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O-GlcNAcylation promotes malignant phenotypes of bladder cancer cells

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O-GlcNAcylation (O-GlcNAc) is a posttranslational modification that is mediated by O-GlcNAc-transferase (OGT) and reversed by O-GlcNAcase (OGA). Increasing evidence indicates that protein O-GlcNAcylation is increased in various types of cancer. In the present study, we aimed to evaluate the expression and function of both OGT and OGA in bladder cancer cells *in vitro* and *in vivo*. Expression data of OGT and OGA at the mRNA level was obtained from the Oncomine database. Effects of OGT and OGA on cell proliferate, invasive, and migratory abilities were assessed using MTT, wound healing, cell invasive assay, and cell cycle analysis. *In vivo* assay was also performed in nude mice. The results revealed that the expression of OGT in bladder cancer tissues was higher than that of normal tissues, while the OGA level was found to be lower in cancer tissues. We also found that knockdown of OGT could inhibit cell proliferation, migration, invasion, and induce cell cycle arrest, while these are reversed when OGA is inhibited. We also observed that O-GlcNAcylation could promote tumor formation *in vivo*, compared with a negative control. In summary, this study describes the oncogenic role of O-GlcNAcylation in bladder cancer cells.

Key words: O-GlcNAcylation, OGT, OGA, bladder cancer

Bladder cancer is the second most common genitourinary malignancy and accounts for approximately 20% of incidence and mortality from genitourinary tumors worldwide [1]. More than 95% of bladder cancers are urothelial carcinomas arising from transitional epithelial tissue. Patients with bladder cancer are staged based on the invasiveness of cancer. Unfortunately, non-invasive bladder carcinoma (NMIBC) may gradually progress into muscle invasive bladder carcinoma (MIBC), which accounts for 25% of bladder cancer cases, with five-year survival rates of less than 60% [2, 3]. However, little progress had been made regarding the treatment of bladder cancer until recently.

Like most cancers, bladder cancer cells are acquisitive of nutrients, including glucose and glutamine, and primarily produce their energy through aerobic glycolysis, also termed the Warburg effect, which is different from that of normal cells, which produce energy through oxidative phosphorylation that efficiently produces adenosine triphosphate (ATP) [4]. However, aerobic glycolysis confers cancer cells the advantage of proliferating through an increase in the generation of additional metabolites [4]. In addition to its role in the biosynthesis of cancer cells, glucose and glutamine are implicated in O-linked-β-N-acetylglucosamine modification of proteins (O-GlcNAcylation), a post-translational modification that proceeds through the hexosamine biosynthetic pathway (HBP) [5]. As the final product of HBP, UDP-N-acetylglucosamine (UDP-GlcNAc) is in turn used as a substrate with serine or threonine residues by O-GlcNAc-transferase (OGT). O-GlcNAcylation of proteins is a reversible modification in which the GlcNAc moiety can be removed from O-GlcNAc modified proteins by glycoside hydrolase O-GlcNAcase (OGA). Sometimes O-GlcNAcylation can regulate protein function in competition with phosphorylation sites on the same protein.

Increasing evidence indicates that protein O-GlcNAcylation is increased in different types of cancer and that it may be involved in tumor development and progression. At the same time, dysregulation of OGT and/or OGA expression has been reported in various cancers, including bladder cancer [6–8].

Wang et al. recently found that repression of OGT expression inhibits bladder cancer cell proliferation [9]. In addition to inhibition of OGT function, O-GlcNAcylation also can be suppressed by 6-Diazo-5-oxo-L-norleucine (DON),

a glutamine antagonist, by inhibiting the production of UDP-GlcNAc in HBP [10]. On the contrary, O-GlcNAcylation can be enhanced by Thiamet G, an inhibitor of OGA [11]. In the present study, we evaluated the role of O-GlcNAcylation in bladder cancer using pharmacological or genetic methods and found that O-GlcNAcylation could markedly enhance cell growth and invasion.

Patients and methods

Oncomine. The Oncomine database (www.oncomine.org) was used to analyze the expression of *oga* and *ogt* mRNA in superficial bladder cancer, infiltrating bladder cancer and normal bladder tissues.

Sample collection. A total of 21 paired bladder cancer tissues and normal adjacent tissues (located 2 cm outside of the visible lesions) were collected in the Second Affiliated Hospital of Soochow University between January 2017 and December 2017. All the tissues were immersed in RNAlater (Thermo Fisher Scientific, Inc., USA) and stored in liquid nitrogen. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. Written informed consent was obtained from all patients.

RNA extraction, cDNA synthesis, and real time-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Thermo Fisher Scientific) was used to extract RNA from cells and tissues according to the manufacturer's instructions. Then 1 μg RNA was used to get cDNA with performing reverse transcription using the PrimeScript™ RTreagent kit (Takara Bio, Inc.). Subsequently, RT-qPCR was performed with Premix Ex Taq™ II (Takara Bio, Inc.) on a Roche light cycler 480 Real-Time PCR system (Roche Applied Science, Penzberg, Germany). GAPDH was used as an internal control and the expression was analyzed via the $2^{-\Delta\Delta Cq}$ method. RT-qPCR analysis was performed in triplicate.

