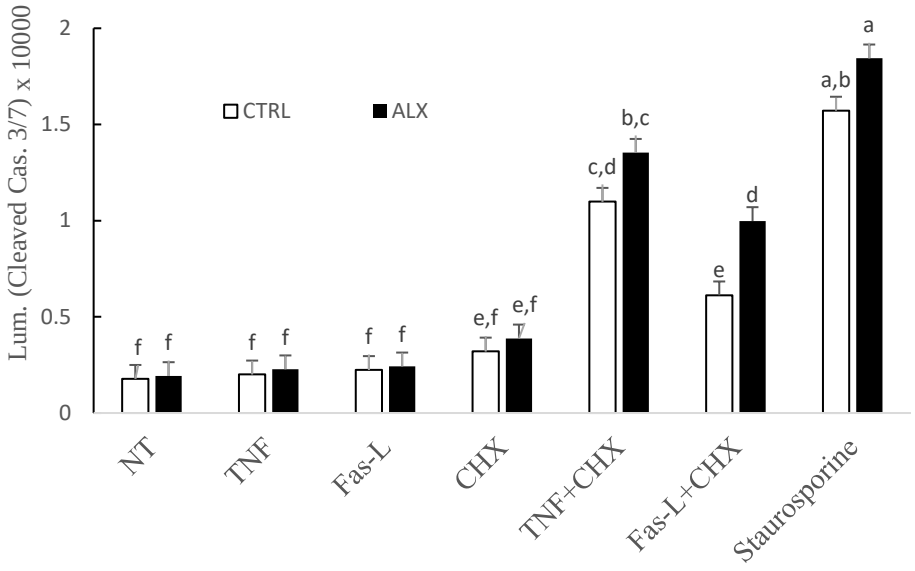

Figure 5: Both control cells (vehicle) and hypo-O-GlcNAcylated (ALX) cells responded to cytokine treatment ($P<0.05$). However, reduced O-GlcNAcylation increased SiHa cells susceptibility to FasL ($P<0.01$).

Discussion

Hypo-O-GlcNAcylation impairs cervical cancer cell proliferation, invasion, and migration

Studies of the similar cervical cancer cell line (HeLa), have shown defects in G1 progression following treatment with the GFAT1 inhibitor, DON, to reduce global O-GlcNAcylation (Chad Slawson et al., 2005). However, the same study also found increased O-GlcNAcylation to cause changes to the cell cycle, possibly through the induced changes to OGT and OGA expression. Low glucose conditions were also found to down-regulate Sp1 activity through hyper-O-GlcNAcylation as opposed to hypo-O-GlcNAcylation in the same cell line (Kang, Ju, Cho, & Hwang, 2003). More consistently, hyper-O-GlcNAcylation has been implicated in increased proliferation in both breast (Chaiyawat et al., 2014) and ovarian cancer (Kwei, Baker, & Pelham, 2012). These findings illuminate the complex nature of O-GlcNAcylation and highlight its role as nutrient sensor, but do not address the effects of OGT inhibition, directly. And with recent scrutiny of the reliability of the HeLa cell as a model for cervical cancer, we felt repeating proliferation studies in a new cell line to be of importance.

SiHa cell proliferation was assessed monitoring growth curves through the death phase in control cultures (~120 hours; Figure 1). The lack of O-GlcNAcylation (Hypo-O-GlcNAc) impaired SiHa cell proliferation ($p<0.0001$) compared to control cultures over the entire length of the experiment. SiHa cells in control cultures grew an average of 2.2 (+/- 0.96) generations with an average generation time of 2.5 (+/- 0.12) days (Figure 1). Conversely, Hypo-O-GlcNAcylated SiHa cells did not double at all in culture and the generation time extended beyond the seven days of the experiment. Overall, the doubling rate for controls was four-fold

higher per day than the Hypo-O-GlcNAcylated cultures (0.4 vs 0.1; $p<0.0001$) when averaged across the experiment. Notably, while proliferation rate was significantly impaired by the loss of O-GlcNAcylation, viability of the SiHa cells was unaffected; viability in both treatment groups was ~95% as determined by Trypan blue exclusion (results not shown). These results implicate O-GlcNAcylation as an important regulator of the cell cycle, as concluded in the above mentioned studies. More specifically, inhibition of OGT has a negative effect on proliferation, and future studies should focus on the mechanisms responsible.

Observing that O-GlcNAcylation impaired cell proliferation but not viability, we then investigated the effect of this post-translational modification on aspects of cellular metastasis of SiHa cells by measuring relative invasiveness and migration potential. Both global and specific protein and transcription factor O-GlcNAcylation has been linked to increased migration/invasion in both breast (Sa Caldwell et al., 2010; Huang et al., 2013) and ovarian cancer (F. Jin, Yu, Zhao, Wu, & Yang, 2013). O-GlcNAc cycling enzymes are also known to be up-regulated in the myometrial invasive cells of endometrial cancer (Krześlak et al., 2012). In this way, our findings are consistent with the literature in similar tissue types. Control cultures of SiHa cells displayed an aggressive phenotype, characterized by cell penetration through the Transwell® polycarbonate membrane insert within 6 hours of culture (Figure 2). The extent of cell penetration increased linearly over the next 12 hours and then plateaued at 24 hours (Figure 2B). For purposes of comparison, the 24 hour endpoint was selected to further evaluate SiHa cell invasiveness in Control and Hypo-O-GlcNAc cultures for this study. Hypo-O-GlcNAcylation reduced cell invasion compared to controls as depicted in Figure 2 ($p<0.05$), suggesting that GlcNAcylation influences the mobility of the cells. A wound-healing assay, which evaluates cell migration, was conducted to corroborate this. Hypo-O-GlcNAcylation also

impaired the migration potential of SiHa cells compared to control, as evidenced by a larger wound area (55mm2 vs. 4.1mm2) for Hypo vs. control, respectively, $P<0.001$), 48 hours after wound creation (Figure 3). This inability of Hypo cells to accomplish wound closure extended beyond 96 hours of culture, wherein only 60% wound closure was achieved at this time point (Figure 3).

O-GlcNAcylation may stabilize K18 filaments resulting in increased cell survival

Morphological differences were observed between SiHa cells grown under control conditions and those in which O-GlcNAcylation is impaired. Hypo-O-GlcNAcylated cells showed a decrease in cytoplasmic area and were more spindle-like in shape compared to normally glycosylated cells. To further investigate this observation, cells were grown on coverslips, fixed, and stained for cytokeratin 18 (figure 4), the main cytokeratin expressed in the cervix. K18 decreased in perinuclear localization and increased in aggregation. Representative images from 4 independent experiments suggest hypo-O-GlcNAcylated cells exhibit a change in K18 organization. Control cells exhibited a filamentous K18 network, while those of the hypo-O-GlcNAcylated cells were granular, suggesting increased solubility, which is consistent with findings in human hepatocytes, where K8/18 is the sole intermediate filament (Srikanth et al., 2010b).

K18 expression has been implicated in cell survival mechanisms in hepatocytes (N.-O. Ku et al., 2010) and granulosa cells (Trisdale, Schwab, Hou, Davis, & Townson, 2016), and previous research conducted with HeLa cells indicated K18 filaments were protective against Fas-mediated apoptosis (Brian T. Sullivan, MS, Jessica A Cherry, MS, Hideo Sakamoto, PhD, Luiz E Henkes, PhD, David H. Townson, PhD, and Bo Rueda, 2010). The current study saw a

similar increase in susceptibility to FasL in the presence of an OGT inhibitor (figure 5), but no increase in susceptibility to TNF-α. These results are congruent with studies on hepatocytes that found disruption of K8/18 filaments by a point mutation resulted in increased susceptibility to Fas-induced, but not TNF-induced apoptosis (N. O. Ku, Soetikno, & Omary, 2003). This supports the hypothesis that O-GlcNAcylation stabilizes K18 filaments in cervical cancer cells, affording cell survival mechanisms.

Conclusion

O-GlcNAcylation of cervical cancer cells augments tumorigenicity by improving cell proliferation rate, migration and invasion potential. Additionally, a reduction of O-GlcNAcylation resulted in increased solubility of cytokeratin 18 and subsequently, an increased susceptibility to Fas-mediated cell death. This work suggests a connection between expression of K18 and O-GlcNAcylation levels in several tumorigenic mechanisms of cervical cancer and warrants further investigation.

CHAPTER 3

O-GLCNACYLATION ENHANCES THE TUMORIGENIC PROPERTIES OF CERVICAL CANCER CELLS IN VITRO

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Introduction

Cervical cancer is among the most common types of cancer world-wide, and is the most readily diagnosed form of cancer for women in developing countries (Vaccarella & Bray, 2015). Infection by human papillomavirus (HPV) is the primary cause of cervical cancer, with approximately 97% of all cervical cancer patients testing positive for HPV DNA (Munoz N, Bosch FX, de Sanjose S, Herrrero R, Castetellaasague X, 2003). Fifteen types of HPV are classified as high-risk (HR-HPV) based upon their prevalence and oncogenic nature (Munoz N, Bosch FX, de Sanjose S, Herrrero R, Castetellaasague X, 2003), with two types (HPV 16 and 18) being closely related and contributing to the majority of all cervical cancer tumors (Crow, 2012). In less-developed nations, cervical cancer continues to be the leading cause of cancer-related death in women (Crow, 2012; Woodman et al., 2007). Prophylactic measures to prevent cervical cancer in these parts of the world present logistical challenges, so there is increasing urgency to gain fundamental insight about disease progression and to develop therapeutic methods that will ultimately eliminate the problem.

In many epithelial cancers, disease onset and progression are associated with increases in post-translational modification of cellular proteins, including β-N-acetylglucosaminylation (O-GlcNAcylation) (Fardini et al., 2013; Z. Ma & Vosseller, 2013; Chad Slawson & Hart, 2011). O-GlcNAcylation is a unique form of glycosylation that results in the addition of a single sugar moiety, β-N-acetylglucosamine (GlcNAc), to proteins at serine and threonine residues. The enzyme, O-GlcNAc Transferase (OGT) adds O-GlcNAc to the proteins via the substrate UDP-

GlcNAc, the end-product of the hexosamine biosynthesis pathway (HBSP). Conversely, the enzyme O-GlcNAcase (OGA) removes O-GlcNAc from the targeted proteins (Bond & Hanover, 2015). In this manner, the reversible and dynamic nature of O-GlcNAc modification is akin to phosphorylation of cellular proteins rather than simply glycosylation. Because O-GlcNAcylation occurs on serine and threonine residues, it can dynamically influence O-phosphorylation (Comer & Hart, 2000). Proteins can be reciprocally modified by O-GlcNAc and O-phosphate (e.g., estrogen receptor β (Cheng & Hart, 2000)), while in other instances O-GlcNAc competes with phosphorylation physiologically via steric hindrance (Ngoh et al., 2010). Still other proteins, for example many cytokeratin intermediate filaments, exhibit both forms of modifications, but at distant sites (Hart et al., 2011).

The most common type of cytokeratin intermediate filament expressed in simple epithelial cells is the heterodimer, cytokeratin 8/18 (K8/18). The type I monomer, cytokeratin 18 (K18), is O-GlcNAcylated at three serine residues in the head domain, while still highly-phosphorylated in the tail domain (N.-O. Ku et al., 2010; Omary et al., 2006). The major biological function of cytokeratins is structural, but K8/18 filaments are also important in many cellular functions including apoptosis, mitosis, cell cycle progression, and cell signaling (Brian T. Sullivan, MS, Jessica A Cherry, MS, Hideo Sakamoto, PhD, Luiz E Henkes, PhD, David H. Townson, PhD, and Bo Rueda, 2010; Pan et al., 2013; Y.-R. Weng, Cui, & Fang, 2012). Additionally, K18 of K8/18 filaments is widely-used as a diagnostic tool and biomarker for many epithelial cancers (Fortier, Asselin, & Cadrin, 2013b; Toivola et al., 2015; Y.-R. Weng et al., 2012), and is known to promote metastasis (Fortier et al., 2013b). Importantly, the dynamic equilibrium of K8/18 filament stability between soluble and filamentous forms is crucial in

determining cellular function, and this stability is regulated by both site-specific phosphorylation and O-GlcNAcylation (Srikanth et al., 2010a).

High intracellular concentrations of O-GlcNAc and high levels of O-GlcNAcylation are common among many cancers, yet the role of this form of glycosylation in disease progression is less understood. Additionally, there are a variety of endpoints to measure metastatic potential, but the extent to which O-GlcNAcylation impacts these measures in cervical cancer is unknown. The objective of the current study was to investigate the impact of global O-GlcNAcylation on cervical cancer cells, hypothesizing that O-GlcNAcylation augments metastatic potential, in part by influencing K8/18 filaments and cytoskeletal re-organization.

Materials and Methods

Cell Culture and Reagents. The human cervical cancer cell line, SiHa (ATCC® HTB35™), derived from the cervical tumor of a HR-HPV 16-infected patient was used (Friedl et al., 1970). At present, there is no information available indicating the state of O-GlcNAcylation or other forms of glycosylation within this cell line. For culture, the SiHa cells were maintained in Eagle's Minimum Essential Medium (EMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic, all purchased from Thermo Fisher Scientific (Waltham, MA). The cells were incubated in a humidified, 5% CO_2 environment at 37°C. Global inhibition of O-GlcNAcylation (Hypo- O-GlcNAcylation) was achieved by exposing the cells to the OGT inhibitor, OSMI-1 (50μM) as described previously by others (M. Kim et al., 2016; Ortiz-Meoz et al., 2015), and was verified by immunoblotting (described below). The OSMI-1 was purchased from Aobious (Gloucester, MA) dissolved in DMSO (Thermo Fisher), and the cells were exposed for a period of 9-24 hours depending upon the experimental endpoint (described below).

Additional cultures of SiHa cells were exposed to an equivalent amount of DMSO (1µl/ml) as a negative control.

Immunoblotting for Global O-GlcNAcylation. Whole cell lysates of SiHa cells cultured in 25cm^2 flasks (Corning, Corning, NY) for 48 hours, were harvested by scraping, and separated on pre-stained SDS-Page gels (BioRad, Hercules, CA) and transferred to PVDF membrane. Membranes were then probed for global O-GlcNAcylation (CST-O-GlcNAc 110.6 antibody; Cell Signaling Technology, Inc.; Beverly, MA) and then secondary antibody (Anti-rabbit IgG HRP-linked Antibody, Cell Signaling Technology, Inc.), followed by Clarity Western ECL substrate (BioRad). Blots were imaged on the BioRad ChemiDoc Imaging System.

Cell Proliferation Assay. In a set of 3 independent experiments, cells were seeded in duplicate in flat-sided Thermo Fisher Scientific™ Nunc™ Cell Culture Tubes at an initial seeding density of 60k cells/ml of culture medium. Treatment groups included cultures of cells continuously exposed to vehicle (DMSO-Control) or 50µM OSMI-1. The conditioned culture medium was exchanged without/with treatment daily, and the cells for each group were harvested for counting at 24-hour intervals over a seven-day period.

Flow Cytometric Analysis of Cell Cycle Progression. In a set of 3 independent experiments, SiHa cells were seeded at a density of 100,000 cells/ml in T-25 flasks (Corning) and treated without or with OSMI-1 in culture through the exponential growth phase before being enzymatically harvested and fixed/permeabilized with 70% ethanol. The cells were then stained with propidium iodide (PI) and fluorescence was measured with a 488nm laser (610/20 BP Filter) on a BD LSRII Flow Cytometer. Data were analyzed with Flow Jo Analysis Software.

Caspase-Glo 3/7 Assay for Fas-Induced Apoptosis. In a set of 3 independent experiments, SiHa cells were seeded in triplicate into 96-well plates (Corning™ Costar™ 96-Well White Clear-Bottom Plates) at a density of 20,000 per well in 100µL of complete media and then cultured without or with OSMI-1 (50 µM) for 24 hours. The cells were then pretreated for 30 min with the protein synthesis inhibitor, 30µg/ml Cycloheximide (CHX; Sigma-Aldrich), followed by 1µg/ml Fas-L antibody (CH11; EMD Millipore), or 10µM Staurosporine (Sigma-Aldrich) for 8 hours. The incidence of apoptosis was determined using a caspase enzyme assay, following the manufacturer's recommendations (Caspase-Glo 3/7 Assay; Promega, Madison, WI).

Cell Migration Invasion Assay. Three independent *in vitro* cell migration-invasion experiments were conducted to measure metastatic potential of the SiHa cells. The assays were conducted using Corning® Transwell® polycarbonate membrane cell culture inserts for 24 well plates (VWR, Radnor, PA) containing a Tissue Culture-treated polycarbonate membrane filter (6.5mm diameter, 8µm pore size). This approach has been described previously by others in similar experiments (Hu & Verkman, 2006; Kramer et al., 2013; Qian et al., 2012). Briefly, the upper chamber of each well was seeded with SiHa cells (50K cells/well) in serum-free medium without or with OSMI-1 (50 µM) and the lower chamber contained medium supplemented without or with 10% FBS (chemoattractant). Treatments for each experiment were conducted in triplicate. After 24 hours of incubation at 37°C and 5% CO_2, the cells on the top of the membrane (non-invasive) were removed with a cotton swab, and the inserts were fixed and stained with 600µl of crystal violet (Thermo Scientific). Invasive cells that had penetrated the membrane were photographed (20X magnification). Invasiveness was also quantified by de-staining the

membranes in 600ul of 70% ethanol for 10 minutes and then measuring the absorbance at 540nm.

Immunocytochemistry of Cytoskeletal Proteins. SiHa cells were cultured in 12 well plates (100K cells/well) on glass cover slips using conditions described above for 24 hours, then fixed and permeabilized with 100% methanol. Primary antibodies for the detection of K18 in the K8/18 filaments (CY90 antibody, Cell Signaling Technology, Inc.), α-tubulin (236-10501 antibody, Thermo Fisher Scientific, Waltham, MA), and β-actin (13E5 antibody, Cell Signaling Technology, Inc.) were applied, followed by wash steps and detection using the secondary antibody (Anti-rabbit or IgG, HRP-linked Antibody, Cell Signaling Technologies) and AEC substrate kit (Vector Laboratories, Burlingame, CA). The cells were counterstained with hematoxylin (Vector), mounted onto slides, and imaged at 40X.

