Signal Transduction

Molecular Cancer Research

mTOR/MYC Axis Regulates O-GlcNAc Transferase Expression and O-GlcNAcylation in Breast Cancer

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Abstract

Cancers exhibit altered metabolism characterized by increased glucose and glutamine uptake. The hexosamine biosynthetic pathway (HBP) uses glucose and glutamine, and directly contributes to O-linked-β-N-acetylglucosamine (O-GlcNAc) modifications on intracellular proteins. Multiple tumor types contain elevated total O-GlcNAcylation, in part, by increasing O-GlcNAc transferase (OGT) levels, the enzyme that catalyzes this modification. Although cancer cells require OGT for oncogenesis, it is not clear how tumor cells regulate OGT expression and O-GlcNAcylation. Here, it is shown that the PI3K–mTOR–MYC signaling pathway is required for elevation of OGT and O-GlcNAcylation in breast cancer cells. Treatment with PI3K and mTOR inhibitors reduced OGT protein expression and decreased levels of overall O-GlcNAcylation. In addition, both AKT and mTOR activation is sufficient to elevate OGT/O-GlcNAcylation. Downstream of mTOR, the oncogenic transcription factor c-MYC is required and sufficient for increased OGT protein expression in an RNA-independent manner and c-MYC regulation of OGT mechanistically requires the expression of c-MYC transcriptional target HSP90A. Finally, mammary tumor epithelial cells derived from MMTV-c-myc transgenic mice contain elevated OGT and O-GlcNAcylation and OGT inhibition in this model induces apoptosis. Thus, OGT and O-GlcNAcylation levels are elevated via activation of an mTOR/MYC cascade.

Implications: Evidence indicates OGT as a therapeutic target in c-MYC–amplified cancers. *Mol Cancer Res; 13(5); 923–33. ©2015 AACR.*

Introduction

Cancer cells alter cellular metabolic pathways to support the energetic demands of increased biomass and replication (1). Otto Warburg first noted cancer cells rapid conversion of glucose to lactate, even in the presence of sufficient oxygen concentrations, now termed the "Warburg Effect" (2). This term encompasses altered utilization of glucose required to supply necessary building blocks for sustained proliferation. Oncogenic drivers alter gene expression that sustain changes in metabolic pathways, including genes involved in intermediate steps regulating metabolism (3).

Although the majority of glucose taken up by the cell is metabolized through the glycolytic pathway, 3% to 5% is diverted into the hexosamine biosynthetic pathway (HBP; ref. 4), where it is converted to glucosamine-6-phosphate (GlucN-6-P) by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (5). Subsequently, GlucN-6-P is converted to uridine-5-diphosphate-N-acetylglucosamine (UDP-GlcNAc), which then serves as a substrate for N-linked glycosylation of proteins in the ER and Golgi as well as O-linked glycosylation of nuclear and cytoplasmic proteins (6). The enzyme O-GlcNAc (O-linked-β-N-acetylglucosamine) transferase (OGT) uses UDP-GlcNAc as a substrate to add O-linked sugar moieties onto serine and threonine residues of a diverse range of nuclear and cytoplasmic proteins (7). The process of O-GlcNAc cycling is highly dynamic, and the addition and removal of O-GlcNAc is performed solely by the two enzymes OGT and O-GlcNAcase (OGA), respectively. The addition of this unique O-linked sugar is an important posttranslational modification with the ability to influence many biologic processes by altering protein stability, interaction capabilities, and phosphorylation status (8). The role of O-GlcNAc extends to diverse cellular processes, as it is known to modify a wide range of proteins, including transcription factors, signaling proteins, and receptors (9). O-GlcNAcylation plays an important role in normal biology and is deregulated in a wide range of pathologies, including cardiovascular and neurodegenerative diseases as well as metabolic syndromes such as diabetes (10).

Critical regulators of oncogenesis, including the c-Myc oncogene (11), the tumor suppressor p53 (12), and viral oncoprotein SV40 large T antigen (13), have been previously shown to be O-GlcNAcylated lending the idea that O-GlcNAcylation may play a key role in the pathogenesis of tumors (14, 15). On the basis of this earlier work, as well as more recent data showing the critical role of O-GlcNAcylation on NF-κB (16) function, our laboratory provided the first evidence that total O-GlcNAcylation and OGT was elevated in cancer cells and that OGT was required for tumor

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growth *in vitro* and *in vivo* (17). A number of groups have subsequently shown that OGT and O-GlcNAcylation levels are elevated in various epithelial cancers, including breast (18, 19), prostate (20, 21), lung (22), colon (23), liver (24), bladder (25), as well as in chronic lymphatic leukemia (26). A critical role for O-GlcNAcylation in cancer metabolism has emerged as it has been shown to regulate a major glycolytic driver, hypoxia-inducible factor (HIF1α), along with its transcriptional target glucose transporter 1 (GLUT1). This mechanism is critical for breast cancer cell survival both *in vitro* and *in vivo* (27). O-GlcNAcylation has also recently been shown to influence the pentose phosphate pathway through regulation of phosphofructokinase 1 activity (22). In some cancers, increased total O-GlcNAcylation may be due to increased OGT levels and/or decreased OGA levels as seen in breast (27, 28), liver (24), and colon (29) cancers. Although it has become evident that OGT and O-GlcNAc play critical roles in cancer metabolism and survival, it remains unclear how OGT and O-GlcNAcylation levels are elevated in cancer cells.

The PI3K–AKT pathway directly contributes to altered metabolism of cancer cells by inducing cells to take up excess glucose via regulation of glucose transporters (30, 3) and via activation of the mTOR signaling pathway (31). The mTOR pathway senses the energy status of a cell in response to a number of environmental cues and in turn alters cell growth and metabolism. Once induced, mTOR increases cell growth and proliferation through activation of two effector molecules p70S6K and 4EBP1 leading to a global increase in protein translation (31). One key transcription factor regulated by mTORC1 signaling is c-MYC (32). The oncogene c-MYC is involved in the regulation of cell-cycle progression, cell growth, and glycolysis (33) and is commonly amplified in breast cancer. Moreover, MYC overexpression is associated with highly aggressive clinical features correlating with poor patient outcome (34).

Here, we show that the master nutrient signaling PI3K–AKT–mTOR pathway is required for elevation of OGT and O-GlcNAcylation in breast cancer cells. We also demonstrate that hyperactivation of AKT or mTOR is sufficient to elevate OGT protein and O-GlcNAcylation levels. Downstream of mTOR activation, we observe that c-MYC is required and sufficient to drive OGT protein elevation and increase O-GlcNAcylation in cancer cells via c-MYC regulation of its transcriptional target HSP90A. Importantly, we show that Myc-driven cancer cells elevate OGT and O-GlcNAcylation and require OGT activity for cancer cell survival. Our data are the first to link mTOR/MYC activity to increased OGT/O-GlcNAcylation that contributes to the oncogenic phenotype seen in multiple cancers.