Antibodies, chemicals, and plasmids. The antibody against GAPDH (#5174) for immunoblot was purchased from Cell Signaling Technology (Danvers, MA, USA). The monoclonal antibody against O-GlcNAc (RL2, #MA1072) for immunoblot and immunohistochemistry was purchased from Thermo Fisher Scientific (Rockford, IL, USA). DON and TG were purchased from Sigma-Aldrich (St. Louis, MO, USA). The pcDNA6.2-myc construct containing OGT, OGA or null control, and the pLKO shRNA construct containing OGT, OGA or negative control, were purchased from Addgene (Cambridge, MA, USA).

Cell lines and DNA transfection. Bladder cancer cell line 5637 and RT4 (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, in a humidified incubator containing 5% CO_2 at 37 °C. 5637 and RT4 cells were transfected with shRNA-OGT, shRNA-OGA, shRNA-CTRL or OGT-OE, OGA-OE, CTRL to establish stable cell lines.

Immunoblotting. Immunoblotting was performed as previously described [12, 13]. The cell lysate was prepared by extracting proteins with RIPA buffer (Fermentas, Ontario, Canada) supplemented with 1% protease inhibitors (Sigma). The cells were lysed in boiling sample buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted for the proteins of interest. Primary antibody (anti-GAPDH or O-GlcNAc) at a 1:1,000 dilution was used for immunoblot analysis. Secondary antibody (goat anti-mouse IgG (H+L, #A32729, Thermo Fisher Scientific, 1:5,000 dilution) was used for the immunoblot analysis. Membranes were blocked with 0.25% gelatin in TBST, followed by incubation with primary antibodies overnight at 4 °C. Membranes were washed three times with TBST, followed by the incubation with secondary antibody for 2 h at room temperature. Membranes were washed three times with TBST and developed with enhanced chemiluminescence (Fermentas). A total of 30 mg of protein was used for immunoblotting, unless indicated otherwise. GAPDH was used as a loading control.

MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The MTT assay was carried out as previously described [13] to assess the proliferation of bladder cancer cells.

Cell colony formation assays. After transfection for 48 h, approximately 600 5637 or RT4 cells (PBS, DON, TG; sh-CTRL, sh-OGT, sh-OGA; CTRL, OGA-OE, OGT-OE) were suspended in 2 ml of medium and then plated in six-well plates (500 cells/well) in triplicates. The cells were maintained in a moist atmosphere (5% CO_2) at 37 °C for 14 days. Following fixation, the cells were stained using Giemsa solution for 20 min. The colonies that were 50 μm in diameter or larger were counted and photographed.

Cell cycle analysis. 70% ethanol was used to fix cells overnight at −20 °C. Then, the cells were treated with a DNA staining solution containing 3.4 mM Tris-Cl (pH 7.4), propidium iodide, 0.1% Triton X-100 buffer and 100 mg/ml RNase A. The cell cycle was analyzed using flow cytometry (BD, USA).

Wound healing assay. A wound healing assay was performed to assess the cell migratory ability. Approximately 600,000 cells were seeded into a 6-well plate. 24 h later, the cells were transfected with sh-OGT, sh-OGA or sh-NC. After a further 6 h, an artificial wound was created by scratching on the well surface with a 200 μl pipette tip. We supplied pure DMEM medium without fetal bovine serum to incubate cells for 24 h before the assessments were performed. A digital camera system was used to acquire images of the scratches at 0 and 24 h. The experiments were performed in triplicate and repeated at least three times.

Cell invasion assay. The invasive ability of the cells was assessed using Transwell chambers (6.5 mm; Corning, NY, USA) with 8 μm pore membranes, with the upper face of the membrane covered with 70 μl Matrigel (1 mg/ml, BD Biosciences, NJ, USA). The lower chamber was filled with 600 μl of

lower medium (medium with 20% FBS). The cells (2×10^4) were suspended with 100 µl of upper medium (medium with 1% FCS) and plated into the upper chamber. After 20 h, the number of cells showing crystal violet staining on the undersurface of the polycarbonate membrane was scored visually in five random fields at ×100 magnifications, which was used to indicate the invasive ability. All experiments were conducted in triplicate.

Tumorigenesis in nude mice. 5637 and RT4 cells (sh-CTRL, sh-OGT, sh-OGA; 1×10^7 cells/ml) were injected subcutaneously into the right shoulder of nude mice (6-week-old, 18 male mice and 3 in each group, with the weight of about 16–20 g). The nude mice were purchased from the Institute of Zoology, the Chinese Academy of Sciences of Shanghai. After injection, the mice were housed at the temperature of 26 °C with a relative humidity of 45–50% and an aseptic environment. Enough illumination (12 h for light/dark cycle), food and water were provided. Six weeks later, the mice were sacrificed, and the tumor that had developed in each mouse was removed and weighed. Tumor volume was calculated with the formula, $0.5 \times \text{length} \times \text{width}^2$. All animal protocols were approved by the Animal Care Committee of the Second Affiliated Hospital of Soochow University.

Immunohistochemistry. Immunohistochemistry was performed on the TMAs using a DAKO Liquid DAB Substrate Chromogen System (DakoCytomation, CA, USA) and an antibody against O-GlcNAc (RL2). The Fromowitz standard was used to semi-quantitatively assess the staining of O-GlcNAcylation.