Statistical Analysis. All experiments were independently repeated a minimum of three times, using a fresh aliquot of SiHa cells (passage 4-7) to initiate each experiment. Data were analyzed by one- or two-way analysis of variance (ANOVA), followed by a Student's t post-test for multiple comparisons; differences among means were considered significant at $p<0.05$.

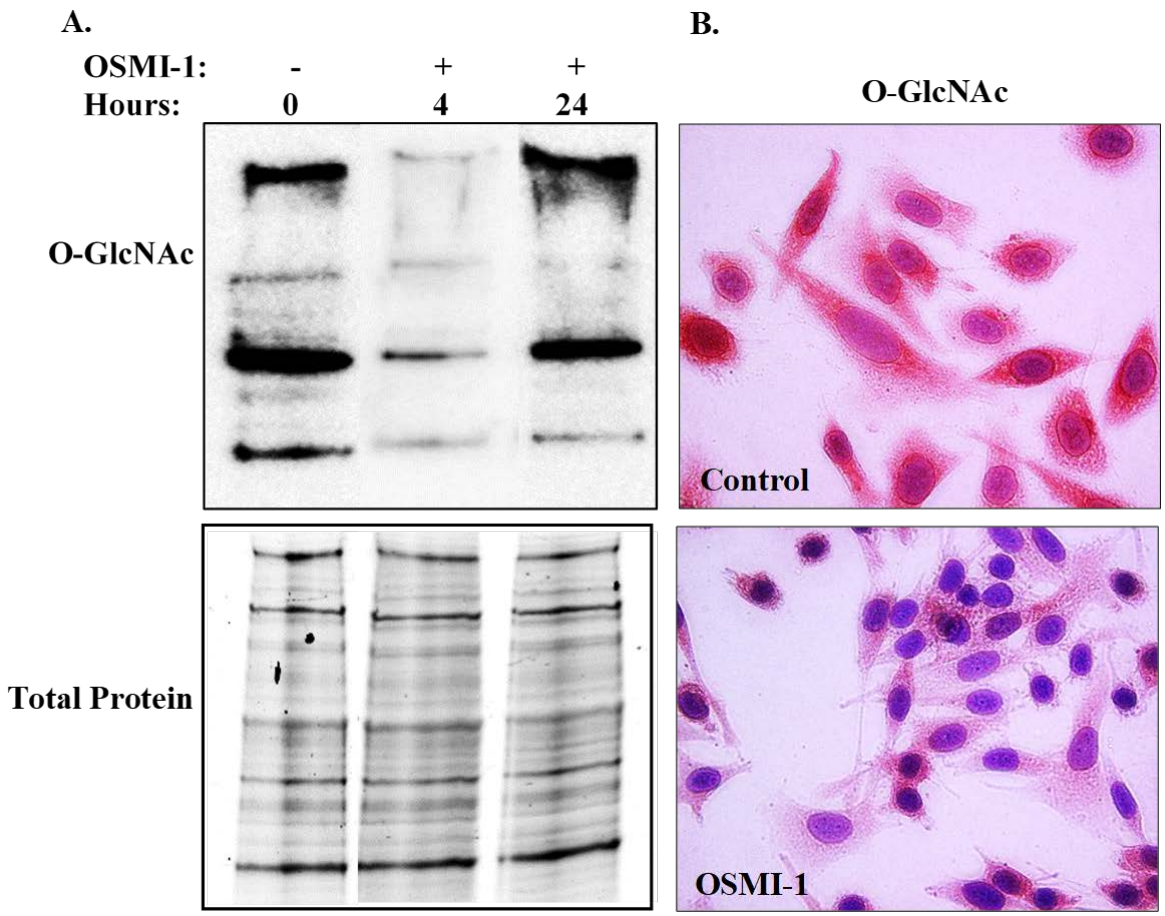

Figure 1. Inhibition of global O-GlcNAcylation by OSMI-1. [A] Immunoblot analysis of global O-GlcNAc in SiHa whole cell lysates following treatment with OSMI-1 for 4 and 24 hours. O-GlcNAcylation of cellular proteins declined within 4 hours of OSMI-1 treatment and remained diminished through 24 hours. There was no effect of OSMI-1 on total protein expression, as indicated in the lower panel **[B]** Impaired O-GlcNAcylation was also evident in fixed, cultured cells, wherein immunostaining for O-GlcNAcylation was reduced throughout the cytoplasm of OSMI-1 treated cells, 24 hours after treatment (40X magnification). Representative images of 3 independent experiments.

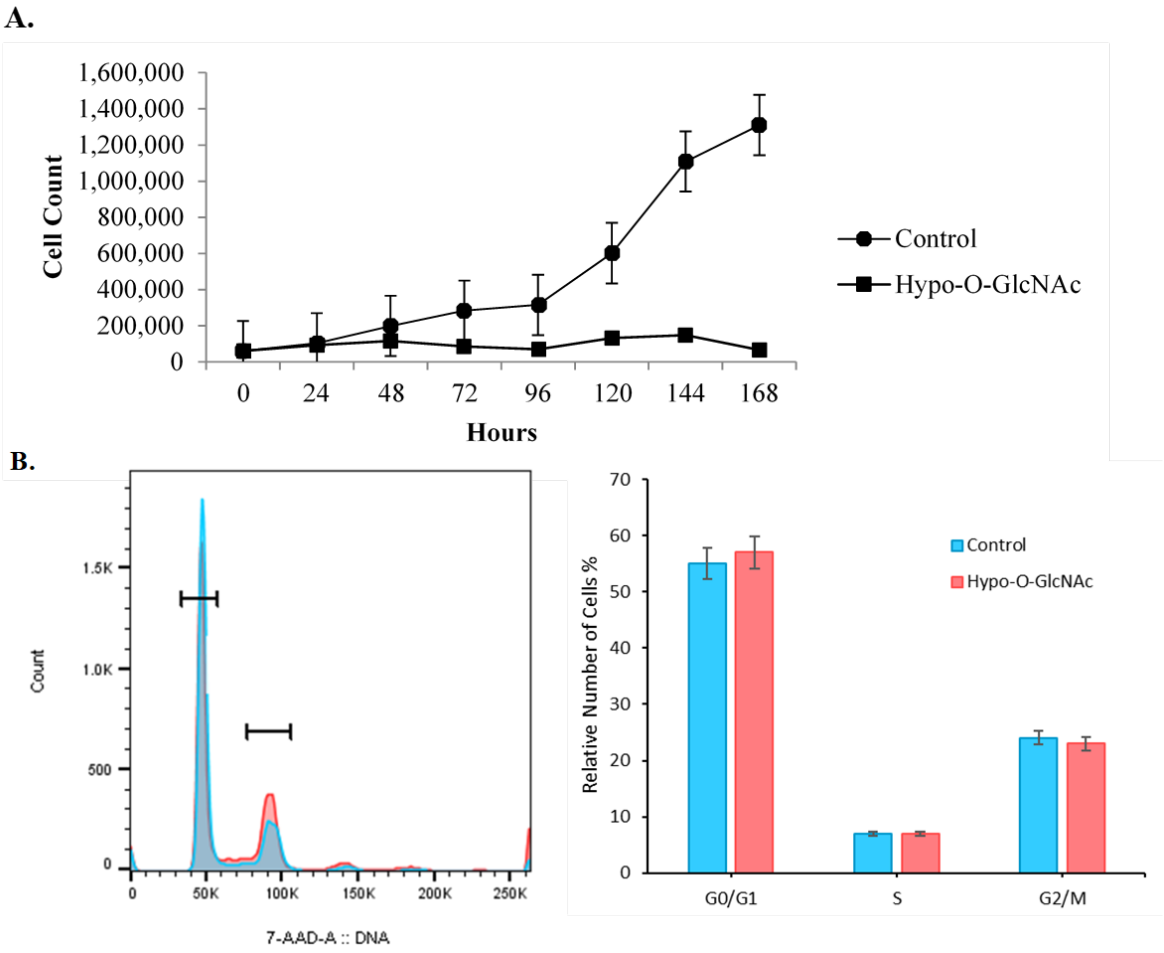

Figure 2. SiHa cell proliferation is impaired by Hypo-O-GlcNAcylation. [A] SiHa cell proliferation was impaired ($p<0.01$) by Hypo-O-GlcNAcylation following OSMI-1 exposure. Total number of generations, multiplication rate, and generation time for control versus Hypo-O-GlcNAcylated cells were all reduced following 3 independent experiments. **[B]** Flow cytometric analysis of parameters of cell cycle progression revealed no effect of Hypo-O-GlcNAcylation when comparing control and OSMI-1 treated cultures.

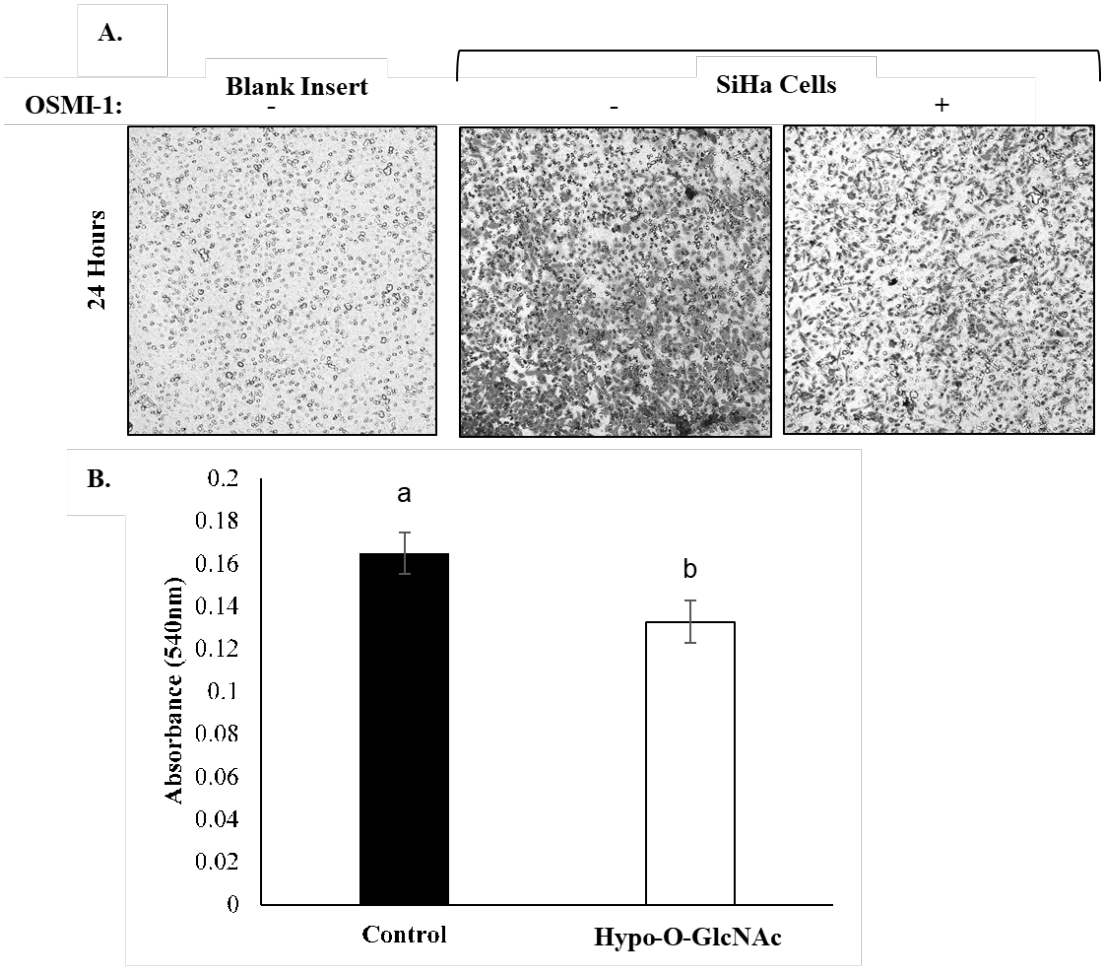

Figure 3. Effect of Hypo-O-GlcNAcylation on SiHa cell invasion. [A] Representative images (10X) depicting SiHa cells that have penetrated the Transwell inserts in response to chemoattractant. Note that OSMI-1 treatment impaired cell penetration. **[B]** A bar graph comparing the invasiveness of SiHa cells in control and Hypo-O-GlcNAcylated cultures for 3 independent experiments (Mean ±SEM) is shown. Bars with different letters denote differences ($p<0.05$).

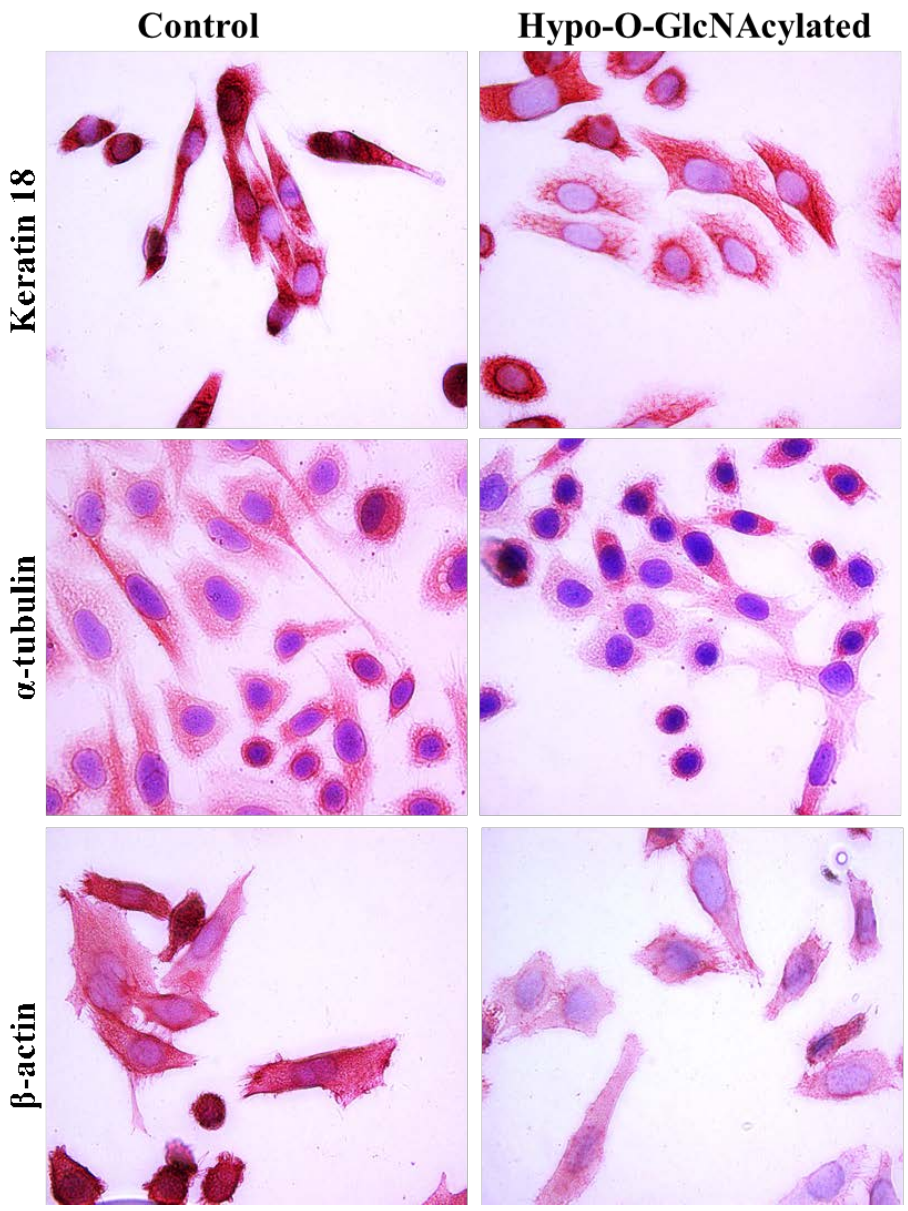

Figure 4. Effects of Hypo-O-GlcNAcylation on SiHa cell morphology and cytoskeletal expression. Representative images (40X) of immunocytochemical staining for keratin 18, α-tubulin, and β-actin in SiHa cells are shown. Hypo-O-GlcNAcylation reduced both Keratin 18 and β-actin expression. There were no obvious alterations of α-tubulin expression observed. These outcomes were evaluated following three independent experiments.

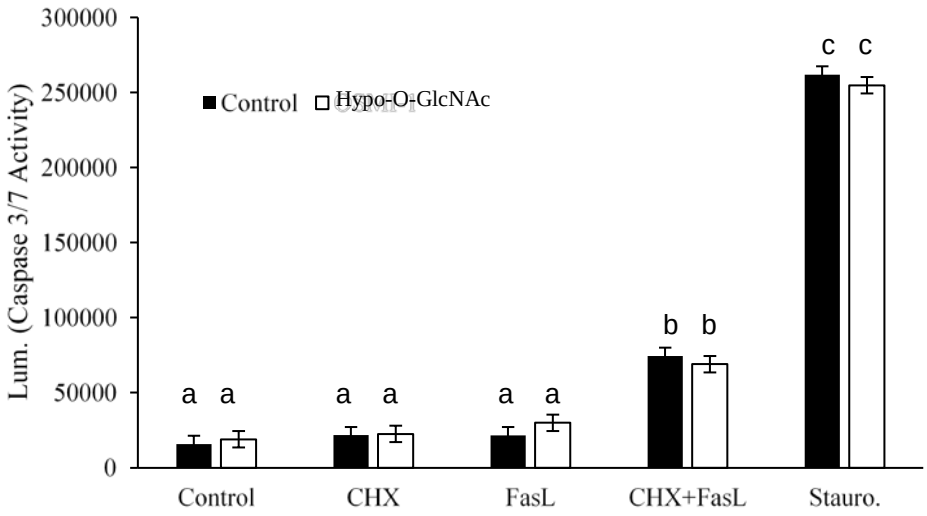

Figure 5: Effect of Hypo-O-GlcNAcylation on SiHa cell sensitivity to apoptosis. A bar graph depicts the relative sensitivity of SiHa cells (Mean ±SEM) to cytokine- (Fas-L) and chemotherapeutic- (Staurosporine) induced apoptosis (as measured by cleaved caspase 3/7 activity) following three independent experiments. Cytokine in the presence of the protein synthesis inhibitor, cycloheximide (CHX), induced apoptosis ($p<0.05$), but impairment of O-GlcNAcylation did alter the sensitivity of the cells to FasL. Staurosporine was used as the positive control for the assay. Bars with different letters denote differences ($p<0.05$).