Materials and Methods

Cell lines

MCF-10A, SKBR-3, MDA-MB-231, SUM-159, and MCF-7 cells were acquired from the ATCC and cultured following ATCC instructions. Wild-type (WT) and $TSC2^{-/-}$ MEFS were a gift from Aristotelis Astreinidis, Drexel University College of Medicine. CommaD cells were a gift from Senthil Muthuswamy, University of Toronto. MCF-10A-ErbB2 (NeuT; ref. 17), MCF-10A-AKT (Myr-AKT1; ref. 35), and MCF-10A-MEK2 (MEK2-DD; ref. 35) cells have been previously described. The pWZL-Blast-c-MYC plasmid (kindly provided by Michael Amatangelo, Drexel University) was used to make MCF-10A cells stably overexpressing c-MYC (MCF-10A-c-MYC). Cells were infected with retrovirus and selected as previously described (17).

Inhibitors and treatments

Cells were seeded at 5×10^6, were treated for 16 hours with either; 0.1% DMSO (Sigma), LY294002 30 μmol/L (Promega), rapamycin 50 nmol/L (Enzo Biochem), or U0126 30 μmol/L (Promega). OGT inhibitor Ac-5SGlcNAc has been previously described (36) and HSP90 inhibitor 17-AAG (Selleck Biochem) was used at indicated concentrations. Lactacystin (Calibiochem) was used at 10 μmol/L in combination with indicated treatments (16 hours).

Animals and establishing MMTV-Myc cells

MMTV-c-Myc transgenic females were procured from the Mouse Models of Human Cancer Consortium (MMHCC; stock O1XG2) under protocols approved by the University of Tennessee Health Science Center. Mammary epithelial tumor cell (MTEC) lines were generated from late-stage carcinomas, grown in DMEM/F12 + 2% FBS and then routinely passaged in culture using a 3:1 ratio of dispaseII/tryspin as previously described (37).

Western blot analysis

Cells were collected in RIPA lysis buffer (150 mmol/L NaCl, 1% NP40, 0.5% DOC, 50 mmol/L Tris-HCl at pH 8, 0.1% SDS, 10% glycerol, 5 mmol/L EDTA, 20 mmol/L NaF, and 1 mmol/L Na_3VO_4, 1 μg/mL each of pepstatin, leupeptin, and aprotinin, 200 μg/mL phenyl-methylsulfonyl-fluoride). Lysates were cleared by centrifugation at $14{,}000 \times g$ for 20 minutes at 4°C and analyzed by SDSPAGE and autoradiography. Western blots were then analyzed with the following antibodies; anti-actin, anti–c-MYC, anti-ERK2 (D-2), anti–O-GlcNAc (RL2), anti-Cyclin D1 (Santa Cruz Biotechnology), anti–phospho-Akt (Ser473), anti-P70 S6 Kinase, anti-Tuberin/TSC2, anti-AKT, anti–phospho-P70-S6 Kinase (T389), and anti–phospho-4EBP1 (T70), anti-4EBP1 (53H17), cleaved caspase-3 (D175), anti-BIM, anti-CHOP (L63F7), anti–phospho-eIF2α, anti-eIF2α (Cell Signaling Technology), anti–phospho-ERK (T185/Y187; Invitrogen Corporation), anti-OGT, anti–O-GlcNAc (CTD110.6; Sigma), anti-HSP90 alpha (Enzo Life Sciences), and anti-MGEA5 (OGA; Proteintech Group). Densitometry was performed using ImageJ Software (NIH, Bethesda, MA).

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol for monolayer cell RNA extraction. Levels of OGT, HSP90, and cyclophilinA (PPIA) were determined using the Applied Biosystems 7500, with the Brilliant II qRT-PCR Master Mix Kit (Stratagene) according to the manufacturer's protocol. TaqMan gene expression assay primer probes for cyclophilin A (Hs99999904_m1), OGT (Hs00914634_g1), c-MYC (Hs00905030_m1), and HSP90AA1 (Hs00743767_sH) were purchased from Applied Biosystem. Expression levels were analyzed using Data Assist v2.0 (Life Technologies).

RNA interference

Stable cell lines were generated by infection with the lentiviral vector pLKO.1-puro carrying shRNA sequence: control-scrambled CCTAAGGTTAAGTCGCCCTCGCTCTAGCGA

GGGCGACTTAACCTT (Addgene), c-Myc#1 CCGGCAGGAACTATGACCTCGACTACTCGAGTAGTCGAGGTCATAGTTCCTGTTTTTG, c-Myc#2 CCGGCAGGAACTATGACCTCGACTACTCGAGTAGTCGAGGTCATAGTTCCTGTTTTTG, HSP90 #1 CCGGGAAGGATGGTGACAAGAAGAACTCGAGTTCTTCTTGTCACCATCCTTCTTTTTG, HSP90#2 CCGGTATGGCATGACAACTACTTTACTCGAGTAAAGTAGTTGTCATGCCATATTTTG (Sigma Aldrich). Lentivirus was generated and cells were infected and selected as described previously (17).

Statistical analysis

All results shown as averages are presented as mean ± SE from three or more independent experiments. Unless otherwise noted, *P* values were calculated using the Student two-tailed test: (*, $P < 0.05$).

