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14. ABSTRACT

Cancer cells upregulate glycolysis, increasing glucose uptake to meet energy needs. Approximately 2-5% of a cell's glucose enters the hexosamine biosynthetic pathway (HBP) which regulates levels of O-linked ß-N-acetylglucosamine (O-GlcNAc), a carbohydrate post-translational modification of diverse nuclear and cytosolic proteins. We have discovered that breast cancer cells upregulate the HBP, including increased O-GlcNAcation and elevated expression of O-GlcNAc transferase (OGT), the enzyme catalyzing addition of O-GlcNAc to proteins. Reduction of O-GlcNAcation via RNAi of OGT in breast cancer cells leads to inhibition of tumor growth in vitro as well as in vivo and associated with decreased cell cycle progression and increased expression of the cell cycle inhibitor $p27^{Kip1}$. Elevation of $p27^{Kip1}$ was associated with decreased expression and activity of the oncogenic transcription factor FoxM1 a known regulator of $p27^{Kip1}$ stability via transcriptional control of Skp2. Reducing O-GlcNAc levels in breast cancer cells decreased levels of FoxM1 protein and caused decrease in multiple FoxM1-specific targets including Skp2. Moreover, reducing O-GlcNAcation decreased cancer cell invasion and was associated with downregulation of MMP-2, a known FoxM1 target. Lastly, pharmacological inhibition of OGT in breast cancer cells had similar anti-growth and anti-invasion effects. These findings identify O-GlcNAc as a novel mechanism through which alterations in glucose metabolism regulate cancer growth and invasion and suggest that OGT may represent novel therapeutic targets for breast cancer.

15. SUBJECT TERMS
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Introduction

Tumor cells have altered carbohydrate metabolism, producing ATP primarily through glycolysis, even under normal oxygen concentrations (6). This metabolic shift in cancer cells, termed the "Warburg effect", involves increased glucose uptake and is critical in supporting cancer phenotypes (27). Changes in tumor glucose uptake and metabolism also alter distinct nutrient signaling pathways, including mTOR, AMPK,and HBP (15). Indeed, there is growing evidence that abnormalities within the mTOR and AMPK pathways can lead to abnormal growth and cancer (8, 22). The majority of glucose enters glycolysis, producing ATP, while approximately 2-5% of a cell's glucose enters the HBP (16), resulting in the end product uridine diphosphate (UDP)-N-acetylglucosamine (UDP-GlcNAc) (10). While flux through the HBP is likely increased in tumor cells as a result of upregulated glucose uptake, a role for the HBP in oncogenesis has not been explored.

UDP-GlcNAc is a donor substrate in the enzymatic covalent addition of a single monosaccharide (GlcNAc) onto serine or threonine residues. In contrast with all other types of glycosylation, O-GlcNAc modifies a wide variety of cytosolic and nuclear proteins. O-GlcNAc acts as novel regulatory switch mechanism analogous to phosphorylation (28). Cytosolic and nuclear enzymes dynamically catalyze addition (O-GlcNAc transferase or OGT) and removal (O-GlcNAcase) of O-GlcNAc in response to various stimuli, including tyrosine kinase receptor activation (24). OGT is unique among glycosyltransferases in its high affinity for UDP-GlcNAc. As a consequence, OGT activity responds to physiological changes in UDP-GlcNAc (13), thus leading to elevated O-GlcNAc modifications in response to increased flux through the HBP (3). Accordingly, OGT is positioned to act as a molecular sensor of enhanced HBP nutrient flux, which would be expected in cancer cells. O-GlcNAc is known to influence protein-protein interactions (21); therefore, modulations of O-GlcNAc may alter the formation of specific protein complexes involved with oncogenic signaling. Modulation of O-GlcNAc levels has been linked to growth/survival phenotypes such as cell cycle progression and altered mitotic phosphorylation patterns (2, 19, 23, 30), demonstrating that a proper balance of O-GlcNAc and phosphorylation is required for normal cell growth. Recently, it was shown that p53-deficient mouse embryonic fibroblasts, which elevate glycolysis, display increased O-GlcNAcation on a number of proteins (11). Thus, abnormal levels of O-GlcNAc in cancer cells may contribute to deregulated post-translational control of protein function linked to oncogenic phenotypes.

A number of transcription factors are known to be modified by O-GlcNAc, suggesting this glucose-sensing mechanism can directly link nutrient status to gene expression (5). Elevated expression or activity of FoxM1 is associated with development and progression of numerous cancers (18, 29). FoxM1 serves as a key regulator of cell proliferation during organ development by controlling transcription of genes critical for G1/S and G2/M progression (18), including Skp2 during G1/S and Nek2, Survivin and PLK1 during G2/M. Recently, FoxM1 overexpression was found to correlate with ErbB2 (HER2) status in breast cancers (1). FoxM1 has also been shown to regulate cellular invasion via transcriptional regulation of matrix metalloproteinases (26). Thus, targeting FoxM1 or its regulators has been proposed as a viable therapeutic strategy for treating cancer (17).

During the first year of funding we provide the first evidence that OGT and O-GlcNAc levels are elevated in breast cancer cells, and that reducing abnormally high O-GlcNAcation inhibits cancer cell growth in vitro and in vivo, and also reduces breast cancer cell invasion. Decreasing O-GlcNAc levels through knockdown of OGT in cancer cells promotes elevation of cell cycle regulator $p27^{Kip1}$ and reduces expression of FoxM1, in addition to a number of FoxM1 targets. Indeed, regulation of FoxM1 may provides a mechanism through which decreased levels of O-GlcNAc inhibit breast cancer phenotypes, as we also found that inhibition of invasion by targeting OGT was associated with reduction in FoxM1 transcriptional target MMP-2. Our data suggests that tumor progression is associated with elevated O-GlcNAcation, which deregulates

critical factors in oncogenic growth and invasion. Additionally, we show that pharmacological inhibition of OGT may be a valuable strategy for normalizing oncogenic phenotypes in breast cancer transformation.

Body

Task 1: Establish whether modulation of O-GlcNac levels, through pharmacological inhibition or genetic knock-down of enzymes that add or remove O-GlcNAc, can inhibit ErbB2-mediated oncogenic phenotypes in vitro in the absence of toxicity in non-transformed cells (months 1—6, PI:Reginato):

We will test effects of OGT inhibitor (XI) and OGT RNAi (lowering of O-GlcNAc) as well as inhibitors PUGNAc and 9d and O-GlcNAcase RNAi (elevation of O-GlcNAc) on:

a. Colony formation (growth in soft agar) of ERBB2 expressing MCF-10A cells and breast cancer cell lines (SKBR3 and MDA-MB-453). (months 1-3)

Our studies completed Breast cancer cell lines upregulate O-GlcNAc and OGT levels. To determine whether levels of O-GlcNAc modified proteins are altered in cancer cells, we compared normal epithelial cells to established breast cancer cells or oncogene-overexpressing cells. We found that MCF-10A cells overexpressing the activated form of ErbB2 (NeuT), and the breast cancer cell lines SKBR3 and MDA-MB-453, contain elevated levels of O-GlcNAc-modified proteins compared to normal human immortalized mammary epithelial MCF-10A cells (Fig. 1A). We then examined whether the increase in O-GlcNAc-modified proteins in breast cancer cell lines was related to altered expression of OGT, the enzyme responsible for catalyzing O-GlcNAc addition to proteins. We found that OGT is overexpressed in five different breast cancer cell lines when compared to normal MCF-10A and MCF-12A cells (Fig. 1B). Our data thus shows, for the first time, that breast cancer cells contain elevated levels of O-GlcNAc and OGT.