Discussion

Hypo-O-GlcNAcylation of SiHa cells impairs cell proliferation and invasion

A variety of studies have demonstrated that the state of global O-GlcNAcylation in cells, including epithelial-derived cancer cells, can have profound physiological effects on growth and development. In the current study, the effect of global O-GlcNAcylation on proliferation, invasion, and cytoskeletal organization was assessed in a cervical cancer cell line (SiHa cells). To our knowledge, this is the first report of the effects of the highly-specific O-GlcNAc Transferase (OGT) inhibitor, OSMI-1, on cervical cancer cells. The ability of OSMI-1 to globally impair O-GlcNAcylation, without affecting SiHa cell viability, was clearly evident in these experiments, and was sustained for an extended period (Figure 1). Cell proliferation was assessed by monitoring growth curves through the death phase, and revealed that the lack of O-GlcNAcylation (Hypo-O-GlcNAc) impaired proliferation ($p<0.01$) over the entire length of the experiment (Figure 2A). Overall, the doubling rate for controls was more than three-fold higher per day than the Hypo-O-GlcNAcylated cultures (0.7 vs 0.2; $p<0.01$) when averaged across the experiment. These results suggest O-GlcNAcylation regulates mitosis, possibly via the cell cycle (Chad Slawson et al., 2005). Indeed, others have reported the cell cycle is accelerated ($G_1 \rightarrow S$) following exposure to the GFAT1 inhibitor, DON, which reduces global O-GlcNAcylation (Sa Caldwell et al., 2010); whereas the same study determined that increased O-GlcNAcylation delays G_2/M progression, possibly through induced changes to OGT and OGA activity. These findings illuminate the complex, sometimes unpredictable, nature of O-GlcNAcylation in cells. In the current study, however, hypo-O-GlcNAcylation via OSMI-1 had no effect on parameters of cell cycle progression (Figure 2B), indicating that SiHa proliferation is impaired in some other way.

Observing that hypo-O-GlcNAcylation impaired cell proliferation, but not viability, we then investigated the effect of this post-translational modification on aspects of cellular metastasis by evaluating the relative invasiveness of SiHa cells. Both global and specific O-GlcNAcylation of proteins and transcription factors are linked to increased cell invasiveness/migration in breast and ovarian cancers (Huang et al., 2013; F.-Z. Jin, Yu, Zhao, Wu, & Yang, 2013; Steenackers et al., 2016). Similarly, upregulation of O-GlcNAc enzymes is associated with myometrial invasive cells in endometrial cancer (Krześlak et al., 2012). Our results are consistent with these findings. Control cultures of SiHa cells exhibited an aggressive phenotype, characterized by cell penetration through the Transwell® polycarbonate membrane insert within 24 hours of culture (Figure 3A). Conversely, hypo-O-GlcNAcylation reduced cell invasion ($p<0.05$; Figure 3B), suggesting that O-GlcNAcylation augments the mobility of the cells. Overall, the results indicate that O-GlcNAcylation, specifically through the actions of OGT, heightens the tumorigenic potential of cervical cancer cells by accelerating both proliferation and invasion capabilities. These observations agree with a recent study of OGT manipulation in colon cancer cell lines, wherein genetic silencing of OGT impaired cell invasiveness and migration (Steenackers et al., 2016).

Hypo-O-GlcNAcylation affects cytoskeletal morphology, but not Fas-induced apoptosis

Cytoskeletal proteins are fertile targets for post-translational modification, including O-GlcNAcylation, so it is plausible for O-GlcNAcylation to affect cell morphology. Indeed, SiHa cells exhibited pronounced changes in morphology following OSMI-1 treatment compared to control cells (Figure 4). Notably, immunostaining for K18 and β-actin proteins was diminished in Hypo-O-GlcNAcylated cells, and the cells generally had a flattened, stellate morphology compared to the more spindle-like appearance of control cells (Figure 4). For K18, in particular,

staining was uniformly detectable throughout the cytoplasm in control cells; whereas it remained primarily perinuclear, and somewhat aggregated as it extended to the periphery in Hypo-O-GlcNAcylated cells (Figure 4). Similar forms of filament reorganization have been noted in other epithelial cancers (Busch et al., n.d.), and are believed to be a direct result of K8 phosphorylation in K8/18 filaments (Fois, Weimer, Busch, & Felder, 2013). In the current study, there was no evidence that α-tubulin expression was affected by hypo-O-GlycNAcylation (Figure 4), despite the observation that K18 and β-actin expression were both diminished. Others have observed that α-tubulin and β-actin expression in colon cancer cells are unaffected by inhibition of OGT, yet the morphology of these cells is "stocky and stunted" (Steenackers et al., 2016). Although K8/18 filament expression was not evaluated, the results of the previous study did support the concept that hypo-O-GlcNAcylation disrupts cytoskeletal organization. Beyond a structural role, K8/18 filaments also influence cell survival, specifically providing resistance to Fas-induced apoptosis (Brian T. Sullivan, MS, Jessica A Cherry, MS, Hideo Sakamoto, PhD, Luiz E Henkes, PhD, David H. Townson, PhD, and Bo Rueda, 2010; N.-O. Ku et al., 2010; Trisdale et al., 2016). In the present study, we found that SiHa cells are indeed resistant to Fas-induced apoptosis, but such resistance is not influenced by hypo-O-GlyNAcylation (Figure 5; $p>0.05$), or associated with a loss of K8/18 filament expression (Figure 4). This suggests the mechanism(s) of Fas-resistance in SiHa cells involve processes other than O-GlyNAcylation and proteins other than K8/18 filaments. Additionally, as noted above, the overall viability of the SiHa cells remained unaffected by hypo-O-GlyNAcylation, as measured by caspase 3/7 enzyme activity (Figure 5; Control vs. Hypo-O-GlycNAcylated, $p>0.05$). Thus while other investigations have concluded O-GlcNAcylation is fundamental to cell viability, especially in

embryonic stem cells (Shafi et al., n.d.), the extent of impairment of O-GlcNAcylation in the current study was insufficient to hinder cell survival.

Conclusion

Overall, O-GlcNAcylation enhances the tumorigenic properties of SiHa cells by accelerating cell proliferation and augmenting invasive and migratory capabilities. Conversely, hypo-O-GlcNAcylation alters cellular morphology and cytoskeletal organization, in part by reducing the expression of K8/18 and β-actin filament proteins. The results support the hypothesis that O-GlcNAcylation augments metastatic potential of cervical cancer cells and suggest there is a physiologic connection between O-GlcNAcylation and cytoskeletal reorganization, ostensibly involving the modulation of O-phosphorylation of K8/18 filaments, but this warrants further study.

CHAPTER 4

HYPER-O-GLCNACYLATION PROMOTES EPITHELIAL-MESENCHYMAL TRANSITION IN ENDOMETRIAL CANCER CELLS

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HYPER-O-GLCNACYLATION PROMOTES EPITHELIAL-MESENCHYMAL TRANSITION IN ENDOMETRIAL CANCER CELLS

Introduction

β-N-acetylglucosaminylation (O-GlcNAcylation) is a unique form of glycosylation that occurs on serine and threonine residues of proteins throughout the cytoplasmic and nuclear compartment of cells. It is a monosaccharide that is dynamically cycled akin to that of O-phosphorylation. The enzyme O-GlcNAc transferase (OGT) adds O-GlcNAc to proteins via the substrate UDP-GlcNAc, while O-GlcNAcase (OGA) is responsible for its removal. O-GlcNAc influences the extent of O-phosphorylation of proteins through shared binding sites and steric hindrance (Bond & Hanover, 2015). As the end-product of the Hexosamine Biosynthesis pathway (HBP), UDP-GlcNAc and subsequent O-GlcNAcylation is considered a nutrient sensor to the overall metabolic status of the cell. Two to five percent of the glucose that enters the cell is channeled into the HBP. As such, it is not surprising that increased O-GlcNAcylation is implicated in the development of insulin resistance (T Issad, Masson, & Pagesy, 2010). Aberrant O-GlcNAcylation is a characteristic of heart disease, neurodegenerative disorders such as Alzheimer's disease, and is a hallmark of many cancers, including endometrial cancer (Krześlak et al., 2012). A relationship between glycosylation and metastasis is also evident in lung cancers, wherein epithelial-mesenchymal transition (EMT) acts through the HBP as an inducer of aberrant glycosylation (Lucena et al., 2016). Despite these observations, very little is known about the mechanistic actions of O-GlcNAcylation in cancer, specifically, in the EMT process.

Many illnesses associated with aberrant O-GlcNAcylation are also co-morbidities of Type 2 Diabetes (T2D). For instance, women with T2D have a 2-fold greater risk of developing endometrial cancer than their healthy cohorts (Friberg et al., 2007). While the incidence of many forms of cancer is declining, endometrial cancer remains among those increasing for all women (Cote, Ruterbusch, Olson, Lu, & Ali-Fehmi, 2015). Endometrial cancer results from the abnormal growth, migration, and invasion of cells that line the uterus. Common genetic alterations in endometrial tumors include *PTEN*, *PIK3CA*, *CTNNB1* (beta-catenin), and *KRAS*, all of which are related to metabolism (Byrne et al., 2014). Moreover, all of these genes influence epithelial-mesenchymal transition (EMT) signaling pathways, and recent studies indicate that the E-cadherin repressors *Slug*, *ZEB1*, and *HMGA2* are preferentially expressed along the myometrial invasive edge of tumors (Stewart & Mccluggage, 2013). Snail1, a key regulator of EMT, is stabilized by O-GlcNAcylation in several cell types (Sang Yoon Park et al., 2010), and the mRNA of O-GlcNAc cycling enzymes (OGT and OGA) is up-regulated in endometrial tumors(Krześlak et al., 2012), suggesting that O-GlcNAcylation influences metastasis.

The gold standard for treatment of endometrial cancer is radiation therapy and surgery; however, 5-30% of women with endometrial cancer are premenopausal and under the age of 50 at the time of diagnosis. For these women, fertility-sparing treatments, such as progestin therapy, are an option (Jeong-Yeol Park, 2015). A recent meta-analysis determined that women treated with hormonal therapy methods had a pooled regression rate of 76.2%, with 28% live births reported; however, a 40.6% relapse rate was also noted (Gallos et al., 2012). These findings underscore the importance of identifying basic mechanisms by which metabolism and O-GlcNAcylation influence the progression of endometrial cancer, with the goal of improving

fertility-sparing treatments. Therefore, the objective of this study was to determine these mechanisms, specifically focusing on the manipulation of O-GlcNAc cycling enzymes (OGT and OGA) and the impact on molecular and cellular aspects of Epithelial-Mesenchymal Transition (EMT).

Materials and Methods

Cell Culture/Reagents. Endometrial Cancer cells (Ishikawa) were obtained from Sigma Aldrich (cat. #99040201). Cell Line authentication was performed before, during, and after experimentation in the University of Vermont Cancer Center Advanced Genome Technologies Core and was supported by the University of Vermont Cancer Center, Lake Champlain Cancer Research Organization, and the University of Vermont College of Medicine. O-GlcNAcylation in cells was manipulated by the OGA inhibitor, ThmG (1μM, Fisher Scientific), the OGT inhibitor, OSMI-1 (50μM, Aobious), and supplementation of excess glucose (25Mm). ThmG and OSMI-1 were dissolved in DMSO (Fisher Scientific) at a concentration of 1000X, thus control and glucose treated cells received 0.1% DMSO (vehicle). Cell culture media (EMEM, 10% FBS and 10μl/mL antibiotic-anti-mycotic (Fisher Scientific) was exchanged every 24 hours unless otherwise specified.

Immunoblotting. Cell Lysates were harvested via trypsin digestion and dissolved in RIPA buffer, and then passaged 5 times through a 26G needle. Protein concentration was measured by BCA Assay (BioRad) following the manufacturer's protocol. Pre-stained SDS-PAGE gels were loaded with 20μg of proteins per lane. Protein was then transferred to PVDF membrane (Millipore). Membranes were then probed with antibodies associated with EMT (EMT antibody kit), the Akt Pathway (Akt Pathway Antibody Kit), O-GlcNAc (CTD 110.6), OGT, and OGA.

Goat-anti-rabbit and goat-anti-mouse HRP conjugated secondary antibodies were used in combination with Clarity Western ECL Clotting Substrate (BioRad) for imaging on the BioRad ChemiDoc Imager. All antibodies were purchased from Cell Signaling unless otherwise specified.

Immunohistochemistry. Cells were seeded at 50,000 cells/mL in black-walled 96 wells plates and incubated in treatments described above, with or without 5ng/mL TGF-β (R&D Systems), for 72 hours. Media was exchanged daily. Cells were then fixed with 4% paraformaldehyde in PBS (Fisher Scientific) and stained with Alexaflour-488 Phalloidin (Molecular Probes, Eugene, OR) and DAPI (Molecular Probes). Cells were imaged with the Olympus CKX53 at 200X.

Proliferation and Migration. A wound-healing assay was used to measure cell proliferation of Control cells (vehicle, DMSO), Hyper-O-GlcNAcylated cells (25Mm Glucose or 1μM ThmG), and Hypo-O-GlcNAcylated cells (50μM OSMI-1). Confluent monolayers of treated cells grown in 24 well plates in EMEM with 10% FBS. “Wounds” were created by running a 200μL pipette tip across the monolayer. At 0 hours and every 24 hours following, the “wound” area was measured with ImageJ software and percent wound closure was calculated. Three independent wound-healing assays were also conducted to assess the migration potential of treated cells. Confluent monolayers of treated cells grown in 24 well plates were serum starved for 24 hours before “wounds” were created by running a 200μL pipette tip across the monolayer. Cells were maintained in serum free conditions to inhibit proliferation and were incubated at 37°C and 5% CO_2 for 18 hours. The “wound” area was measured with ImageJ software at 0 and 18 hours to calculate percent wound closure.

Invasion. Corning Matrigel Biocoat Invasion Chambers (24 well, 0.8μ) (Corning, Bedford, MA) were used to assess the invasion potential of treated cells compared to control. The upper chamber was seeded with 100,000 serum-starved cells stimulated with 5ng/ml TGF-β. Cells were exposed to O-GlcNAc-modifying treatments at the time of seeding. The lower chamber contained 5% FBS as a chemoattractant. Cells were incubated for 48 hours at 37°C and 5% CO_2. Cells were then removed from the upper chamber and the Transwell membranes were fixed with 100% methanol and stained with Crystal Violet. Invaded cells were then counted by two independent researchers and the results were averaged.

RT PCR Array. RNA was extracted from treated cell pellets with the RNeasy Mini-Kit (Qiagen) with DNase digestion (RNAse free DNase Kit, QIAGEN) following the manufacturer protocols. The cDNA synthesis and RT^2 Profiler PCR Arrays (QIAGEN, PAHS-090Z) (Supp. Table 1) were performed in the University of Vermont Cancer Center Advanced Genome Technologies Core and was supported by the University of Vermont Cancer Center, Lake Champlain Cancer Research Organization, and the University of Vermont College of Medicine. Results were analyzed via the GeneGlobe Data Analysis Center (QIAGEN).

Oncogenomic Data Analysis. Multidimensional cancer genomic data analysis was performed using online data-mining tool from the cBioPortal for Cancer Genomics (http://cbioportal.org) and the data sets from the cBioPortal for Cancer Genomics and the TCGA research Network (http://cancergenome.nih.gov) according to recently published protocols.26, 27 Tumor types and data sets are chosen in accordance with the publication guidelines from TCGA (last update:30 September 2014). Genomic alterations were identified when the following occurred: (1) gene mutations; (2) putative copy number alteration (amplification or deletion); (3) RNA expression

Z-scores (RNA Seq Version 2 RSEM) with Z-score thresholds±2.0; and (4) protein/phospho-protein level (RPPA) with Z-score thresholds±2.0.

Data Analysis. A minimum of 3 independent replicates were completed for each experiment, reported as mean +/- SEMs. Statistical analysis was conducted using 2-Way ANOVA and Student's t-tests with JMP Pro 13 Statistical Analysis Software. Fold-change differences in mRNA expression were calculated with the GeneGlobe Data Analysis Center (QIAGEN) for RT^2 Profiler PCR Arrays.

Results

The O-GlcNAc cycling enzymes, OGT and OGA, are altered in endometrial cancer

Analysis of gene alterations for OGT and OGA (*OGT* and *MGEA5*, respectively) using data from the RNAseq and Microarray databases available in cBioPortal (cBioPortal.org) revealed that of the 18 female reproductive cancer databases available, Uterine/Endometrial Cancer ranks highest for gene alterations to *OGT* and *MGEA5* , including both mutational and amplification modifications (Figure 1A). Additionally, both genes are upregulated in participants with Diabetes Mellitus compared to non-Diabetic controls (Figures 1B). These findings are consistent with the previous assertion that O-GlcNAc enzyme gene expression is increased along the myometrial invasive edge of endometrial tumors (Krześlak et al., 2012). Similarly, elevated expression of *OGT* and *MGEA5* mRNA is consistent with the nutrient-sensing nature of O-GlcNAcylation. High nutrient intake, hyperglycemia, and other metabolic abnormalities all promote the flow of glucose into the HBP, resulting in elevated O-GlcNAcylation. In this way, the current findings of the meta-analysis add support to the concept that O-GlcNAc modification

has a role in the development of diabetic complications and cancer (Copeland, Han, & Hart, 2013; T Issad et al., 2010; Ruan et al., 2013).