Results

O-GlcNAcylation and OGT levels require PI3K and mTOR activation in cancer cells

We have previously demonstrated that breast cancer cells contain elevated OGT and O-GlcNAcylation levels compared to normal mammary epithelial cells (17). In addition, stable overexpression of the active form of the receptor tyrosine kinase (RTK) ErbB2/HER2 in nontransformed mammary epithelial MCF-10A cells resulted in elevated OGT and O-GlcNAc levels (Supplementary Fig. S1; ref. 17). Because ErbB2 activation stimulates numerous signaling cascades, including PI3K–AKT, Ras–MEK–ERK, and mTOR (34), we examined which signaling pathway was responsible for increased OGT and O-GlcNAc levels in breast cancer cells. We tested a panel of breast cancer cells that represent different breast cancer subtypes, including estrogen receptor–positive (MCF-7 cells), ErbB2/HER2–positive (SKBR-3 cells), and triple-negative/basal breast cancers (MDA-MB-231 and SUM-159 cells; ref. 38). Cells were treated with vehicle control or pharmacologic inhibitors against the PI3K, mTOR, and MEK signaling pathways. Treatment of all breast cancer cells with PI3K (LY294002) and mTOR inhibitor (Rapamycin) reduced O-GlcNAc levels compared with control (Fig. 1A–E) and decreased OGT levels (Fig. 1E, Fig. 1A–D). Treatment of breast cancer cells with PI3K inhibitor (LY294002) significantly reduced OGT levels in MDA-MB-231, SKBR-3, MCF-7 but not SUM-159 cells (Fig. 1D and E). Inhibition of MEK (U0126) had negligible effects on OGT levels and O-GlcNAcylation (Fig. 1A–E). These data suggest that the PI3K–mTOR pathway is a key regulator of OGT and O-GlcNAc levels in multiple breast cancer cell lines with different genetic origins. To determine whether the PI3K–mTOR pathway regulates OGT at the level of RNA, quantitative real-time PCR (qRT-PCR) was performed. Treatment of breast cancer cells MDA-MB-231, MCF-7, SKBR-3, and SUM-159 (Supplementary Fig. S2) with inhibitors of PI3K, mTOR, or MEK pathways did not significantly inhibit OGT RNA levels, suggesting that PI3K/mTOR-mediated regulation of OGT protein levels is RNA independent. Interestingly, protein levels of OGA were not elevated in breast cancer cells treated with PI3K or mTOR inhibitors (Supplementary Fig. S3), suggesting that the decrease in O-GlcNAcylation caused by inhibitors of the PI3K–mTOR pathway is primarily a result of diminished OGT protein levels. Thus, OGT protein and global cellular O-GlcNAcylation levels are maintained in breast cancer cells via PI3K and mTOR activation independent of OGT mRNA regulation.

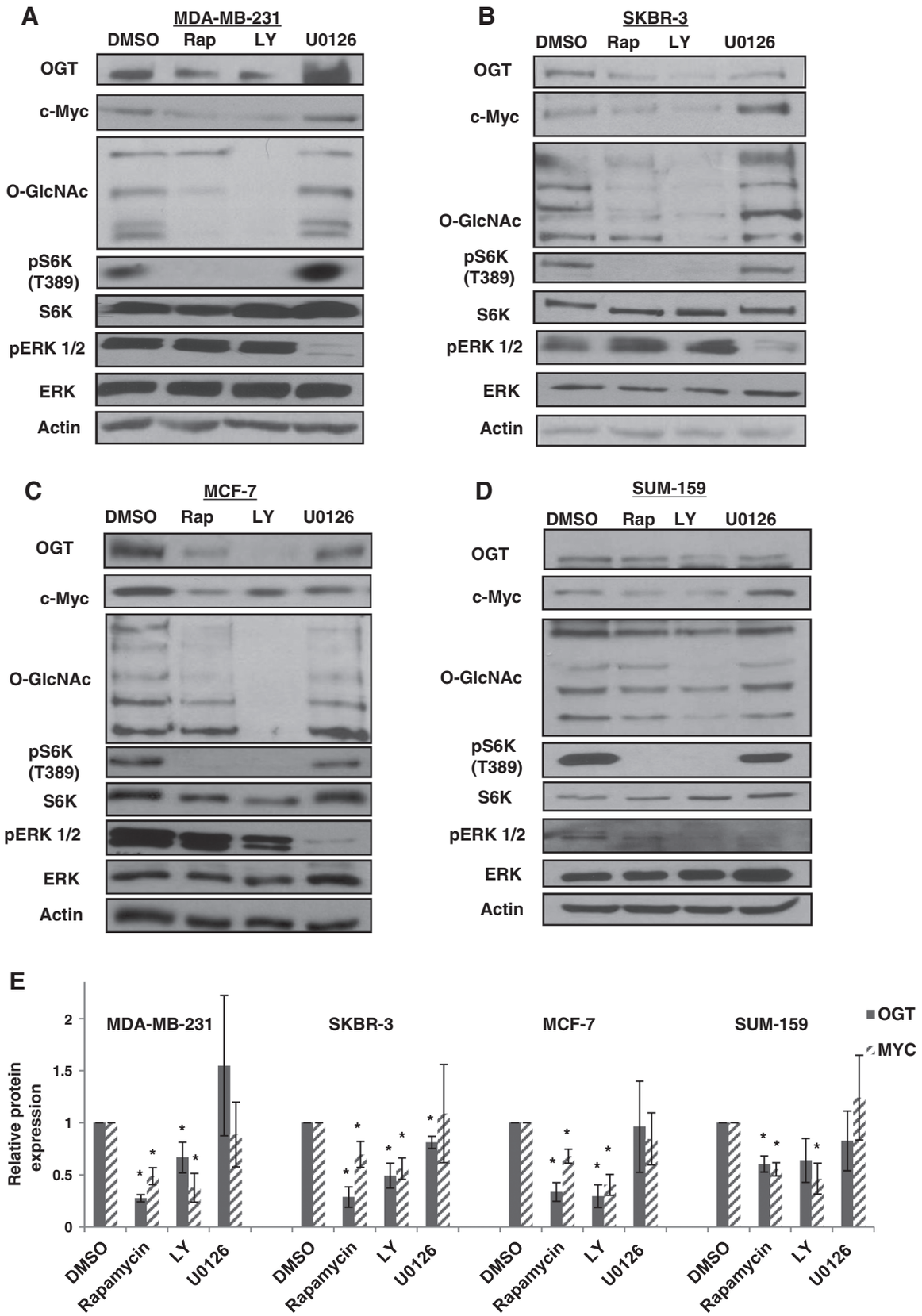
AKT or mTOR activation is sufficient to elevate OGT protein expression and O-GlcNAcylation

Because cancer cells require PI3K and mTOR activation to maintain OGT and O-GlcNAc levels, we examined whether these pathways were sufficient to increase OGT and O-GlcNAcylation levels. PI3K can activate many pathways, including the kinase AKT (protein kinase B; ref. 39). To determine whether AKT activation alone was sufficient to increase OGT and O-GlcNAc levels in epithelial cells, we stably overexpressed an active form of AKT1 (Myr-AKT1) in the normal mammary epithelial cell line MCF-10A. Cells stably expressing Myr-AKT contained increased phosphorylated AKT and activated mTOR pathway as measured by phosphorylation of p70 S6 kinase (S6K; T389) as well as elevated OGT protein levels and increased overall protein O-GlcNAcylation compared with control cells (Fig. 2A). Consistent with the MEK inhibitor results, MCF-10A cells overexpressing a constitutively active form of MEK2 (MEK2-DD) did not alter OGT and protein O-GlcNAcylation levels (Supplementary Fig. S4A). This result is notable because MCF-10A-MEK2-DD cells have a pronounced phenotype when cultured in a three-dimensional (3D) culture system (Supplementary Fig. S4B) that allows bypass of growth suppression and anoikis resistance (40). Thus, OGT and O-GlcNAcylation levels are not elevated simply as a result of hyperproliferation, but are specifically regulated by the PI3K–AKT pathway. We conclude that activation of the AKT pathway, but not the MEK–ERK pathway, is sufficient to elevate OGT and O-GlcNAcylation levels in human breast epithelial cells.

