

ORIGINAL ARTICLE

Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1

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Cancer cells upregulate glycolysis, increasing glucose uptake to meet energy needs. A small fraction of a cell's glucose enters the hexosamine biosynthetic pathway (HBP), which regulates levels of O-linked β -N-acetylglucosamine (O-GlcNAc), a carbohydrate posttranslational modification of diverse nuclear and cytosolic proteins. We discovered that breast cancer cells upregulate the HBP, including increased O-GlcNAcation and elevated expression of O-GlcNAc transferase (OGT), which is the enzyme catalyzing the addition of O-GlcNAc to proteins. Reduction of O-GlcNAcation through RNA interference of OGT in breast cancer cells leads to inhibition of tumor growth both *in vitro* and *in vivo* and is 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 through transcriptional control of Skp2. Reducing O-GlcNAc levels in breast cancer cells decreased levels of FoxM1 protein and caused a decrease in multiple FoxM1-specific targets, including Skp2. Moreover, reducing O-GlcNAcation decreased cancer cell invasion and was associated with the downregulation of matrix metalloproteinase-2, a known FoxM1 target. Finally, 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.

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Introduction

Tumor cells have altered carbohydrate metabolism, producing ATP primarily through glycolysis, even under normal oxygen concentrations (Dang and Semenza, 1999). This metabolic shift in cancer cells, termed the 'Warburg effect', involves increased glucose uptake and is critical in supporting cancer phenotypes (Warburg, 1956). Changes in tumor glucose uptake and metabolism also alter distinct nutrient signaling pathways, including mammalian target of rapamycin, AMP-activated protein kinase and hexosamine biosynthetic pathway (HBP) (Marshall, 2006). Indeed, there is growing evidence that suggests that abnormalities within the mammalian target of rapamycin and AMP-activated protein kinase pathways can lead to abnormal growth and cancer (Shaw, 2006; Guertin and Sabatini, 2007). The majority of glucose enters glycolysis, producing ATP, whereas approximately 2–5% of a cell's glucose enters the HBP (Marshall *et al.*, 1991), resulting in the end product uridine diphosphate (UDP)-N-acetylglucosamine (UDP-GlcNAc) (Hart *et al.*, 2007). Although 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 yet 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-linked β -N-acetylglucosamine (O-GlcNAc) modifies a wide variety of cytosolic and nuclear proteins. O-GlcNAc acts as novel regulatory switch mechanism analogous to phosphorylation (Wells *et al.*, 2001). Cytosolic and nuclear enzymes dynamically catalyze both the addition (O-GlcNAc transferase or OGT) and the removal (O-GlcNAcase) of O-GlcNAc in response to various stimuli, including tyrosine kinase receptor activation (Vosseller *et al.*, 2002). OGT is unique among glycosyltransferases in its high affinity for UDP-GlcNAc. As a consequence, OGT activity responds to physiological changes in UDP-GlcNAc (Lubas and Hanover, 2000), thus leading to elevated O-GlcNAc modifications in response to increased flux through the HBP (Buse *et al.*, 2002). Accordingly, OGT is positioned to function as a molecular sensor of enhanced HBP nutrient flux, which would be expected in cancer cells.

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O-GlcNAc is known to influence protein–protein interactions (Roos *et al.*, 1997); therefore, modulations of O-GlcNAc may alter the formation of specific protein complexes involved in oncogenic signaling. Modulation of O-GlcNAc levels is linked to growth/survival phenotypes such as cell-cycle progression and altered mitotic phosphorylation patterns (Boehmelt *et al.*, 2000; Zhu *et al.*, 2001; O'Donnell *et al.*, 2004; Slawson *et al.*, 2005), showing that a proper balance between O-GlcNAcylation and phosphorylation is required for normal cell growth. Recently, it was shown that p53-deficient mouse embryonic fibroblasts, which increase glycolysis, display increased O-GlcNAcylation on a number of proteins (Kawauchi *et al.*, 2009). Thus, abnormal levels of O-GlcNAc in cancer cells may contribute to deregulated posttranslational control of protein function linked to oncogenic phenotypes.

A number of transcription factors are known to be modified by O-GlcNAc, suggesting that this glucose-sensing mechanism can directly link nutrient status to gene expression (Comer and Hart, 1999). Elevated expression or activity of FoxM1 is associated with the development and progression of numerous cancers (Wonsey and Follettie, 2005; Myatt and Lam, 2007). 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 (Myatt and Lam, 2007), 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 (Bektas *et al.*, 2008). FoxM1 has also been shown to regulate cellular invasion through the transcriptional regulation of matrix metalloproteinases (MMPs) (Wang *et al.*, 2007). Thus, targeting FoxM1 or its regulators has been proposed as a viable therapeutic strategy for treating cancer (Myatt and Lam, 2008).

In this study, we provide the first evidence that OGT and O-GlcNAc levels are elevated in breast cancer cells, and that reducing abnormally high O-GlcNAcylation 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 the cell-cycle regulator p27^{Kip1} and reduces expression of FoxM1, in addition to a number of FoxM1 targets. Indeed, regulation of FoxM1 may provide 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 the FoxM1 transcriptional target MMP-2. Our data suggest that tumor progression is associated with elevated O-GlcNAcylation, which deregulates critical factors in oncogenic growth and invasion. In addition, we show that pharmacological inhibition of OGT may be a valuable strategy for normalizing oncogenic phenotypes in breast cancer transformation.

Results

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

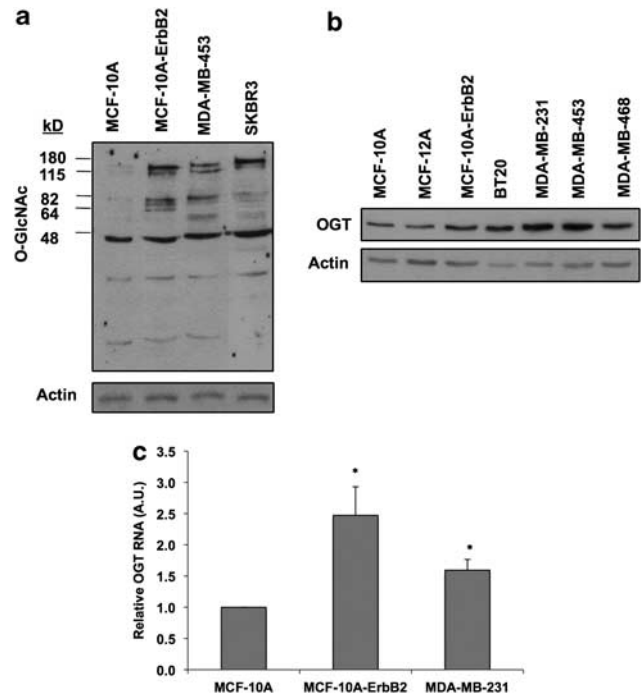


Figure 1 Breast cancer cells contain elevated O-GlcNAcylation and OGT levels. (a) Protein lysates from indicated cell lines were collected for immunoblot analysis and probed with indicated antibodies. (b) Normal mammary epithelial cells MCF-10A and MCF-12A and breast cancer cells MCF-10A-ErbB2, BT20, MDA-MB-231, MDA-MB-453 and MDA-MB-468 were lysed and subjected to immunoblot analysis with indicated antibodies. (c) Total RNA was harvested from MCF-10A, MCF-10A-ErbB2 and MDA-MB-231 cells, and levels of OGT mRNA were quantified by QRT-PCR and normalized to cyclophilin A. Normalized OGT mRNA levels are presented relative to MCF-10A. Values represent mean and s.e. of at least three independent experiments, * represents Student's *t*-test, *P*-value < 0.05.

mammary epithelial cells with 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 with normal human immortalized mammary epithelial MCF-10A cells (Figure 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 with normal MCF-10A and MCF-12A cells (Figure 1b). The increase in OGT protein levels may be due to an increase in RNA levels, as we found that ErbB2-overexpressing cells and MDA-MB-231 cells contain elevated OGT RNA levels compared with normal MCF-10A cells (Figure 1c). Furthermore, we searched the Oncomine database and found OGT levels elevated in invasive ductal carcinoma compared with normal breast tissue (Supplementary Figure 1). We thus show, for the first time, that breast cancer cells have elevated levels of O-GlcNAc and OGT.

OGT is required for malignant growth of transformed breast cancer cells in vitro

To test whether reducing high levels of O-GlcNAcation alters breast cancer phenotypes, we targeted OGT through RNA interference (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 with cells infected with control (scrambled) shRNA sequence (Figure 2a). We then tested whether the reduction of OGT led to a global decrease in O-GlcNAcation. Cells were treated with or without the specific O-GlcNAcase inhibitor 9D (Macauley *et al.*, 2005) to block enzymatic removal of O-GlcNAc. MCF-10A-ErbB2 cells infected with control shRNA showed a significant increase in O-GlcNAcation in the presence of 9D (Figure 2b). However, cells infected with RNAi targeting OGT had significantly decreased basal O-GlcNAcation, and completely blocked the 9D-induced elevation of O-GlcNAcation (Figure 2b); decrease in OGT levels led to a 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 a dramatic inhibition of oncogenic phenotypes, including decreased cell growth and an eight-fold decrease in cell number at day 12 (Figure 2c) compared with control RNAi cells. In addition, reduction of OGT levels in MCF-10A-ErbB2 cells showed a similar decrease in colony formation in soft agar assays (Supplementary Figure 2). To test whether the 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 (Figure 2d) and resulted in significant inhibition of growth under 3D conditions compared with control-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 (Supplementary Figure 3D), suggesting that reducing OGT levels in nontransformed 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 showed that OGT and abnormally elevated levels of O-GlcNAc are required for and may contribute to transformed growth of breast cancer cells *in vitro*.

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 (Figure 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 with control cells at the end of the 8-week experiment (Figure 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 (Figure 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 with tumors from control cells (Supplementary Figure 4A). Importantly, tumors that eventually grew from OGT knockdown cells restored OGT expression (Figure 3d) and had a similar Ki-67 expression (Supplementary Figure 4B), suggesting a strong selective pressure against tumor cells deficient in OGT. These data indicate the importance of OGT in tumor cell growth *in vivo*.