Detection and manipulation of O-GlcNAcylation in the endometrial cancer line, Ishikawa

Immunodetection of global O-GlcNAcylation in Ishikawa cells revealed this form of protein modification was upregulated (Hyper-O-GlcNAcylation) in cells by supplementing complete media with 25mM Glucose or by inhibiting OGA with Thiamet-G (1μM; ThmG) (Figures 1C and 1D), and downregulated (Hypo-GlcNAcylation) by inhibiting OGT with OSMI-1 (50μM) (Figures 1C and 1 D). These manipulations of O-GlcNAcylation were then utilized in all subsequent experiments to determine effects of aberrant O-GlcNAcylation on phenotypic changes in Ishikawa cells (i.e., cell proliferation/migration and invasion), as well as morphological and molecular alterations associated with EMT.

Hyper-O-GlcNAcylation supports endometrial cancer cell proliferation/migration, and promotes invasion

A wound healing assay demonstrated that Hyper-O-GlcNAcylation supported Ishikawa cell proliferation and migration, with no difference in wound closure observed among Control, Glucose and ThmG-treated cultures ($p>0.05$; Figures 2A and B). Hence, Hyper-O-GlcNAcylation was conducive to wound closure. Conversely, Hypo-O-GlcNAcylation (via OSMI-1) impaired cell proliferation/migration ($p<0.05$), resulting in wounds that failed to close within the time constraints of the experimental period (~40% wound closure after 72- hours of culture; Figures 2A and B). The inhibition was serum-dependent, however, as no difference in wound closure was observed among the treatment groups when FBS was excluded from the culture medium ($p>0.05$; Figure 2C).

Although Ishikawa cells are considered relatively-low metastatic cells (Kaori Ohtani, Hideki Sakamoto, Thomas Rutherford, Zhaocong Chen, Kazuo Satoh, 1999), treatment with Hyper-O-GlcNAcylation (ThmG treatment) augmented invasiveness compared to Control and Hypo-O-GlcNAcylated (OSMI-1-treated) cells ($p<0.05$; Figures 2D and E). Glucose supplementation provided an intermediate response, midway between Control and Hyper-O-GlcNAcylated cells (Figure 2E). The Hypo-O-GlcNAcylated cells (OSMI-1-treated) exhibited no invasion potential, comparable to Controls ($p>0.05$; Figures 2D and E).

Hyper-O-GlcNAcylation promotes the EMT phenotype

The EMT process was examined in Ishikawa cells at both the mRNA and protein levels by manipulating O-GlcNAc modification (as previously described). Phalloidin staining of stress filaments in these cells (F-actin) revealed diffuse and epithelial-like staining in Hypo-O-GlcNAcylated (OSMI-1- treated) cells (Figure 3A). Conversely, Hyper-O-GlcNAcylation (Glucose and ThmG-treatment) of cells resulted in conspicuous F-actin staining throughout the cytoplasm and stress fiber-like bundles along the marginal borders of the cells (white arrows, Figure 3A), indicative of mesenchymal cell morphology.

Despite the observed morphological changes in Hypo- and Hyper-O-GlcNAcylated cells, there was no evidence of changes in transcript abundance for key EMT markers (*ZEB1*, *CDH1*, *CDH2*, *CTNNB1*, *VIM*, *SNAI1*, *SNAI2*, and *CLDN1*) among the treatment groups as assessed by qPCR analysis (Table 2). Similarly, immunoblots indicated no change in E-Cadherin (CDH1) expression compared to Control cells; (Figures 3B and 3C). Although, expression of the E-Cadherin suppressor, Snail, was supported by all treatments, Hyper-O-GlcNAcylation via ThmG increased Snail expression to a greater extent than that of high glucose ($p<0.05$; ThmG vs.

Glucose, respectively, Figure 3C). The expression of β-Catenin was down-regulated by Hypo-O-GlcNAcylation (OSMI-1), while Hyper-O-GlcNAcylation (ThmG) sustained β-Catenin expression ($p<0.05$; Figure 3C). Claudin-1, a major constituent of tight junction complexes, was noticeably decreased by Hyper-O-GlcNAcylation (ThmG) and Hypo-O-GlcNAcylation (OSMI-1) compared to Control cells ($p<0.05$; Figure 3B). None of the other detected EMT markers (N-Cadherin, ZO-1, Vimentin, Slug, ZEB1) were affected by O-GlcNAcylation status ($p>0.05$; Figure 3B).

Microarray analysis corroborates dysregulation of O-GlcNAcylation as a mechanism of EMT in endometrial cancer

The above-described phenotypic changes were supported by mRNA analysis of 86 genes important to the process of EMT. The EMT RT^2 Profiler PCR Array and GeneGlobe Data Analysis Center analysis tool (Qiagen), was used to measure changes in gene expression (2 fold or greater) compared to Control cells (Supp. Table 2). Three independent experiments yielded the same mRNA expression profile for both Hyper-O-GlcNAcylation treatments, with increased expression of *FOXC2* and *WNT5B*, both promoters of EMT, and *KRT14*, a cytoskeletal intermediate filament monomer (Figure 4). Hypo-O-GlcNAcylated cells also had increased *WNT5B* and *KRT14*, however, Hypo-O-GlcNAcylation decreased the expression of *AHNAK*, *CALD1*, *FGFBP1*, *TGFB2*, *TFPI2* genes (Figure 4). The *AHNAK*, *CALD1*, and *TGFB2* genes are all typically up-regulated during EMT, while *FGFBP1* and *TFPI2* are commonly downregulated during this process (Chang et al., 2016).

Figure 1.

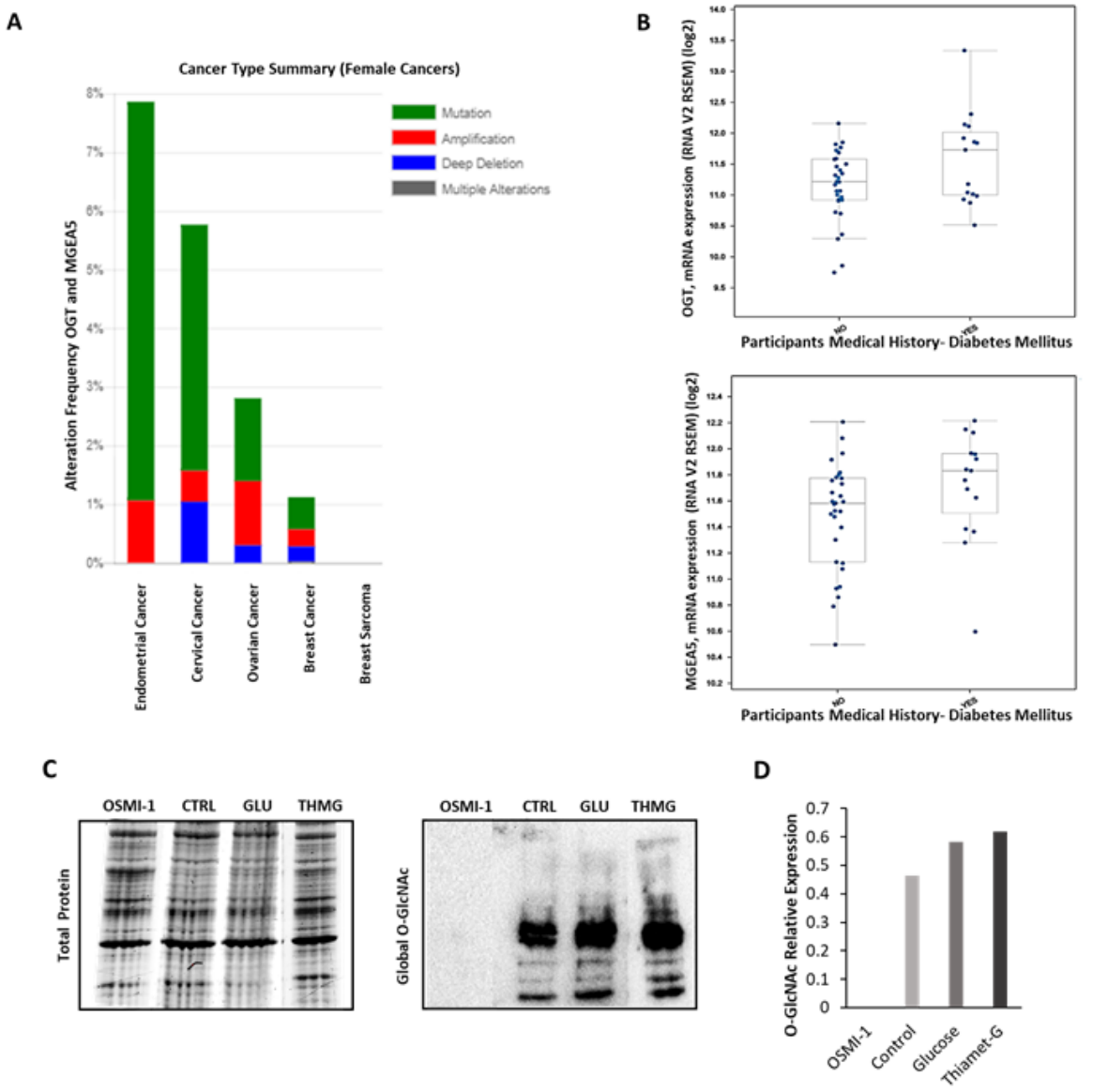


Figure 1: Meta-analysis of O-GlcNAc alteration in female cancers and Validation of global O-GlcNAc modification in Ishikawa cells. Cancer genomics data analysis depicting a cross-cancer O-GlcNAc enzyme (OGT, MGEA5) alteration summary of female cancers. [A] Histograms depicting the level of gene amplification, mutation, or deletion in each data set. Data was mined from the TCGA database and was analyzed with the cBioPortal web analysis tool. Of these 18 female cancer datasets, uterine/endometrial cancer ranked the highest for gene alterations of OGT and MGEA5. [B] Box plots comparing the relative mRNA expression of OGT and MGEA5 in patients with or without a diagnosis of diabetes mellitus (DM). Additional analysis of the data set revealed that OGT and MGEA5 mRNA was more highly expressed in endometrial cancer patients with DM. [C] Western Blot analysis of global O-GlcNAc modification, relative to total protein, in whole cell Ishikawa lysates treated with the OGT inhibitor, OSMI-1, the OGA inhibitor, Thiamet-G, or supplemented with 25mM Glucose. Both Glucose and Thiamet-G increased O-GlcNAcylation; no O-GlcNAcylation was detected in the OSMI-1 treated cells.

Figure 2.

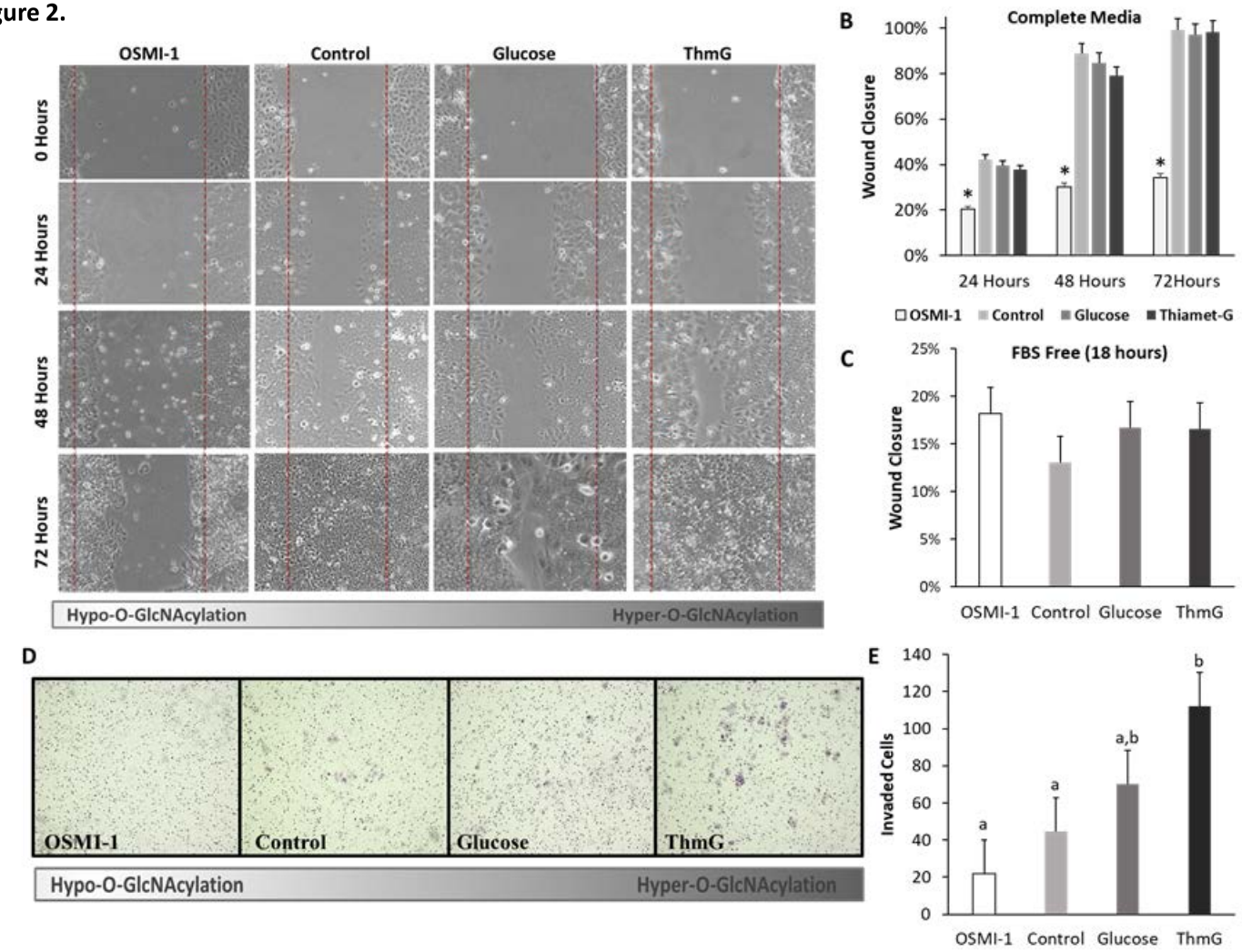


Figure 2. O-GlcNAcylation is required for proliferation and migration of Ishikawa cells. [A] Representative images of the effects of Thiamet-G, high glucose, OSMI-1, or vehicle on migration/proliferation of Ishikawa cells (10%FBS). "Wounds" were imaged every 24 hours for 72 hours. [B] Quantitative data of the wound healing assay. Each bar in the graph represents the mean +/- SEM of 3 biological replicates (n=3). An asterisk (*) indicates a significant difference ($p<0.05$) within a corresponding time point. [C] Results for the wound-healing assay repeated under serum-free conditions for 18 hours. Bars depict the mean +/- SEM (n=3). [D] Representative images of invasive cells following a Biocoat Matrigel Transwell Invasion assay (n=4). Purple foci depict invasive cells. [E] Quantitative analysis of the invasion assay in which each bar represents the mean number of invaded cells (+/- SEM) after 48 hours of culture. Different letters denote differences ($p<0.05$) among the treatments (n=4).