To test whether mTOR activation is also sufficient to increase levels of OGT and O-GlcNAcylation, we examined OGT/O-GlcNAc levels in mouse embryo fibroblasts (MEF) containing deletion of *TSC2*. TSC1 and TSC2 are components of a complex that negatively regulates mTORC1 nutrient signaling and inactivation of either *TSC1* or *TSC2* results in elevated mTOR activity, is associated with uncontrolled cell growth and division, and is linked to benign tumor growth (31). To determine whether mTOR activation was sufficient to increase OGT and O-GlcNAc levels, WT and *TSC2*-deficient MEFs were analyzed. As expected, basal phosphorylation levels of mTOR targets p70 S6K (T389) and 4EBP1 (T70) were elevated in the *TSC2*-null cells compared with WT MEFs (Fig. 2B) verifying constitutive mTOR activation. Interestingly, *TSC2*-null cells showed elevated OGT and O-GlcNAc levels (Fig. 2B) further implicating the mTOR pathway in the regulation of O-GlcNAc cycling. However, *TSC2*-null cells also displayed significantly lower OGA levels (Supplementary Fig. S5), suggesting that increased O-GlcNAcylation in these cells may be due to both elevation of OGT and reduction of OGA. To determine whether OGT and O-GlcNAc levels were directly controlled by deregulated mTOR activity in these cells, *TSC2*-null MEFs were treated with rapamycin. Similar to the cancer cells, inhibition of mTOR in *TSC2*-null MEFs resulted in a decrease in OGT and O-GlcNAcylation (Fig. 2C). These results reveal that, in addition to AKT, activation of mTOR is also sufficient to increase OGT protein levels and increase O-GlcNAcylation.

c-MYC regulates O-GlcNAcylation and OGT protein expression in cancer cells

Many pathways are altered by activation of mTOR (31). Because OGT is required for metabolic pathways in cancer cells (27), we hypothesized that pathways downstream of mTOR associated with metabolic regulation may be involved in OGT/



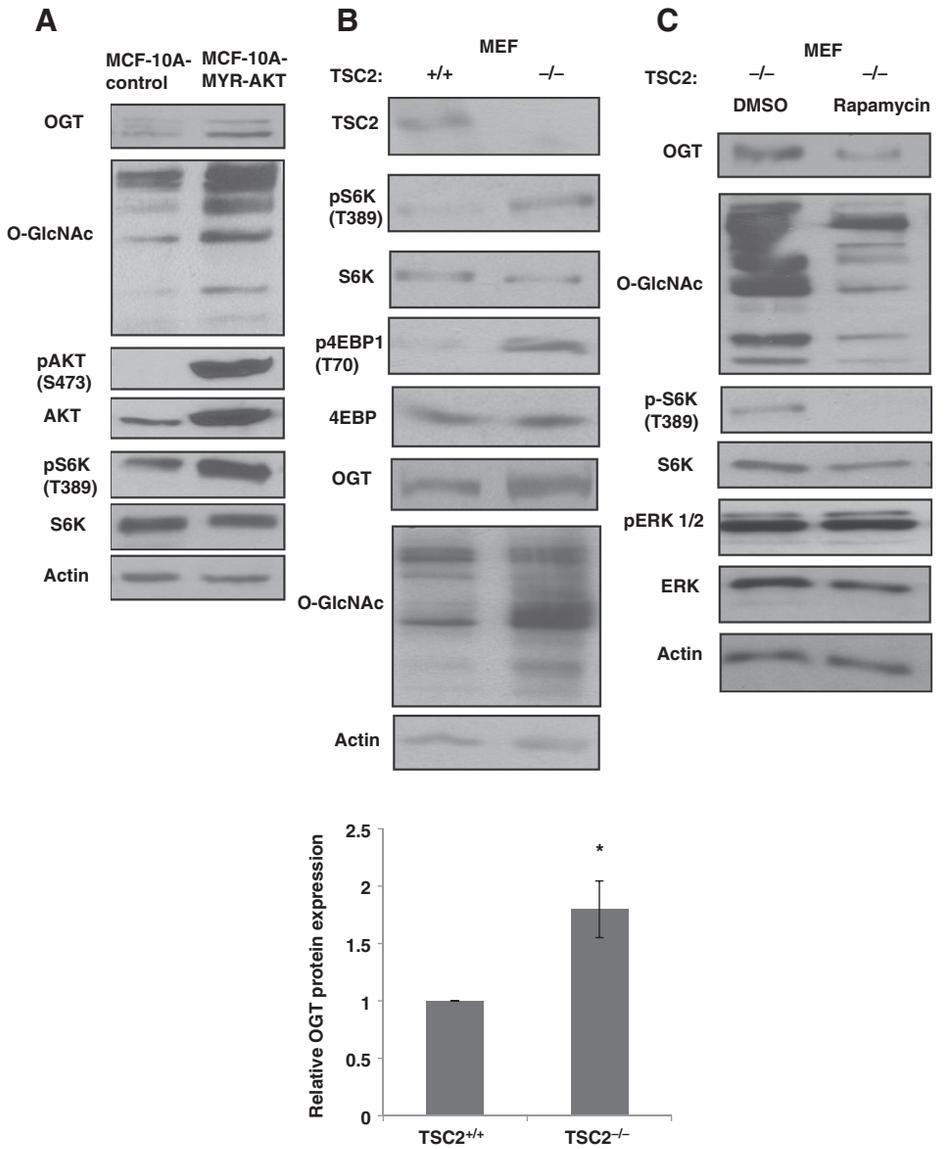


Figure 2.
AKT and mTOR activation is sufficient to elevate OGT and O-GlcNAc levels. A, protein lysates were collected from MCF-10A control (pBabe) or MCF-10A-MYR-AKT cells and analyzed using Western blot analysis with indicated antibodies. B, top, lysates from WT MEFs or *TSC2*$^{-/-}$-null MEFs were collected for immunoblot analysis and probed with the indicated antibodies. Bottom, levels of OGT protein were compared between WT and *TSC2*-null MEFs and quantified graphically. Mean ± SE; *, $P < 0.05$. C, *TSC2*$^{-/-}$ MEFs were treated for 16 hours with 0.1% DMSO or 50nmol/L rapamycin. Protein lysates were collected for immunoblot analysis and probed with the indicated antibodies.