OGT is required for malignant growth of transformed breast cancer cells in vitro. To test whether reducing abnormally high levels of O-GlcNAcation alters breast cancer phenotypes, we targeted OGT via RNAi in MCF-10A-ErbB2 cells. The efficiency of two different OGT shRNA lentiviral constructs was confirmed by western blotting. We detected at least a 50% knockdown of OGT protein compared to cells infected with Control (scrambled) shRNA sequence (Fig. 2A). We then tested if reduction of OGT led to global decrease in O-GlcNAcation. Cells were treated with or without the specific O-GlcNAcase inhibitor 9D (14) to block enzymatic removal of O-GlcNAc. MCF-10A-ErbB2 cells infected with control shRNA lentivirus displayed a significant increase in O-GlcNAcation in the presence of 9D (Fig. 2B). However, cells infected with RNAi targeting OGT had significantly decreased basal O-GlcNAcation, and completely blocked the 9D-induced elevation of O-GlcNAcation (Fig. 2B); the decrease of OGT levels led to significant reduction in O-GlcNAc modified proteins. These cells were then placed in three-dimensional (3D) culture assays or soft agar assays to determine the effect of reducing OGT levels on cancer cell growth. Under 3D conditions, reduction of OGT by RNAi in MCF-10A-ErbB2 cells caused dramatic inhibition of oncogenic phenotypes, including decreased cell growth and an eight-fold decrease in cell number at day 12 (Fig. 2C) compared to control RNAi cells. Additionally, reduction of OGT levels in MCF-10A-ErbB2 cells shows a ten-fold decrease in colony formation in soft-agar compared to control cells (data not shown). To test whether reduction of abnormally high levels of O-GlcNAcation could alter breast cancer phenotypes independent of ErbB2, we knocked down OGT levels in the highly transformed breast cancer cell line MDA-MB-231, which does not overexpress ErbB2. MDA-MB-231 cells stably infected with RNAi against OGT showed a three-fold decrease in soft agar colony formation (Fig. 2D) and resulted in significant inhibition of growth in 3D conditions compared to control shRNA infected cells (data not shown). Knockdown of OGT in parental MCF-10A cells did not significantly block growth or ability to form acinar structures in 3D culture (data not shown) suggesting that reducing OGT levels in non-transformed cells is not cytotoxic. Consistent with the idea that elevated OGT contributes to tumor cell growth, overexpression of OGT in MCF-10A-ErbB2 cells increased the number of soft agar colonies (data not shown). Thus, we show that OGT and abnormally elevated levels of O-GlcNAc are required and may contribute to transformed growth of breast cancer cells in vitro.

Our data completed so far validates our initial hypothesis that targeting protein O-GlycNAc modifications in breast cancer cells inhibits tumor phenotypes.

b. Proliferation and invasion through extracellular matrix by MCF-10A-ERBB2 cells and extend our preliminary results to breast cancer cell lines using 3D culture assay. (months 2-5)

Inhibition of OGT decreases cell cycle progression.
To further investigate the growth-inhibitory effect of OGT knockdown in breast cancer cells, we performed cell cycle analysis by propidium iodide staining and flow cytometry. Reduction of OGT in MCF-10A-ErbB2 cells caused a significant accumulation of cells in G1 phase within 48 hours compared to control shRNA infected cells; 72% G1 content in OGT shRNA infected cells relative to 47% in control shRNA cells (Fig. 4A). With OGT shRNA, we also observed a significant decrease in S and G2/M phase population compared to control. In addition, we found a two-fold decrease in Ki-67 staining in MCF-10A-ErbB2 and MDA-MB-231 cells expressing OGT shRNA (Supplementary Fig. 5A). We did not detect an increase in the sub-G1 population of cells nor did we detect a significant change in DNA fragmentation at this time point (data not shown), suggesting that targeting OGT had minimal effects on apoptosis. Reducing OGT in normal MCF-10A cells caused a slight increase in G1 population, but neither this (data not shown) nor changes in Ki-67 staining (data not shown) were statistically significant.

OGT regulates breast cancer cell invasion. We observed that knockdown of OGT in breast cancer cells produced fewer invasive protrusions when cultured under 3D conditions (Fig. 5A), suggesting that reduction of elevated O-GlcNAcation may inhibit cellular invasion. To test this directly, we placed MCF-10A-ErbB2 cells targeted with OGT or control shRNA in transwell invasion assays. Knockdown of OGT in MCF-10A-ErbB2 cells led to a three-fold decrease in invasion compared to controls (Fig. 5B). Breast cancer invasion and metastasis is associated with elevated levels of MMP-2 (7). Since FoxM1 regulates expression of MMP-2 in pancreatic cancer cells (26), we examined levels of MMP-2 in OGT knockdown cells. Indeed, we found a two-fold decrease in expression of MMP-2 at both the mRNA (Fig. 5C) and protein level (Fig. 5D) in MCF-10A-ErbB2 cells when OGT is knocked down. Our data suggest that OGT regulates cancer cell invasion by modulating MMP-2 expression, possibly via regulation of FoxM1.

c. Anoikis sensitivity of breast cancer cells. (months 3-6)

Knockdown of OGT in ErbB2 overexpressing MCF-10A cells or in MDA-MB-231 cells did not induce apoptosis in attached or cells suspended for 48 hours (data not shown). Thus, reducing OGT in breast cancer cells does not increase anoikis sensitivity.

d. Normal growth of mammary epithelial cells (parental MCF-10A cells) in standard 2D and 3D conditions. (months 3-6)

Knockdown of OGT in parental MCF-10A cells did not significantly block growth or ability to form acinar structures in 3D culture (data not shown) suggesting that reducing OGT levels in non-transformed cells is not cytotoxic.

Outcomes: We expect to establish that breast cancer phenotypes in vitro can be inhibited by modulating O-GlcNAc levels, while in parallel studies showing a lack of toxicity in normal mammary epithelial cells.

We have achieved this outcome.

Task 2: In collaboration with Dr. Senthil Muthuswamy (Cold Spring Harbor Labs), we will test whether targeting OGT, the enzyme that adds O-GlcNAc to proteins (with OGT RNAi or OGT inhibitor) inhibits oncogenic phenotypes in vivo (months 6-12, PI:Reginato):
Preliminary studies indicate elevated O-GlcNAc in ERBB2 transformed cells, and suggest that lowering O-GlcNAc levels inhibits oncogenic phenotypes. Thus, lowering of O-GlcNAc by inhibition or knockdown of OGT will be the focus of this aim.
a. Using a mammary fat pad transplantation mouse model, we will inject MCF-10A cells overexpressing ERBB2 into thoracic mammary fat pad of 8-week-old anesthetized mice. Cells will be stably expressing control shRNA vector (pLKO-scrambled), or OGT shRNA. We will inject one fat pad with MCF-10A-ERBB2 cells containing control shRNA and the contra lateral fat pad with cells containing the shRNA to OGT (13 mice). Twenty one days later, mice will be sacrificed and we will quantify tumor mass, and determine O-GlcNAc and OGT levels.