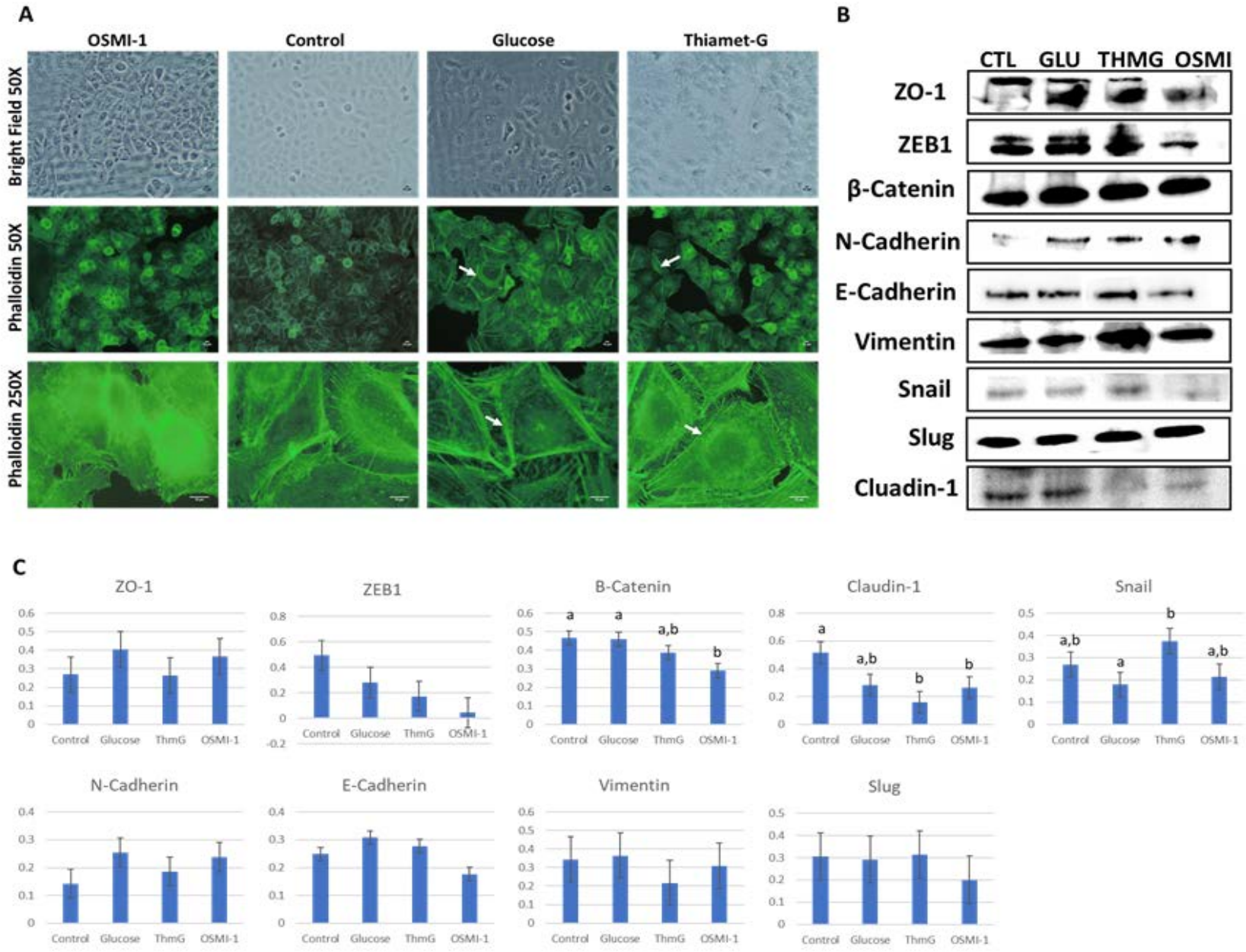
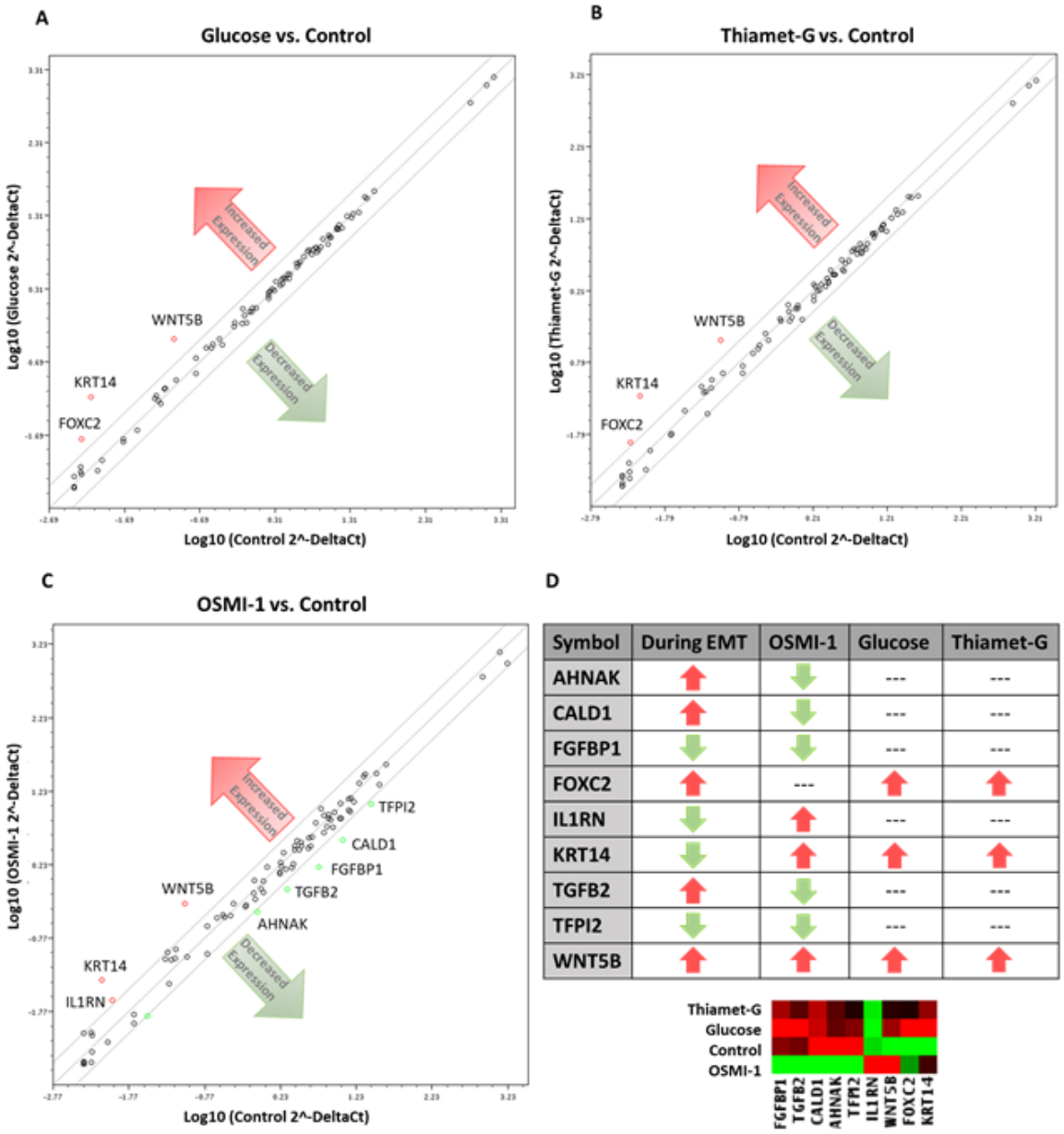


Figure 3. Disruption to O-GlcNAc signaling alters cell morphology and EMT protein expression. Representative images of fixed Ishikawa cells treated with OSMI-1, Thiamet-G, 25mM glucose, or vehicle for 48 hours. Immunofluorescent staining of actin with phalloidin was asses for signs of EMT, including stress fibers (white arrows), and focal adhesions (yellow arrows). [B] Representative images of Western Blot analysis (n=3) of whole cell lysates probed for epithelial and mesenchymal protein markers associated with EMT in Ishikawa cells treated as stated above for 48 hours. [C] Densitometry analysis of Western Blots [B], bars represent the mean +/- SEM (n=3), different letters denote statistically significant differences in density ($p<0.05$).

Figure 4.



Symbol	During EMT	OSMI-1	Glucose	Thiamet-G
AHNAK	↑	↓	---	---
CALD1	↑	↓	---	---
FGFBP1	↓	↓	---	---
FOXC2	↑	---	↑	↑
IL1RN	↓	↑	---	---
KRT14	↓	↑	↑	↑
TGFB2	↑	↓	---	---
TFPI2	↓	↓	---	---
WNT5B	↑	↑	↑	↑

Figure 4. Disruption of O-GlcNAc alters gene expression in EMT related genes in Ishikawa cells. Cells were cultured with OSMI-1, Thiamet-G, 25mM glucose, or vehicle for 48 hours before being harvested for RNA isolation. The mRNA was the quantified by RT-PCR (n=3) and depicted is relative expression of cells grown in [A] 25mM glucose, [B] Thiamet-G, and [C] OSMI-1 compared to vehicle (control). The lines encompass 2-fold changes. Genes up-regulated are denoted by red circles, whereas genes down-regulated appear as green circles. Black circles constitute genes that were relatively unchanged. [D] The chart and heatmap summarize the results compared to anticipated outcomes for EMT based upon the current literature.

Table 1.

PCR Array Catalog #:	PAHS-090Z					
Position	**Unigene**	**Refseq**	**Symbol**	**Description**	**Gname**	**RT2 Catalog**
A01	Hs.502756	NM_024060	AHNAK	AHNAK nucleoprotein	AHNAKRS	PPH18485A
A02	Hs.525622	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1	AKT/CWS6/PKB/PKB	PPH00088B
A03	Hs.1274	NM_006129	BMP1	Bone morphogenetic protein 1	OI13/PCOLC/PCP/PC	PPH00515B
A04	Hs.73853	NM_001200	BMP2	Bone morphogenetic protein 2	BDA2/BMP2A	PPH00549C
A05	Hs.473163	NM_001719	BMP7	Bone morphogenetic protein 7	OP-1	PPH00527A
A06	Hs.490203	NM_004342	CALD1	Caldesmon 1	CDM/H-CAD/HCAD/L-	PPH21139A
A07	Hs.731383	NM_018584	CAMK2N1	Calcium/calmodulin-dependent protein kinase II i	PRO1489	PPH14215B
A08	Hs.212332	NM_001233	CAV2	Caveolin 2	CAV	PPH12406B
A09	Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Arc-1/CD324/CDHE/E	PPH00135F
A10	Hs.464829	NM_001792	CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	CD325/CDHN/CDw325	PPH00636F
A11	Hs.489142	NM_000089	COL1A2	Collagen, type I, alpha 2	OI4	PPH01918B
A12	Hs.443625	NM_000090	COL3A1	Collagen, type III, alpha 1	EDS4A	PPH00439F
B01	Hs.445827	NM_000393	COL5A2	Collagen, type V, alpha 2	EDSC	PPH00781A
B02	Hs.712929	NM_001904	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88	CTNNB/MRD19/armad	PPH00643F
B03	Hs.95612	NM_004949	DSC2	Desmocollin 2	ARVD11/CDHF2/DG2/	PPH10581B
B04	Hs.519873	NM_004415	DSP	Desmoplakin	DCWHKTA/DP/DPI/DI	PPH17443A
B05	Hs.488293	NM_005228	EGFR	Epidermal growth factor receptor	ERBB/ERBB1/HER1/I	PPH00138B
B06	Hs.118681	NM_001982	ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene h	ErbB-3/HER3/LCCS2/	PPH00463B
B07	Hs.744830	NM_000125	ESR1	Estrogen receptor 1	ER/ESR/ESRA/ESTR	PPH01001A
B08	Hs.517293	NM_016946	F11R	F11 receptor	CD321/JAM/JAM1/JAI	PPH02605A
B09	Hs.1690	NM_005130	FGFBP1	Fibroblast growth factor binding protein 1	FGF-BP/FGF-BP1/FG	PPH07093A
B10	Hs.203717	NM_002026	FN1	Fibronectin 1	CIG/ED-B/FINC/FN/FN	PPH00143B
B11	Hs.436448	NM_005251	FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead	FKHL14/LD/MFH-1/MI	PPH01971A
B12	Hs.173859	NM_003507	FZD7	Frizzled family receptor 7	FzE3	PPH02420B
C01	Hs.83381	NM_004126	GNG11	Guanine nucleotide binding protein (G protein), ga	GNGT11	PPH02836C
C02	Hs.440438	NM_173849	GSC	Goosecoid homeobox	SAMS	PPH01920A
C03	Hs.445733	NM_002093	GSK3B	Glycogen synthase kinase 3 beta	-	PPH00787C
C04	Hs.462998	NM_001552	IGFBP4	Insulin-like growth factor binding protein 4	BP-4/HT29-IGFBP/IBP	PPH00286B
C05	Hs.81134	NM_000577	IL1RN	Interleukin 1 receptor antagonist	DIRA/ICIL-1RA/IL-1RN	PPH00555G
C06	Hs.706355	NM_004517	ILK	Integrin-linked kinase	HEL-S-28/ILK-1/ILK-2/	PPH00737F
C07	Hs.505654	NM_002205	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polyp	CD49e/FNRA/VLA-5/V	PPH00176C
C08	Hs.436873	NM_002210	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polyp	CD51/MSK8/VNRA/VT	PPH00628C
C09	Hs.643813	NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polype	CD29/FNRB/GPIIA/MI	PPH00650B
C10	Hs.224012	NM_000214	JAG1	Jagged 1	AGS/AHD/AWS/CD33	PPH06022B
C11	Hs.654380	NM_000526	KRT14	Keratin 14	CK14/EBS3/EBS4/K1	PPH02389A
C12	Hs.654568	NM_002276	KRT19	Keratin 19	CK19/K19/K1CS	PPH01004E
D01	Hs.411501	NM_005556	KRT7	Keratin 7	CK7/K2C7/K7/SCL	PPH08502E
D02	Hs.335079	NM_005909	MAP1B	Microtubule-associated protein 1B	FUTSCH/MAP5/PPP1	PPH02398A
D03	Hs.513617	NM_004530	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa g	CLG4/CLG4A/MMP-2/	PPH00151B
D04	Hs.375129	NM_002422	MMP3	Matrix metallopeptidase 3 (stromelysin 1, progela	CHDS6/MMP-3/SL-1/S	PPH00235F
D05	Hs.297413	NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa g	CLG4B/GELB/MANDB	PPH00152E
D06	Hs.713679	NM_002444	MSN	Moesin	HEL70	PPH13452B
D07	Hs.517973	NM_002447	MST1R	Macrophage stimulating 1 receptor (c-met-related	CD136/CDw136/PTK8	PPH07170B
D08	Hs.370414	NM_018055	NODAL	Nodal homolog (mouse)	HTX5	PPH01944B
D09	Hs.495473	NM_017617	NOTCH1	Notch 1	AOS5/AOVD1/TAN1/h	PPH00526C
D10	Hs.533657	NM_015901	NUDT13	Nudix (nucleoside diphosphate linked moiety X)-t	-	PPH20529A
D11	Hs.592605	NM_002538	OCLN	Occludin	BLCPMG/PPP1R115	PPH02571B
D12	Hs.509067	NM_002609	PDGFRB	Platelet-derived growth factor receptor, beta polyp	CD140B/IBGC4/IMF1/	PPH00477C
E01	Hs.170473	NM_016445	PLEK2	Pleckstrin 2	-	PPH13458F
E02	Hs.570455	NM_015704	DESI1	PPPDE peptidase domain containing 2	D15Wsu75e/DESI2/DJ	PPH23010A
E03	Hs.395482	NM_005607	PTK2	PTK2 protein tyrosine kinase 2	FADK/FAK/FAK1/FRN	PPH02827A
E04	Hs.227777	NM_003463	PTP4A1	Protein tyrosine phosphatase type IVA, member	HH72/PRL-1/PRL1/PT	PPH14671C
E05	Hs.413812	NM_006908	RAC1	Ras-related C3 botulinum toxin substrate 1 (rho f	MIG5/Rac-1/TC-25/p2	PPH00733F
E06	Hs.78944	NM_002923	RGS2	Regulator of G-protein signaling 2, 24kDa	G0S8	PPH02231A
E07	Hs.414795	NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasm	PAI/PAI-1/PAI1/PLAN	PPH00215F
E08	Hs.652307	NM_003616	GEMIN2	Survival of motor neuron protein interacting protei	SIP1/SIP1-delta	PPH10871A
E09	Hs.12253	NM_005901	SMAD2	SMAD family member 2	JV18/JV18-1/MADH2/I	PPH01949F
E10	Hs.48029	NM_005985	SNAI1	Snail homolog 1 (Drosophila)	SLUGH2/SNA/SNAH/I	PPH02459B
E11	Hs.360174	NM_003068	SNAI2	Snail homolog 2 (Drosophila)	SLUG/SLUGH1/SNAIL	PPH02475A
E12	Hs.673548	NM_178310	SNAI3	Snail homolog 3 (Drosophila)	SMUC/SNAIL3/ZNF29	PPH15155C
F01	Hs.376984	NM_006941	SOX10	SRY (sex determining region Y)-box 10	DOM/PCWH/WS2E/W	PPH02458C
F02	Hs.111779	NM_003118	SPARC	Secreted protein, acidic, cysteine-rich (osteonect	BM-40/ON	PPH01175A
F03	Hs.313	NM_000582	SPP1	Secreted phosphoprotein 1	BNSP/BSPI/ETA-1/OP	PPH00582E
F04	Hs.463059	NM_003150	STAT3	Signal transducer and activator of transcription 3	ADMIO/APRF/HIES	PPH00708F
F05	Hs.61635	NM_012449	STEAP1	Six transmembrane epithelial antigen of the prost	PRSS24/STEAP	PPH02268C
F06	Hs.371282	NM_003200	TCF3	Transcription factor 3 (E2A immunoglobulin enha	E2A/E47/ITF1/TCF-3/V	PPH06916G
F07	Hs.742885	NM_003199	TCF4	Transcription factor 4	E2-2/ITF-2/ITF2/PTHS	PPH02770A
F08	Hs.438231	NM_006528	TFPI2	Tissue factor pathway inhibitor 2	PP5/REF1/TFPI-2	PPH02580A
F09	Hs.645227	NM_000660	TGFB1	Transforming growth factor, beta 1	CED/DPD1/LAP/TGFB	PPH00508A
F10	Hs.133379	NM_003238	TGFB2	Transforming growth factor, beta 2	LDS4/TGF-beta2	PPH00524B
F11	Hs.713281	NM_003239	TGFB3	Transforming growth factor, beta 3	ARVD/ARVD1/RNHF/	PPH00531F
F12	Hs.522632	NM_003254	TIMP1	TIMP metallopeptidase inhibitor 1	CLGI/EPA/EPO/HCI/T	PPH00771C
G01	Hs.598100	NM_003692	TMEFF1	Transmembrane protein with EGF-like and two fo	C9orf2/CT120.1/H736	PPH17229A
G02	Hs.118552	NM_178031	TMEM132A	Transmembrane protein 132A	GBP/HSPA5BP1	PPH11409A
G03	Hs.364544	NM_014399	TSPAN13	Tetraspanin 13	NET-6/NET6/TM4SF13	PPH17916A
G04	Hs.66744	NM_000474	TWIST1	Twist homolog 1 (Drosophila)	ACS3/BPES2/BPES3	PPH02132A
G05	Hs.643801	NM_004385	VCAN	Versican	CSPG2/ERVR/GHAP/	PPH06098D
G06	Hs.455493	NM_003380	VIM	Vimentin	CTRCT30/HEL113	PPH00417F
G07	Hs.459790	NM_033305	VPS13A	Vacuolar protein sorting 13 homolog A (S. cerevis	CHAC/CHOREIN	PPH09443A
G08	Hs.108219	NM_004626	WNT11	Wingless-type MMTV integration site family, mer	HWNT11	PPH02399C
G09	Hs.643085	NM_003392	WNT5A	Wingless-type MMTV integration site family, mer	hWNT5A	PPH02410A
G10	Hs.306051	NM_032642	WNT5B	Wingless-type MMTV integration site family, mer	-	PPH02447C
G11	Hs.124503	NM_030751	ZEB1	Zinc finger E-box binding homeobox 1	AREB6/BZP/DELTAEI	PPH01922A
G12	Hs.34871	NM_014795	ZEB2	Zinc finger E-box binding homeobox 2	HSPC082/SIP-1/SIP1/	PPH09021B
H01	Hs.520640	NM_001101	ACTB	Actin, beta	BRWS1/PS1TP5BP1	PPH00073G
H02	Hs.534255	NM_004048	B2M	Beta-2-microglobulin	-	PPH01094E
H03	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD/HEL-S-1	PPH00150F
H04	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT/HPRT	PPH01018C
H05	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0	L10E/LP0/P0/PRLP0/I	PPH21138F
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination	HIGX1A	
H07	N/A	SA_00104	RTC	Reverse Transcription Control	RTC	PPX63340A
H08	N/A	SA_00104	RTC	Reverse Transcription Control	RTC	PPX63340A
H09	N/A	SA_00104	RTC	Reverse Transcription Control	RTC	PPX63340A
H10	N/A	SA_00103	PPC	Positive PCR Control	PPC	
H11	N/A	SA_00103	PPC	Positive PCR Control	PPC	
H12	N/A	SA_00103	PPC	Positive PCR Control	PPC	

Table 2.

