Critical Role of O-Linked β-N-Acetylglucosamine Transferase in Prostate Cancer Invasion, Angiogenesis, and Metastasis

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Background: Cancer cells display altered metabolism and expression of the nutrient sensor O-linked β-N-acetylglucosamine transferase (OGT).

Results: Through regulation of FoxM1, OGT contributes to increased invasion, angiogenesis, and metastasis of prostate cancer cells.

Conclusion: OGT plays a critical role in prostate cancer.

Significance: OGT may provide a novel therapeutic target for treating prostate cancer.

Cancer cells universally increase glucose and glutamine consumption, leading to the altered metabolic state known as the Warburg effect; one metabolic pathway, highly dependent on glucose and glutamine, is the hexosamine biosynthetic pathway. Increased flux through the hexosamine biosynthetic pathway leads to increases in the post-translational addition of O-linked β-N-acetylglucosamine (O-GlcNAc) to various nuclear and cytosolic proteins. A number of these target proteins are implicated in cancer, and recently, O-GlcNAcylation was shown to play a role in breast cancer; however, O-GlcNAcylation in other cancers remains poorly defined. Here, we show that O-GlcNAc transferase (OGT) is overexpressed in prostate cancer compared with normal prostate epithelium and that OGT protein and O-GlcNAc levels are elevated in prostate carcinoma cell lines. Reducing O-GlcNAcylation in PC3-ML cells was associated with reduced expression of matrix metalloproteinase (MMP)-2, MMP-9, and VEGF, resulting in inhibition of invasion and angiogenesis. OGT-mediated regulation of invasion and angiogenesis was dependent upon regulation of the oncogenic transcription factor FoxM1, a key regulator of invasion and angiogenesis, as reducing OGT expression led to increased FoxM1 protein degradation. Conversely, overexpression of a degradation-resistant FoxM1 mutant abrogated OGT RNAi-mediated effects on invasion, MMP levels, angiogenesis, and VEGF expression. Using a mouse model of metastasis, we found that reduction of OGT expression blocked bone metastasis. Altogether, these data suggest that prostate cancer cells alter glucose and glutamine levels, O-GlcNAc modifications and OGT levels become elevated and are required for regulation of malignant properties, implicating OGT as a novel therapeutic target in the treatment of cancer.

Cancer cells characteristically display altered metabolism, producing ATP independent of the oxygen concentration (1), in a phenomenon known as the Warburg effect (2). Cancer cells have increased utilization rates of glucose and glutamine, which are established hallmarks of tumor metabolism (3, 4). As glycolysis is much less efficient in generating ATP than is oxidative phosphorylation, increased glucose flux must occur to compensate. To serve the less efficient energy-producing glycolytic route, tumor cells increase glucose uptake; clinical tumors generally show an order of magnitude increase in uptake (5). Changes in tumor cell glucose uptake and metabolism may also alter distinct nutrient signaling pathways, including the mTOR (mammalian target of rapamycin) pathway, the AMP-activated protein kinase pathway, and the hexosamine biosynthetic pathway (HBP) (6).

The HBP is highly dependent on glucose and glutamine and functions as a minor branch of the glycolytic pathway, as ~3–5% of total glucose enters this pathway (7). The rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase uses glutamine to convert fructose 6-phosphate into glucose 6-phosphate, which is then utilized further to synthesize the end product, UDP-GlcNAc (7). UDP-GlcNAc is required for the biosynthesis of many classes of extracellular glycopolymers, including N- and O-glycans (8). UDP-GlcNAc is the obligatory substrate for O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT). This enzyme adds the O-GlcNAc moiety to the free hydroxyl of select serine and threonine residues on a diverse population of nuclear and cytosolic proteins (9). Increased O-GlcNAc cycling has been suggested to play a key role in insulin resistance, as many key substrates involved in the insulin signaling pathway are modified by O-GlcNAc (10), and OGT overexpression in liver promotes insulin resistance in mice (11). Moreover, a number of cancer-associated proteins, including p53 (12), IκB kinase (13), c-Myc (14), and Snail (15), are regulated by O-GlcNAcylation, yet the role of O-GlcNAc in cancer biology is just now being defined.

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2The abbreviations used are: HBP, hexosamine biosynthetic pathway; O-GlcNAc, O-linked β-N-acetylglucosamine; OGT, O-GlcNAc transferase; MMP, matrix metalloproteinase; HUVEC, human umbilical vein endothelial cell; qRT-PCR, quantitative RT-PCR; APC/C, anaphase-promoting complex/cyclosome.