OGT is required for tumorigenic growth of human breast cancer cells in vivo. We next examined a role for OGT in promoting oncogenic phenotypes in vivo. To test this, we performed orthotopic xenografts of

MDA-MB-231 cells stably expressing either OGT shRNA or control shRNA. OGT knockdown and decreased basal O-GlcNAcation were verified by western blot analysis at the time of injection (Fig. 3A). Control and OGT knockdown cells were then injected directly into contralateral mammary fat pads of immunocompromised Nu/Nu mice to avoid inter-animal variations. A four-fold decrease in tumor volume was observed in mice injected with OGT knockdown cells compared to control cells at the end of 8-week experiment (Fig. 3B). At necropsy, of mice injected with cells expressing scrambled shRNA, 84% developed visible tumors that could be excised; only 41% of mice injected with cells containing OGT-1 shRNA (Fig. 3C) and 40% of mice injected with cells containing OGT-2 developed visible tumors (data not shown). Tumor mass measurements from OGT knockdown cells showed a similar four-fold reduction compared to tumors from control cells (data not shown). Importantly, tumors that eventually grew from OGT knockdown cells restored OGT expression (Fig. 3D) and had similar Ki-67 expression (data not shown), suggesting a strong selective pressure against tumor cells deficient in OGT. These data indicate the importance of OGT in tumor cell growth in vivo.

b. We will repeat fat pad injections of ERBB2-overexpressing cells treated with vehicle control or OGT inhibitor XI (dose to be determined from in vitro studies). Fat pad will be injected every five days with additional dose of OGT inhibitor. Analysis of tumor weight, mass and O-GlcNAc levels will be carried out at day 21.

We have been unable to perform these experiment since dose used in vitro to inhibit O-GlcNAcation was between 200-500 μM and thus not able to achieve this dose in vivo. Currently collaborating with chemist Dr. Suzanne Walker (Harvard Medical School) and testing second generation OGT inhibitors that are more potent and soluble in vitro before testing them in vivo.

Outcomes: We expect to establish that breast cancer tumor formation in vivo can be inhibited by lowering O-GlcNAc levels through targeting of the enzyme which adds O-GlcNAc to proteins.

We have achieved this outcome.

Task 3: Identify ERBB2-mediated signaling pathways regulated by O-GlcNAc (months 3-9, PI:Reginato/Vosseller):
a. Test effects of PUGNAc, 9d, OGT inhibitor XI, and OGT RNAi on ERBB2-mediated signaling pathways by using activation state specific phosphospecific antibodies against following targets: ERBB2, ERBB1, ERBB3, ERBB4, AKT, MEK1/2, ERK1/2, and IkBa. (months 3-4)

Inhibition of OGT induces $p27^{Kip1}$ expression via regulation of FoxM1 in breast cancer cells.

Increase in the population of cells in G1 suggestes that cell cycle regulators may be altered by reducing OGT expression. Knockdown of OGT results in a significant induction of $p27^{Kip1}$ levels and reduction of PCNA in MCF-10A-ErbB2 cells (Fig. 4B), as well as in MDA-MB-231 cells (data not shown), consistent with cell cycle arrest at G1. The regulation of $p27^{Kip1}$ is highly complex; it is well established that oncogenic signaling, including receptor tyrosine kinase (RTK), c-Src, and MAP kinase activation in cancer cells is associated with increased $p27^{Kip1}$ proteolysis (4). Yet knockdown of OGT in MCF-10A-ErbB2 cells did not reduce activity of ErbB2, c-Src, Erk (Supplementary Fig. 6), or Akt (Fig. 3B) as measured with respective phospho-specific antibodies. Since $p27^{Kip1}$ mRNA levels were not decreased in cells depleted of OGT (data not shown), we considered alternative pathways regulating $p27^{Kip1}$ degradation.

Degradation of $p27^{Kip1}$ is primarily regulated by the SCF^{SKP2} E3 ubiquitin ligase complex (4). This complex includes the F-Box protein Skp2 that targets CDK inhibitors for degradation during G1/S transition. We find that OGT knockdown in MCF-10A-ErbB2 cells (Fig. 4B) and MDA-MB-231 cells (data not shown) decreases Skp2 expression. One level of Skp2 regulation is through transcriptional activation by FoxM1 (25). We find in MCF-10A-ErbB2 (Fig. 4B) and MDA-MB-231 cells (data not shown) that reducing OGT expression leads to significant decreases in FoxM1 protein levels. FoxM1 can regulate progression from G1 to S phase, but is also known to be a key regulator during G2/M. Indeed, we find that FoxM1 specific targets involved in G2/M phase, including Survivin, Nek2 and PLK1, are also decreased in OGT knockdown cells, both in MCF-10A-ErbB2 (Fig. 4C) and MDA-MB-231 cells (data not shown).

To begin addressing the mechanism of how OGT regulates FoxM1 levels, we examined effects of OGT knockdown in MDA-MB-231 cells stably expressing exogenous FoxM1. Reducing OGT levels caused downregulation of stably-overexpressed wildtype FoxM1 protein (Fig. 4D), suggesting OGT and O-GlcNAcation may regulate FoxM1 post-transcriptionally. Recent studies have identified the N-terminus of FoxM1 as being a substrate for ubiquitin-mediated degradation, contributing to the normal changes in FoxM1 levels across the cell cycle (12) (20). The N-terminus of FoxM1 contains destruction box (D box) and KEN-box sequences, short degradation motifs recognized by the anaphase-promoting complex (APC) E3 ubiquitin ligase (20) (12). FoxM1 regulation by O-GlcNAcation required the N-terminus of FoxM1, as protein levels of a deletion mutant missing the first 209 amino acids of FoxM1 (ΔN-ΔKEN-FoxM1) were no longer decreased by reducing OGT expression as compared to wildtype FoxM1 (Fig. 4D). To test whether overexpression of wildtype or mutant FoxM1 can rescue cell growth defect due to downregulating OGT, we placed these cells in 3D culture. Cells overexpressing wildtype or mutant of FoxM1 were able to partially overcome the inhibitory effect of OGT silencing on cell growth in 3D culture (Fig. 4E). In addition, knockdown of FoxM1 with RNAi in MCF-10A-ErbB2 cells or MDA-MB-231 cells caused increased expression of $p27^{Kip1}$, inhibition of growth in 3D culture and soft agar results similar to that seen in OGT knockdown cells (data not shown). The reduction of FoxM1 protein is not a part of a global alteration in protein degradation, as we did not detect changes in levels of other Fox transcription family members including FOXO3a (data not shown). Moreover, we found that reduction of OGT levels led to no significant change in expression of a number of transcription factors implicated in breast cancer, including p53, c-Myc, and NF-κB (data not shown).

b. Verify changes seen with phosphospecific antibodies by doing in vitro kinase/enzymatic assays on immunoprecipitated ERBB2 signaling enzymes. (months 4-5)

Since we did not detect inhibition of ErbB2-mediated signaling pathways when reducing OGT expression we focused on whether FoxM1 was directly modified by O-GlcNAc..

c. Use western blotting against O-GlcNAc to define O-GlcNAc modification of immunoprecipitated molecules in ERBB2 signaling pathways. (months 4-6)

Since other Fox transcription family members have been shown to be directly modified by O-GlcNAc, we tested whether FoxM1 is modified by O-GlcNAc. FoxM1 immunoprecipitated from MDA-MB-231 cells overexpressing wildtype FoxM1 did not show any O-GlcNAc modifications, while endogenous Sp1, a transcription factor known to be modified by O-GlcNAc (9), was highly modified under similar conditions (data not shown). Thus, our data show that breast cancer cell growth inhibition via targeting OGT is associated with increased cell cycle arrest at G1, elevated expression of $p27^{Kip1}$, and specific post-transcriptional downregulation of the oncogenic transcription factor FoxM1 and its targets. However, FoxM1 is not directly O-GlcNAcated, suggesting that OGT may be regulating FoxM1 indirectly.