25mM Glucose vs. Control (fold change)

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AHNAK 1.00 C	AKT1 -1.02	BMP1 1.09	BMP2 1.21 B	BMP7 1.00 C	CALD1 1.04	CAMK2N1 1.44 B	CAV2 1.24	CDH1 1.19	CDH2 1.23	COL1A2 1.19	COL3A1 1.44
B	COL5A2 -1.01 B	CTNNB1 -1.10	DSC2 -1.00	DSP 1.20	EGFR 1.20	ERBB3 1.13	ESR1 1.03	F11R 1.15	FGFBP1 1.35	FN1 1.18	FOXC2 3.67 B	FZD7 1.58
C	GNG11 1.32	GSC 1.00 C	GSK3B 1.16	IGFBP4 1.43 B	IL1RN 1.01 B	ILK 1.06	ITGA5 1.20	ITGAV 1.18	ITGB1 1.16	JAG1 1.17	KRT14 10.19 B	KRT19 1.15
D	KRT7 1.16 B	MAP1B 1.09	MMP2 -1.14 B	MMP3 1.00 C	MMP9 -1.06 B	MSN 1.05	MST1R 1.20	NODAL 1.02 B	NOTCH1 1.07	NUDT13 1.31	OCLN 1.35	PDGFRB 1.03 B
E	PLEK2 1.24	DESI1 -1.01	PTK2 1.12	PTP4A1 1.08	RAC1 1.29	RGS2 -1.05	SERPINE1 1.40	GEMIN2 1.21	SMAD2 1.25	SNAI1 1.30 B	SNAI2 -1.19 B	SNAI3 -1.14
F	SOX10 1.33 B	SPARC 1.57 B	SPP1 1.02	STAT3 1.04	STEAP1 1.19	TCF3 1.06	TCF4 1.09	TFPI2 -1.04	TGFB1 1.01	TGFB2 1.38	TGFB3 1.29	TIMP1 1.11
G	TMEFF1 1.21	TMEM132 A 1.02	TSPAN13 1.10	TWIST1 1.12	VCAN 1.27	VIM 1.21	VPS13A 1.29	WNT11 1.40 B	WNT5A 1.26	WNT5B 5.06 A	ZEB1 1.41 B	ZEB2 1.42 B

Thiamet-G vs. Control (fold change)

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AHNAK -1.38 C	AKT1 -1.47	BMP1 -1.19	BMP2 -1.37 B	BMP7 -1.04 B	CALD1 -1.09	CAMK2N1 1.16 B	CAV2 -1.02	CDH1 -1.13	CDH2 -1.25	COL1A2 -1.38	COL3A1 -1.13
B	COL5A2 -1.94 B	CTNNB1 -1.01	DSC2 1.18	DSP -1.07	EGFR -1.10	ERBB3 1.13	ESR1 -1.18	F11R -1.12	FGFBP1 1.07	FN1 -1.00	FOXC2 2.32 B	FZD7 1.11
C	GNG11 1.13	GSC -1.38 C	GSK3B -1.27	IGFBP4 1.05 B	IL1RN -1.04 B	ILK -1.19	ITGA5 1.06	ITGAV 1.06	ITGB1 -1.26	JAG1 -1.06	KRT14 7.53 B	KRT19 -1.06
D	KRT7 1.11 B	MAP1B -1.77	MMP2 1.16 B	MMP3 1.02 B	MMP9 -1.21 B	MSN -1.06	MST1R -1.16	NODAL -1.16 B	NOTCH1 -1.28	NUDT13 -1.09	OCLN 1.06	PDGFRB -1.38 C
E	PLEK2 -1.10	DESI1 -1.37	PTK2 -1.26	PTP4A1 -1.18	RAC1 1.06	RGS2 -1.21	SERPINE1 -1.25	GEMIN2 -1.14	SMAD2 -1.20	SNAI1 1.16 A	SNAI2 -1.68 B	SNAI3 -1.56
F	SOX10 -1.10 B	SPARC 1.24 B	SPP1 1.38	STAT3 -1.33	STEAP1 1.13	TCF3 -1.21	TCF4 -1.22	TFPI2 -1.31	TGFB1 -1.20	TGFB2 -1.03	TGFB3 1.03	TIMP1 1.01
G	TMEFF1 -1.14	TMEM132 A -1.15	TSPAN13 -1.18	TWIST1 -1.16	VCAN 1.13	VIM -1.40	VPS13A -1.15	WNT11 1.32 B	WNT5A -1.29	WNT5B 3.58 A	ZEB1 1.48 B	ZEB2 -1.38 C

OSMI-1 vs. Control (fold change)

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AHNAK -1.11 C	AKT1 -1.09	BMP1 -1.14	BMP2 1.04 B	BMP7 -1.05 B	CALD1 -2.53	CAMK2N1 2.41 A	CAV2 -1.19	CDH1 1.41	CDH2 1.37	COL1A2 -1.16	COL3A1 1.56
B	COL5A2 1.67 B	CTNNB1 -1.18	DSC2 1.30	DSP 1.14	EGFR 1.08	ERBB3 1.55	ESR1 1.26	F11R 1.17	FGFBP1 -2.88	FN1 1.04	FOXC2 1.75 B	FZD7 1.61
C	GNG11 -1.15	GSC -1.11 C	GSK3B 1.35	IGFBP4 1.58 B	IL1RN 2.82 B	ILK -1.26	ITGA5 1.11	ITGAV -1.04	ITGB1 1.12	JAG1 1.33	KRT14 7.35 B	KRT19 -1.37
D	KRT7 -1.20 B	MAP1B 1.65	MMP2 -1.70 B	MMP3 2.36 B	MMP9 -1.04 B	MSN -1.32	MST1R 1.43	NODAL -1.42 B	NOTCH1 -1.28	NUDT13 1.27	OCLN 2.03	PDGFRB -1.11 C
E	PLEK2 -1.18	DESI1 1.18	PTK2 1.18	PTP4A1 1.54	RAC1 1.12	RGS2 1.45	SERPINE1 -1.46 A	GEMIN2 1.09	SMAD2 1.20	SNAI1 1.14 B	SNAI2 -1.14 B	SNAI3 1.65
F	SOX10 -1.30 B	SPARC 1.98 B	SPP1 1.12	STAT3 -1.25	STEAP1 -1.06	TCF3 -1.15	TCF4 -1.04	TFPI2 -1.94	TGFB1 1.08	TGFB2 -2.23	TGFB3 -1.06	TIMP1 1.21
G	TMEFF1 1.60	TMEM132 A 1.31	TSPAN13 -1.20	TWIST1 1.14	VCAN 1.11	VIM -1.07	VPS13A 1.52	WNT11 2.03 A	WNT5A -1.14	WNT5B 6.49 A	ZEB1 2.07 B	ZEB2 -1.03 B

Discussion

O-GlcNAcylation is a recently discovered post-translational modification of cellular proteins that is highly abundant and ubiquitous throughout the nuclear and cytoplasmic compartments of the cell, yet its influence on cellular processes is poorly understood. First-discovered about 30 years ago (Torres & Hart, 1984), O-GlcNAc is an end-product of the hexosamine biosynthesis pathway (HBP), where its relative concentration serves as nutrient sensor indicative of metabolic status(Palin et al., 2014). Indeed, elevated O-GlcNAcylation (i.e., Hyper- O-GlcNAcylation), for example, is a hallmark of high metabolism seen in many types of cancers (Singh et al., 2015), including cancers of the breast (S a Caldwell et al., 2010), liver(GUO et al., 2012), bladder (Rozanski et al., 2012), prostate (Itkonen et al., 2013), lung(Mi et al., 2011), colon (Kamigaito et al., 2013; Mi et al., 2011), and endometrium(Krześlak et al., 2012). Thus, O-GlcNAc, and by extension O-GlcNAcylation, may constitute an important regulator of cancer development and progression, while simultaneously providing a potential, novel connection between the onset of T2D and the increased risk of certain cancers (Gallagher & LeRoith, 2015). Endometrial cancers fall into this category of increased risk attributable to T2D (Byrne et al., 2014; Friberg et al., 2007; Zelenko & Gallagher, 2014), and the current study is the first, to our knowledge, to reveal that aberrant O-GlcNAcylation mechanistically-augments tumorigenicity of endometrial cancer cells.

One mechanism responsible for increased tumorigenicity in many cancers is the phenomenon of epithelial-mesenchymal transition (EMT). Although most scientists acknowledge that EMT is a critical process of embryogenesis, this same process enhances tumor development and metastasis (Kondaveeti, Guttilla Reed, & White, 2015; Tam & Weinberg, 2013; Yilmaz & Christofori, 2009). Some of the key characteristics of EMT include the loss of

cell polarity and cell-cell adhesions, and an increase in invasive properties (Larue & Bellacosa, 2005; Rojas-Puentes et al., 2016). In the current study, Hyper-O-GlcNAcylation supported cell proliferation, induced reorganization of the cytoskeleton, and promoted invasion of endometrial cancer (Ishikawa) cells.

Intercellular adhesion, mediated largely by cadherin and catenin proteins, is a critical obstacle for epithelial cells to overcome as they de-differentiate and become more tumorigenic. A decrease in E-Cadherin expression, for instance, is among the initiating steps of EMT (Onder et al., 2008; Yao, Dai, & Peng, 2011), and is a notable consequence of Hyper-O-GlcNAcylation in many cell types (Fardini et al., 2013). Snail1, an E-Cadherin suppressor, is stabilized by O-GlcNAc at Ser112 under hyperglycemic culture conditions (Sang Yoon Park et al., 2010), and O-GlcNAcylation of E-Cadherin can occur directly via the cytoplasmic domain, which consequently inhibits its transport to the cell surface and prevents intercellular adhesion(Zhu et al., 2012). In the current study, Hyper-O-GlcNAcylation increased the expression of Snail1, but the effect was modest and had no overall impact on E-Cadherin expression or the expression of any other EMT protein markers. This suggests that the alteration of E-Cadherin expression is perhaps less critical for EMT in Ishikawa cells, or that the period of time required for this type of transition is much longer than that evaluated in the current experiments (48 hrs).

A major group of cellular signaling pathways associated with EMT are the Wnt signaling pathways. Of the Wnt pathways, 3 have been characterized; the canonical pathway, the noncanonical pathway, and the noncanonical Wnt/Calcium pathway (Yilmaz & Christofori, 2009). In the canonical pathway, Wnt induces the expression of the mesenchymal-like protein, Beta-catenin. Beta-catenin accumulates in the cytoplasm and is translocated to the nucleus, where it acts as a transcriptional activator of TCF/LEF family transcription factors, which in turn

induces a cellular response (Polakis, 2000). A recent study investigating the role of high glucose concentrations in endometrial cancer found that high glucose increased the flux of glucose into the HBP, in turn increasing O-GlcNAcylation of proteins (Zhou et al., 2016). Hyper-O-GlcNAcylation also increased the expression of B-catenin (Zhou et al., 2016). In contrast, an upregulation of the non-canonical Wnt ligand, WNT5B, by any form of O-GlcNAc dysregulation (i.e., Hyper- and Hypo-O-GlcNAcylation), occurred in the current study, and this outcome did not affect the canonical Wnt ligands (i.e. Wnts -1, -2, -3, -8a, -8b, -10a, -10b)(Siar et al., 2012) or β-catenin expression (Figures 4 and 3B, respectively). The non-canonical Wnt pathways are not as well understood as the canonical, partly due to the diversity and sheer number of pathways. However, one such intracellular pathway activated by Wnt5b is the Planar Cell Polarity (PCP) pathway, which controls tissue polarity and cell migration (Spandidos & Ethnikon Hidryma Ereunōn, 1994). WNT5B is up-regulated in MCF-7 cells(Panepistēmio tēs Krētēs. & Katoh, 2002) and is associated with cell migration and proliferation in lung cancer cells (Harada et al., 2016). In preadipocytes, WNT5B overexpression partially inhibits canonical Wnt/β-catenin signaling, thereby promoting adipogenesis (Kanazawa et al., 2005). In addition to its role in cancer and metastasis, non-canonical Wnt signaling also promotes metabolic dysfunction and adipose tissue inflammation, which is critical to the development of insulin resistance (Fuster et al., 2015). Collectively, these observations lead us to suggest that, in Ishikawa cells, dysregulation of O-GlcNAc signaling promotes EMT through activation of non-canonical Wnt signaling by WNT5B overexpression, which may in turn inhibit Wnt/β-catenin signaling. Such a mechanism might also help explain the connection between insulin resistance and increased risk of endometrial cancer.

In addition to increased WNT5B expression, the transcription factor, Forkhead box protein C2 (FOXC2) is highly-expressed in Hyper-O-GlcNAcylated Ishikawa cells. In accordance with these results, there is often increased expression of FOXC2 during EMT, and over-expression of FOXC2 is associated with highly-metastatic cancers (Hollier et al., 2013), some of which can be reduced by shRNA therapy (Paranjape et al., 2016). In podocytes, for instance, increased FOXC2 is associated with increased vimentin expression, cytoskeletal reorganization, disruption to ZO-1 localization, and increased cell motility (Datta, Lindfors, Miura, Saleem, & Lehtonen, 2016). Thus, there is support for the concept that Hyper-O-GlcNAcylation of Ishikawa cells induces EMT and metastasis via a FOXC2-mediated mechanism.

Cytoskeletal re-organization is key feature of EMT. Cytokeratin intermediate filaments are often considered biomarkers of cancers, but are more frequently gaining attention as mediators of disease (Karantza, 2011; Pan et al., 2013). Perhaps the best example of this is Keratin 18, a component of cytokeratin 8/18 intermediate filaments, which has long been used as a marker for apoptosis in cancers (Lomnytska & Souchelnytsky, 2007), but the loss of keratin 18 expression in cells is associated with EMT (Fortier et al., 2013a). In our previous work utilizing cervical cancer cells, we determined that Hypo-O-GlcNAcylation reduces and re-organizes cytokeratin 8/18 filaments, which suggests that O-GlcNAcylation influences filament expression and stability (Jaskiewicz, et al, 2017). Similar results were noted in the current study, as both Hyper- and Hypo-O-GlcNAcylation increased the expression of Keratin 14 mRNA in endometrial cells. Others have shown that co-expression of vimentin and keratin in MCF-7 breast cancer cells increases cell invasiveness (Hendrix, Seftor, Seftor, & Trevort, 1997). Hyper-O-GlcNAcylation in Ishikawa cells in the current study increased Keratin 14 expression, had no

effect on vimentin expression, and yet increased metastatic potential. These findings are intriguing and warrant further investigation into the potential regulation of keratin filaments by O-GlcNAcylation, and a possible role in disease progression.

In summary, dysregulation of O-GlcNAcylation supports EMT and cytoskeletal re-organization in endometrial cancer cells, potentially through activation of the noncanonical Wnt signaling pathway by WNT5B. Additionally, Hyper-O-GlcNAcylation induces the expression of FOXC2 and augments the invasion potential of the cells. These findings indicate Hyper-O-GlcNAcylation, such as that observed in diabetic individuals (Medford et al., 2012), could enhance the aggressiveness of endometrial cancer. Conversely, Hypo-O-GlcNAcylation impaired endometrial cancer cell proliferation and wound healing, and down-regulated expression of pro-EMT genes (AHNAK, CALD1, and TGFB2). These observations suggest that metabolic status, particularly as it relates to O-GlcNAcylation, might be an important consideration in therapeutic approaches to endometrial cancer. Future studies should focus on aberrant O-GlcNAcylation as a potential marker for more aggressive disease in human tissues, and the inhibition of Hyper-O-GlcNAcylation as a potential treatment in diabetic patients.

CHAPTER 5

HYPER-O-GLCNACYLATION IMPAIRS THE ANTI-PROLIFERATIVE AND ANTI-INVASIVE PROPERTIES OF PROGESTERONE IN ENDOMETRIAL CANCER CELLS

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Introduction

Hyper-O-GlcNAcylation of proteins is a subsequent artifact of metabolic disorder (Keembiyehetty et al., 2015) and is indicative of many cancers (Ma & Vosseller, 2013; Onodera, Nam, & Bissell, 2014), including endometrial cancer (Krześlak, Wójcik-Krowiranda, Forma, Bieńkiewicz, & Bryś, 2012). While the incidence of most types of cancer has declined in the U.S., endometrial cancer remains on the rise, disproportionately affecting black women (Cote, Ruterbusch, Olson, Lu, & Ali-Fehmi, 2015). The mean age for onset of endometrial cancer is 60 years, but at least 14% of cases diagnosed are women of reproductive age (Evans-Metcalf, Brooks, Reale, & Baker, 1998). These women have the option to postpone surgical treatment in favor of fertility-sparing progestin therapy (Jeong-Yeol Park, 2015; Kesterson & Fanning, 2012; Ramondetta, Burke, Broaddus, & Jhingran, 2006), but there are inevitable risks associated with this alternative, including the risk for undiagnosed synchronous or metastatic cancer. In a meta-analysis of 27 studies, 76% of the women responded favorably to progestin treatment, however, 24% of these patients had recurring disease within 19 months post treatment (Ramirez, Frumovitz, Bodurka, Sun, & Levenback, 2004). Additionally, many endometrial cancer patients are obese and/or suffer from metabolic disorders like insulin resistance and hyper-glycemia (Byrne et al., 2014), which results in global hyper-O-GlcNAcylation (Trinca & Hagan, 2017) and can confound therapeutic approaches.