This article contains supplemental Figs. 1–7 and additional references.
Recently, we and others found that OGT and O-GlcNAcylation are elevated in breast cancer cells and that reducing OGT levels blocks breast cancer growth in vitro and in vivo (16) and reduces metastasis in mouse breast cancer cells (17). Metabolic alterations in breast cancer cells also increase O-GlcNAc cycling, and this has profound effects on cancer phenotypes, including growth and invasion; however, reducing OGT and O-GlcNAc levels has minimal effects on growth and differentiation of immortalized mammary epithelial cells (16). OGT regulation of breast cancer growth is dependent on regulation of the oncogenic transcription factor FoxM1, as reducing O-GlcNAc levels increases degradation of FoxM1. Because altered metabolism is a general hallmark of many cancers and because FoxM1 is frequently overexpressed in a number of human malignancies, including pancreatic (18), lung (19), liver (20), and prostate (21) cancer, we examined whether elevation of O-GlcNAc and OGT occurs in other epithelial cancers.

Here, through examination of Oncomine™ microarray databases, we have found that OGT is overexpressed in prostate cancer tissue compared with normal prostate epithelium and that elevated OGT levels are associated with poor clinical outcome. Moreover, we provide the first evidence that the expression of OGT and the levels of O-GlcNAc modifications are elevated in prostate cancer cell lines compared with non-transformed prostate cells. Using RNAi targeting OGT expression in the metastatic prostate cancer cell line PC3-ML, we inhibited growth, which was associated with decreased FoxM1 levels and increased expression of the cyclin-dependent kinase inhibitor p27kip1. In addition, we observed a reduction in invasive phenotypes by reducing O-GlcNAcylation in PC3-ML cells, as well as a reduction in matrix metalloproteinase (MMP)-2 and MMP-9 expression. We show for the first time that reducing OGT levels inhibits angiogenic potential and VEGF expression in PC3-ML cells, which is dependent on FoxM1. Finally, reducing OGT expression in human prostate cancer cells inhibits metastasis to bone. Thus, by regulating the growth, invasion, angiogenesis, and metastasis of prostate cancer cells, OGT is positioned as a novel target for therapeutic intervention in the treatment of human prostate cancer.

**EXPERIMENTAL PROCEDURES**

**Materials**—Growth factor-reduced Matrigel was purchased from BD Biosciences. The O-GlcNAcase inhibitor 9D was a kind gift from D. Vocadlo (Department of Biochemistry, Simon Fraser University). The OGT inhibitor ST060266 was purchased from TintTec (Newark, DE). Lactacytin was purchased from Sigma. The antibodies used were anti-actin and anti-FoxM1 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-MGEA5 (O-GlcNAcase) from Abcam (Cambridge, MA); anti-MMP-2 and anti-MMP-9 from Cell Signaling (Danvers, MA); anti-OGT from Sigma; anti-p27, anti-integrin α5, and anti-integrin α6 from BD Biosciences; and anti-VEGF from NeoMarkers (Fremont, CA). The anti-O-GlcNAc antibody (CTD110.6) has been described previously (22), and the anti-Sp1 antibody was a kind gift provided by J. Clifford-Azizkhan (Department of Biochemistry and Molecular Biology, Drexel University College of Medicine).

**Cell Culture**—RWPE-1, NPTX-1532, PC3, PC3-ML, and DU145 cell lines were kindly provided by M. Starns (Department of Pathology, Drexel University College of Medicine). RWPE-1 and NPTX-1532 cells were routinely cultured in Keratinocyte-SFM (Invitrogen) supplemented with bovine pituitary extract, 10 ng/ml EGF, and 1% penicillin/streptomycin. PC3, PC3-ML, and DU145 cells were cultured in DMEM (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum, 1% 1-glutamine, and 1% penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs) were a kind gift of P. Lelkes (School of Biomedical Engineering, Science, and Health Systems, Drexel University) and were routinely cultured in Clonetics complete endothelial cell growth medium. The degradation-resistant FoxM1 mutant pbabe-FLAG-FoxM1-DN/AKEN was described previously (16). Retroviruses were packaged and used to stably transduce PC3-ML cells with pbabe or pbabe-FLAG-FoxM1-DN/AKEN virus as described previously (16, 17, 23, 24). Bioluminescent PC3-ML cells were generated by stable transfection with firefly luciferase in a pWZL-Hygro vector (kindly provided by M. Murphy, Fox Chase Cancer Center).

**Immunoblotting**—Cell lysates from 1 × 106 cells were prepared in radioimmune precipitation assay lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 50 mM Tris-HCl (pH 8), 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF, and 1 mM Na3VO4) supplemented with 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 200 µg/ml PMSF. Lysates were cleared by centrifugation at 16,000 × g for 20 min at 4 °C and analyzed by SDS-PAGE and autoradiography. Proteins were analyzed by immunoblotting using primary antibodies as indicated above.

**Three-dimensional Morphogenesis Assays**—Assays were performed as described previously (16). Briefly, RWPE-1 or PC3-ML cells were seeded at a concentration of 1.25 × 104/well in 8-well chamber slides (BD Falcon™) that had been previously coated with 50 µl of growth factor-reduced Matrigel. Cells were then overlaid with the respective growth medium supplemented with 2% growth factor-reduced Matrigel. Cells were harvested and counted at the indicated times. Immunofluorescence of three-dimensional structures was performed as described (16).