Outcomes: We expect to identify specific steps in ERBB2 signaling pathways that are altered by modulation of O-GlcNAc levels which will suggest links between these biochemical changes and phenotypic effects on transformed cells in task 1 and 2. We also expect to identify kinases/signaling molecules directly modified by O-GlcNAc, which would suggest specific O-GlcNAc regulatory potential in altered states of signaling observed.

We have linked phenotypic changes to regulation of key oncogenic transcription facto FoxM1. We are currently trying to determine how O-GlcNAcation regulates FoxM1 stability.

Task 4. Quantitatively identify O-GlcNAc sites, phosphorylation sites, and binding partners of selected ERBB2 signaling molecules in transformation and in response to modulation of O-GlcNAc (months 4-18, PI:Vossseller):

a. Immunoprecipitate O-GlcNAc modified ERBB2 signaling molecules and their complexes and identify sites of O-GlcNAc/phosphorylation and interacting proteins with mass spectrometry (months 4-12).

We have carried out mass spec analysis on FoxM1 under conditions of elevated O-GlcNAcation and found no evidence of direct O-GlcNAcation (data not shown). This data, along with lack of O-GlcNAcation following FoxM1 IP, suggest that FoxM1 is not directly modified. We are currently examining whether proteins that regulate FoxM1 stability are directly modified/regulated by O-GlcNAc.

b. Use differential isotopic labeling with iTRAQ reagents to measure site-specific changes in O-GlcNAc, phosphorylation, or protein interactions in response to cellular transformation and/or modulation of O-GlcNAc. (months 8-18).

-*We have not started this task during this funding period.*

Outcomes: We expect to identify specific O-GlcNAc and phosphorylation sites, and binding partners of ERBB2 signaling molecules that may be altered in states of transformation and/or modulated O-GlcNAc. Such results may reveal functional interplay between O-GlcNAc, phosphorylation, and protein-protein interactions which would suggest models of O-GlcNAc involvement in oncogenic signaling which may underlie phenotypic effects of O-GlcNAc modulation on transformed cells observed in task 1 and 2.

Task 5. Determine functions of O-GlcNAc sites mapped on selected ERBB2 signaling molecule(s) (months 12-24, PI:Vosseller/Reginato):

a. Express selected ERBB2 signaling molecule(s) with O-GlcNAc site(s) mutagenized, and characterize effect of site-specific loss of O-GlcNAc on protein-protein interactions, phosphorylation events, localization, activity, and transformed phenotypes in cells. (months 12-24).

-*We have not started this task during this funding period.*

b. Specifically determine the effects of MEK2 O-GlcNAc modification at threonine 396 (by site-directed mutagenesis) on negative regulatory phosphorylation of MEK2 at threonine 394 (using phosphospecific antibody and mass spec), MEK2 activity, MEK association with MP1, and the downstream activation of MAP kinase.

-*We have not started this task during this funding period.*

c. If time allows, in support of Task 5a, we will generate recombinant forms of selected ERBB2 signaling molecule(s) either unmodified or O-GlcNAc modified and characterize effect of O-GlcNAc on protein-protein interactions, activity, or specific phosphorylation events in vitro (months 14-24).

-*We have not started this task during this funding period.*

Outcomes: We expect to define specific regulatory roles of site-specific O-GlcNAc modification events in ERBB2 signaling. relevant to effects of modulated O-GlcNAc on transformed phenotypes.

Key Research Accomplishments

-We have for the first time shown that reducing OGT and O-GlcNAc levels in breast cancer cells block growth in vitro and in vivo.
-We have for the first time shown that chemical inhibitors of OGT block tumor growth and invasion in vitro.
-We have shown that that reducing OGT induces G1 cell cycle arrest and associated with increased expression of Cdk inhibitor p27.
-We have shown that reducing OGT did not reduce ErbB2mediated signaling thus we have focused on mechanisms of regulation of critical trscription factor FoxM1.
-We have found that targeting OGT reduces FoxM1 expression at the level of protein.

Reportable Outcomes

-We have validated OGT as a potential therapeutic target for treating breast cancer.

-Targeting OGT blocks breast cancer growth in vivo.

-Targeting OGT blocks cancer growth and invasion in vitro.

-We have found one novel mechanism of how OGT regulates breast cancer phenotype by, in part, regulating the critical oncogenic transcription factor FoxM1.

Conclusion

During this initial funding period (Sept. 08-Aug.-09) we have completed tasks 1, 2, and 3. We hope to complete tasks 4 and 5 during next funding period. The work performed to date has supported the novel hypothesis that O-hyper-O-GlcNAcation occurs generally in cancer and represents a valid and novel potential therapeutic target in cancer. Of significance, is establishing a new connection between well known alterations in cancer metabolism (e.g. the Warburg effect) and "downstream" molecular sensing of these metabolic changes by the post-translational modification O-GlcNAc which contributes to oncogenic signaling. We've established some of the consequences of this connection, as cell cycle control through p27 and FoxM1 is directly altered by reversing hyper-O-GlcNAcation in breast cancer. This knowledge introduces the enzymes that regulate O-GlcNAc levels as novel therapeutic targets in cancer. Importantly, the universality of metabolic alterations in cancer indicate that our findings are not breast cancer specific, but will likely be relevant in the context of cellular transformation in general, and thus be of importance in extrapolation to other cancers. Thus, our conclusions are likely to have far reaching influence with other researchers. The important work that remains in the breast cancer system, and which is consistent with the work plan, is to determine site-specific mechanisms of how elevated O-GlcNAc in breast cancer contribute to oncogenesis.

Figures:

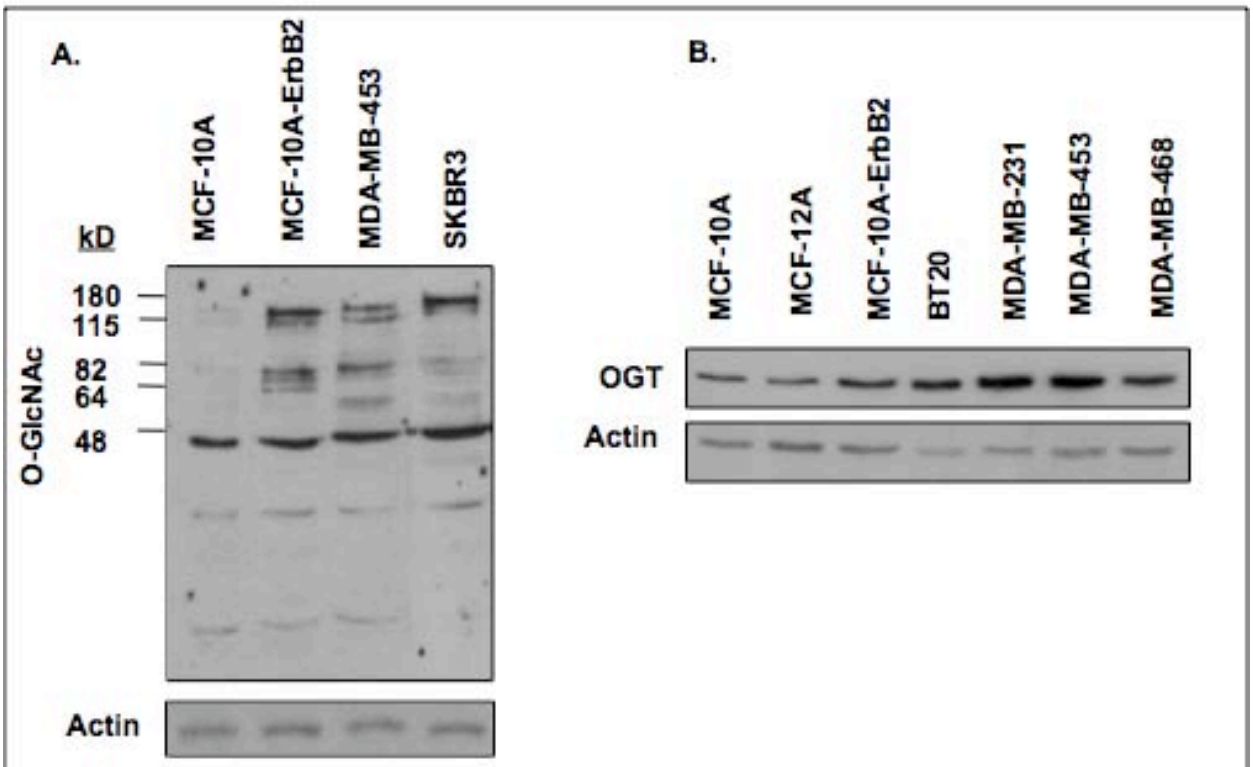
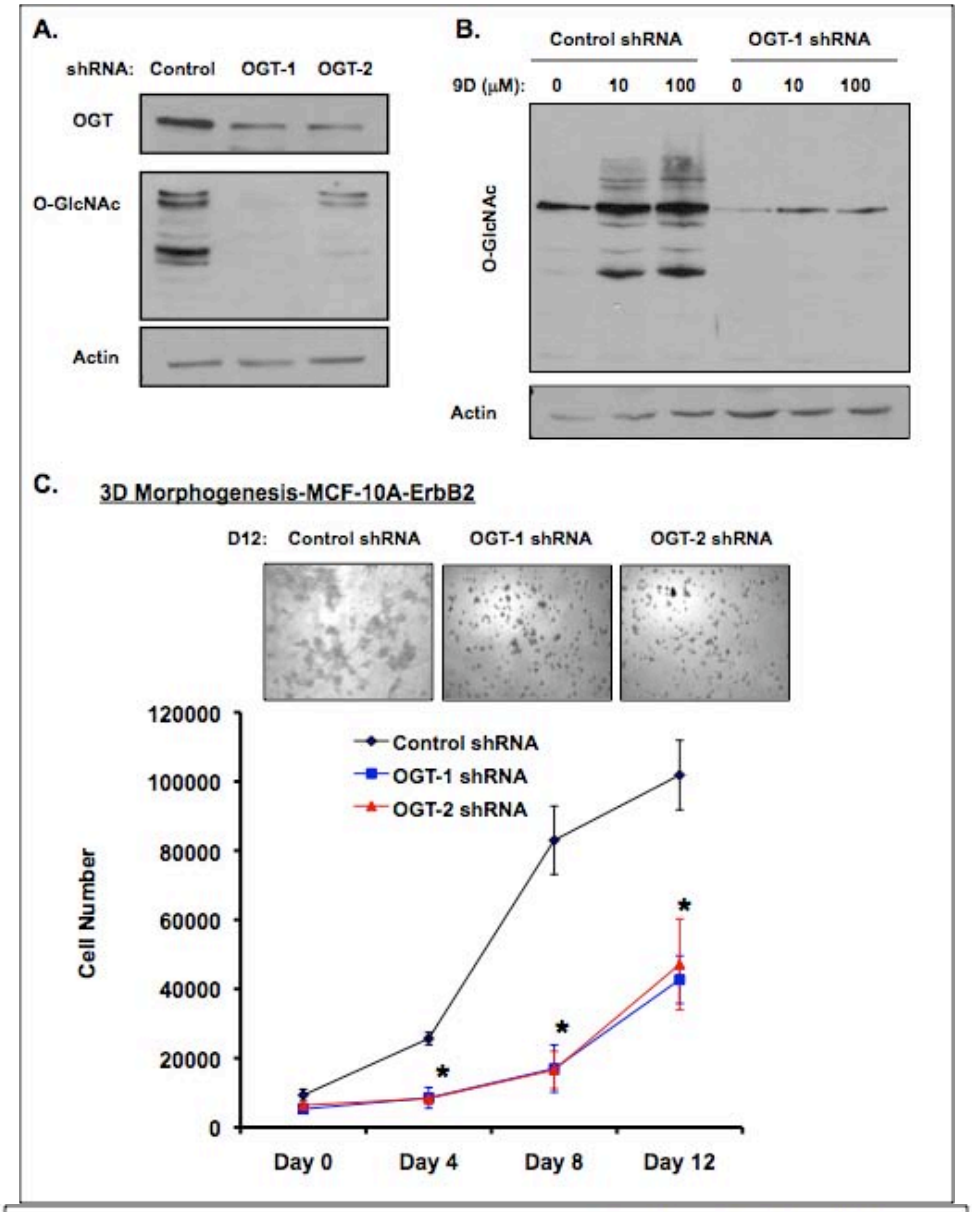


Figure 1. Breast cancer cells contain elevated O-GlcNAcation and OGT levels. **(a)** Protein lysates from indicated cell lines were collected for immunoblot analysis and probed with indicated antibodies. **(b)** Normal mammary epithelial cells MCF-10A and MCF-12A and breast cancer cells MCF-10A-ErbB2, BT20, MDA-MB-231, MDA-MB-453, and MDA-MB-468 were lysed and subjected to immunoblot analysis with indicated antibodies.