Immunofluorescence. Fixation, permeabilization and blocking were performed as described above. The sections were stained with a primary antibody (anti-LC3, #12741, Cell Signaling Technology, Danvers, MA, USA), at a dilution of 1:200 in PBS at 4 °C overnight, and incubated the cells with a secondary antibody (anti-rabbit IgG (H+L), #4414, Cell Signaling Technology) for 1 h at room temperature in the dark, at a dilution of 1:500. The nuclei were hematoxylin counterstained. Indirect immunofluorescence examination was performed using a NIKON ECLIPSE TE2000-Efluorescence microscope.

Statistical analysis. ANOVA was used for statistical analyses between three groups. The effect of each treatment was then compared with the control using unpaired t-test or Dunnett t-tests for multiple comparisons. A p-value <0.05 was considered to be statistically significant. Statistical analyses were performed using IBM SPSS Statistics software version 22 (IBM SPSS, Armonk, NY, USA).

Results

***Ogt* mRNA is overexpressed in bladder cancer tissues.** Based on the sequencing data of bladder cancer tissues and normal tissues obtained from Oncomine, it was shown that the expression of *ogt* in both superficial and infiltrating bladder cancer tissues is significantly higher than that in normal bladder tissues, and the fold change in superficial and infiltrating bladder cancer tissues was 2.46 and 1.45, respectively, of normal tissues (Figure 1A, p<0.0001). The expression of *oga* in superficial and infiltrating bladder cancer tissues is significantly lower than that of normal bladder tissues, and the fold change in superficial and infiltrating bladder cancer tissues was –1.46 and –1.60, respectively, of normal tissues (Figure 1B). Results of RT-qPCR in tissues also revealed that relative expression of *ogt* mRNA in bladder cancer tissues was higher than which in normal tissues (5.98±0.33 vs. 1.00±0.54, p<0.001, Supplementary Figure S1). And expression of *oga* mRNA in bladder cancer tissues is lower than normal tissues (0.19±0.22 vs. 1.00±0.40, p<0.001, Supplementary Figure S1).

Validation of knockdown of targeted genes. RT-qPCR and western blot were performed to validate the knockdown rate of OGT and OGA. The results revealed that the expression of *ogt* and *oga* mRNA was significantly lower in cells transfected with shRNA compared with cells transfected NC. In Supplementary Figure S2A, after transfection of shRNA, expression of *ogt* mRNA in cells transfected with sh-ogt1 vs. sh-ogt2 vs. shNC was 0.21±0.02 vs. 0.25±0.04 vs. 1.01±0.17 for 5637 cells and 0.12±0.01 vs. 0.13±0.03 vs. 1.00±0.05 for RT4 cells. In Supplementary Figure S2B, expression of *oga* mRNA in cells transfected with sh-oga1 vs. sh-oga2 vs. shNC was 0.11±0.02 vs. 0.02±0.00 vs. 1.00±0.01 for 5637 cells and 0.25±0.03 vs. 0.03±0.01 vs. 1.04±0.11 for RT4 cells (**p<0.01, ***p<0.001). Results of western blot indicated similar results, which were shown in Supplementary Figures S2C and S2D; sh-oga2 and sh-ogt2 were used in the following research.

O-GlcNAcylation accelerates bladder cancer cell viability and proliferation. O-GlcNAcylation levels were found to be altered in 5637 bladder cancer cells when analyzed using pharmacological or genetic methods (Figure 2A), which indicated the effect of treating bladder cancer with chemicals, such as 6-diazo-5-oxo-norleucine (DON), thiamet-G (TG) or by manipulation of O-GlcNAc transferase (OGT) or O-GlcNAcase (OGA). In order to determine whether O-GlcNAcylation affects cell viability, 5637 and RT4 cells were treated with PBS, DON (50 µM) or TG (10 µM), and 48 h later an MTT assay was done. As shown in Figure 2B, cell viability in the DON group was notably inhibited, while there was some increase in the TG group, compared with the PBS group. Similarly, cell viability in sh-OGT and OGA-OE cells (5637 and RT4) was obviously inhibited, and some promotion was found in sh-OGA and OGT-OE cells (both 5637 and RT4), compared with the control groups (Figures 2C, 2D). Next, cell proliferation was detected using a colony formation assay in 5637 and RT4 cells with different O-GlcNAcylation conditions. As shown in Figures 2E and 2F, the colony numbers of the lower O-GlcNAcylation groups were lower than that of the control ones. On the contrary, the higher O-GlcNAcylation groups showed much higher colony numbers.