**shRNA Infections**—RWPE-1 and PC3-ML cells were infected with control (scrambled sequence), OGT-1, and OGT-2 shRNA construct-containing lentiviruses and stably selected as described previously (16).

**Quantitative RT-PCR (qRT-PCR)**—Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer’s protocol for monolayer animal cell RNA extraction. The levels of OGT, MMP-2, MMP-9, VEGF, and cyclphin A were determined using a Stratagene Mx3000P QPCR device with a Brilliant II QRT-PCR Master Mix kit (Stratagene) according to the manufacturer’s protocol. Data analysis was performed with the MxPro software package (Stratagene). TaqMan gene expression assay primers/probes were purchased from Applied Biosystems (Foster City, CA) for cyclphin A (Hs00999904_m1), OGT (Hs00914634_g1), MMP-2 (Hs00234472_m1), MMP-9 (Hs00234579_m1), and VEGF.
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(Hs00173626_m1). Expression levels were normalized to cyclophilin A.

**Soft Agar Colony Formation Assays**—Base layers consisting of growth medium containing a 1% low melting point agarose (ICS BioExpress, Kaysville, UT) made in 10 mM HEPES (pH 7.5) were poured onto 6-well plates and allowed to solidify. Cells (1 × 10^4/well) were plated in triplicate on top layers consisting of growth medium containing 0.3% agarose and 5 mM HEPES. The top layer was also allowed to set and then further overlaid with 1 ml of medium. Colonies were stained after 14 days with 500 μl of 0.05% p-iodonitrotetrazolium violet overnight, and colonies measuring >50 μm were counted manually.

**Flow Cytometry**—At 48 h post-infection with control or OGT shRNA lentivirus, cells were harvested and washed with PBS. Pellets were fixed in cold 70% ethanol and maintained at −20 °C for at least 2 h. Propidium iodide (Sigma) in a solution of Triton X-100 (Sigma) and DNase-free RNase A (Fisher) was used for cell cycle distribution staining, and an FITC mouse anti-human Ki-67 kit (BD Pharmingen) was used according to the manufacturer’s protocol for Ki-67 staining. All data were collected and analyzed using a Guava EasyCyte Plus system and CytoSoft (version 5.3) software (Millipore). Ki-67 data are presented as the mean fluorescence intensity of gated cells, normalized to the isotype control, and are expressed as intensity relative to the appropriate control shRNA.

**Transwell Invasion Assays**—Invasion assays were performed using BD BioCoat growth factor-reduced Matrigel invasion chambers (BD Biosciences) following the manufacturer’s protocol, utilizing serum-free medium in the upper chamber and chambers (BD) following the manufacturer’s protocol, utilizing serum-free medium in the upper chamber and complete medium in the lower chamber over a 12-h incubation period. Invasive cells were visualized with DAPI (5 μg/ml; Sigma); cells in four fields were counted for each chamber.

**Endothelial Tube Formation Assay**—Endothelial tube formation assays were performed as described (18) with the following modifications. Antibiotic-selected PC3-ML cells stably expressing control or OGT shRNA were used to condition RPMI 1640 medium (Invitrogen) for 24 h and passed through 0.45-μm syringe filters. Tube length was quantified using iVision-Mac (version 4.0.14) software (BioVision Technologies, Exton, PA) from digital images taken using an inverted microscope.

**In Vivo Bioluminescent Xenograft Model of Metastasis**—Male severe combined immunodeficient mice (5–6 weeks old) were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) and inoculated in the left cardiac ventricle with 5 × 10^5 PC3-ML cells stably expressing luciferase containing either OGT or control shRNA in 100 μl of serum-free DMEM/F-12 (Invitrogen) using a 30-gauge needle. Mice were injected intraperitoneally with 200 μl of d-luciferin solution (9 mg/ml; Caliper Life Sciences, Hopkinton, MA), and bioluminescence imaging was done 30 min after the intracardiac injection to detect the distribution of prostate cancer cells; mice were imaged weekly. Images were acquired with an IVIS 200 imaging system, and results were analyzed using Living Image software (Caliper Life Sciences). Metastatic lesions were counted for each mouse at week 5 and averaged for both groups. All protocols involving the use of animals were approved by the Institutional Animal Care and Use Committee at the Fox Chase Cancer Center.

**Histological Analysis of Mouse Tissues**—To confirm the presence of PC3-ML cells in the bone metastatic regions, tissues were excised from the mice at necropsy. Tissue was prepared and analyzed by the Pathology Diagnostic Laboratory at Drexel University College of Medicine. Briefly, tissues were fixed in 4% formaldehyde, cut at the midpoint, and embedded in paraffin blocks. Serial paraffin longitudinal sections were stained with hematoxylin/eosin.