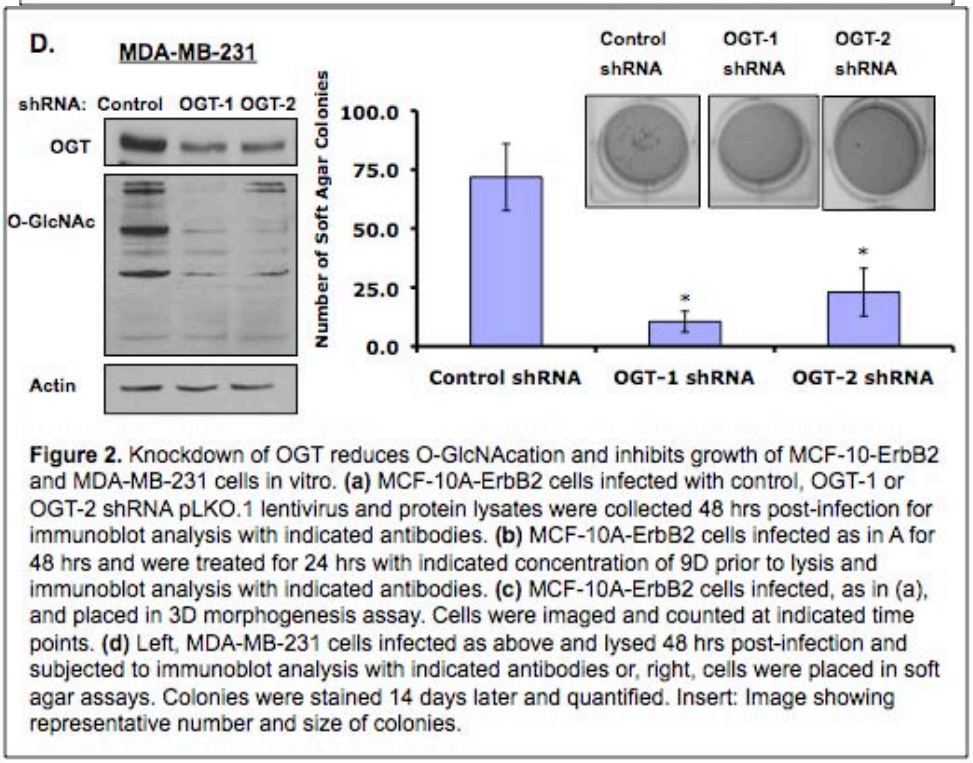


Figure 2. Knockdown of OGT reduces O-GlcNAcation and inhibits growth of MCF-10-ErbB2 and MDA-MB-231 cells in vitro. **(a)** MCF-10A-ErbB2 cells infected with control, OGT-1 or OGT-2 shRNA pLKO.1 lentivirus and protein lysates were collected 48 hrs post-infection for immunoblot analysis with indicated antibodies. **(b)** MCF-10A-ErbB2 cells infected as in A for 48 hrs and were treated for 24 hrs with indicated concentration of 9D prior to lysis and immunoblot analysis with indicated antibodies. **(c)** MCF-10A-ErbB2 cells infected, as in (a), and placed in 3D morphogenesis assay. Cells were imaged and counted at indicated time points. **(d)** Left, MDA-MB-231 cells infected as above and lysed 48 hrs post-infection and subjected to immunoblot analysis with indicated antibodies or, right, cells were placed in soft agar assays. Colonies were stained 14 days later and quantified. Insert: Image showing representative number and size of colonies.

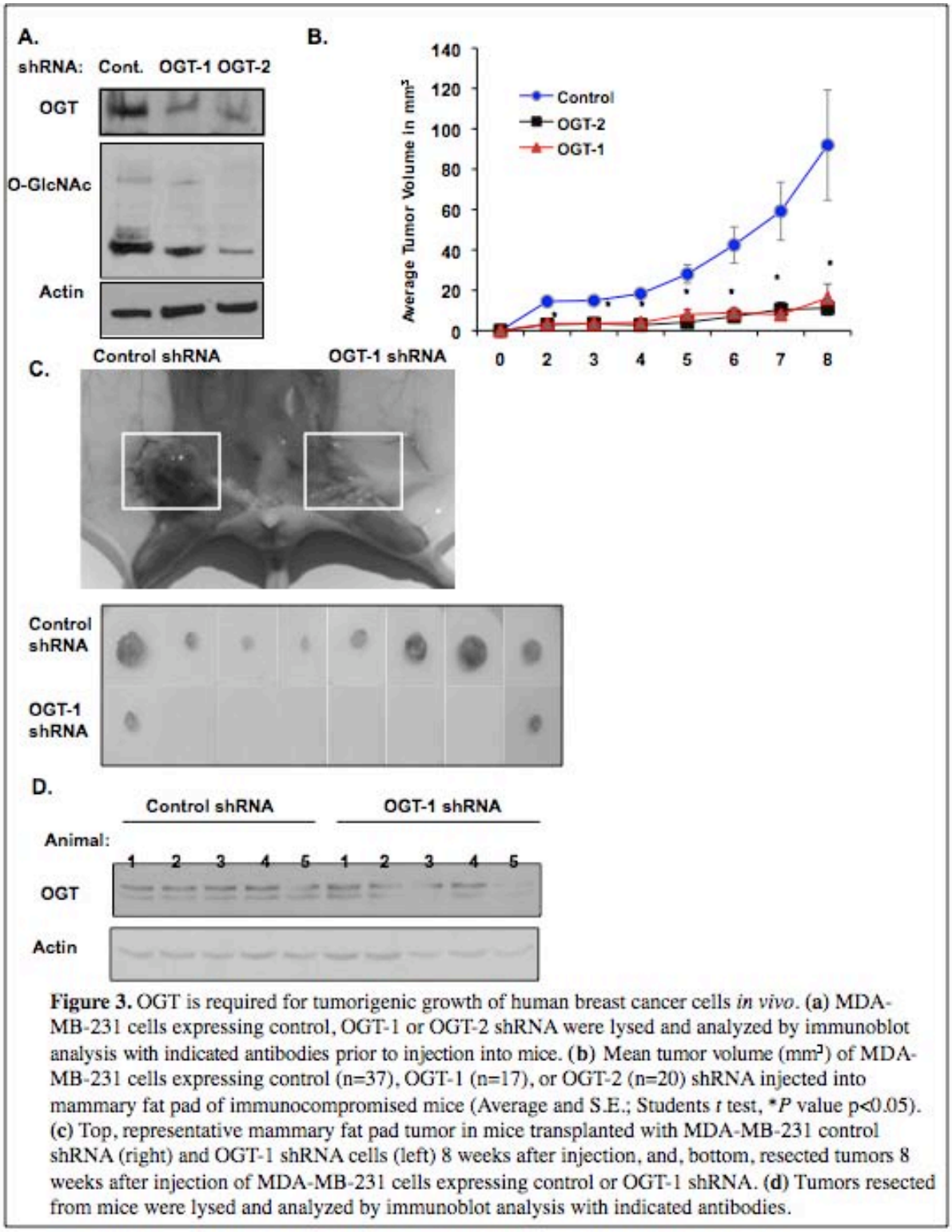
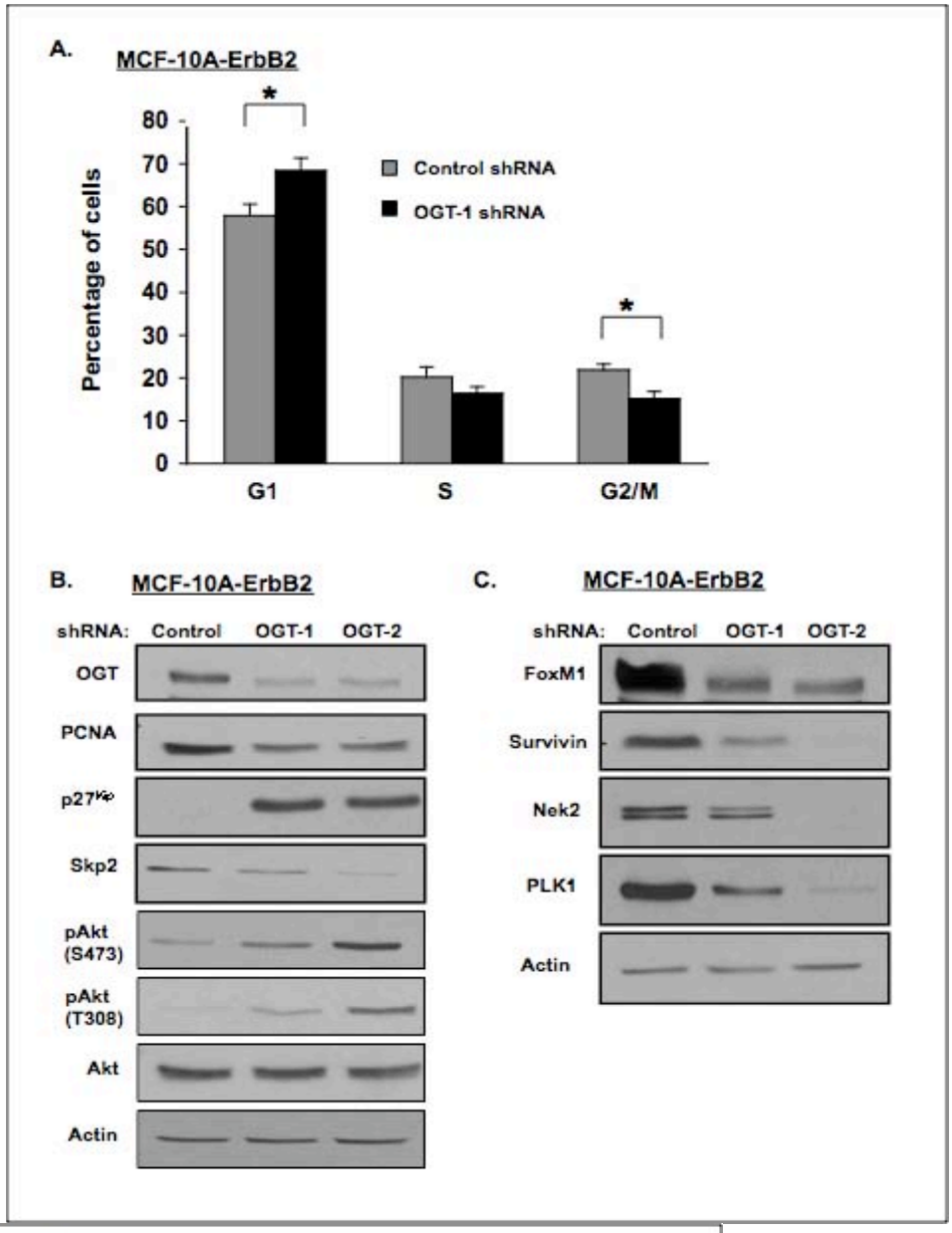