O-GlcNAc regulation. Because c-MYC is a key metabolic transcription factor downstream from the mTOR pathway (32), we examined whether this transcription factor is involved in OGT and O-GlcNAc regulation in cancer cells. c-MYC is amplified in multiple cancers and plays a significant role in cancer cell proliferation, growth, and apoptosis and has been strongly implicated in cancer metabolism (33). To verify that c-MYC expression is downstream of mTOR in breast cancer cells, we examined c-MYC levels in breast cancer cells after treatment with rapamycin or LY294002. Inhibition of PI3K and mTOR, but not MEK, significantly reduced c-MYC expression in MDA-MB-231, SKBR-3, MCF-7, and SUM-159 breast cancer cells and correlated with reduced OGT protein levels in these cells (Fig. 1A–E). To test whether c-MYC was required for OGT expression in breast cancer cells, we targeted c-MYC with two different lentiviral shRNA constructs and determined that decreasing MYC expression in MDA-MB-231 resulted in significantly reduced OGT levels and an overall reduction in O-GlcNAcylation compared with cell containing control shRNA (Fig. 3A). Consistent with our data regarding OGT expression under conditions of inhibition of PI3K and mTOR, there was no significant difference in OGT mRNA in cells expressing c-MYC RNAi as compared with control cells

Figure 1.
The PI3K and mTOR pathways regulate OGT and O-GlcNAc levels in cancer cells. A, MDA-MB-231, SKBR-3 (B), MCF-7 (C), and SUM-159 (D) cells were treated for 16 hours with 0.1% DMSO, 30 μmol/L LY294002 (PI3K inhibitor), 50 nmol/L Rapamycin (mTOR inhibitor), or 30 μmol/L U0126 (MEK inhibitor). Protein lysates were collected for immunoblot analysis and probed with the indicated antibodies. E, levels of OGT and c-MYC protein were quantified between different treatments in indicted breast cancer cells and normalized to actin. Mean ± SE represents at least three independent experiments; *, $P < 0.05$.

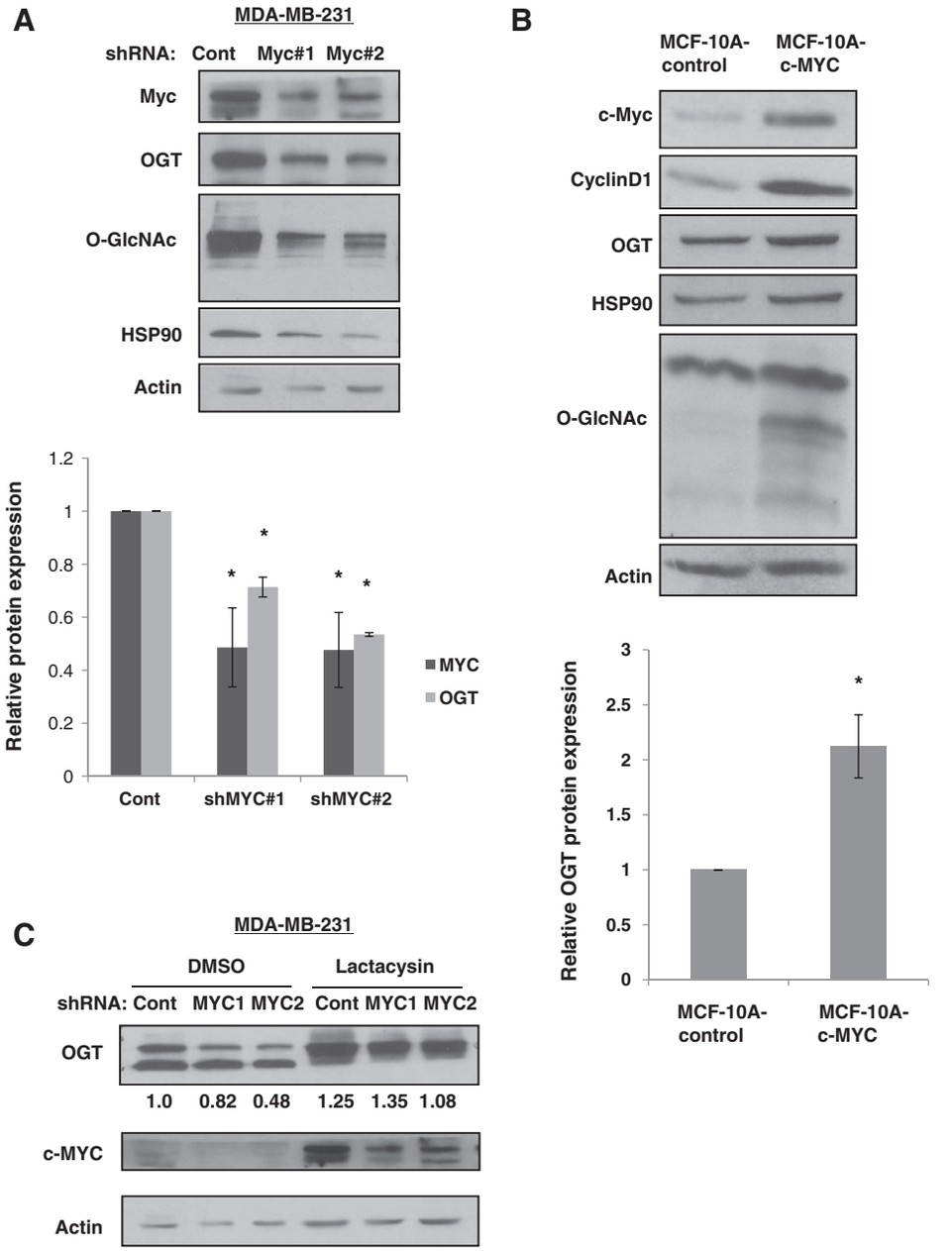


Figure 3.
OGT expression in breast cancer cells requires c-MYC. A, MDA-MB-231 cells stably expressing either control, MYC#1 or MYC#2 shRNA were lysed and immunoblotted with indicated antibodies. Expression of OGT and c-MYC is quantified below. B, protein lysates were collected from MCF-10A control (pWZL) or MCF-10A-c-MYC and were analyzed using Western blot analysis with indicated antibodies. Data are quantified ($n = 3$); *, $P < 0.05$; C, cell lysates from control or c-Myc shRNA–containing MDA-MB-231 treated with control (DMSO) or lactacysin (10 μmol/L) were collected and analyzed by immunoblotting. Quantification of OGT/actin is quantified below OGT blot.