**Data Mining**—OGT gene expression data from four independent prostate cancer studies were obtained using the Oncomine™ Database. In the four studies retrieved, we compared samples of normal prostate tissue and prostate carcinoma. These data were regraphed using GraphPad Prism software (GraphPad Software, La Jolla, CA) and are presented as scatter plots, including the mean ± S.E. Survivorship data were additionally acquired through the Oncomine™ Database; OGT expression data were stratified as either being high (above the dataset mean) or low (below the dataset mean) and regraphed to represent probability of survival at the indicated time point. Tissue-specific OGT expression data were acquired through the National Center for Biotechnology Information Gene Expression Omnibus and graphed to depict average OGT expression for the indicated tissue type.

**Statistical Analysis**—All results are from at least three independent experiments. Statistical significance was ascertained with Student’s t test, with a p value threshold of ≤0.05.

**RESULTS**

**OGT Levels Are Elevated in Prostate Cancer**—We and others have recently demonstrated that OGT and O-GlcNAc modifications are overexpressed in breast cancer (16, 17). To determine whether OGT is overexpressed in other tumor types, we examined OGT expression using the Oncomine™ Database. We found four independent microarray gene expression studies showing elevated OGT mRNA expression patterns in human prostate carcinoma compared with normal adjacent tissue samples (supplemental Fig. 1). A survey of the National Center for Biotechnology Information Gene Expression Omnibus (25, 26) indicated a positive correlation (r^2 = 0.996) between high OGT expression and metastatic progression in normal, primary tumor, and metastatic prostate tumor tissues (Fig. 1A). Furthermore, an additional study (27) of 94 patient tumor samples, when stratified by the level of OGT expression, indicated that disease-free survival 5 years post-treatment for prostate cancer was higher (75% probability of survival) in patients with a low OGT expression profile compared with patients with increased OGT expression (25% probability of survival) (Fig. 1B).

To examine whether prostate cancer cells contain elevated OGT expression levels, we compared the expression of OGT and total O-GlcNAc modifications among a panel of normal and prostate carcinoma cell lines. In three-dimensional culture, the non-transformed immortalized prostate RWPE-1 cells formed organized prostate duct-like acinar structures, which closely resembled their native architecture in vivo, as evidenced by acinus-like architecture, including the basal localization of integrin α6 and the formation of hollowed lumens (supplemental Fig. 3A). In contrast, the prostate cancer cell line PC3-ML...
formed highly disorganized structures, which displayed features of invasive phenotypes and loss of integrin α6 basal localization (supplemental Fig. 3A). The RWPE-1 cells, as well as the normal prostate epithelial cell line NPTX-1532 (29), exhibited lower levels of both O-GlcNAc and OGT compared with the prostate carcinoma cell lines PC3, PC3-ML, and DU145 (Fig. 1C). Furthermore, PC3-ML cells contained ∼2-fold more OGT mRNA compared with RWPE-1 cells as quantified by qRT-PCR (Fig. 1D). In addition to global O-GlcNAcylation and OGT expression being elevated in prostate carcinoma cell lines, we also observe decreased O-GlcNAcase protein levels in prostate carcinoma cell lines compared with normal prostate epithelial cells (Fig. 1C). Thus, these results show that prostate cancer cell lines and tissue contain elevated levels of OGT RNA and protein, as well as increased total O-GlcNAcylation, and that the elevation of OGT levels in prostate cancer may be associated with poor clinical prognosis.

Targeting OGT Decreases Prostate Cancer Growth—A lentiviral delivery system was used to stably introduce either control (scrambled sequence) or two different OGT (OGT-1 or OGT-2) shRNA constructs into PC3-ML cells. The level of OGT knockdown and decrease in O-GlcNAcylation was confirmed by immunoblotting for OGT and global O-GlcNAc levels (Fig. 2A). These cells were subsequently cultured in soft agar for 16 days, and the resulting colonies were stained and counted; there was an ∼80% reduction in the anchorage-independent growth of OGT shRNA-expressing cells compared with control PC3-ML cells (supplemental Fig. 2). The ability of PC3-ML cells expressing OGT shRNA to grow in three-dimensional culture was also significantly impaired by ∼65% compared with control cells (Fig. 2B); however, non-transformed RWPE-1 cells expressing OGT shRNA, which contained decreased O-GlcNAc levels (supplemental Fig. 3B), did not demonstrate significant changes in growth during three-dimensional culture (supplemental Fig. 3C).
To further examine the growth inhibitory effect of OGT knockdown in prostate cancer cells, we examined the possible effects on cell cycle progression using propidium iodide staining, followed by flow cytometry. OGT knockdown in PC3-ML cells induced a statistically significant \((p < 0.05)\) accumulation of cells in G1 phase and a slight decrease in S phase (Fig. 2C). There was also a concomitant reduction in the proliferation marker Ki-67 in PC3-ML cells expressing OGT shRNA constructs compared with those expressing control shRNA (Fig. 2D). Consistent with cell cycle arrest, we observed a decrease in expression of the cell cycle- and proliferation-associated transcription factor FoxM1 and an increase in the cyclin-dependent kinase inhibitor p27Kip1 (Fig. 2A). Interestingly, we did not detect an increase in G1 arrest (supplemental Fig. 4B) or a decrease in Ki-67 positivity (supplemental Fig. 4C) in the non-transformed RWPE-1 cells expressing OGT shRNA (supplemental Fig. 4A). The effects on PC3-ML growth and signaling by targeting OGT with RNAi are dependent upon the catalytic activity of OGT, as treatment of PC3-ML cells with a previously characterized OGT inhibitor (16) reduced O-GlcNAcylation, reduced growth in three-dimensional morphogenesis, decreased the expression of FoxM1, and increased the expression of p27Kip1 (supplemental Fig. 7).