Inhibition of OGT decreases cell-cycle progression and induces p27^{Kip1} expression through regulation of FoxM1 in breast cancer cells

To investigate further the growth-inhibitory effect of OGT knockdown in breast cancer cells, we conducted a 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 the G1 phase within 48 h compared with control shRNA-infected cells: 72% G1 content in OGT shRNA-infected cells relative to 47% in control shRNA cells (Figure 4a). With OGT shRNA, we also observed a significant decrease in the S- and G2/M-phase population compared with 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 Figure 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 (Supplementary Figure 3B) nor changes in Ki-67 staining (Supplementary Figure 3C) were statistically significant.

Increase in the population of cells in G1 suggests 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 proliferating cell nuclear antigen in MCF-10A-ErbB2 cells (Figure 4b), as well as in MDA-MB-231 cells (Supplementary Figure 5B), 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, c-Src and mitogen-activated protein

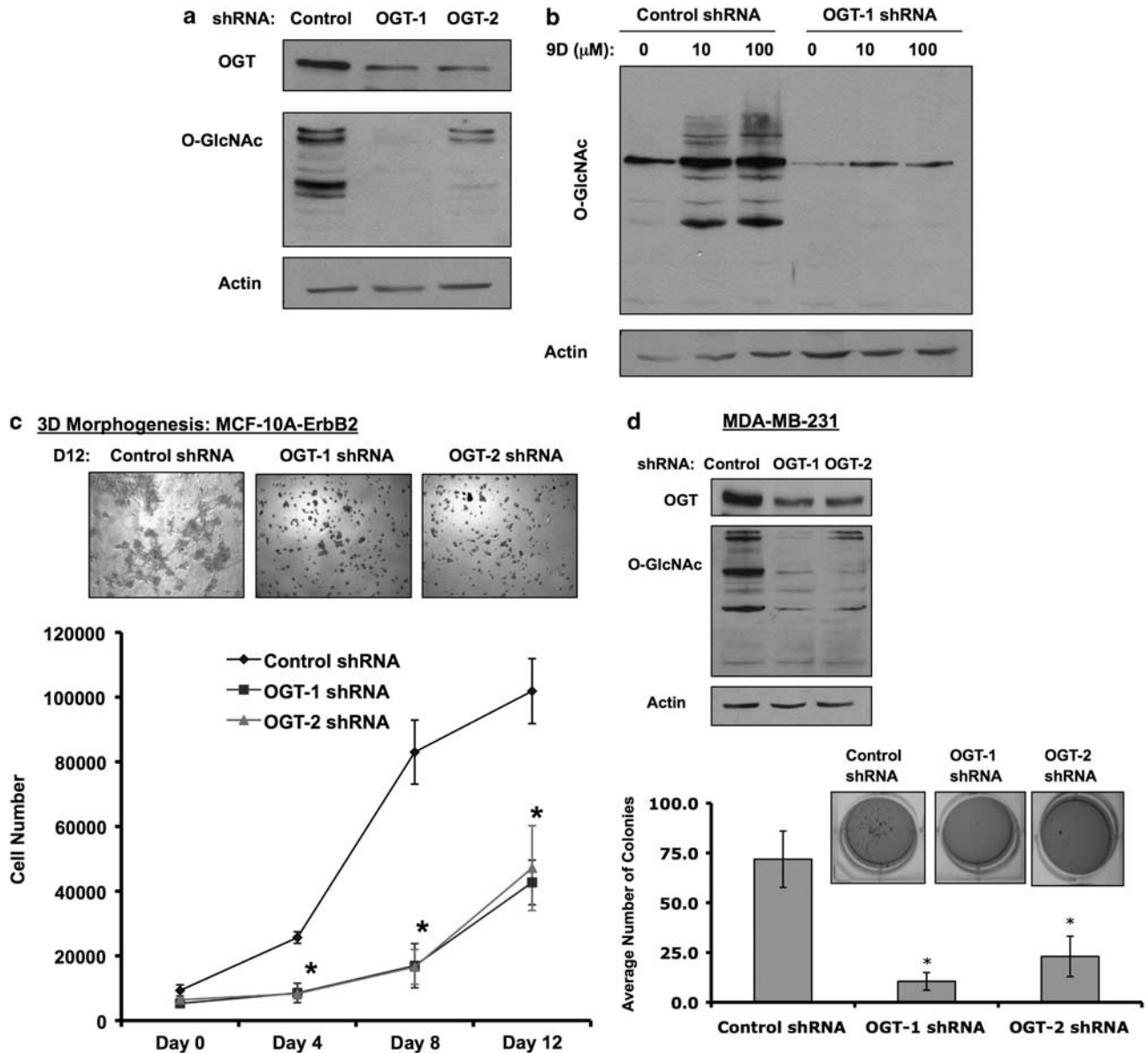


Figure 2 Knockdown of OGT reduces O-GlcNAcation and inhibits growth of MCF-10A-ErbB2 and MDA-MB-231 cells *in vitro*. (a) MCF-10A-ErbB2 cells were infected with control, OGT-1 or OGT-2 shRNA pLKO.1 lentivirus, and protein lysates were collected 48 h after infection for immunoblot analysis with indicated antibodies. (b) MCF-10A-ErbB2 cells infected as described in panel a for 48 h and were treated for 24 h with the indicated concentrations of 9D before lysis and immunoblot analysis with the indicated antibodies. (c) MCF-10A-ErbB2 cells infected, as described in panel a, and placed in a 3D morphogenesis assay. Cells were imaged and counted at indicated time points. (d) MDA-MB-231 cells were infected as above and lysed 48 h after infection and subjected to immunoblot analysis with indicated antibodies (left) or, placed in soft agar assays (right). Colonies were stained 14 days later and quantified. Insert: image showing representative number and size of colonies. Values represent mean and s.e. of at least three independent experiments, * represents Student's *t*-test, *P*-value < 0.05.

kinase activation in cancer cells is associated with increased p27^{Kip1} proteolysis (Chu *et al.*, 2008). Yet, knockdown of OGT in MCF-10A-ErbB2 cells did not reduce activity of ErbB2, c-Src, Erk (extracellular signal-regulated kinase) (Supplementary Figure 6) or Akt (Figure 3b) as measured with respective phospho-specific antibodies. As 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 (Chu *et al.*, 2008). This complex includes the F-Box protein Skp2 that targets cyclin-dependent kinase inhibitors for degradation during the G1/S transition. We found that OGT knockdown in MCF-10A-ErbB2 cells (Figure 4b) and MDA-MB-231 cells (Supplementary Figure 5b) decreases Skp2 expression. One level of Skp2 regulation is through transcriptional activation by FoxM1 (Wang *et al.*, 2005). We found that in MCF-10A-ErbB2

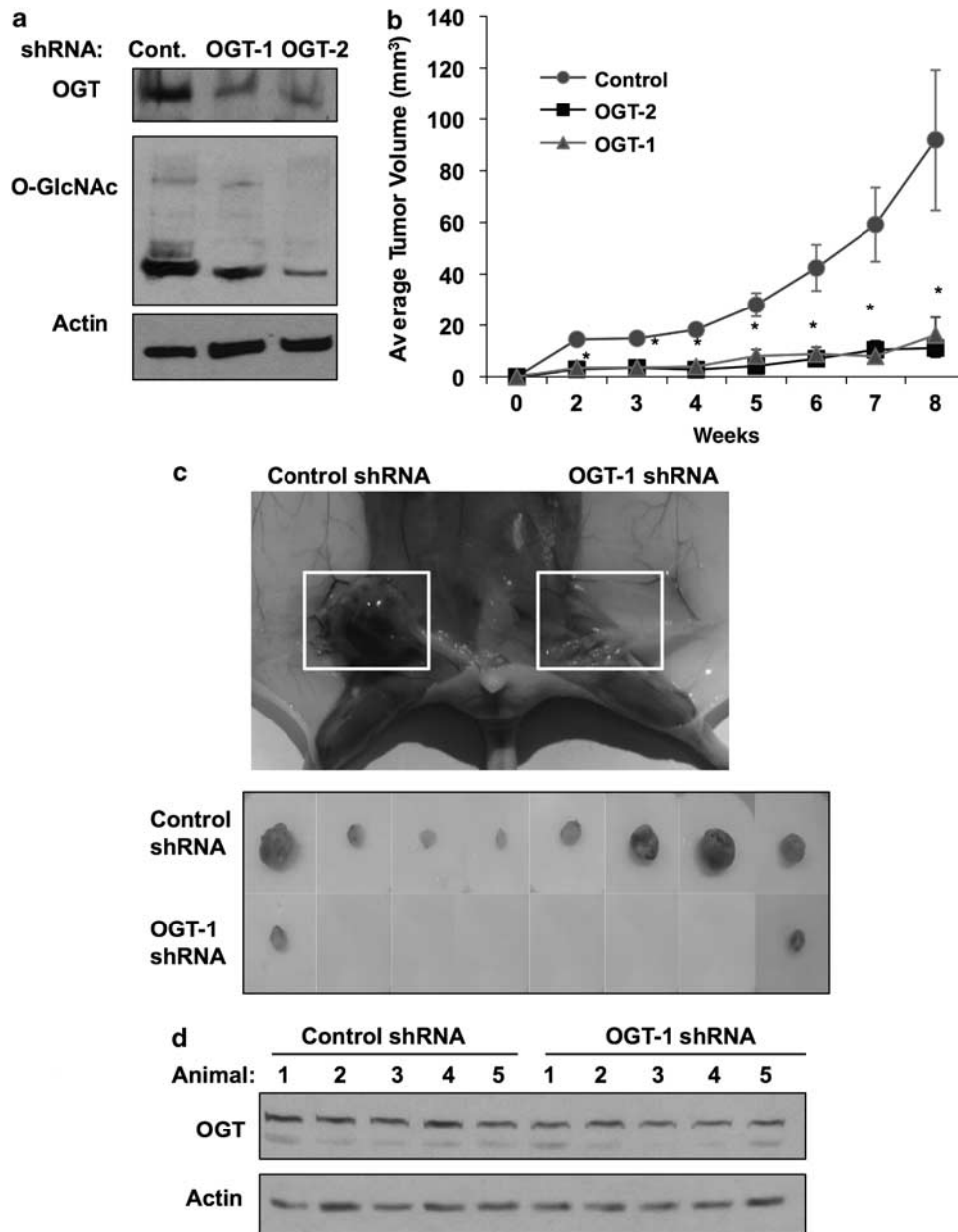


Figure 3 OGT is required for tumorigenic growth of human breast cancer cells *in vivo*. (a) MDA-MB-231 cells expressing control, OGT-1 or OGT-2 shRNA were lysed and analyzed by immunoblot analysis with indicated antibodies before injection into mice. (b) Mean tumor volume (mm³) of MDA-MB-231 cells expressing control ($n = 37$), OGT-1 ($n = 17$), or OGT-2 ($n = 20$) shRNA injected into mammary fat pad of immunocompromised mice at indicated week. Values represent mean and s.e. of independent experiments, * represents Student's *t*-test, P -value < 0.05. (c) Top, representative mammary fat pad tumor in mice transplanted with MDA-MB-231 control shRNA and OGT-1 shRNA cells 8 weeks after injection, and, bottom, resected tumors 8 weeks after injection of MDA-MB-231 control shRNA and OGT-1 shRNA cells. (d) Tumors resected from mice were lysed and analyzed by immunoblot analysis with indicated antibodies.