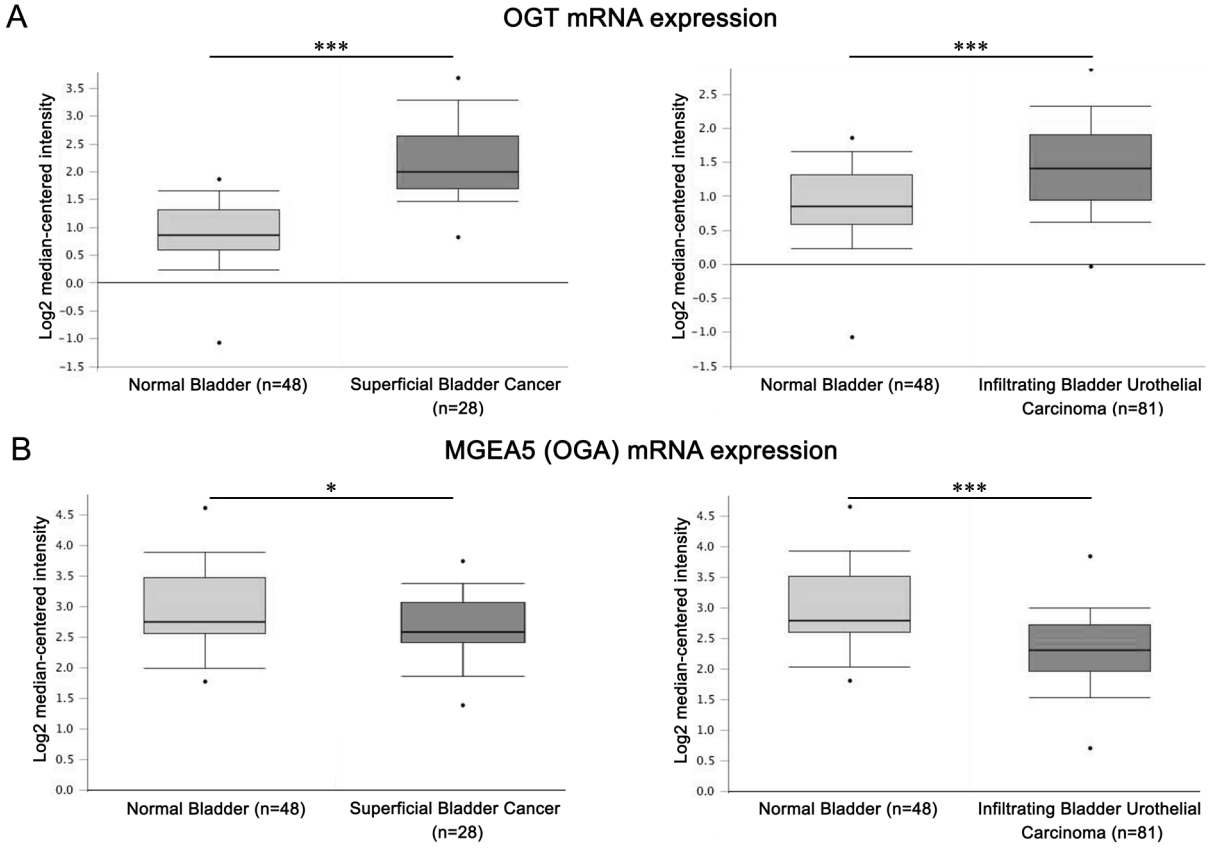

Figure 1. Expression of OGT and OGA at mRNA level in tissues from the Oncomine database. A) Expression of OGT at mRNA level is higher in bladder cancer tissues than that of normal tissues. B) Expression of OGA at mRNA level in bladder cancer tissues is lower than that of normal tissues. (*p<0.05, ***p<0.0001)

O-GlcNAcylation promotes migration and invasion of bladder cancer cells. In order to determine whether O-GlcNAcylation is associated with cellular migration, we detected cell migration ability in the context of altered O-GlcNAcylation, using a monolayer wound healing assay. The cells with lower O-GlcNAcylation migrated slower than control ones, while the higher O-GlcNAcylation cells migrated faster (Figure 3A). We used the Transwell invasion assay to mimic the basement membrane and examined the role of O-GlcNAcylation on cell invasion *in vitro*. Compared with the control, the lower O-GlcNAcylation cells significantly inhibited their ability to invade Matrigel, whereas higher O-GlcNAcylation cells invaded Matrigel faster (Figures 3B, 3C).

O-GlcNAcylation modulates the bladder cancer cells cycle. In order to determine whether inhibition of cell proliferation by low O-GlcNAcylation is related to cell cycle arrest, the phase distribution of the cell cycle was analyzed using flow cytometry. We found that the 5637/sh-OGT group had a larger majority of cells that stayed at the G0/G1 phase, a significant decrease in the G2/M phase, and no obvious changes in the S phase. Compared with the control, the 5637/sh-OGA group had fewer cells that stayed at the G0/G1 phase, an increase in the G2/M phase, and no obvious change in the S phase (Figure 4A). Similar results were obtained using RT4 cells (Figure 4B).

O-GlcNAcylation promotes tumor formation. In order to test whether O-GlcNAcylation regulates tumor formation *in vivo*, 5637 and RT4 cells that stably expressed OGT shRNA (sh-OGT), OGA shRNA (sh-OGA) or control shRNA (sh-CTRL) were subcutaneously injected into nude mice. Tumor formations were periodically monitored (at least every 2 days). After animals were sacrificed, the tumors were excised. Images of the tumors are shown in Figures 5A and 5B. Median tumor weight was 1.81±0.16 g for 5637/sh-CTRL mice, a lower value of 0.98±0.11 g for 5637/sh-OGT mice ($p<0.01$), and a higher value of 2.30±0.27 g was obtained for 5637/sh-OGA mice ($p<0.05$). We also obtained similar results using RT4 cells: RT4/sh-CTRL 1.77±0.17 g, RT4/sh-OGT 0.92±0.15 g ($p<0.01$, compared with sh-CTRL) and RT4/sh-OGA 2.18±0.20 g ($p<0.01$, compared with sh-CTRL) (Figure5C).