Targeting OGT Reduces Invasion of PC3-ML Cells and Is Associated with Decreased MMP-2 and MMP-9 Expression—When placed in three-dimensional basement membrane culture, PC3-ML cells exhibited a morphological phenotype that is consistent with invasive potential (supplemental Fig. 2); however, when cultured under three-dimensional conditions, PC3-ML cells expressing OGT shRNA exhibited reduced invasive protrusions compared with control shRNA-expressing cells (Fig. 3A). This reduction in invasive morphology upon decreased OGT expression led us to assay the invasive potential of PC3-ML cells in Transwell invasion chamber inserts. As shown in Fig. 3B, there was an \(\sim 60\%\) reduction in the ability of PC3-ML cells to invade through the Matrigel coating of the Transwell membrane inserts with decreased OGT expression compared with control cells. Because it has previously been established that MMP-2 and MMP-9 are associated with prostate cancer progression and metastasis (30, 31), we examined the expression of MMP-2 and MMP-9 in the context of OGT knockdown. We observed an \(\sim 70\%\) reduction in MMP-2 and

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**FIGURE 2.** Targeting OGT decreases malignant growth and proliferation in vitro. A, PC3-ML cells stably expressing control, OGT-1, or OGT-2 shRNA were lysed and immunoblotted with the indicated antibodies. B, PC3-ML cells stably expressing control or OGT shRNA were seeded on a bed of Matrigel, overlaid with dilute Matrigel in three-dimension culture medium, and assayed for proliferation as measured by cells/well (depicted graphically). Data are expressed as average cells/well; error bars indicate S.E. for \(n = 3\), \(p < 0.05\). C, PC3-ML cells described in A were stained with propidium iodide for cell cycle analysis. Data are presented as percentage of the gated population in the indicated phase; error bars indicate S.E. for \(n = 3\), \(p < 0.05\). D, PC3-ML cells stably expressing control, OGT-1, or OGT-2 shRNA were incubated with FITC-conjugated antibodies against Ki-67 or an isotype control and analyzed by flow cytometry. Data are presented as percentage of the gated population positive for Ki-67; error bars indicate S.E. for \(n = 3\), \(p < 0.01\).
an ~50% reduction in MMP-9 mRNA levels by qRT-PCR (Fig. 3C) in PC3-ML cells with reduced OGT expression compared with control cells. Additionally, MMP-2 and MMP-9 protein levels were decreased upon knockdown of OGT (Fig. 3D).

It has been previously shown that targeting OGT in breast cancer results in a reduction of the oncogenic transcription factor FoxM1 (16), and it has also been previously established that FoxM1 regulates expression of MMP-2 in breast cancer cells (32); however, the role of FoxM1 in prostate cancer invasion and MMP-2 regulation had not been examined. Thus, we examined whether FoxM1 can also regulate expression of MMP-2 and regulate invasion in prostate cancer cells. Knockdown of FoxM1 in PC3-ML cells with stably expressed shRNA constructs resulted in a reduction of MMP-2 protein expression (supplemental Fig. 5A), invasive structures in three-dimensional culture (supplemental Fig. 5B), and inhibition of invasion through Matrigel-coated Transwell invasion chambers (supplemental Fig. 5C). Consistent with these data, we observed a marked reduction in FoxM1 protein levels in PC3-ML cells stably expressing OGT shRNA (Fig. 2A).