(Figure 4b) and MDA-MB-231 cells (Supplementary Figure 5B), reducing OGT expression leads to significant decreases in FoxM1 protein levels. FoxM1 can regulate progression from the G1 to S phase, and is also known to be a key regulator during G2/M transition. 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 (Figure 4c) and MDA-MB-231 cells (Supplementary Figure 5B).

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 wild-type FoxM1 protein (Figure 4d), suggesting that OGT and

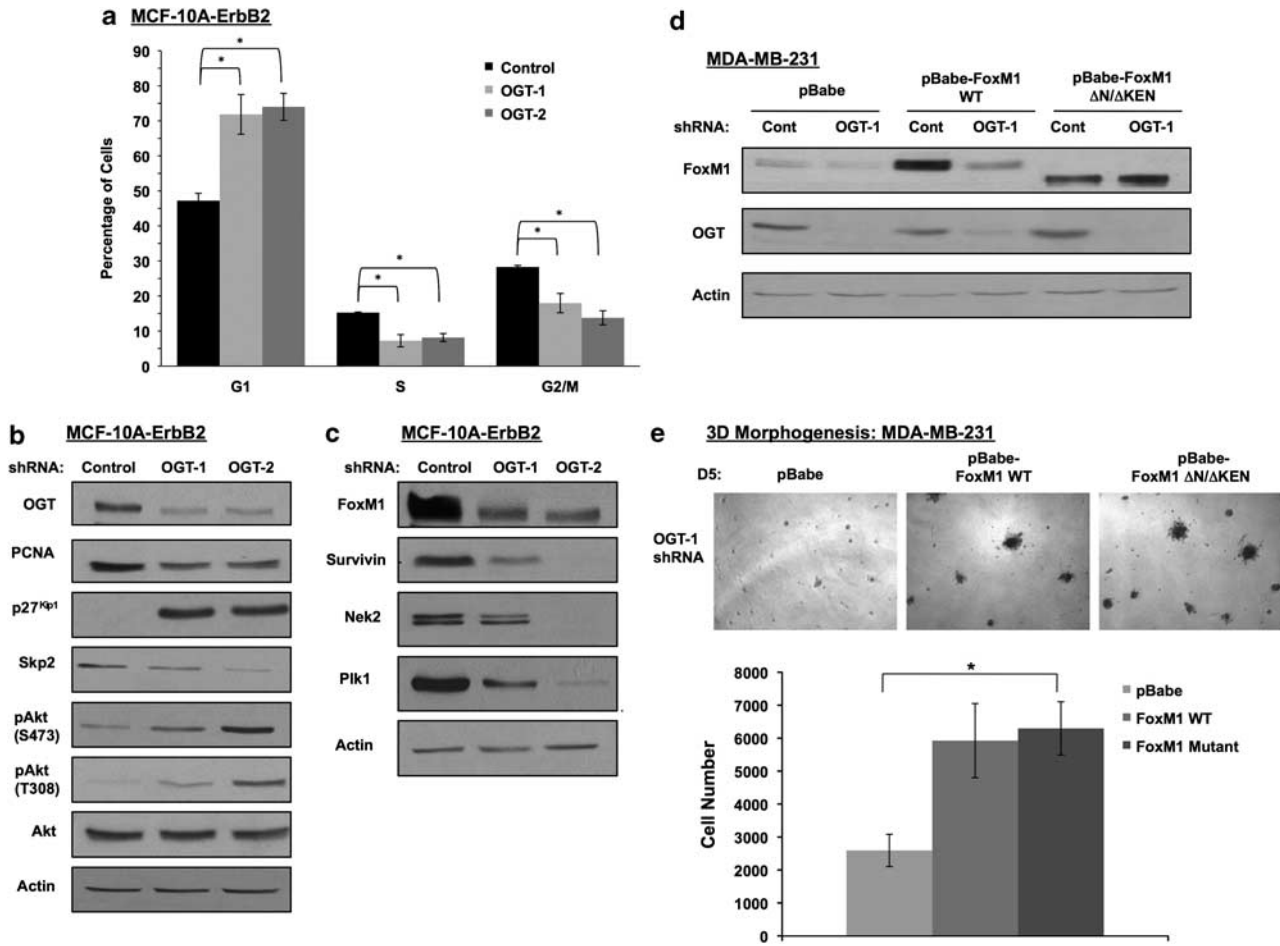


Figure 4 Knockdown of OGT inhibits cell-cycle progression, elevates p27^{Kip1} expression and reduces FoxM1 expression in breast cancer cells. (a) Cell-cycle analysis of MCF-10A-ErbB2 cells 48 h after lentiviral infection with control, OGT-1 or OGT-2 shRNA. Cells were collected, stained with propidium iodide and analyzed by flow cytometry. Cell-cycle distribution was determined using Guava Cytosoft Software (Millipore, Billerica, MA, USA). (b, c) Cell lysates were collected from MCF-10A-ErbB2 cells 48 h 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 (pBabe), wild-type FoxM1 (pBabe-FoxM1-WT) or mutant FoxM1 (pBabe-FoxM1-ΔN/ΔKen). After stable selection, cells were infected with lentivirus containing control or OGT-1 shRNA for 48 h, lysed and analyzed by immunoblot analysis with indicated antibodies. (e) MDA-MB-231 cells stably expressing control vector, wild-type FoxM1 or mutant FoxM1 were infected with OGT-1 shRNA lentivirus. Cells were then placed in 3D morphogenesis assay, imaged at day 5 and counted at day 8. Values represent mean and s.e. of at least three independent experiments, * represents Student's *t*-test, *P*-value < 0.05.

O-GlcNAcylation may regulate FoxM1 posttranscriptionally. 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 (Laoukili *et al.*, 2008) (Park *et al.*, 2008). The N terminus of FoxM1 contains destruction box (D box) and KEN-box sequences, short degradation motifs recognized by the anaphase-promoting complex E3 ubiquitin ligase (Park *et al.*, 2008) (Laoukili *et al.*, 2008). FoxM1 regulation by O-GlcNAcylation 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 with wild-type FoxM1 (Figure 4d). To test whether overexpression of wild-type or mutant FoxM1

can rescue cell growth defect caused by downregulating OGT, we placed these cells in 3D culture. Cells overexpressing either the wild type or mutant of FoxM1 were able to partially overcome the inhibitory effect of OGT silencing on cell growth in 3D culture (Figure 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 observed 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 (Supplementary Figure 7). Moreover, we found that reduction of OGT levels led to no significant change in the expression of a number of

transcription factors implicated in breast cancer, including p53, c-Myc and nuclear factor- κ B (Supplementary Figure 7). As 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 wild-type FoxM1 did not show any O-GlcNAc modifications, whereas endogenous Sp1, a transcription factor known to be modified by O-GlcNAc (Han and Kudlow, 1997), was highly modified under similar conditions (data not shown). Thus, our data show that breast cancer cell growth inhibition by targeting OGT is associated with increased cell-cycle arrest at G1, elevated expression of p27^{Kip1} and specific posttranscriptional 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.

OGT regulates breast cancer cell invasion

We observed that breast cancer cells with OGT knockdown produced fewer invasive protrusions when cultured under 3D conditions (Figure 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 led to a three-fold decrease in invasion compared with controls (Figure 5b). Breast cancer invasion and metastasis is associated with elevated levels of MMP-2 (Duffy *et al.*, 2000). As FoxM1 regulates expression of MMP-2 in pancreatic cancer cells (Wang *et al.*, 2007), we examined levels of MMP-2 in OGT knockdown cells. Indeed, we found a two-fold decrease in the expression of MMP-2 at both mRNA (Figure 5c) and protein levels (Figure 5d) in MCF-10A-ErbB2 cells when OGT is knocked down. Knockdown of FoxM1 in MCF-10A-ErbB2 cells also leads to decreased MMP-2 levels and invasion (data not shown). Thus, our data suggest that OGT regulates cancer cell invasion by modulating MMP-2 expression, possibly by the regulation of FoxM1.

OGT inhibitor blocks breast cancer growth and invasion

We have recently identified novel inhibitors of OGT catalytic activity (Gross *et al.*, 2005). OGT inhibitor (OGTi) treatment of MCF-10A-ErbB2 cells reduced O-GlcNAc levels (Figure 6a) and dramatically decreased growth in soft agar (Figure 6b) and 3D culture assays (Figure 6c). Pharmacological inhibition of OGT led to decreased FoxM1 expression, which correlated with elevation of p27^{Kip1} levels (Figure 6a). Similar to OGT knockdown, a decrease in invasive protrusions from cells treated with OGTi in 3D culture was observed, and a six-fold decrease in cell invasion of MCF-10A-ErbB2 cells was observed in response to treatment with OGTi using transwell invasion assays (Figure 6d). Similar inhibitory effects on FoxM1 levels, cell growth and invasion were observed in MDA-MB-231 cells treated with OGTi (Supplementary Figure 8). Treatment of

parental MCF-10A acinar structures with OGTi at day 14 for 48 h did not cause cytotoxic effects or disruption of acinar architecture (data not shown).