Figure 3. OGT is required for tumorigenic growth of human breast cancer cells *in vivo*. (**a**) MDA-MB-231 cells expressing control, OGT-1 or OGT-2 shRNA were lysed and analyzed by immunoblot analysis with indicated antibodies prior to injection into mice. (**b**) Mean tumor volume (mm^3) of MDA-MB-231 cells expressing control (n=37), OGT-1 (n=17), or OGT-2 (n=20) shRNA injected into mammary fat pad of immunocompromised mice (Average and S.E.; Students *t* test, **P* value $p<0.05$). (**c**) Top, representative mammary fat pad tumor in mice transplanted with MDA-MB-231 control shRNA (right) and OGT-1 shRNA cells (left) 8 weeks after injection, and, bottom, resected tumors 8 weeks after injection of MDA-MB-231 cells expressing control or OGT-1 shRNA. (**d**) Tumors resected from mice were lysed and analyzed by immunoblot analysis with indicated antibodies.



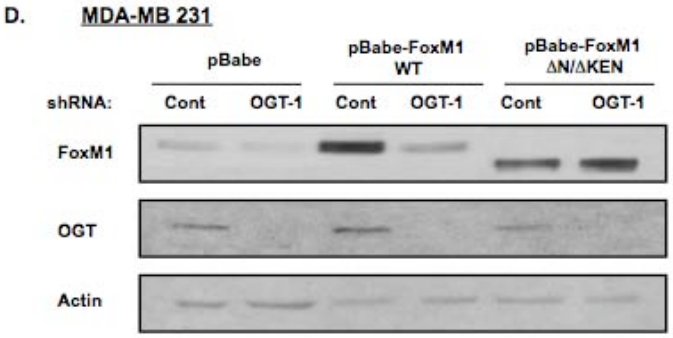


Figure 4. Knockdown of OGT inhibits cell cycle progression, elevates $p27^{Kip}$ expression, and reduces FoxM1 expression in breast cancer cells. **(a)** Cell cycle analysis of MCF-10A-ErbB2 cells 48 hours after lentiviral infection with control, OGT-1, or OGT-2 shRNA. Cells were collected, stained with Guava Cell Cycle Reagent, and analyzed by flow cytometry. Cell cycle distribution was determined by Guava Cytosoft Software. **(b), (c),** Cell lysates were collected from MCF-10A-ErbB2 cells 48 hours after lentiviral infection with control, OGT-1, or OGT-2 shRNA. Lysates were analyzed by immunoblot analysis with indicated antibodies. **(d)** MDA-MB-231 cells were infected with retroviruses encoding control vector, wildtype FoxM1, or DN-DKEN-FoxM1. Following stable selection, cells were infected with lentivirus containing control or OGT-1 shRNA for 48 hrs, lysed and analyzed by immunoblot analysis with indicated antibodies.