Targeting OGT Reduces Angiogenic Potential and VEGF Expression in PC3-ML Cells—FoxM1 has been shown to regulate VEGF expression in breast (32), pancreatic (18), and gastric (33) cancer, suggesting that decreasing OGT may also inhibit angiogenic potential and VEGF expression in prostate cancer cells. We first determined if reducing levels of OGT in PC3-ML cells alters endothelial tube formation, an indirect measure of angiogenesis (18). After 24 h of stably expressing control or OGT shRNA in PC3-ML cells, we collected the conditioned medium. This conditioned medium was then used to stimulate HUVECs in an endothelial tube formation assay; HUVECs were seeded onto a bed of Matrigel and then overlaid with PC3-ML cell-conditioned medium. After 6 h, the HUVECs were imaged, and tube length was quantified using iVision-Mac software. As shown in Fig. 4A, there was a statistically significant (p < 0.05) reduction in the ability of the conditioned medium from PC3-ML cells expressing OGT shRNA to stimulate tube formation in HUVECs compared with the medium from control shRNA-expressing cells. RNA was harvested from the same PC3-ML cells used to condition the media, and the levels of VEGF RNA were determined. As expected, the levels of OGT RNA were decreased in cells expressing OGT shRNA; however, VEGF RNA levels were also significantly (p < 0.05) decreased, by ~50% (Fig. 4B), consistent with the decreased ability of OGT knockdown cells to stimulate endothelial tube formation (Fig. 4A). Because FoxM1 had not been previously shown to induce VEGF expression in prostate cancer cells, we examined VEGF levels in PC3-ML cells in which we knocked down FoxM1 levels with RNAi. PC3-ML cells stably expressing FoxM1 shRNA compared with control cells con-
tained reduced levels of VEGF (supplemental Fig. 5A). Thus, reducing OGT inhibits angiogenic potential and VEGF levels in prostate cancer cells.

**Degradation-resistant FoxM1 Mutant Rescues Angiogenic Potential and Invasive Capacity of OGT Knockdown Cells**—To investigate the O-GlcNAcylation status of FoxM1 in PC3-ML cells, we immunoprecipitated endogenous FoxM1 without and with 9D treatment to enhance total cellular O-GlcNAcylation and subjected the resulting proteins to immunoblotting with anti-O-GlcNAc antibody. As shown in supplemental Fig. 6, FoxM1 protein did not appear O-GlcNAcylated, unlike the well known O-GlcNAcylated transcription factor Sp1 (34). Previous work in breast cancer cells has indicated that the N terminus of FoxM1, which contains degron destruction D-box and KEN-box sequences (35), is essential for regulation by OGT (16, 17, 23, 24) and suggests that OGT regulates FoxM1 via proteasome-mediated degradation. Because decreasing OGT expression also decreased FoxM1 protein levels in PC3-ML cells (Fig. 2A), we tested directly whether OGT regulates FoxM1 via the proteasome. Treatment of PC3-ML cells with the proteasome inhibitor lactacystin for 6 h reversed FoxM1 inhibition in OGT knockdown cells (Fig. 5A), suggesting that OGT regulation of FoxM1 protein levels is a proteasome-mediated process. Consistent with these data, the levels of a non-degradable FoxM1 mutant lacking the N terminus, which contains D-box and KEN-box motifs, overexpressed in PC3-ML cells were not altered by OGT knockdown compared with control RNAi (Fig. 5B). To test whether FoxM1 regulation by OGT is required for alterations of PC3-ML angiogenic and invasive potential, we examined whether the non-degradable FoxM1 mutant could reverse the phenotypes of PC3-ML cells caused by down-regulating OGT. PC3-ML cells overexpressing non-degradable mutant FoxM1 (Fig. 5B) were able to fully overcome the inhibition of angiogenesis mediated by OGT silencing (Fig. 5D), as well as the reverse inhibition of VEGF expression (Fig. 5C).

Expression of the non-degradable FoxM1 mutant in PC3-ML cells coexpressing OGT shRNA was also able reverse inhibition of invasive protrusions in cells cultured in three-dimensional basement membranes (Fig. 6A) and nearly completely restored invasion through Transwell invasion inserts to control levels (Fig. 6B). Furthermore, expression of MMP-2 RNA was restored to near control levels, and expression of MMP-9 RNA was partially restored (Fig. 6C). Thus, we have demonstrated that the decrease in PC3-ML angiogenic potential and invasive capacity upon decreased OGT expression is at least partially dependent upon FoxM1 function.

**Reducing OGT Levels Inhibits Prostate Cancer Metastasis to Bone**—Depletion of OGT in prostate cancer cells leads to decreased invasion and angiogenesis, suggesting that OGT may also regulate prostate cancer metastasis. To test directly whether OGT signaling is functionally important for human
prostate cancer metastasis, we injected the highly bone metastatic PC3-ML cells stably overexpressing control or OGT RNAi intracardially into immunocompromised mice. We found that OGT shRNA-expressing PC3-ML cells showed a reduction in OGT expression of 60% compared with control PC3-ML cells as analyzed by Western blotting (Fig. 7A). The progression of bone metastasis after left ventricle intracardiac injection of tumor cells was monitored by bioluminescence imaging using a stably expressed firefly luciferase reporter. After 5 weeks, 56% animals injected with control shRNA-expressing PC3-ML cells developed metastatic foci to bone, whereas 13% animals injected with OGT shRNA-expressing PC3-ML cells developed metastatic foci in either the mandibles or hind limbs (Fig. 7B). Animals injected with OGT knockdown PC3-ML cells had 4.7-fold less bone metastases to mandibles and hind limbs (Fig. 7C). Histological analysis of mandibles and hind limbs from injected mice confirmed that the luciferase signal corresponded with the presence of metastatic PC3-ML cells within bone lesions in mice injected with control cells but not cells depleted of OGT (Fig. 7D). These results support the functional role for OGT in prostate bone metastasis.