Thus, we show in this study for the first time that OGT and O-GlcNAcation is elevated in cancer cells, and that normalization of these levels by two independent methods (RNAi knockdown of OGT and pharmacological inhibition) reduces tumor growth and invasion. Elevated O-GlcNAcation and OGT levels appear to contribute to cancer cell growth and invasion, at least in part by regulating the stability of the oncogenic transcription factor FoxM1 and its downstream targets.

Discussion

Glucose flux through the HBP, leading to modifications of nuclear and cytoplasmic proteins by O-GlcNAc, is emerging as a key regulator for many biological processes and disease states. OGT regulation of the insulin pathway has been implicated in insulin resistance associated with type II diabetes (Vosseller *et al.*, 2002) (Yang *et al.*, 2008), and O-GlcNAc alterations has also been associated with neurodegenerative diseases, including Alzheimer's disease (Liu *et al.*, 2004). In this study, we show for the first time that OGT and O-GlcNAc modifications are elevated in cancer cells, and that normalization of these levels reduces tumor growth and invasion. Elevated O-GlcNAc and OGT may contribute to cancer cell growth and invasion, in part by regulating the oncogenic transcription factor FoxM1.

Most cancers exhibit altered metabolism, including increased aerobic glycolysis and a dependence on glycolytic pathways for ATP generation. To serve the less-efficient energy-producing glycolytic route, tumor cells increase glucose uptake. Increased glucose consumption may lead to increased shunting to the HBP. Consistent with this idea, a recent study has shown that elevated glycolysis associated with loss of p53 in mouse embryonic fibroblasts leads to increased O-GlcNAc modifications (Kawauchi *et al.*, 2009). Our results show that breast cancer cells known to have increased glycolysis, such as MDA-MB-231 (Gatenby and Gillies, 2004; Gallagher *et al.*, 2007), contain elevated O-GlcNAc modifications and increased OGT levels. However, it is not clear whether elevation of glycolysis in cancer cells directly leads to increased flux through the HBP and consequential increase in O-GlcNAc modifications, or whether transformation by oncogenes or loss of tumor suppressors may regulate OGT expression or activity. Nonetheless, our data indicate that elevated O-GlcNAcation links cancer cell alterations of metabolic pathways to transformed cell growth and invasion signals.

The induction of p27^{Kip1} by knockdown of OGT is significant, as many breast cancer therapies directly upregulate p27^{Kip1} protein. Furthermore, the magnitude of breast cancer cell growth inhibition by therapies including the anti-ErbB2 antibody Herceptin closely parallels the level of p27^{Kip1} induced (Yakes *et al.*, 2002).

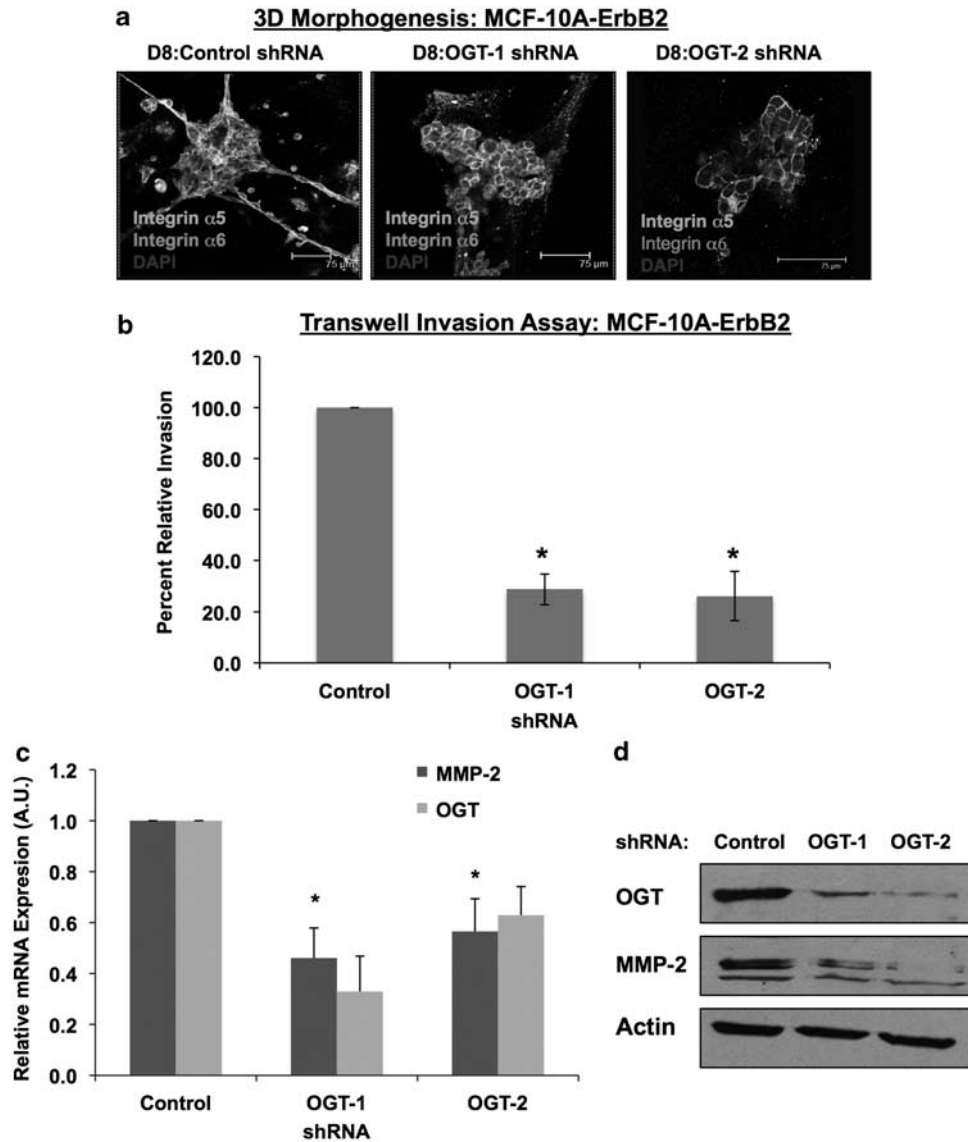


Figure 5 OGT knockdown blocks invasion and reduces MMP-2 expression in breast cancer cells. (a) MCF-10A-ErbB2 cells expressing control, OGT-1 or OGT-2 shRNA were placed in 3D culture. At day 8, cells were fixed and stained for confocal microscopy with indicated antibodies. (b) MCF-10A-ErbB2 cells infected with control, OGT-1 or OGT-2 shRNA were placed in transwell invasion chambers for 24 h. Invading cells were DAPI stained and counted. (c) Total RNA from MCF-10A-ErbB2 cells infected with control, OGT-1 or OGT-2 shRNA were collected and assayed for OGT and MMP-2 expression 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 immunoblotting with indicated antibodies. Values represent mean and s.e. of at least three independent experiments, * represents Student's *t*-test, *P*-value < 0.05.

However, we found little change in signaling pathways associated with ErbB2 activation or breast cancer in general in OGT knockdown cells compared with controls, suggesting that decreased O-GlcNAcylation alters p27^{Kip1} stability through mechanisms independent of inhibiting ErbB2 signaling. Nevertheless, it is almost certain that additional functionally significant effects of OGT knockdown/inhibition on cellular signaling are occurring, and it will be important to elucidate these.

FoxM1 is a well-characterized regulator of p27^{Kip1} and cell growth and transcriptionally regulates Skp2, the specific recognition factor for p27^{Kip1} ubiquitination. In

glioma cells, RNAi knockdown of FoxM1 led to increased p27^{Kip1} levels associated with a decrease in Skp2 protein (Liu *et al.*, 2006). In pancreatic cells, RNAi against FoxM1 led to a decrease in metastasis and angiogenesis, correlating with a reduction of MMP-2 and vascular endothelial growth factor expression (Wang *et al.*, 2007). Consistent with these data, OGT knockdown in breast cancer cells led to a reduction in invasion and downregulation of the FoxM1 target MMP-2. As FoxM1 is highly expressed in proliferating tumor cells and contributes to metastasis, it is currently being considered as a viable therapeutic target for a