Progesterone (P4) therapy reduces cell growth and invasion in endometrial cancer through the up-regulation of the cyclin dependent kinase inhibitors, p21 and p27 (Dai, Wolf, Litman, White, & Leslie, 2002; Shimizu et al., 2009); mechanisms that can also be influenced by O-GlcNAcylation. The tumor suppressor, p27 is highly O-GlcNAcylated, and an O-GlcNAc modification at Ser2 promotes the accumulation of p27 in the cytoplasm of HEK293 and hepatocellular carcinoma (HCC) cell lines (Qiu et al., 2017). Furthermore, the PI3K/Akt pathway is heavily influenced by O-GlcNAcylation (Gandy, Rountree, & Bijur, 2006; Jóźwiak, Forma, Bryś, & Krześlak, 2014), and can regulate the expression of both p21 (Lawlor & Rotwein, 2000; Rössig et al., 2001) and p27 (Graff et al., 2000) to further augment human cancers (Vivanco & Sawyers, 2002).

In the current study, the objective was to investigate the influence of hyper-O-GlcNAcylation on the efficacy of progestin therapy in endometrial cancer cells. Ishikawa cells (an immortal endometrial cancer cell line) were exposed to the O-GlcNAcase inhibitor, Thiamet-G (ThmG), to induce hyper-O-GlcNAcylation and then test the efficacy of a therapeutic concentration of P4 (100nM) to inhibit Ishikawa cell proliferation and invasion.

Materials and Methods

Cell Culture and Reagents. Endometrial cancer cells (Ishikawa) were obtained from Sigma Aldrich (cat. #99040201). Cell Line authentication was performed before, during, and after experimentation at the University of Vermont Cancer Center Advanced Genome Technologies Core and was supported by the University of Vermont Cancer Center, Lake Champlain Cancer Research Organization, and the University of Vermont College of Medicine. O-GlcNAcylation within the cells was manipulated by the OGA inhibitor, ThmG (1μM, Fisher Scientific) dissolved in DMSO (Fisher Scientific) at a concentration of 1000X. Control cells received an

equivalent concentration (0.1%) of DMSO as vehicle. Progesterone was administered to cells at 100nM as reported by others (Dai et al., 2002), and dissolved in ethanol (0.1%). Cell culture media (EMEM, 10% FBS and 10µl/mL antibiotic-anti-mycotic) (Fisher Scientific) was supplemented with 10nM estradiol (E2, #E1132, Sigma,; a gift from Dr. Catherine Combelles, Middlebury College) to support progesterone receptor expression as described (Holinka, Hata, Kuramoto, & Gurpide, 1986). Culture medium was exchanged every 24 hours unless otherwise specified.

Immunoblotting. Whole cell lysates of Ishikawa cells cultured in 25cm^2 flasks (Corning, Corning, NY) for 48 hours, were harvested by trypsin and lysed with RIPA buffer and passage through a 26G needle. Proteins were then separated on pre-stained SDS-Page gels (BioRad, Hercules, CA) and transferred to PVDF membrane. Membranes were then probed for global O-GlcNAcylation (CST-O-GlcNAc 110.6 antibody; Cell Signaling Technology, Inc.; Beverly, MA), p21 (p21 Waf1/Cip1 (12D1) Rabbit mAb #2947, Cell Signaling), and p27 (p27 Kip1 (D69C12) XP® Rabbit mAb #3686, Cell Signaling) proteins. Detection was achieved using secondary antibody (Anti-rabbit IgG HRP-linked Antibody, Cell Signaling Technology, Inc.), followed by Clarity Western ECL substrate (BioRad). Blots were imaged on the BioRad ChemiDoc Imaging System, and densitometry was calculated with Image J.

Cell Proliferation Assay. In a set of 3 independent experiments, cells were seeded in duplicate in flat-sided Thermo Fisher Scientific™ Nunc™ Cell Culture Tubes at an initial seeding density of 50k cells/ml of culture medium. Treatment groups included cultures of cells continuously exposed to vehicle (DMSO-Control), 1 µM Thiamet-G, and 100nM P4 accordingly. The conditioned culture medium was exchanged without/with treatment daily, and the cells for each group were harvested for counting at 24-hour intervals over a three-day period.

Matrigel Invasion Assay. Corning Matrigel Biocoat Invasion Chambers (24 well, 0.8μ) (Corning, Bedford, MA) were used to assess the invasion potential of treated cells compared to control. The upper chamber was seeded with 100,000 serum-starved cells. Cells were exposed to O-GlcNAc-modifying treatments and progesterone at the time of seeding. The lower chamber contained 5% FBS as a chemoattractant. Cells were incubated for 48 hours at 37°C and 5% CO_2. Cells were then removed from the upper chamber and the Transwell membranes were fixed with 100% methanol and stained with Crystal Violet. Invaded cells were then counted by two independent researchers and the results were averaged.

Results

Proliferation and Invasion.

A three-day cell proliferation assay confirmed the anti-proliferative effects of 100nM progesterone reported in the literature (Dai et al., 2002), ($p<0.05$ P4 treatment extended the doubling time by 21%; however, simultaneous treatment with ThmG negated this effect and resulted in a doubling time comparable to control (19.50 +/- 0.48 hours vs. 19.52 +/- 0.35 hours, respectively; Figure 1). In contrast, P4 did not decrease the invasion potential of Ishikawa cells compared to control, however ThmG treatment with or without progesterone did increase the invasiveness compared to progesterone treatment alone, ($p<0.05$, Figure 2).

Inhibition of OGA augments O-GlcNAcylation in Ishikawa cells; an effect impaired by P4 and downregulation of OGT expression.

OGA inhibition by Thiamet-G increased O-GlcNAcylation by 3-fold compared to control cells. Interestingly, and reduced OGT expression as reported in the literature. Progesterone treatment reduced the expression of OGT compared to control, and this was further

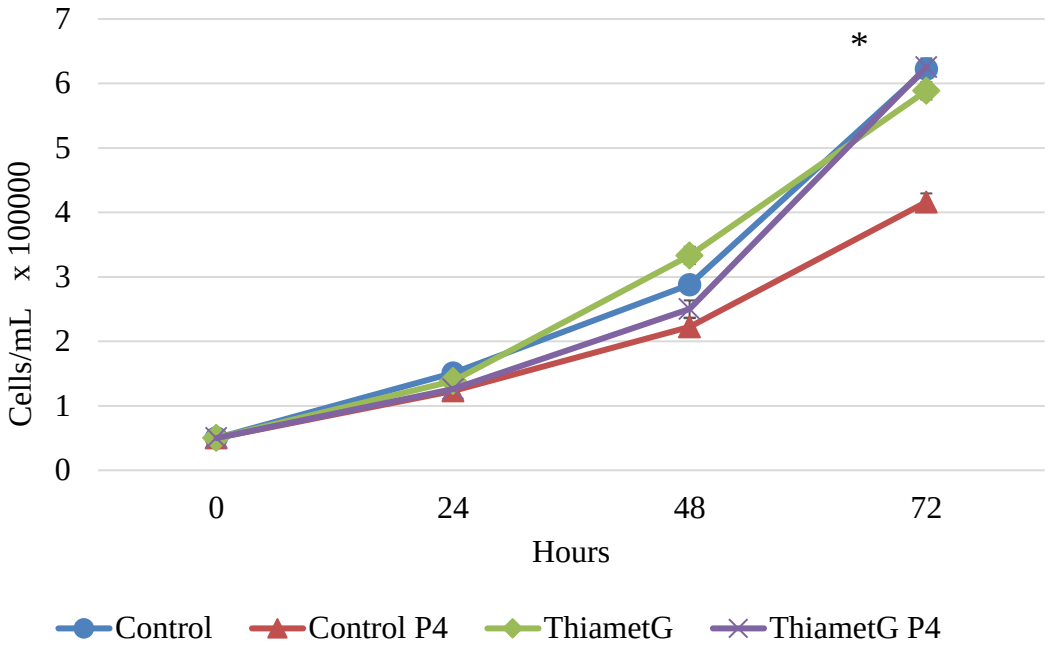
reduced by ThmG and P4 in combination. Subsequently, the level of O-GlcNAc modification was also reduced compared to cells treated with ThmG alone ($p<0.05$, Figure 3).

Inhibition of OGA by ThmG, reduces PR A and B and p27 expression in Ishikawa cells.

Progesterone treatment maintained PR A and PR B expression while, hyper-O-GlcNAcylation, induced by ThmG treatment, reduced both PR A and PR B expression ($p<0.05$), even with progesterone treatment ($p<0.05$). Progesterone reduced p21 expression compared to control cells ($p<0.05$, Figure 3). Interestingly, p21 expression was increased compared to control cells in hyper-O-GlcNAcylated cells (ThmG) that were also treated with P4 ($p<0.05$, Figure 3). Progesterone treatment maintained control levels of p27, but hyper-O-GlcNAcylation reduced p27 expression compared to control cells, regardless of P4 treatment. However, p27 expression was increased compared to ThmG treatment alone when P4 was added to the culture media ($p<0.05$, Figure 3).

Figure 1.

A.



B.

	Doubling Time (Hours)	**+/- SEM**
Control (DMSO, etOH)	19.52	0.35
P4 (100nM)	23.73 *	0.63
Thiamet-G (1µM)	19.94	0.069
P4 + Thiamet-G	19.50	0.48

C.

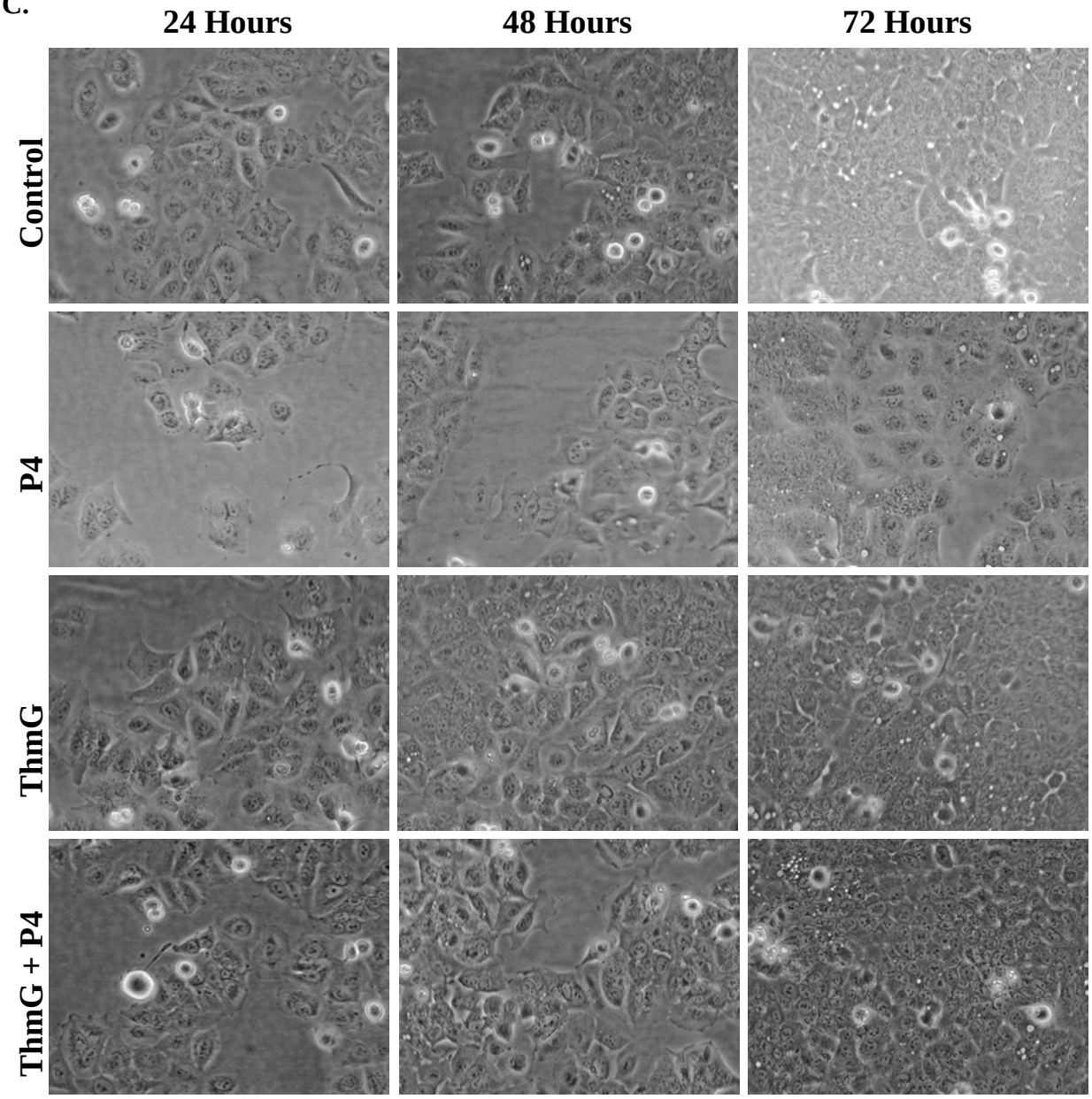


Figure 1. [A] Growth curve depicting the average (n=3) daily total cell counts for cells grown in control conditions (vehicle, DMSO and etOH), 100nM P4, 1μM ThmG, or a combination of ThmG and P4. [B] Doubling time +/-SEM calculated from growth curve data. [C] Representative images (n=3) of cell culture confluency at 24, 48, and 72 hours imaged at 20X magnification. * Specifies statistical significance ($p<0.05$).

Figure 2.

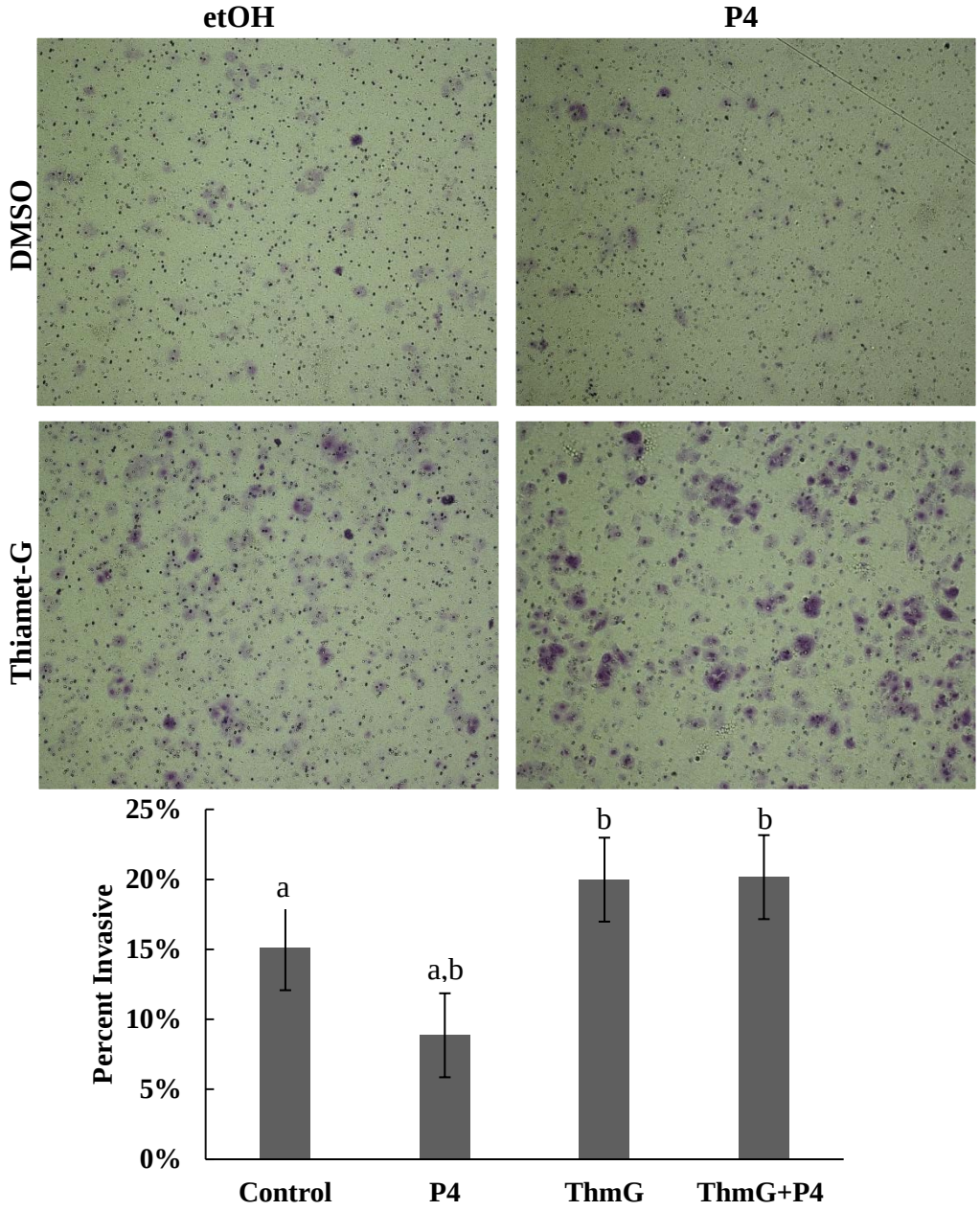


Figure 2. [A] Representative images of Transwell invasion assay membranes (n=4), fixed invasive cells stained with Crystal Violet (20X). [B] Bar graph denoting average (n=4) percent invasive population counted by 2 independent researchers. Different letters denote significant statistical differences ($p<0.05$).