Overall, we have shown that OGT and O-GlcNAcylation are elevated in prostate cancer cells and are required for growth, invasion, angiogenesis, and metastasis and that this phenotype requires, in part, regulation of FoxM1, as a degradation-resistant mutant was able to rescue invasion and angiogenesis defects. Thus, OGT may serve as a novel therapeutic target for treatment of primary and metastatic prostate cancers.

**DISCUSSION**

The data presented here describe a critical role for the elevated expression of OGT and O-GlcNAcylation observed in prostate cancer in the invasion, angiogenesis, and metastasis of such cancer cells. Reducing the expression of OGT via RNAi attenuated not only the invasive phenotype of PC3-ML cells in three-dimensional culture but also the ability of PC3-ML cells
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A. PC3-ML:

\[ \text{pBabe} \quad \text{pBabe-FoxM1-ΔN/ΔKEN} \]

\[ \text{Control shRNA} \quad \text{OGT-1 shRNA} \quad \text{OGT-2 shRNA} \]

B. Relative Invasion

\[ \begin{array}{ccc}
\text{Control} & \text{n.s.} & \ast \\
\text{OGT-1 shRNA} & \ast & \ast \\
\text{OGT-2 shRNA} & \ast & \ast \\
\end{array} \]

C. Relative Expression

\[ \begin{array}{ccc}
\text{Control} & \text{n.s.} & \n.s. \\
\text{OGT-1} & \n.s. & \n.s. \\
\text{OGT-2} & \n.s. & \n.s. \\
\text{pBabe-FoxM1-ΔN/ΔKEN} & \n.s. & \n.s. \\
\end{array} \]

FIGURE 6. Expression of non-degradable FoxM1 mutant (FoxM1-ΔN/ΔKEN) partially rescues invasive capacity of OGT shRNA-expressing PC3-ML cells. A, representative bright-field micrographs comparing day 12 control, OGT-1, or OGT-2 shRNA-expressing PC3-ML-pBabe or PC3-ML-pBabe-FoxM1-ΔN/ΔKEN cells maintained in three-dimensional culture systems. B, PC3-ML cells as described in A were subjected to Transwell invasion assays. Error bars indicate S.E. for \( n = 3 \). *, \( p < 0.05 \). C, PC3-ML cells as described in A were subjected qRT-PCR for MMP-2, MMP-9, and OGT and normalized to the respective control. Error bars indicate S.E. for \( n = 3 \), n.s., not significant.

to invade through a reconstituted extracellular matrix-coated membrane. Furthermore, targeting OGT decreased VEGF expression and the ability of PC3-ML cells to stimulate endothelial tube formation, suggesting that targeting OGT may block angiogenesis in prostate cancer cells. Taken together, these data suggest that OGT plays a role in the metastatic potential of human prostate cancer, and indeed, we observed a decrease in the ability of PC3-ML cells to establish metastases in an intracardiac injection mouse model system. These observations concur with and expand upon a previously reported association between OGT expression and metastasis in breast cancer (17).

Increasing evidence suggests that OGT plays a critical role during oncogenesis and tumor progression in numerous cancers; indeed, several reports have implicated OGT overexpression and elevated O-GlcNAcylation in cancers ranging from solid tumors (including breast (16, 18), lung (23), colon (23), liver (36), and now prostate) to chronic myeloid leukemia (16, 17, 23, 24). More recently, expression of the enzyme that removes O-GlcNAcylation, O-GlcNAcase, has been found to be reduced in breast (37) and liver (36) cancer. Knockdown of O-GlcNAcase in liver cancer cells increases total O-GlcNAcylation levels and increases the migration and invasion of these cells. In addition, the O-GlcNAcase gene is localized in frequently altered chromosomal regions, critical in the pathogenesis of breast and ovarian cancer (38). Thus, increased OGT and decreased O-GlcNAcase levels in cancers may lead to a general increase in O-GlcNAc cycling that has diverse tumor-promoting functions.

Despite emerging evidence for the role of O-GlcNAc in various cancers, it is not immediately clear as to how OGT expression is regulated in cancer. Here, we have shown that both OGT protein (Fig. 1C) and RNA (Fig. 1D) are up-regulated in prostate cancer, but the exact mechanism by which cancer cells up-regulate OGT and O-GlcNAcylation is currently not clear. It is known, however, that cancer cells up-regulate the flux of glucose and glutamine, both substrates of the HBP (39). The HBP generates the activated substrate UDP-GlcNAc for OGT and, in this manner, may serve as a potential mechanism by which global O-GlcNAcylation occurs in cancer cells. The UDP-GlcNAc generated by HBP can also be used as a substrate for N-linked glycosylation, which has also been found to be elevated in cancer cells (40). Recent studies have implicated flux through the HBP as playing a key role in coordinating glucose and glutamine metabolism by regulating the surface expression of growth factor receptors via N-linked glycosylation (41). Whether cancer cells have increased flux through the HBP has not been directly examined; future studies will be aimed at elucidating the origins of elevated O-GlcNAcylation in cancer cells.