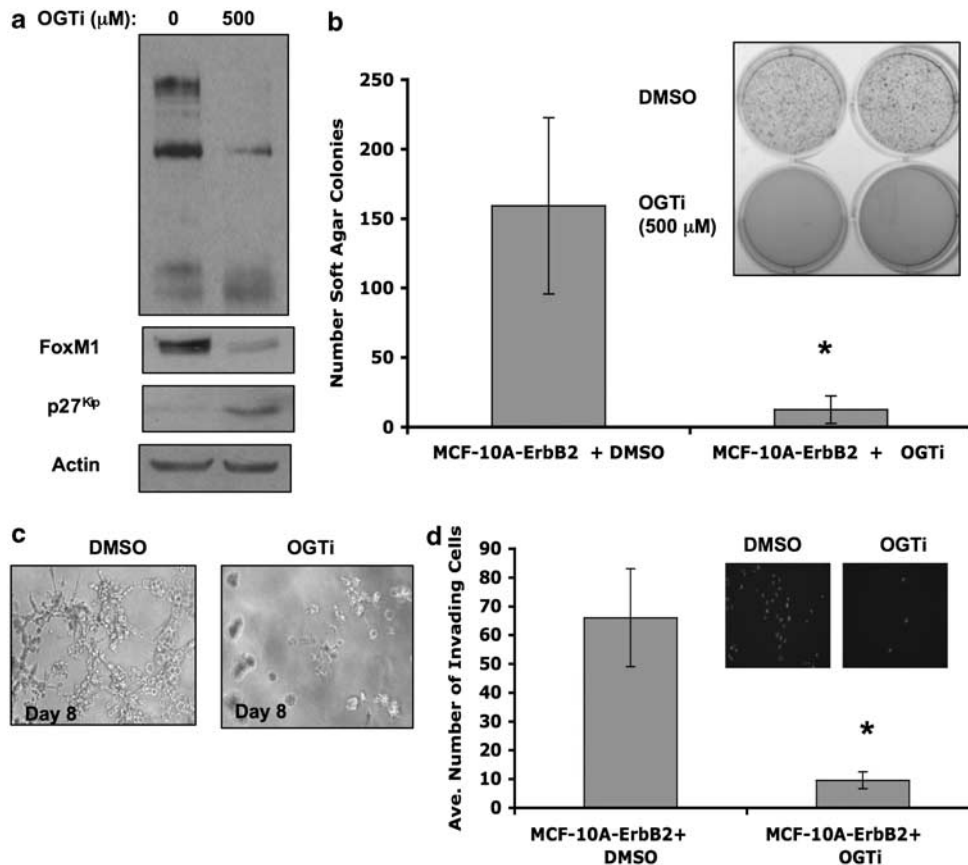


Figure 6 Pharmacological OGT inhibition reduces O-GlcNAcation and blocks growth and invasion of MCF-10A-ErbB2 cells. (a) MCF-10A-ErbB2 cells were treated with control (DMSO) or 500 μM OGTi for 48 h. Cells were lysed and proteins analyzed by immunoblotting with indicated antibodies. (b) MCF-10A-ErbB2 cells were placed in soft agar assay and treated with control or 500 μM OGTi for 14 days. Cells were stained, colonies were counted and imaged (inset). (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 acquired. (d) MCF-10A-ErbB2 cells were placed in transwell invasion slides in the presence of control or 500 μM OGTi. Values represent mean and s.e. of at least three independent experiments, * represents Student's *t*-test, *P*-value < 0.05.

number of cancers (Gartel, 2008). Our results show that targeting OGT with first-generation OGTis may be a novel way to modulate FoxM1 expression in breast and perhaps other cancers.

FoxM1 expression increases during the G1 to S phase after cyclin E/cyclin-dependent kinase 2-mediated (Major *et al.*, 2004) and Ras/Mek/Erk kinase-mediated phosphorylation (Ma *et al.*, 2005). However, it is unlikely that the increase in G1-phase cells and down-regulation of FoxM1 expression in response to OGT knockdown is due to loss of Mek/Erk signaling, as no decrease in Erk activation is observed in OGT knockdown cells. Recent studies have identified FoxM1 as being a substrate for ubiquitin-mediated degradation, contributing to the normal changes in FoxM1 levels across the cell cycle (Laoukili *et al.*, 2008) (Park *et al.*, 2008). O-GlcNAcation of p53 has been shown to protect against ubiquitin-mediated degradation (Yang *et al.*, 2006) and increase the half-life of steroid nuclear receptors (Cheng and Hart, 2001). However, although the Fox family member FoxO1 has recently been shown to be modified by O-GlcNAc (Housley *et al.*, 2008), we

were unable to detect O-GlcNAc modifications on FoxM1. One possibility is that hyper-O-GlcNAcation of regulators of FoxM1 in breast cancer cells blocks FoxM1 degradation, increasing FoxM1 protein levels and contributing to transformation. In this model, as O-GlcNAc levels are decreased, FoxM1 becomes susceptible to proteosomal degradation, accounting for its decreased expression level and inhibition of transformed phenotypes. Our data show that the N terminus of FoxM1 is required for regulation by OGT. Recent studies have shown that the N terminus of FoxM1 contains both D-box D- and KEN-box sequences that are required for proteolytic targeting by anaphase-promoting complex-Cdh1 adaptor (Park *et al.*, 2008) (Laoukili *et al.*, 2008), thus suggesting that changes in O-GlcNAcation may indirectly regulate FoxM1 degradation.

In summary, this study is the first to link OGT and O-GlcNAcation to cancer cell growth and invasion and identifies novel regulation of the oncogenic transcription factor FoxM1 by altered O-GlcNAcation. Thus, the nutrient-sensing roles of OGT may link abnormal

metabolic states in cancer cells to deregulation of critical growth and transformation factors such as FoxM1, and pharmacological modulation of enzymes regulating O-GlcNAcylation may be a novel therapeutic strategy in cancer.

Materials and methods

Materials

The O-GlcNAcase inhibitor, 9D, was provided by David Vocadlo (Simon Fraser University, Burnaby, BC, Canada). Growth-factor-reduced Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Antibodies such as anti-actin, anti-FoxM1 and anti-proliferating cell nuclear antigen were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho-Akt (Ser473), anti-phospho-Akt (T308), anti-AKT and anti-MMP-2 were purchased from Cell Signaling (Danvers, MA, USA); anti-OGT was obtained from Sigma (St Louis, MO, USA); anti-p27^{Kip1}, anti-Nek2, anti-PLK1 and anti-integrin- α 5 were purchased from BD-Biosciences; anti-Skp2 was from Zymed (Carlsbad, CA, USA); anti-integrin- α 6 was obtained from Chemicon (Billerica, MA, USA). Anti-O-GlcNAc antibody (CTD110.6) (Comer *et al.*, 2001) and OGTi (Gross *et al.*, 2005) have been described previously.

Cell culture and viral infections

MCF-10A, MCF-12A SKBR-3, MDA-MB-231, MDA-MB-453, MDA-MB-468, MCF-7 and BT-20 cells were acquired from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured following the instructions of ATCC. MCF-10A cells were grown as described previously (Reginato *et al.*, 2003). Constitutively active ErbB2 mutant (pBabe-NeuT) was kindly provided by Danielle Carroll (Harvard Medical School, Boston, MA, USA). pBabe-Flag-FoxM1 and pBabe-Flag- Δ N- Δ KEN-FoxM1 (was created by cloning Flag-FoxM1 cDNA insert from pcDNA3-Flag-FoxM1 and pcDNA3-Flag- Δ N- Δ KEN-FoxM1 plasmids (Laoukili *et al.*, 2008) (kindly provided by Rene H Medema, University Medical Center, Utrecht, The Netherlands) into the *Bam*HI and *Eco*RI sites of pBabe-puro. MDA-MB-231 cells over-expressing FoxM1 were generated using vesicular stomatitis virus G protein-pseudotyped retroviruses and were infected and selected as described previously (Reginato *et al.*, 2003).

Immunoblotting

Cell lysates from $1-5 \times 10^6$ cells were prepared in RIPA lysis buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM Tris-HCl at pH 8, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF and 1 mM Na₃VO₄) supplemented with 1 μ g/ml each of pepstatin, leupeptin, aprotinin and 200 μ g/ml phenyl-methyl-sulfonyl-fluoride. Lysates were cleared by centrifugation at 16000 *g* for 20 min at 4 °C and analyzed by SDS-PAGE and autoradiography. Proteins were analyzed by immunoblotting using primary antibodies indicated above.

RNA interference

Stable cell lines for shRNA knockdowns were generated by infection with the lentiviral vector pLKO.1-puro carrying shRNA sequence for scrambled (Addgene, Cambridge, MA, USA) or OGT (Sigma). VSVG-pseudotyped lentivirus was

generated by the cotransfection of 293-T packaging cells with 10 μ g of DNA and packaging vectors as described previously (Rubinson *et al.*, 2003). Control-scrambled shRNA sequence used was: CCTAAGGTTAAGTCGCCCTCGCTCTAGCGA GGGCGACTTAACCTT. OGT shRNA sequence used was: for OGT-1, GCCCTAAGTTTGTAGTCCAAATCTCGAGATT TGGACTCAAACCTTAGGGC and for OGT-2, GCTGAGCA GTATTCCGAAAACCTCGAGTTTCTCGGAATACTGCTC AGC. Cells were infected and selected as described previously (Reginato *et al.*, 2003).

3D morphogenesis assay and indirect immunofluorescence

Assays were performed as described previously (Reginato *et al.*, 2005). Briefly, 5×10^3 MCF-10A-ErbB2 or MDA-MB-231 cells, in respective media containing 2% Matrigel, were placed in an 8-well chamber slide (BD Biosciences) coated with 50 μ l of Matrigel. The number of cells was counted in two chambers at indicated time points and the mean of each determined. Immunofluorescence of 3D structures was performed as described previously (Reginato *et al.*, 2005) using antibodies to integrin- α 5 and integrin- α 6 then stained with 4',6-diamidino-2-phenylindole. Fluorescent secondary antibodies coupled with Alexa-Fluor dyes (Molecular Probes, Carlsbad, CA, USA) were used. Confocal analysis was performed by using the Leica DM6000B Confocal Microscope (Leica). Images were generated using the Leica Imaging Software (Wetzlar, Germany) and converted to Tiff format.