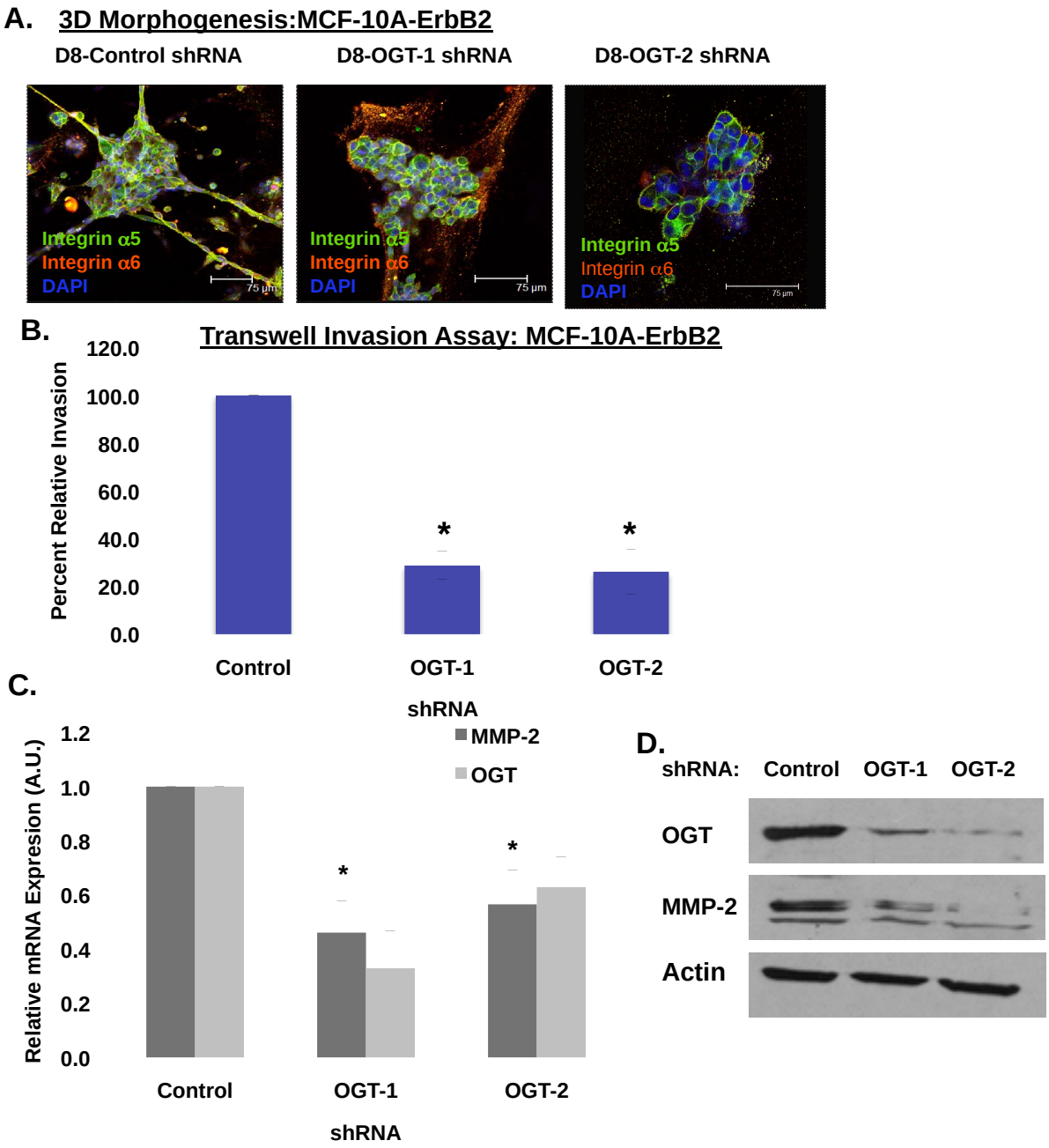


Figure 5. OGT knockdown blocks invasion and reduces MMP-2 expression in breast cancer cells. **(a)** MCF-10A-ErbB2 cells expressing control, OGT-1, or OGT-2 shRNA were placed in 3D culture. At day 8, cells were fixed and stained for confocal microscopy with indicated antibodies. **(b)** MCF-10A-ErbB2 cells infected with control, OGT-1, or OGT-2 shRNA pLKO.1 were placed in transwell invasion slides for 24 hrs. Cells in bottom of well were DAPI stained and counted. **(c)** Total RNA from MCF-10A-ErbB2 cells infected with control, OGT-1 or OGT-2 shRNA pLKO.1 were collected and used to assay OGT and MMP-2 transcripts using QRT-PCR, normalized to Cyclophilin A. Data expressed as normalized expression relative to control shRNA. **(d)** Cell lysates from MCF-10A-ErbB2 cells expressing control, OGT-1 or OGT-2 shRNA were collected and analyzed by immunoblot analysis with indicated antibodies.

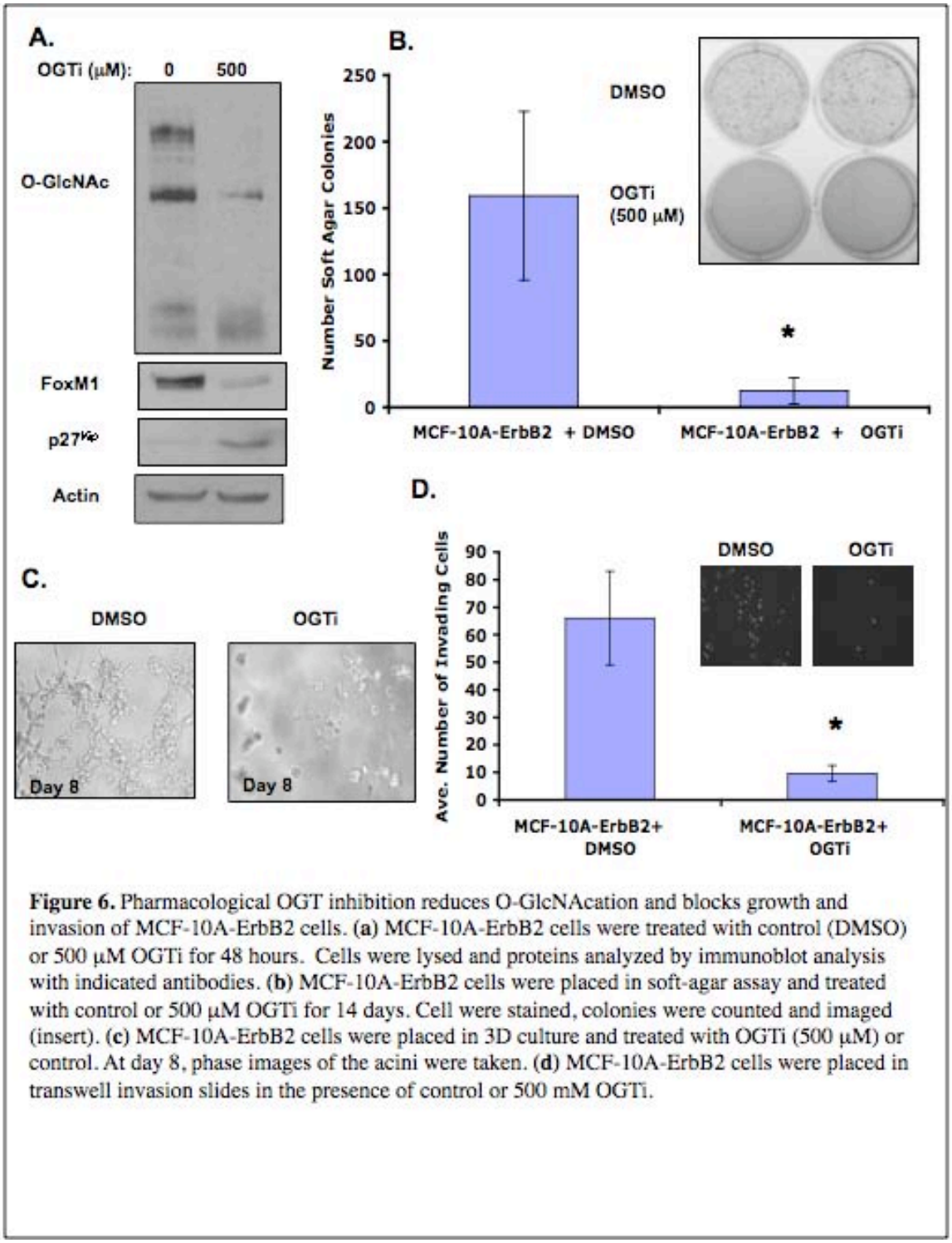


Figure 6. Pharmacological OGT inhibition reduces O-GlcNAcation and blocks growth and invasion of MCF-10A-ErbB2 cells. **(a)** MCF-10A-ErbB2 cells were treated with control (DMSO) or 500 μM OGTi for 48 hours. Cells were lysed and proteins analyzed by immunoblot analysis with indicated antibodies. **(b)** MCF-10A-ErbB2 cells were placed in soft-agar assay and treated with control or 500 μM OGTi for 14 days. Cell were stained, colonies were counted and imaged (insert). **(c)** MCF-10A-ErbB2 cells were placed in 3D culture and treated with OGTi (500 μM) or control. At day 8, phase images of the acini were taken. **(d)** MCF-10A-ErbB2 cells were placed in transwell invasion slides in the presence of control or 500 mM OGTi.

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