(Supplementary Fig. S6A). Thus, both PI3K/mTOR and c-MYC regulate OGT protein levels independently of mRNA. To determine whether c-MYC expression is sufficient to regulate OGT expression, we overexpressed c-MYC in MCF-10A cells. Cells overexpressing c-MYC displayed increased expression of its transcriptional target cyclin D1 (Fig. 3B) and significantly upregulated OGT protein levels and O-GlcNAcylation compared with control cells (Fig. 3B). The increase in OGT protein levels in MYC-overexpressing MCF-10A cells was also independent of changes in OGT RNA levels (Supplementary Fig. S6B). Thus, downstream from mTOR, c-MYC is required and sufficient to regulate OGT protein levels and O-GlcNAc cycling in an RNA-independent fashion.

c-MYC regulation of HSP90A is required for OGT expression in cancer cells

Recent studies have shown that the chaperone HSP90A can bind OGT and regulate its proteasomal degradation in endothelial cells (41). Interestingly, HSP90A is a c-MYC transcriptional target and contributes to c-MYC–induced transformation (42); thus, we examined the potential connection between c-MYC, HSP90A, and proteasomal degradation of OGT in cancer cells. To test whether reducing c-MYC levels in cancer cells led to reduction of OGT protein via the proteasome, we treated MDA-MB-231 cells containing c-MYC RNAi with proteasome inhibitor lactacystin. Cells treated with lactacystin reversed MYC-RNAi–induced reduction of OGT protein compared

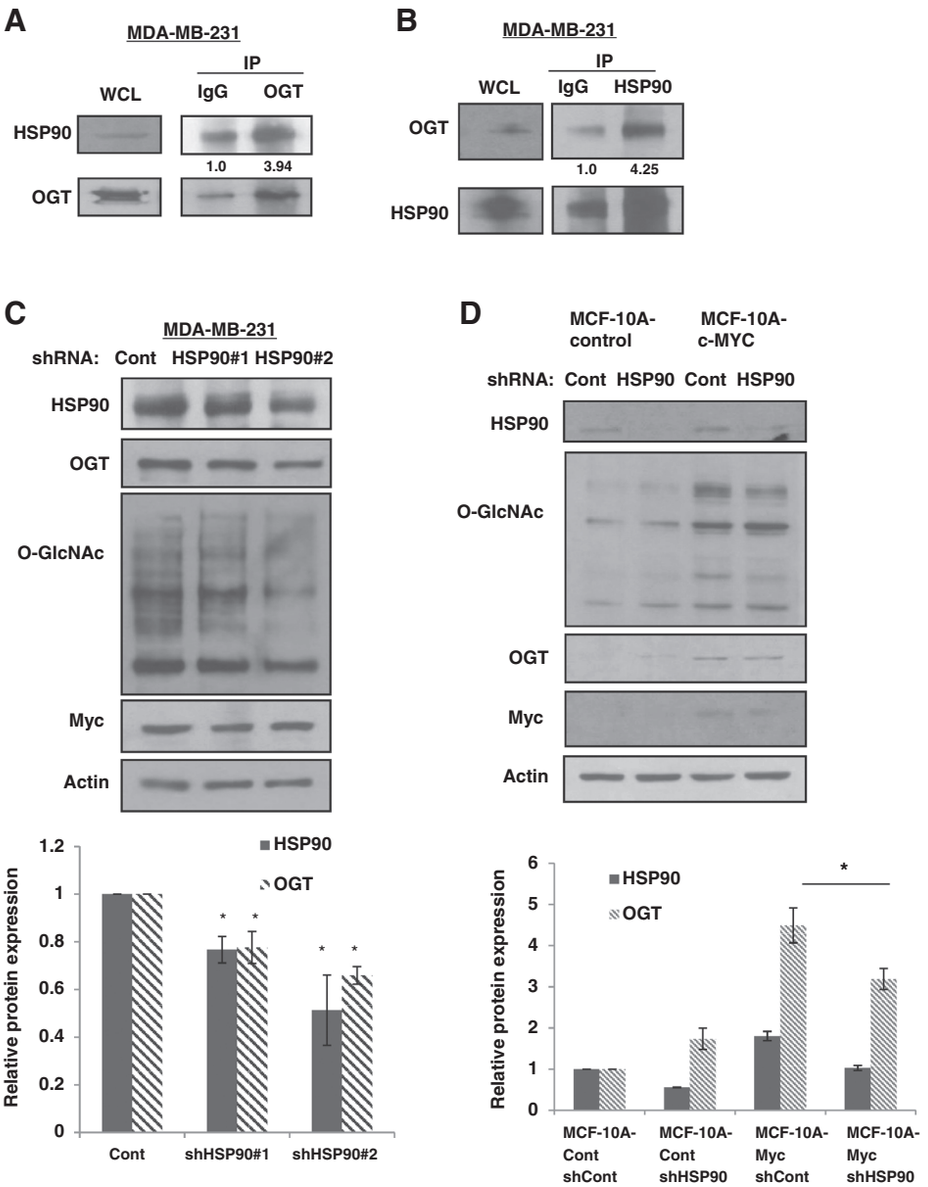
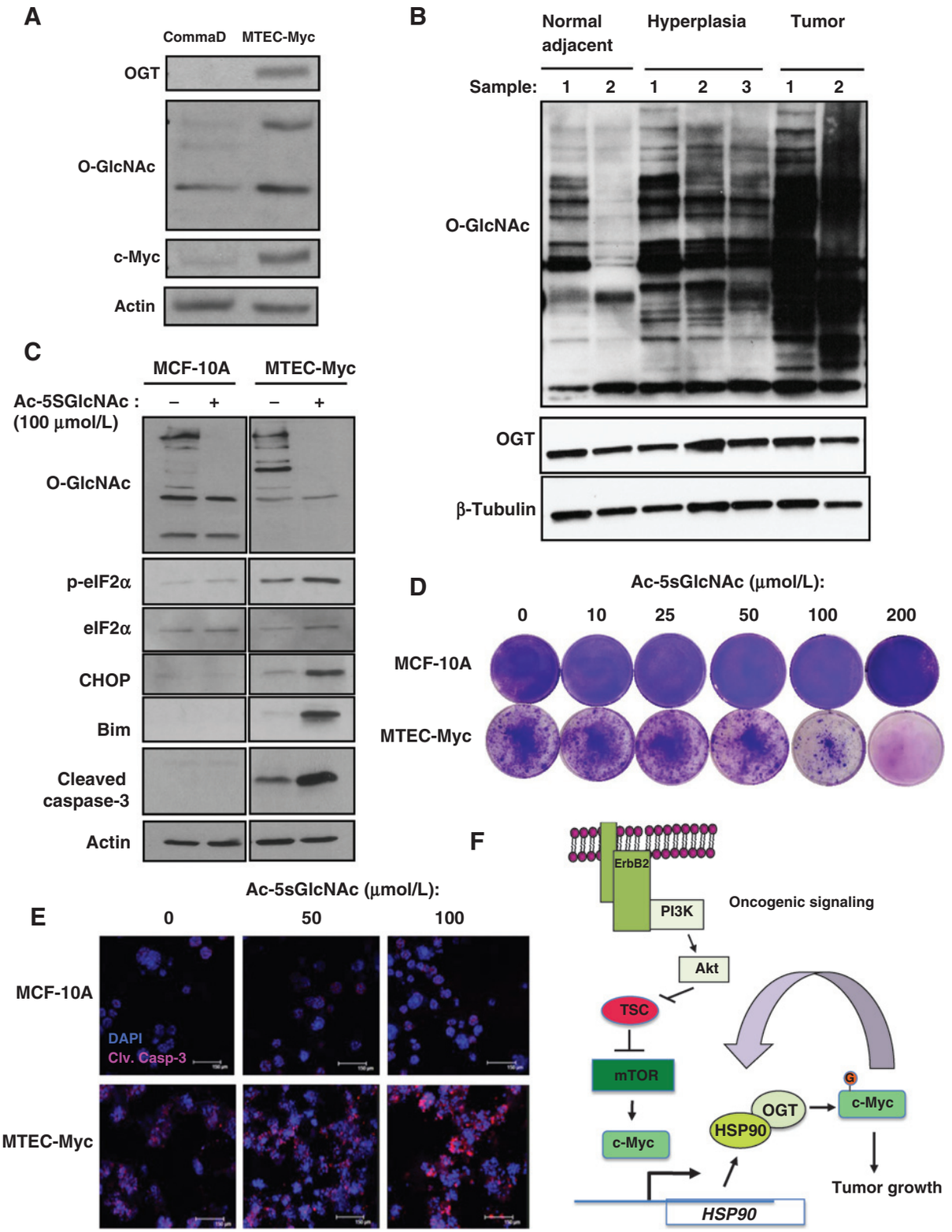