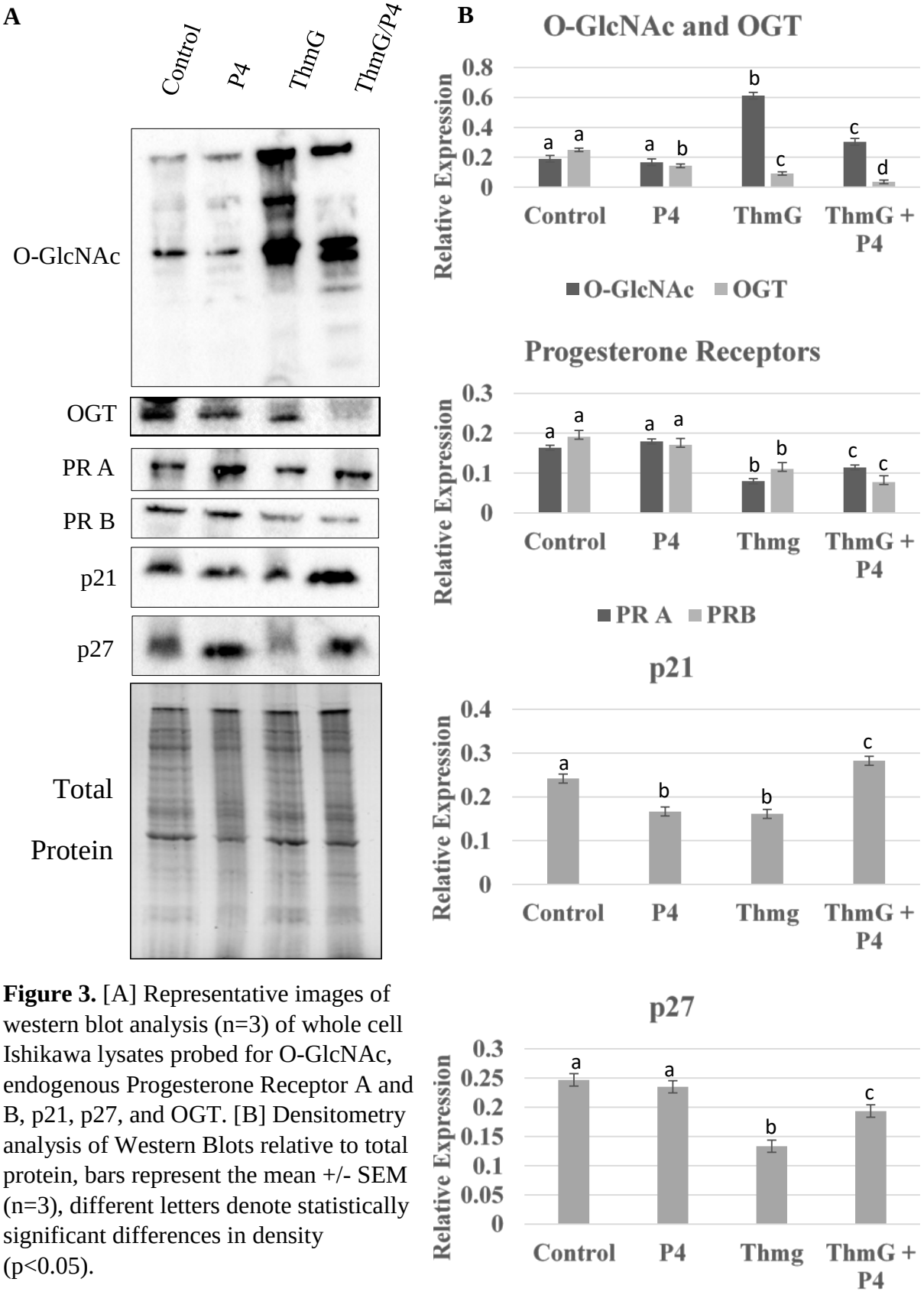


Figure 3. [A] Representative images of western blot analysis (n=3) of whole cell Ishikawa lysates probed for O-GlcNAc, endogenous Progesterone Receptor A and B, p21, p27, and OGT. [B] Densitometry analysis of Western Blots relative to total protein, bars represent the mean +/- SEM (n=3), different letters denote statistically significant differences in density ($p<0.05$).

Discussion

Hyper-O-GlcNAcylation overcomes the inhibitory effects of progesterone therapy on cell proliferation and invasion in Ishikawa cells.

Hyper-O-GlcNAcylation, particularly by the inhibition of OGA, increases cell proliferation in many cell types (Fardini, Dehennaut, Lefebvre, & Issad, 2013). This includes our own previous work in Ishikawa cells, discussed in Chapter 4. In the current study, OGA inhibition by ThmG, increased O-GlcNAcylation and supported both proliferation and invasion despite the therapeutic (i.e., inhibitory) effect of P4 treatment. Unlike previous studies (Dai, Wolf, Litman, White, & Leslie, 2002; Shimizu et al., 2009), however, P4 in the current work had no impact on cell invasion. This result is attributed to likelihood that OGA inhibition (via ThmG) promoted cell invasion through mechanisms associated with Epithelial-to-Mesenchymal Transition (EMT) as discussed in Chapter 4. In previous studies also conducted in Ishikawa cells, P4 was found to reduce EMT in vitro, however loss of PR expression correlated with increased EMT and more progressive disease (Van der Horst, et al., 2012).

Progesterone impairs OGT expression and subsequent O-GlcNAcylation

To our knowledge, the current study is the first to provide evidence that P4 impairs global O-GlcNAcylation in endometrial cancer cells and this effect is associated with the reduction of expression of O-GlcNAc cycling enzymes, namely OGT. P4 reduced the expression of OGT both alone and in combination with the OGA inhibitor, ThmG. While the mechanism for this effect is unclear, P4 does influence carbohydrate metabolism by inducing hyperinsulinemia and promoting glycogen storage in the liver (Kalkhoff, 1982). In this manner, P4 could regulate the amount of glucose shuttled to the Hexosamine Biosynthesis Pathway. A reduction of OGT

expression is associated with reduced proliferation, adhesion, and migration of cells in a fetal human cancer cell line (Steenackers et al., 2016). Thus, it is conceivable that the anti-proliferative and anti-invasive effects of P4 are attributable to a reduction in OGT.

Progesterone Receptor expression is influenced by Progesterone and O-GlcNAcylation

Ishikawa cells expressed Progesterone Receptors A and B in all treatment types, however, ThmG significantly reduced both PR A and PR B regardless of progesterone treatment. A study conducted in 2003 established two stable Ishikawa cell lines, hPR A and hPR B expressing cells, to study the individual effects of PR A and B receptors. Only the cells that expressed PR B alone were growth responsive to progesterone treatment, while the PR A expressing cells continued to proliferate (Smid-Koopman et al., n.d.). In breast cancer, PR A tends to be predominantly expressed, leading to reduced progestin responsiveness (Graham et al., 2005). In congruency with these observation, cell treated with ThmG exhibited reduced PR A and PR B expression and continued to proliferate normally despite P4 treatment. This suggests that the downregulation of PR by OGA inhibition and subsequent hyper-O-GlcNAcylation could protect endometrial cancer cells from the effects of progestin treatment, and possibly even promote growth.

P27 expression can be regulated by both P4 and Hyper-O-GlcNAcylation

The cyclin dependent kinase inhibitor, p27, is generally described as a tumor suppressor. In this study, P4 treatment maintained the expression of p27 compared to control cells, however in similar cell lines P4 promoted the expression of p27 (Dai et al., 2002). Alternatively, treatment with the OGA inhibitor, ThmG, reduced the expression of p27. Decreased expression of p27 has been related to poor prognosis in lung, esophageal, colon, breast, and prostate cancers, of which

O-GlcNAcylation is also largely elevated (Abukhdeir & Park, 2008). When cells were treated with P4 and ThmG in combination, p27 expression did increase, but did not return to control levels (Figure 3).

In this instance, Hyper-O-GlcNAcylation did reduce the anti-proliferative effect of P4 through the reduction of p27 expression, which translated to an increase in cell proliferation (Figure 1) despite P4 treatment. The tumor suppressor p27 is heavily O-GlcNAcylated (Ser2, Ser106, Ser110, Thr157, and Thr198) in HEK293 cells and hepatocellular carcinoma (HCC) cells. In these cells, O-GlcNAcylation and phosphorylation of p27 were found to have a dynamic interplay. O-GlcNAcylation at Ser2 promoted the nuclear export of p27 and increased phosphorylation of p27, both resulting in the accumulation of p27 in the cytoplasm, supporting cell cycle progression (Qiu et al., 2017). Furthermore, increased Akt activity also contributes to increased cell growth through a reduction p27 expression in prostate cancer (Graff et al., 2000), and O-GlcNAcylation is a known activator of Akt signaling (Shi et al., 2012). Further work should assess the nuclear and cytoplasmic expression of p27, as well as Akt activity to further elucidate the mechanism by which OGA inhibition reduces p27 expression and maintains cell cycle progression and proliferation.

Co-treatment with P4 and ThmG increase p21 expression in Ishikawa cells

In previous studies, P4 treatment had been shown to reduce cell proliferation in endometrial cancer cells (Hec50) partially through the up-regulation of the cyclin-dependent kinase inhibitors p21 and p27 (Dai et al., 2002). In contrast, similar experiments conducted in the endometrial cancer cell line, Ishikawa, reduced p21 expression (Figure 3). In other studies conducted in Ishikawa cells, p21 expression was found to be unchanged by estradiol,

progesterone, or hCG (Abıke et al., 2013). Despite the lack of p21 expression, the proliferation rate remained notably reduced in cells treated with P4 (Figure 1, $p<0.05$), suggesting that the inhibition of cell proliferation in P4 treated cells was not related to the expression of cyclin-dependent kinase inhibitors. However, when cells were treated with both P4 and ThmG, p21 expression was increased. A possible explanation for this is that progesterone activates the p21 promoter through an Sp1 site. O-GlcNAcylation of Sp1 may stabilize and activate Sp1 (Ma & Vosseller, 2014), which could in turn promote p21 expression. The expression and function of p21 can be altered through phosphorylation by Akt. In endothelial cells, activated Akt phosphorylates p21, which results in its reduced ability to inhibit Cdk2 activity, presumably by inhibiting p21/cdk2 complex binding (Rössig et al., 2001). This suggests that hyper-O-GlcNAcylation may promote the phosphorylation of p21 by Akt, thus impairing its anti-proliferative function.

Conclusion

While these results are intriguing, it's clear that the relationship between PR and O-GlcNAcylation is complex and warrants further study. We suggest the following experiments to continue this investigation: 1) quantitative cell cycle analysis 2) assessment of cell viability/apoptosis, and 3) further investigation into the potential relationship between O-GlcNAcylation and progesterone and Akt signaling. Understanding the impact O-GlcNAcylation may have on the anti-proliferative and anti-invasive effects of progestin could have significant implications for the treatment of female cancers. Such knowledge could lead to improved treatment protocols and prognoses for patients struggling with metabolic disease and cancers.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

In the present work, global O-GlcNAcylation in cells was manipulated by pharmacological inhibition of O-GlcNAc cycling enzymes to better understand its influence on tumorigenic potential in female cancers. While there is significant literature exists discussing the role of O-GlcNAcylation in breast and ovarian cancer, very little attention has been paid to cervical and endometrial cancer, despite their connection to metabolic disorders. As such, this research project fills an important gap in our literary knowledge, making a modest yet substantial contribution to the fields of glycobiology and reproductive oncology. The finding that Hyper-O-GlcNAcylation consistently supports a more tumorigenic phenotype in multiple cell lines confirms the importance for understanding how nutrition and metabolic health influence chronic disease. This topic will continue to be of great importance to human health as the obesity epidemic progresses (Conway et al., 2018).

While basic science cell culture based studies like this one are critical first steps in understanding molecular mechanisms of disease, they are not without limitations. We must first consider the cell culture model. The SiHa and Ishikawa cell lines utilized for this work are immortalized cell lines that have been cultured for extended periods of time. It is well known that as cells remain in culture, they begin to adapt to the aqueous culture conditions and behave very differently than cells *in vivo* (Kaur & Dufour, 2012). Thus it is important that these cell culture studies be interpreted as such. It is also important to note that no two cell lines are alike, and despite being the same tissue type, different cell lines can produce different results. For

example, the preliminary work for chapters 2 and 3 was completed with the well know cervical cancer cell line, HeLa. While these cells provided interesting and unique results, in surprising contrast to SiHa cells, it was discovered that HeLa cells had undergone such drastic mutation that they were no longer a reliable model of cervical cancer (Landry et al., 2013). In future studies we set out to use multiple cell lines to further validate our work. We originally selected Ishikawa and ECC-1 cells for the experiments described in Chapters 4 and 5, however, genomic validation of the cell lines revealed they were in fact the same cell line (Korch et al., 2012), likely a result of earlier contamination or misidentification. As you can see, there are many potential pitfalls associated with cell culture experimentation; however, it is a critical tool for the early development of studies that lend to progressive work with primary tissues and *in vivo* models.

Additional consideration should be given to use of pharmacological inhibitors. While they are a relatively simple, rapid, and cost-effective way to study the function of a protein, they are not without drawbacks. When working with small molecule inhibitors it is critical to be aware of the specificity of the inhibitor. Inhibitors that do not have a proven selectivity can have unintended effects on cell function, which can over shadow experimental results. An example of this is discussed in chapters 1 and 2, is the OGT inhibitor Alloxan, which was later discovered to have cytotoxic effects beyond the inhibition of OGT. Those experiments were later repeated with the new, more specific small molecule inhibitor, OSMI-1 (Chapter 3). A complimentary technique for validating data collected with a small molecule inhibitor would be the use of knockdown approaches such as si- or shRNA (D. D. Rao et al., 2009) which reduce the expression of the associated genes.

The difficulties presented by the nature of O-GlcNAc modification have been difficult to overcome, but in just over three decades the tools and techniques available to researchers have

grown exponentially, and they likely will continue to do so. Although our laboratory has a particular interest in cytoskeletal filaments, this collection of work focused on global whole-cell O-GlcNAcylation. While this type of broad analysis is vital for our understanding of this nutrient sensing modification, it is my hope that future work will progress to focus on specific proteins and modification sites of interest. As mentioned in chapter 1, this type of work has been done with the implementation of genomic point mutation techniques. With the increased availability of CRISPR/Cas9 gene editing technology this type of research will likely continue to expand, and could potentially lead minimally invasive treatments for O-GlcNAc related diseases.

O-GlcNAcylation's influence on cell metabolism has made it a hot topic issue in diabetes and cancer research, leading to expansive literature; however, there are many areas of human and animal health where O-GlcNAcylation has only minimally been addressed. One such area of interest is mammalian reproduction. Despite a significant connection between metabolic syndrome and reproductive health (Al Awlaqi et al., 2016), O-GlcNAc is not currently on the radar of researchers. Literature on the topic is nearly non-existent, for example, a PubMed search produces only one publication mentioning O-GlcNAcylation in granulosa cells; "Transcriptome profiling of granulosa cells of bovine ovarian follicles during growth from small to large antral size follicles" (Hatzirodos et al., 2014). This study, published in 2014, identified the OGA encoding gene, MGEA5, as a regulator of small follicles, and found it to be down-regulated in large follicles. This suggests that O-GlcNAcylation is an important component in folliculogenesis; however, this concept has not been studied further.

Similarly, there are many aspects of male reproduction that could be influenced by O-GlcNAcylation. For example, many of the same molecules and mechanisms critical to the EMT phenotype, the subject of chapter 4, are also critical in maintaining the Blood-Testes-Barrier

(TBT) (Mruk & Cheng, 2015). This includes, Notch signaling, PI3K, Occludins, claudins, β-catenin, N-Cadherin, and ZO-1. As such, it would be surprising if dysregulation of O-GlcNAcylation did not have an influence on sertoli cells, and subsequently the BTB. Additionally, "leaking" in the BTB is often associated with heat stress, which is implicated in many areas both male and female infertility (Hansen, 2009). As previously mentioned, O-GlcNAcylation is upregulated during times of cellular stress or injury. Stress induced O-GlcNAcylation promotes cell survival through regulation of: PI3K/Akt pathway, heat shock protein expression, calcium homeostasis, levels of reactive oxygen species, ER stress, protein stability, mitochondrial dynamics, and inflammation (Groves et al., 2013). Any of these mechanisms could have a significant influence on reproductive health. Heat stress is of particular concern in livestock species. Given this, understanding the role O-GlcNAcylation may play in the heat stress response could be crucial to agricultural species management, especially as we begin to face the realities of climate change (Sheikh et al., 2017).

Another environmental issue impacting reproduction is our pervasive exposure to environmental chemicals. Many of these chemicals have been shown to influence cell metabolism. Some notable examples include, Bisphenol A, PBDE (a category of flame retardants), some pesticides (DDT/DDE), and PFOA (Heindel et al., 2017), an industrial byproduct that has recently been discovered in the drinking water of many Vermonters. While these chemicals are already on the radar of many, there potential connection to disrupted O-GlcNAc signaling have not been explored, and could be crucial in understanding the full impact these chemicals have on reproductive and metabolic health.

While this project presented me with many challenges and moments of frustration, this work has only fed my intrigue and excitement for glycobiology. I am grateful to have had the

experience of working in a new and excited field, and am especially aware of the unique and rich research opportunities that lay before me as I begin my career in academia. My appreciation for glycobiology has also become a component of my career goals as an educator. As an undergraduate I received minimal to no education in glycoscience, and I am shocked by the lack of glycobiology educational opportunities even at the graduate level. In 2012, the National Academies published a report at the request of the NIH, NSF, DOE, FDA, and the Howard Hughes Medical Institute, investigating the current state of the Glycosciences (Council, 2012). The report found that despite the obvious importance of glycobiology in all aspects of human and animal health, glycoscience is viewed as a niche research area, with limited funding or investment. While this is due to a variety of reasons stemming from the complex nature of the problem, the committee noted specifically, "a pervasive lack of knowledge by non-glycoscientists, at all levels of education, about these types of molecules," (Hart, 2012). I plan to approach my career as a researcher and an educator with at the fore front of my mind. I hope to share my enthusiasm for glycobiology with my students and colleagues in the classroom, laboratory and conference halls.

In conclusion, this body of work contributes significant interdisciplinary findings to the fields of reproduction, cancer biology, and glycobiology. It also provides clear scaffolding for future research in this and related areas of study. The process of compiling this work has illuminated my drastic evolution from an inexperienced graduate student to a productive, independent research scientist. I have acquired a diverse set of both technical and cognitive skills that have prepared me for a career as a scientist and educator, and I am grateful for further opportunities to learn and grow that are presented in a career academia.

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