We also measured tumor volume. Median tumor volume was 996.61±42.80 mm^3 for 5637/sh-CTRL mice, while a

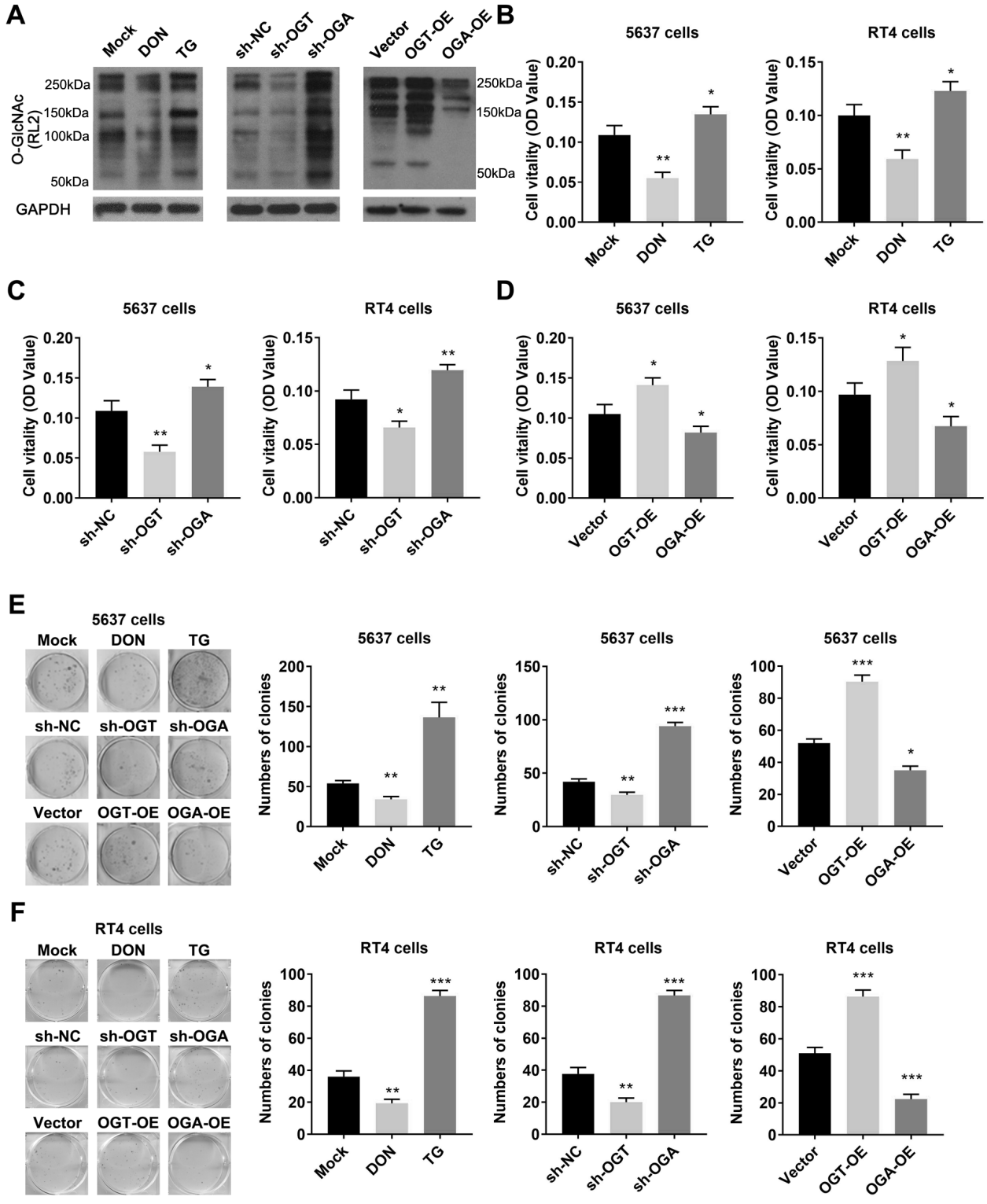

Figure 2. Expression of O-GlcNAc in bladder cancer cells. A) Expression of O-GlcNAc in 5637 cells transfected with sh-OGT/sh-OGA/sh-NC/OGT-OE/OGA-OE/Vector or treated with TG/DON/Mock. GAPDH was used as an internal control. The results show that TG, sh-OGA, and OGT-OE could promote the expression of O-GlcNAc, and DON, sh-OGT and that OGA-OE could inhibit the expression of O-GlcNAc. B–D) Results of the MTT assay revealed that the overexpression of O-GlcNAc could promote the proliferation of 5637 and RT4 cells, while knockdown of O-GlcNAc inhibits the same. E, F) Similar results were observed using the colony formation assay. The colony numbers in lower O-GlcNAcylation groups were less than that of the control group. On the contrary, the higher O-GlcNAcylation groups had a higher colony number. (*p<0.05, **p<0.01)