Orthotopic xenograft model

MDA-MB-231 cells were infected with lentivirus carrying control (scramble) shRNA, OGT-1 and OGT-2 shRNA constructs, as described above. After washes and resuspension in Hank's buffered salt solution (Mediatech, Inc., Manassas, VA, USA), 1.5×10^6 cells in 0.1 ml containing 20% Matrigel were injected subcutaneously through a 27½ gauge needle into the fourth inguinal mammary fat pad pair of each 4–6-week-old female athymic nude Nu/Nu mouse (Charles River, Wilmington, MA, USA). For each individual, control shRNA cells were injected into the right gland and OGT cells into the contralateral gland. After injection, tumors were measured weekly along and perpendicular to the longest dimension using digital calipers (Fowler Co., Inc., Newton, MA, USA). Tumor volumes were calculated as $V = (\text{length}) \times (\text{width})^2 \times 0.52$. After 8 weeks, tumors were excised, weighed and photographed. Tumors were then flash-frozen in liquid N₂ for western blot analysis. Frozen tumor samples were mechanically disrupted and resuspended in ice-cold RIPA buffer and lysed (described above) for immunoblotting.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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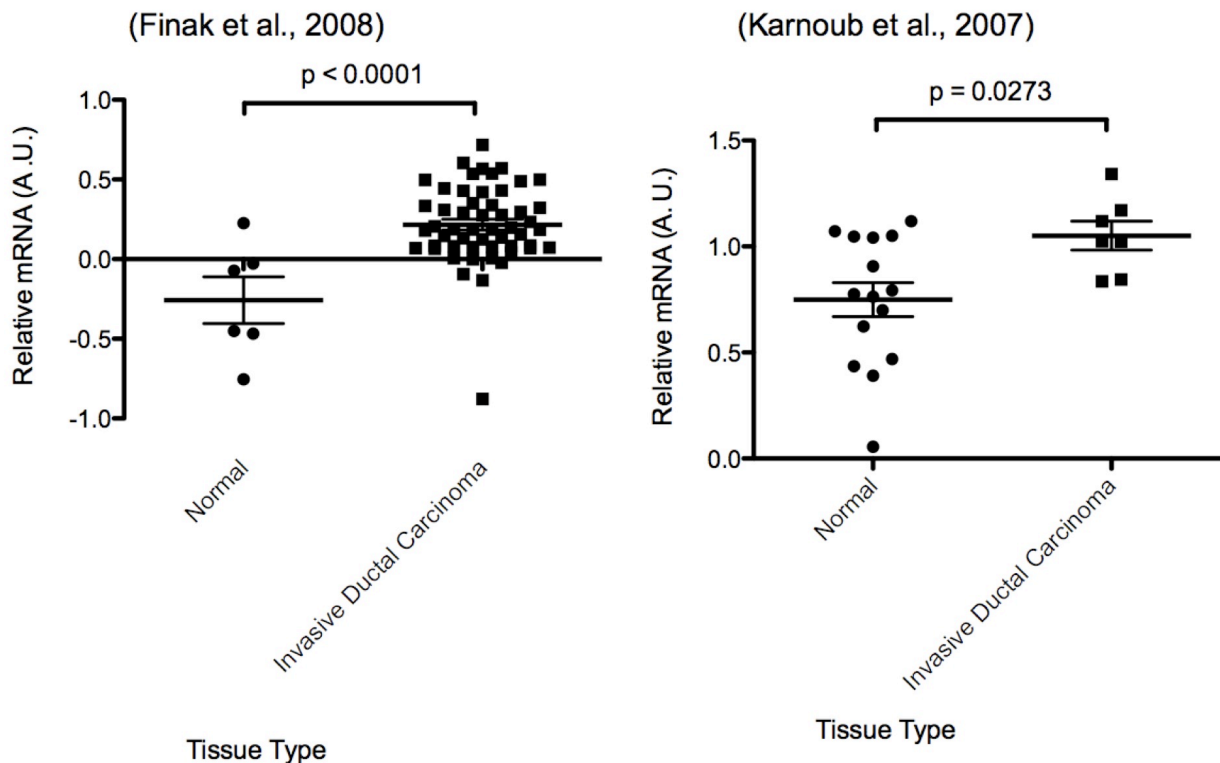
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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)

Caldwell, et. al. Supplementary Figure 1



Supplementary Figure 1. OGT RNA is upregulated in human breast cancer. Gene expression data available through OncoPrint™ from Finak et al. (2008) and Karnoub et al. (2007), re-graphed to show normal breast stroma versus invasive ductal carcinoma, with p-values indicated for Student's t-test. Error bars represent SEM.

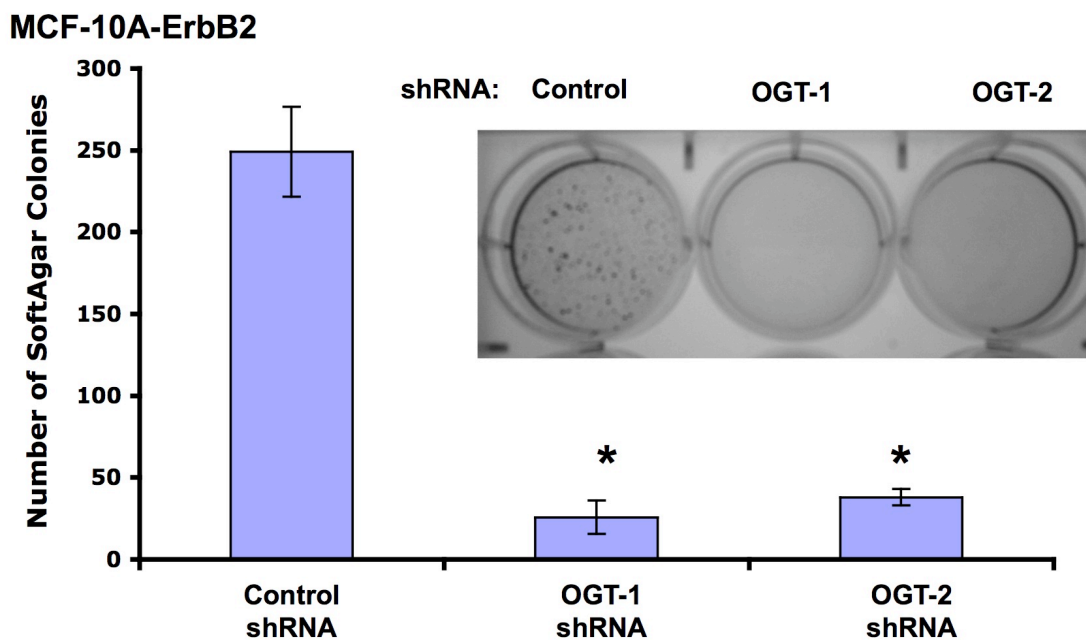
OGT gene expression data from two independent breast cancer studies were obtained using the OncoPrint™ (www.oncoPrint.org) database. The two studies retrieved compared samples of normal breast stroma and invasive ductal breast carcinoma. These data were re-graphed using GraphPad Prism (GraphPad Software, La Jolla, CA) software and presented as scatter plots, including the mean and SEM. Statistical analysis was performed using Student's t-test.

Studies Used:

Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, Chen H, Omeroglu G, Meterissian S, Omeroglu A, Hallett M, Park M. (2008). Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med.* 14:518-27.

Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature.* 449:557-63.

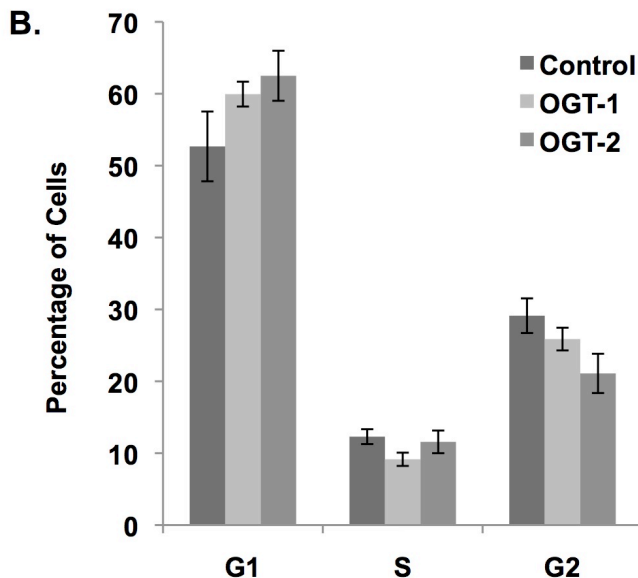
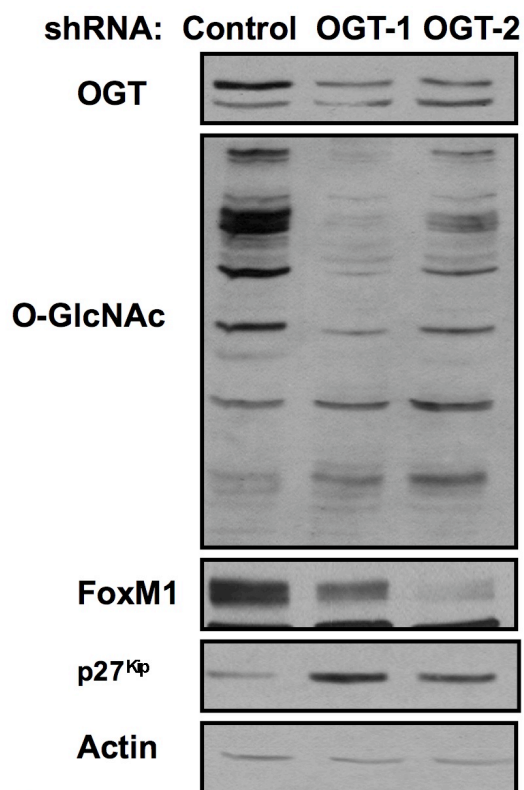
Caldwell, et. al. Supplementary Figure 2



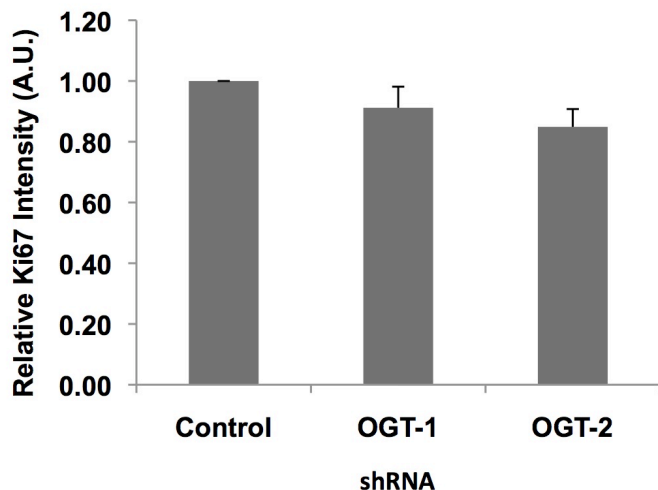
Supplementary Figure 2. OGT knockdown blocks soft agar colony formation of MCF-10A-ErbB2 cells. MCF-10A-ErbB2 cells were infected with lentivirus pLKO-shRNA-Control (Scram), pLKO-shRNA-OGT-1 or OGT-2 were placed in a soft agar assay for 14 days. Cell were stained with INT violet, counted and representative plate was imaged. Values represent mean number of colonies counted in 3 experiments. Bars, Standard Error (S.E.); Student's *t* test, **P* value < 0.05.