Figure 4.
MYC transcriptional target HSP90 is required for OGT/O-GlcNAc regulation. A and B, protein lysates from MDA-MB-231 cells were subjected to immunoprecipitation with the indicated antibodies. Pull down is quantified below immunoblots. C, cell lysates from control or HSP90 shRNA–containing MDA-MB-231 were collected and analyzed by immunobloting. Protein expression is quantified below. D, MCF-10A control or MCF-10A-c-MYC cell lysates stably expressing control or HSP90 shRNA were collected and analyzed by immunobloting. Relative OGT and HSP90 protein levels were normalized to actin and quantified ($n = 3$). Quantification represents mean ± SE of least three independent experiments *, $P < 0.05$.

with control treated cells (Fig. 3C). Consistent with the idea that mTOR regulation of Myc can control OGT via proteasomal degradation, we found that the decrease in OGT protein levels observed with rapamycin treatment was also reversed following lactacystin treatment (Supplementary Fig. S7A and S7B). Thus, mTOR and c-MYC regulation of OGT protein levels is proteasomal dependent.

Consistent with previous findings that c-MYC regulates HSP90A, MDA-MB-231 cells stably expressing c-MYC shRNA contained significant decreases in HSP90A protein levels (Fig. 3A and Supplementary Fig. S8A) and RNA levels (Supplementary Fig. S8B) compared with control RNAi cells. Conversely, MCF-10A cells overexpressing c-MYC contained increased levels of HSP90 compared with control cells (Fig. 3B). To determine whether OGT interacts with HSP90 in breast cancer cells, we immunoprecipitated either OGT or HSP90 and examined interaction between these two proteins. Immunoprecipitating either OGT (Fig. 4A) or HSP90 (Fig. 4B) from MDA-MB-231 cell lysates showed that endogenous OGT and HSP90 interact in breast cancer cells. To test whether HSP90A activity in cancer cells is associated with OGT and O-GlcNAc cycling, we treated MDA-MB-231 cells with HSP90 specific inhibitor 17-demethoxy-17-allyaminogeldanmycin (17-AAG; ref. 43). Inhibition of HSP90 increases ubiquitination and proteasome-dependent degradation of client proteins (44). Consistent with the idea that OGT is an HSP90A client protein, MDA-MB-231 cells treated with 17-AAG displayed decreased OGT and O-GlcNAcylation levels compared with control cells (Supplementary Fig. S8C). This decrease in OGT expression was independent of changes in Myc levels (Supplementary Fig. S8C) and was partially reversed when cells were also treated with lactacystin to block proteasomal degradation (Supplementary Fig. S7). To test directly whether HSP90A is required



for OGT expression in cancer cells, we reduced HSP90A expression in breast cancer cells using two different RNAi constructs. Reducing HSP90A levels in MDA-MB-231 cells resulted in significant decreases in OGT protein levels and reduced O-GlcNAcylation (Fig. 4C). Conversely, overexpressing HSP90 in HEK-293T cells increased both OGT levels and elevated O-GlcNAcylation (Supplementary Fig. S8D). In addition, MCF-10A-MYC–overexpressing cells contain increased HSP90A expression and increased OGT and O-GlcNAcylation as mentioned above (Fig. 3B), suggesting increased association between OGT and HSP90A in a MYC-dependent manner. Reducing HSP90A levels via RNAi in MYC-overexpressing cells resulted in significant inhibition of OGT protein levels and reduced total O-GlcNAcylation (Fig. 4D). Together these results show that MYC regulation of HSP90A controls OGT protein levels and O-GlcNAcylation in cancer cells.

Myc-driven tumor cells contain elevated OGT and O-GlcNAc levels and require OGT for survival

Overexpression of c-Myc under the control of mouse mammary tumor virus (MMTV) results in the development of mammary tumors (45). To determine whether c-MYC–driven tumors show elevated OGT and O-GlcNAcylation, we compared COMMA-D cells, isolated from a mid-pregnant Balb/C mouse, with MTECs isolated from MMTV-MYC transgenic mice. The MTEC-MYC cells displayed elevated c-MYC levels as well as an increase in both OGT and O-GlcNAcylation (Fig. 5A) compared with COMMA-D cells. To examine whether MYC-driven tumors have altered OGT and O-GlcNAcylation *in vivo*, we compared the levels of OGT and O-GlcNAc from mammary tissues harvested over the course of tumor progression from nulliparous female MMTV-MYC transgenic mice. O-GlcNAcylation was elevated in MYC-driven hyperplasic lessions compared with normal mammary glands of Myc^{+} females, and was further increased in late stage carcinomas relative to the hyperplasias (Fig. 5B). Tumor tissue also displayed significantly upregulated OGT expression compared with normal mammary glands (Fig. 5B and Supplementary Fig. S9A).