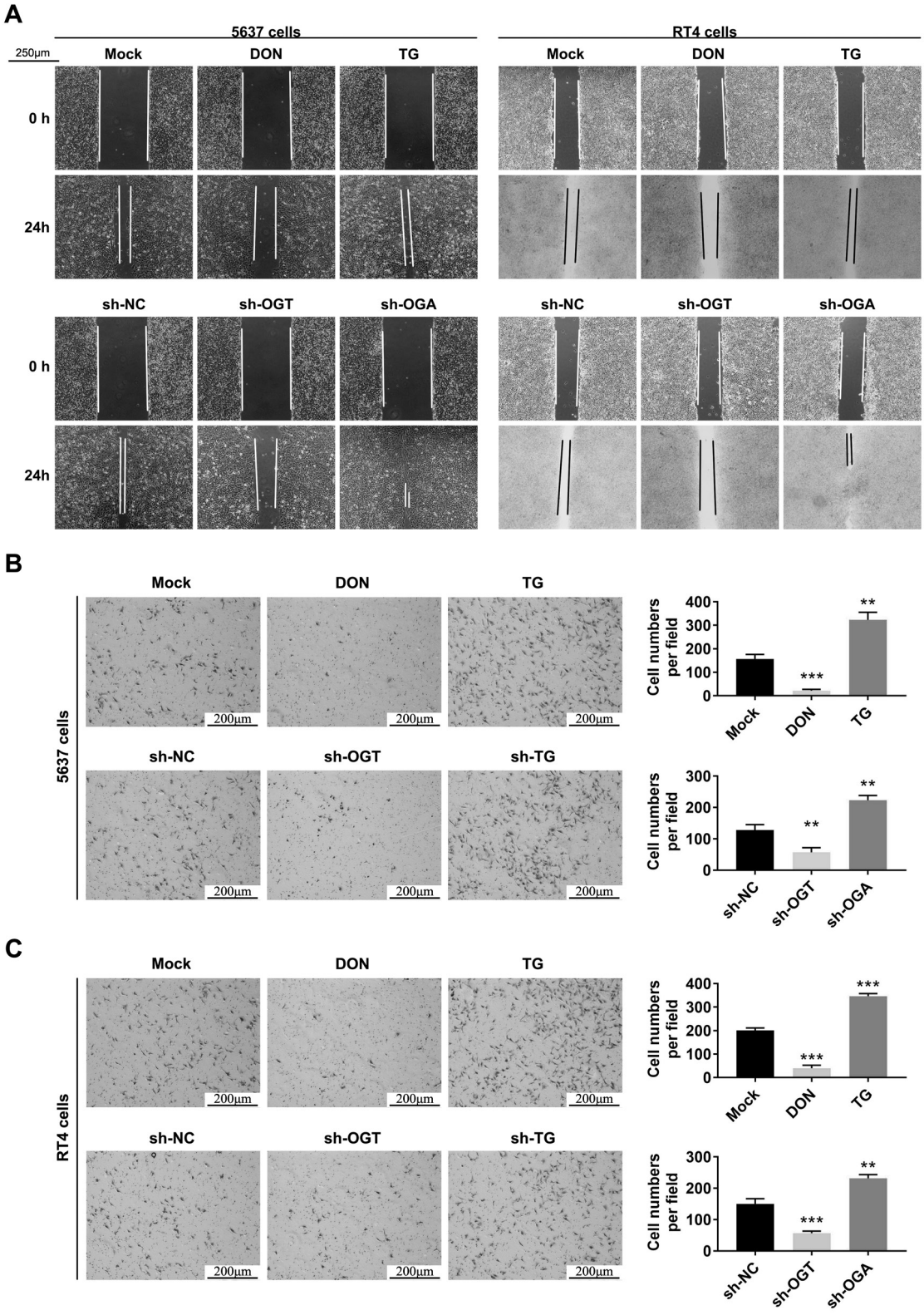

Figure 3. Results of cell migratory and invasive assay. A) Results of the wound healing assay revealed that inhibition of O-GlcNAcylation by transfection with sh-OGT or DON inhibits the migratory ability of 5637 and RT4 cells, while the overexpression of O-GlcNAcylation by transfection with sh-OGA or TG promotes the migration ability of 5637 and RT4 cells. B, C) The invasive assay indicates that O-GlcNAc could promote the invasive ability of 5637 and RT4 cells, while its knockdown inhibits it (p<0.01, ***p<0.0001)**