Caldwell, et. al. Supplementary Figure 3

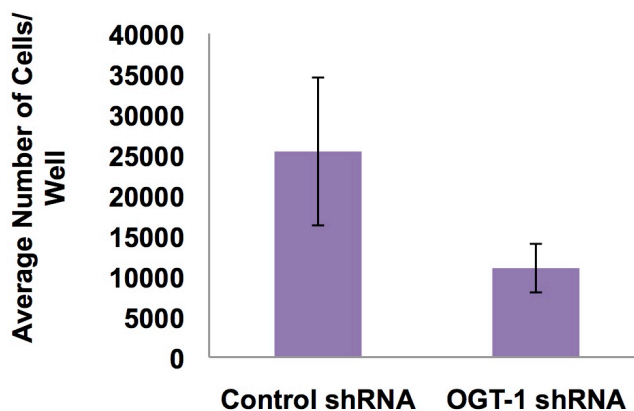
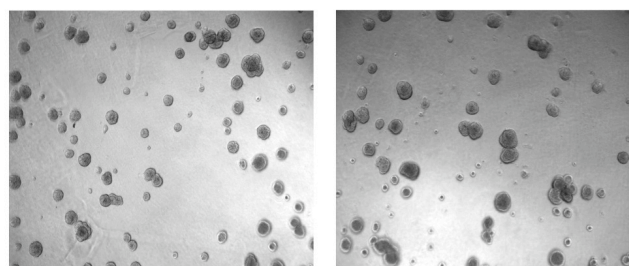
A. MCF-10A:



C.



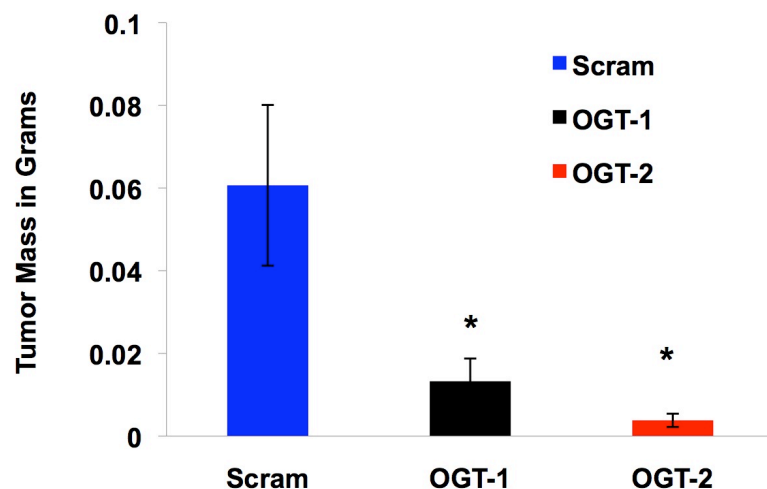
D. MCF-10A: Day 8 3D Morphogenesis



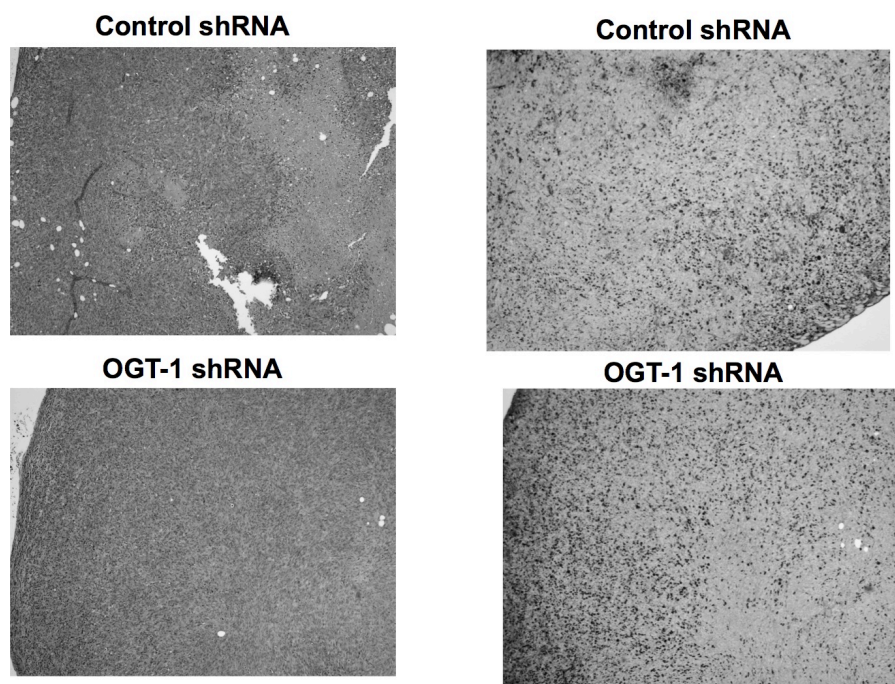
Supplementary Figure 3. Knockdown of OGT in parental MCF-10A does not disrupt growth and morphogenesis in 3D culture. **(a)** MCF-10A cells were infected with lentivirus pLKO-shRNA-Scram, pLKO-shRNA-OGT-1 or pLKO-shRNA-OGT-2 and cells were lysed and proteins analyzed by western blot with indicated antibodies. **(b)** Cell cycle analysis of MCF-10A cells 48 hours after lentiviral infection with control, OGT-1, or OGT-2 shRNA. **(c)** Following infection with lentiviral RNAi MCF-10A cells were harvested and stained with Ki67 and analyzed by flow cytometry. **(d)** Top: MCF-10A cells as above were placed in 3D basement membrane culture and imaged at indicated day (5x). Bottom: Day 8 cell were counted.

Caldwell, et. al. Supplementary Figure 4

A.



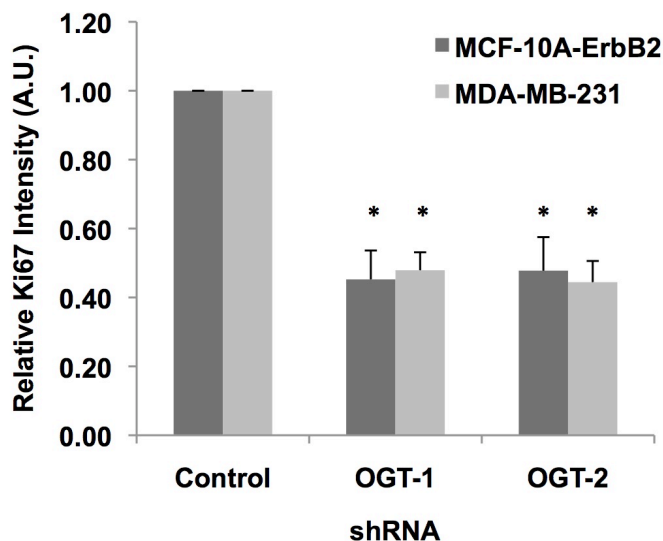
B.



Supplementary Figure 4. Knockdown of OGT Reduces MDA-MB-231 Tumor Weight In Vivo. (a) MDA-MB-231 cells infected with lentivirus pLKO-shRNA-Scram (n=17), pLKO-shRNA-OGT-1 (n=17), or pLKO-shRNA-OGT-2 (n=17) were injected into mammary fat pad of immunocompromised mice and after 8 weeks resected tumors were weighed and the mean tumor weight (\pm S.E.M.) in each group was calculated; Student's *t* test, **P* value < 0.05. (b) Representative tumor section obtained from mouse with MDA-MB-231 cells infected with Scram (Control) or OGT-1 shRNA were fixed, paraffin embedded, sectioned, and stained with H&E (left panel) or Ki-67 (right panel).

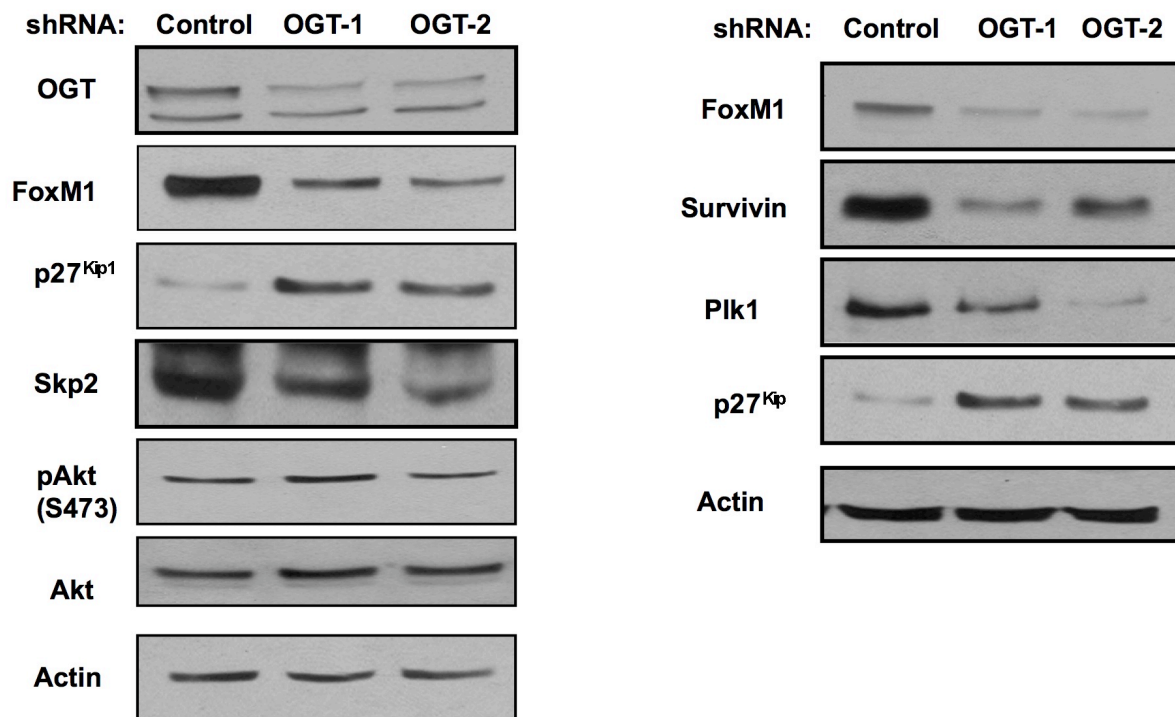
Caldwell, et. al. Supplementary Figure 5

A.