MYC-driven cancers are known to be highly proliferative, but also have high levels of apoptosis (33). We examined whether targeting OGT using a chemical inhibitor could sensitize these cells to apoptosis compared with nontumorigenic mammary epithelial cells. Treatment of MTEC-MYC cells or MCF-10A cells with OGT inhibitor Ac-5SGlcNAc reduced total levels of O-GlcNAcylation (Fig. 5C and Supplementary Fig. S9C). However, Ac-5SGlcNAc treatment increased caspase-3 cleavage (Fig. 5C) and significantly reduced cell viability as measured by crystal violet staining (Fig. 5D and Supplementary Fig. S9B) solely in the MTEC-MYC cells as apoptotic markers and cell viability was not altered in MCF-10A cells upon treatment. Because we have recently shown that reducing OGT in cancer cells leads to ER stress and apoptosis in a CHOP-dependent manner (27), we examined whether inhibiting OGT in MYC-driven cancer cells also activated ER stress. Indeed, treating MTEC-MYC cells with OGT inhibitor Ac-5SGlcNAc induced phosphorylation of eIF2α, increased expression of CHOP, and increased Bcl-2 BH3-only proapoptotic protein Bim (Fig. 5C). Importantly, treating MCF-10A cells with this inhibitor did not activate ER stress or induce expression of these markers (Fig. 5C). This selective cell death of MYC-driven cancer cells was also observed in 3D culture as MTEC-MYC cells treated with the OGT inhibitor contained elevated cleaved caspase-3 staining compared with MCF-10A cells (Fig. 5E). Thus, Myc-driven breast cancer cells increase OGT and O-GlcNAcylation and inhibition of O-GlcNAcylation in these cells induces ER stress and apoptosis.

Discussion

Elevation of O-GlcNAcylation has been implicated in a wide range of cancers. However, the pathways responsible for the regulation of OGT and the O-GlcNAc modification in cancer cells remained unclear. Here, we show for the first time that the PI3K–mTOR pathway via regulation of the oncogenic transcription factor c-MYC is responsible for increased OGT and O-GlcNAcylation levels observed in breast cancer cells. We demonstrate that downstream of the RTK ErbB2, the AKT–mTOR, but not MEK–ERK, pathways were sufficient to elevate OGT and O-GlcNAcylation. In addition, we found that elevation of OGT and O-GlcNAcylation in cancer cells is regulated by the oncogenic transcription factor c-MYC. Indeed, c-MYC is both required and sufficient to elevate OGT protein levels and global O-GlcNAcylation in breast cancer cells. Importantly, we found that c-MYC–driven tumor cells also displayed elevated OGT and O-GlcNAcylation *in vivo*. Our data are consistent with previous work showing that c-MYC can regulate total O-GlcNAcylation as Rat1A $myc^{+/+}$ fibroblasts contained increased O-GlcNAcylation compared with Rat1A $myc^{-/-}$ cells (46). However, this change in O-GlcNAcylation in Rat1A cells was not associated with changes in OGT expression as found in our study. It is likely that c-MYC also contributes to the supply of essential substrates required for hexosamine biosynthesis as c-MYC can increase glucose and glutamine flux in these cells (46) as well as in cancer cells (47).

Interestingly, c-MYC regulates OGT at the posttranscriptional level as we did not detect changes in OGT mRNA expression in cells with altered c-MYC or PI3K–mTOR pathways. This may help explain why OGT RNA levels are not found to be elevated in breast cancer microarray analysis (27). Although c-MYC is commonly known for its role as a transcription factor, it has been shown to stabilize protein levels of the enzyme glutaminase without affecting RNA expression levels (48). c-MYC also directly increases transcription of the HSP HSP90A (42), which plays an important role in protein folding, degradation, and maturation. HSP90A is also known to stabilize a subset of kinases, steroid receptors, and

Figure 5.

Myc-driven tumors contain elevated OGT and O-GlcNAc levels and require OGT for survival. A, CommaD and MTEC-Myc cell lysates were collected and analyzed by immunoblotting with indicated antibodies. B, expression of OGT and O-GlcNAcylated proteins was compared by immunoblotting of HS-WCE (10 μg/lane) prepared from mammary tissue of normal (unaffected) glands, hyperplastic glands, or late-stage carcinomas that originated from four independent MMTV-MYC transgenic females (FVB/N strain). Blots are representative of three normal mammary glands, three hyperplasias, and four carcinomas. C, MCF-10A and MTEC-MYC cells were treated with control (DMSO) or Ac-5s-GlcNAc (100 μmol/L) for 48 hours then protein lysates were collected and analyzed by immunoblotting. D, MCF-10A and MTEC-MYC cells were treated with control or Ac-5s-GlcNAc at indicated doses for 48 hours and then stained with crystal violet. E, MCF-10A and MTEC-MYC cells were placed in 3D basement membrane cultures. On day 5, cells were treated with control (DMSO) or Ac-5s-GlcNAc at indicated doses for 48 hours, fixed, and stained with indicated antibodies and representative images were taken using confocal microscopy. F, model of OGT regulation by mTOR/MYC/HSP90 in cancer cells.

transcription factors, which are overexpressed in cancers (49). It was recently demonstrated that HSP90A directly interacts with OGT, and inhibition of HSP90 reduces the half-life of the OGT protein, leading to decreased global O-GlcNAcylation in endothelial cells (41). Consistent with these data, we showed that MYC–induced regulation of OGT, in part, requires HSP90A expression in cancer cells. Therefore, we propose a model (Fig. 5F) in which MYC regulation of OGT occurs via its transcriptional activation of HSP90A, which facilitates maintenance of OGT protein through stabilization or prevention of degradation by the proteasome.

Previous studies have found that c-MYC can be O-GlcNAcylated (11, 50) in normal cells, and more recently in prostate cancer cells (21). Reducing O-GlcNAcylation in PC-3 prostate cancer cells leads to reduced c-MYC protein O-GlcNAcylation, decreased c-MYC stability, and was associated with decreased growth and survival of prostate cancer cells (21). HSP90A has also been shown to be O-GlcNAcylated (51). These data, together with our results, suggest that c-MYC, HSP90A, and OGT/O-GlcNAcylation may be part of a feed-forward–regulatory loop present in cancer cells. Amplification of c-MYC in cancer cells can transcriptionally induce HSP90A expression leading to increased OGT levels and total O-GlcNAcylation that feeds back to O-GlcNAcylate c-MYC, increasing its stability, and thus enabling cancer cell growth GlcNAcylation (Fig. 5F). Consistent, with this idea, we show that MYC overexpression leads to elevated HSP90A protein levels that are required for increased OGT and O-GlcNAcylation levels. Importantly, we show that OGT is required for MYC-mediated cancer cell survival.

Because increased O-GlcNAcylation can also positively regulate c-MYC and other oncogenic transcription factors, including FoxM1 (17, 20) as well as HIF1α (27), reducing O-GlcNAcylation in cancer cells via OGT inhibition may be an important therapeutic option for a number of cancers. Our results also suggest that OGT may also serve as potential therapeutic target in MYC-amplified breast cancers, and potentially other cancers with amplification of this oncogene, such as prostate, neuroblastoma, and multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V.L. Sodi, S. Khaku, R. Krutilina, L.P. Schwab, T.N. Seagroves
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.L. Sodi, S. Khaku, T.N. Seagroves, M.J. Reginato
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