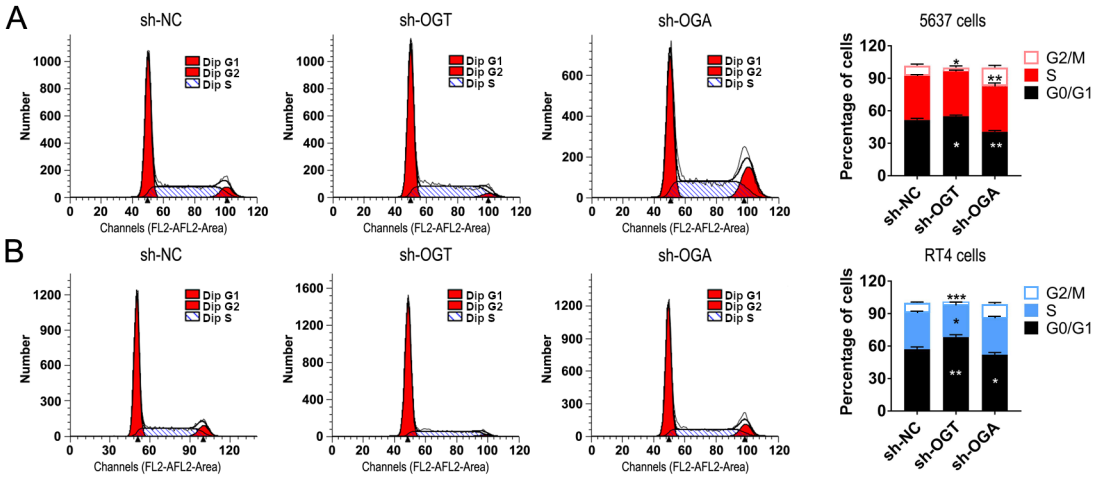

Figure 4. Results of cell cycle analysis using flow cytometry. Inhibition of O-GlcNAcylation by transfection with sh-OGT induced cell cycle arrest, and the overexpression of O-GlcNAcylation by transfection with sh-OGA promotes cell proliferation A) Results in 5637 cells. B) Results in RT4 cells. *p<0.05, **p<0.01)

lower value of 542.40±46.57 mm^3 for 5637/sh-OGT mice and a higher value of 1269.42±155.13 mm^3 was obtained for 5637/sh-OGA mice. We also obtained similar results using RT4 cells: RT4/sh-CTRL 1068.23±147.78 mm^3, RT4/sh-OGT 573.84±100.61 mm^3 and RT4/sh-OGA 1371.80±124.46 mm^3. Similarly, a significant difference in tumor volume was observed among them ($p<0.05$) (Figure 5D). These results indicate that O-GlcNAcylation significantly promotes tumor formation.

Conversely, O-GlcNAcylation regulates the autophagic flux *in vivo*. In addition, the O-GlcNAc (RL2) and LC3-GFP regions were determined in tumor tissues. As shown in Figure 5E, O-GlcNAc decreased in tumors derived from sh-OGT 5637/RT4 cells, compared with that of = sh-CTRL 5637/RT4 cells. In contrast, O-GlcNAc in sh-OGA tumors increased compared with that of the sh-CTRL tumor, which indicates that nude mice models with varying O-GlcNAcylation levels were successfully formed. As shown in Figure 5F, the expression of LC3-GFP, which is amplified in sh-OGT tumors, was found to be reduced in sh-OGA tumors, compared with that of the sh-CTRL tumor. These results support the hypothesis that O-GlcNAcylation may conversely regulate autophagic flux. All protocols involving the use of animals were approved by the Animal Care and Use Committee of our Institution.

Discussion

Nutritional conditions may affect different signaling pathways in tumor cell metabolism. Bladder cancer cells exert a large demand for nutrients from their environment, which leads to an altered metabolic state. In cancer cells, increased glucose intake contributes to increased HBP flux, which also increases the O-GlcNAcylation level of cancer cells. Total O-GlcNAcylation has been found to be increased in multiple cancer cells, including bladder cancer, prostate cancer and breast cancer [9, 14, 15]. O-GlcNAcylation always plays an oncogenic role in these tumors. The results of this study show that O-GlcNAcylation promotes bladder cancer cell proliferation, migration, and invasion, while these functions are reversed by OGA. It is also demonstrated that O-GlcNAcylation significantly enhances bladder cancer cell growth both *in vitro* and *in vivo*. Consistent with most other reports that O-GlcNAcylation tends to increase the entire cell cycle with a decrease at G1/S, our results show that O-GlcNAcylation enhances G2/M transition and cell cycle arrest at G1. Additionally, our results indicate that O-GlcNAcylation enhances the migration and invasion abilities of bladder cancer cells.

Previous studies have shown that hyper-O-GlcNAcylation or high levels of OGT mRNA are associated with poor prognosis of some tumors, which indicates their potential as biomarkers of prognosis [7, 15]. Furthermore, hyper-O-GlcNAcylation and overexpression of OGT are also found in different types of tumors, including prostate, breast, colon, liver, lung, bladder, and endometrial tumors [7, 14-19]. In the present study, we verified that the expression of *ogt* mRNA in bladder cancer tissues is higher than that of normal tissues, while *oga* mRNA expression in bladder cancer tissues is lower than that of normal tissues. Therefore, OGT may be a potential biomarker that can be used for bladder cancer.

The mechanism of O-GlcNAcylation and OGT regulation of cancer cells is always taught to be involved in the autophagic activity. In *Drosophila melanogaster,* it was found that O-GlcNAcylation and OGT could regulate autophagy through the Akt/dFOXO signaling [20], while in *Caenorhabditis elegans*, Guo et al. found that OGT could mediate O-GlcNAcylation of SNAP-29 and regulate cell autophagy [21]. Similar results have also been observed in human cells.