B.

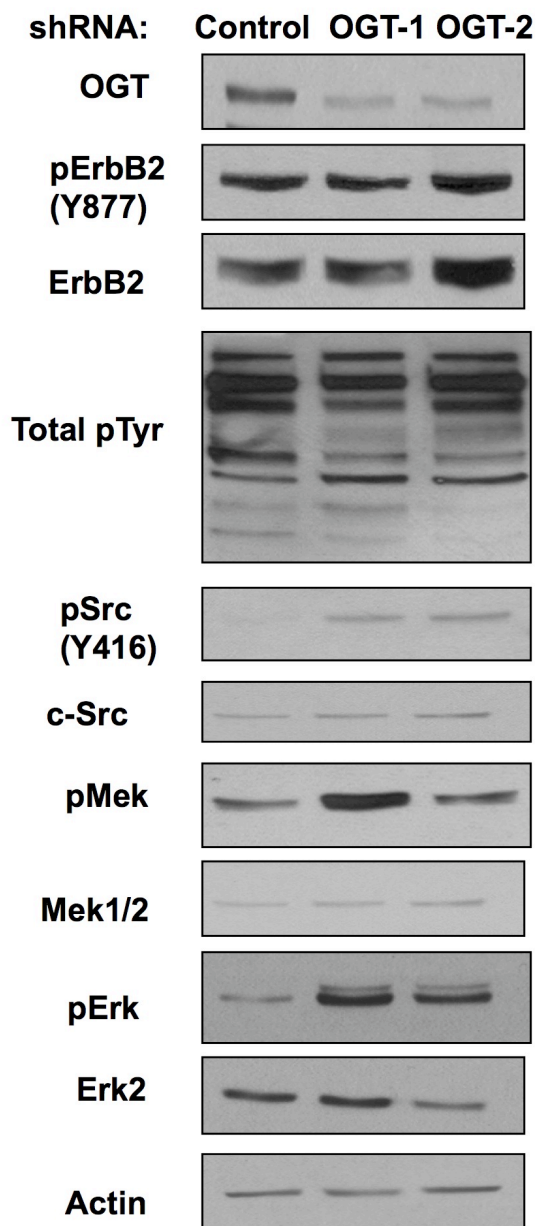
MDA-MB-231



Supplementary Figure 5. Knockdown of OGT in cancer cells alters cell cycle, FoxM1 and its targets. **(a)** At 48 hours post-infection with Control or OGT shRNA lentivirus, MCF-10A-ErbB2 and MDA-MB-231 cells were stained for Ki67 and analyzed via flow cytometry as described in Supplementary methods. **(b)** Lysates were collected from MDA-MB-231 cells treated with Control, OGT-1, or OGT-2 shRNA and analyzed by western blot with indicated antibodies. Antibodies were also used to probe for FoxM1 targets including Survivin, and PIK1.

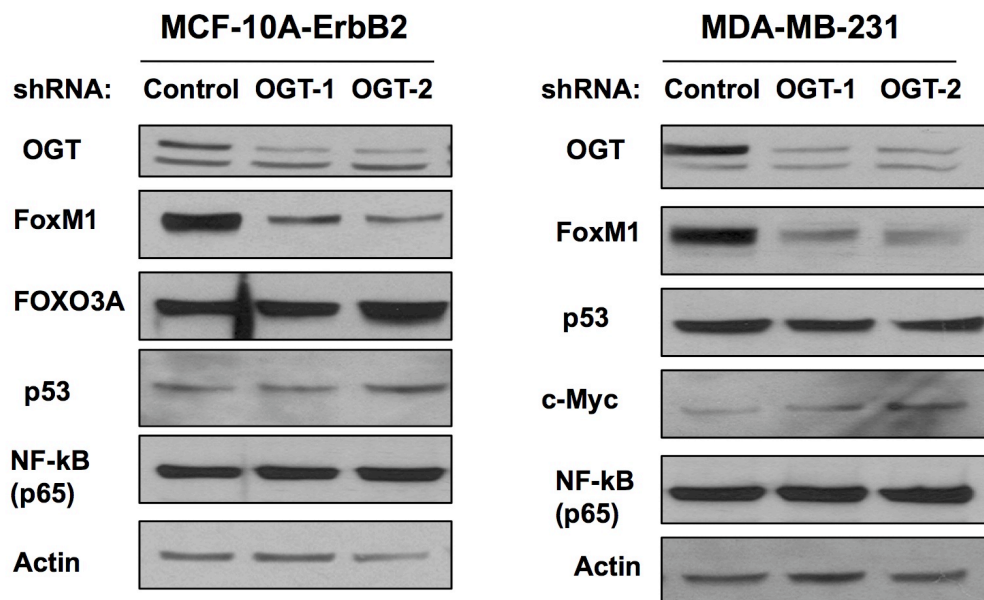
Caldwell, et. al. Supplementary Figure 6

MCF-10A-ErbB2



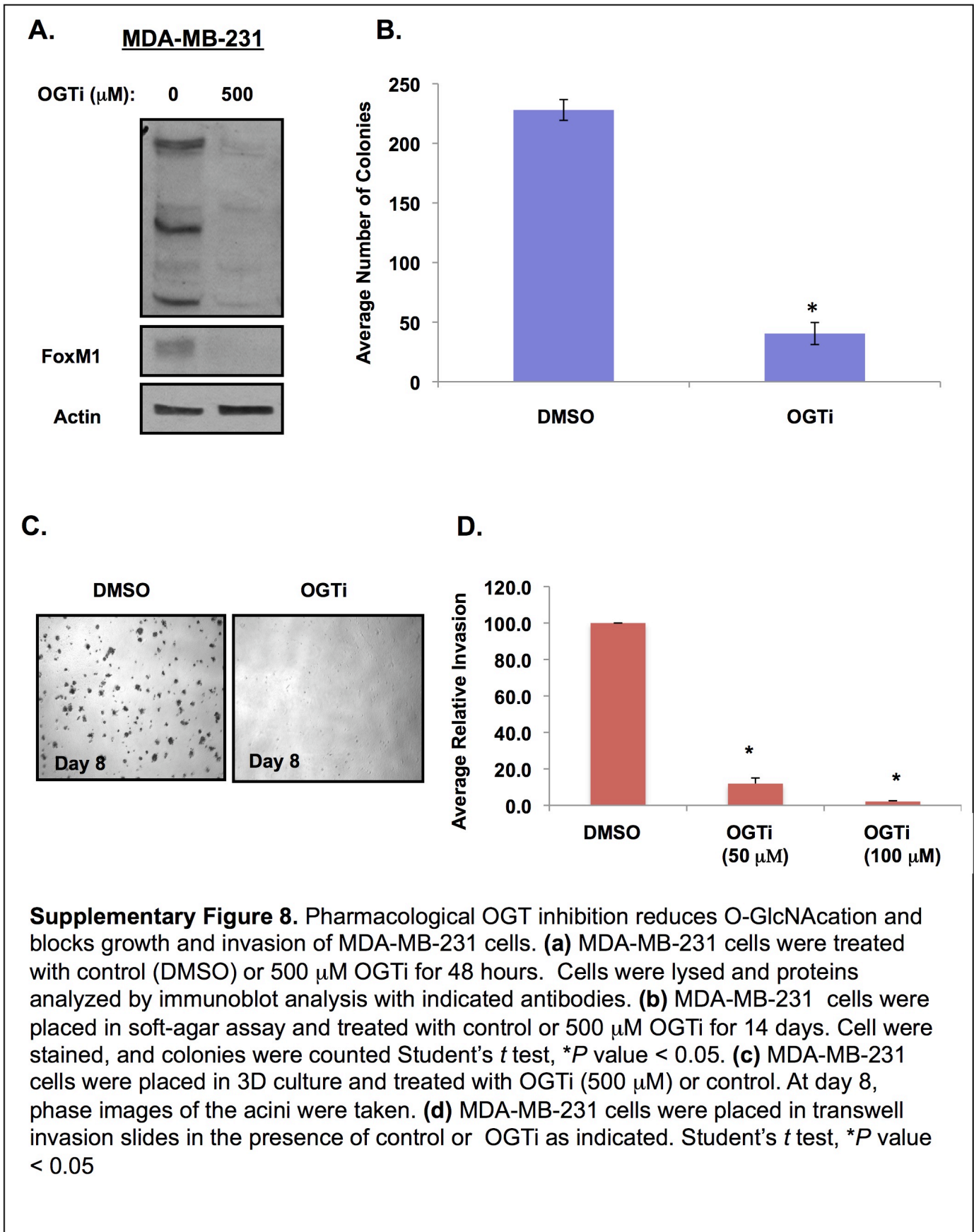
Supplementary Figure 6. OGT RNAi does not block ErbB2-mediated signaling pathways. MCF-10A-ErbB2 cells were infected with lentivirus pLKO-shRNA-Scram, pLKO-shRNA-OGT-1 or OGT-2 and cells were lysed and proteins analyzed by western blot with indicated antibodies.

Caldwell, et. al. Supplementary Figure 7



Supplementary Figure 7. Knockdown of OGT does not alter expression of transcription factors FOXO3A, p53, c-Myc, and NF-kB. MCF-10A-ErbB2 and MDA-MB-231 cells were infected with lentivirus pLKO-shRNA-Scram, pLKO-shRNA-OGT-1 or OGT-2 and cells were lysed and proteins analyzed by western blot with indicated antibodies.

Caldwell, et. al. Supplementary Figure 8



Supplementary Figure 8. Pharmacological OGT inhibition reduces O-GlcNAcation and blocks growth and invasion of MDA-MB-231 cells. **(a)** MDA-MB-231 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)** MDA-MB-231 cells were placed in soft-agar assay and treated with control or 500 μM OGTi for 14 days. Cell were stained, and colonies were counted Student's *t* test, **P* value < 0.05. **(c)** MDA-MB-231 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)** MDA-MB-231 cells were placed in transwell invasion slides in the presence of control or OGTi as indicated. Student's *t* test, **P* value < 0.05