In addition to blocking invasion, angiogenesis, and metastasis, targeting OGT blocked the growth and proliferation of prostate cancer cells. The growth inhibition seen in PC3-ML cells expressing OGT RNAi suggests that targeting OGT blocks not only the ability of PC3-ML cells to metastasize but also the ability of primary tumors to expand and secondary metastases to establish at distant sites. It is possible that reducing OGT in prostate cancer cells induced defects in growth, invasion, and angiogenesis, all contributing to inhibition of bone metastasis. OGT silencing in the mouse breast cancer cell line 4T1 inhibits metastatic nodules to lungs but does not inhibit primary tumor growth (17). The effects of reducing OGT and O-GlcNAc via RNAi are dependent on OGT catalytic activity, as chemical inhibitors of OGT have similar effects on oncogenic phenotypes in vitro. Thus, targeting OGT therapeutically may impair growth at the primary tumor site, the ability of cancer cells to escape primary
The effects of targeting OGT seem to be partly dependent upon proteasomal regulation of the oncogenic transcription factor FoxM1, as inhibition of the proteasome reversed loss of FoxM1 expression in OGT knockdown cells. Moreover, expression of a non-degradable mutant version of FoxM1 rescued OGT depletion-mediated phenotypes; however, it is not clear how OGT regulates FoxM1 degradation. FoxM1 is polyubiquitinated by the anaphase-promoting complex/cyclosome (APC/C)-Cdh1 for degradation by the proteasome (35). APC/C is a critical regulator of cell cycle progression by controlling the ubiquitination-mediated proteolysis of cell cycle regulators and is thought to be active from mitosis to late G1 phase (42). Interestingly, APC/C-Cdh1 substrates are often overexpressed in human cancers, including FoxM1, Skp2, PLK1, cyclin A, and Aurora A, and are associated with poor clinical outcome (43). Recent studies have suggested that Cdh1 may be a haploinsufficient tumor suppressor, as Cdh1 heterozygosity in mice results in tumor development (44). Because OGT does not O-GlcNAcylate FoxM1 directly (supplemental Fig. 6), it is likely that OGT regulates the activity of APC/C-Cdh1 by a yet undefined mechanism.

The reduction in invasion and angiogenesis effectors (MMP-2, MMP-9, and VEGF) was at least partially restored by expression of the FoxM1 mutant in OGT-depleted prostate cancer cells. Reducing FoxM1 expression in multiple cancer cells led to a marked reduction in invasive potential (18, 21, 32), consistent with the OGT-mediated reduction in FoxM1 expression observed here. For example, FoxM1 was shown to stimulate invasion and angiogenesis in pancreatic cancer cells through induction of MMP-2, MMP-9, and VEGF (18). Our studies here are the first to show that FoxM1 is required for prostate cancer invasion and regulation of MMP-2 and VEGF. Moreover, our data are consistent with studies showing that FoxM1 overexpression coincides with metastasis of prostate cancer (25). In total, our data point to a mechanism of OGT-mediated invasion, angiogenesis, and metastasis regulation that is due, in part, to stabilization of FoxM1 expression in prostate cancer.

In summary, we have described a novel role for the metabolic sensor OGT in the growth, invasion, angiogenesis, and metastasis of human prostate cancer cells through, in part, the regulation of FoxM1 expression observed here. For example, FoxM1 was shown to stimulate invasion and angiogenesis in pancreatic cancer cells through induction of MMP-2, MMP-9, and VEGF (18). Our studies here are the first to show that FoxM1 is required for prostate cancer invasion and regulation of MMP-2 and VEGF. Moreover, our data are consistent with studies showing that FoxM1 overexpression coincides with metastasis of prostate cancer (25). In total, our data point to a mechanism of OGT-mediated invasion, angiogenesis, and metastasis regulation that is due, in part, to stabilization of FoxM1 expression in prostate cancer.

In summary, we have described a novel role for the metabolic sensor OGT in the growth, invasion, angiogenesis, and metastasis of human prostate cancer cells through, in part, the regulation of FoxM1 via modulation of downstream effectors, such as MMP-2, MMP-9, and VEGF. Our studies here are the first to show that FoxM1 is required for prostate cancer invasion and regulation of MMP-2 and VEGF. Moreover, our data are consistent with studies showing that FoxM1 overexpression coincides with metastasis of prostate cancer (25). In total, our data point to a mechanism of OGT-mediated invasion, angiogenesis, and metastasis regulation that is due, in part, to stabilization of FoxM1 expression in prostate cancer.

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OGT Regulation of Prostate Cancer

drug design of novel OGT inhibitors will be greatly facilitated. These data suggest OGT as a novel target in the treatment of prostate cancer progression and metastasis.

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