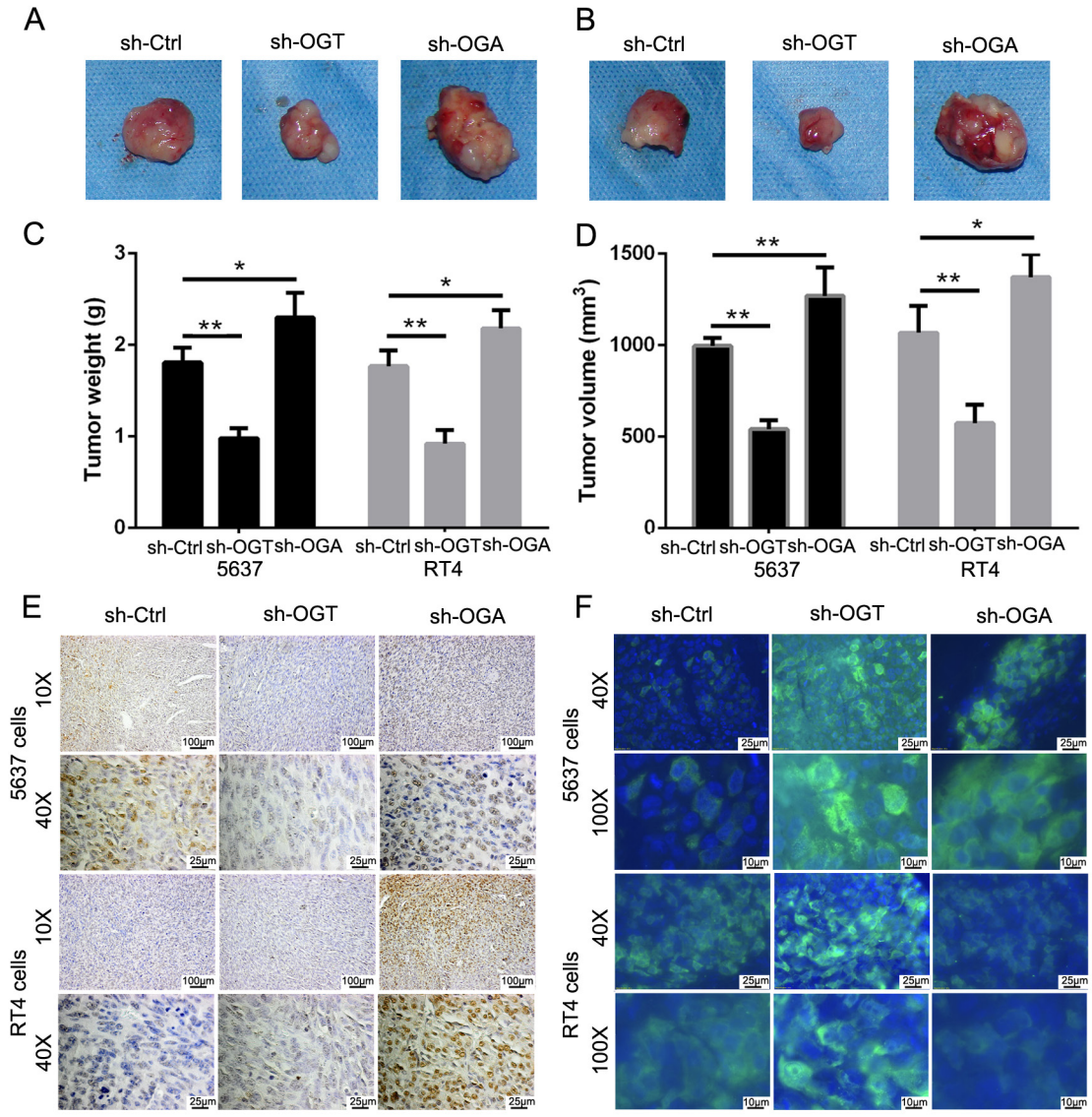

Figure 5. O-GlcNAc could promote bladder cancer development *in vivo*. A) Images of tumors excised from mice injected with sh-Ctrl, sh-OGT or sh-OGA 5637 cells. B) Images of tumors excised from mice injected with sh-Ctrl, sh-OGT or sh-OGA RT4 cells. C, D) Tumor weight and volume in mice of different groups (*p<0.05, **p<0.01). E) Expression of O-GlcNAc in tissues resected from mice of different groups. O-GlcNAc level is higher in mice injected with sh-OGA cells, compared with mice transfected with sh-Ctrl. F) Expression of LC3-GFP was amplified in sh-OGT tumors and reduced in sh-OGA tumors, compared with that of sh-CTRL, which reveals that lower O-GlcNAcylation may induce cell autophagy.

A recent study on the role of OGT in the liver revealed that phosphorylated OGT can promote O-GlcNAc modification and activate Ulk proteins by potentiating AMPK-dependent phosphorylation, and that ablation of OGT could reduce autophagic flux [16]. In ovarian cancer cells, downregulation of OGT induces cell autophagy through O-GlcNAc modification of SNAP-29, which contributes to cisplatin resistance [22]. OGT has also been found to regulate bladder cancer cell autophagy. Knockdown of OGT induces cell autophagy, which plays a 'pro-survival' role, while it also increases the sensitivity of bladder cancer cells to cisplatin, which is different from its function in ovarian cancer [9]. Importantly, our nude mice model confirms that O-GlcNAcylation negatively regulates autophagy in bladder cancer. Taken together, these results indicate that O-GlcNAcylation can advance bladder cancer formation and metastases, while also being able to negatively regulate the autophagy flux. Successful completion of this project will provide important information that can be used to validate the role of O-GlcNAc glycosylation in cancer initiation and progression through disruption of autophagy, and will eventually aid the development of novel, effective therapeutic approaches for bladder cancer.

In the present study, we first described the function of both, OGA and OGT, in bladder cancer cells. Knockdown of

OGT could inhibit cell proliferation, migration, and invasion, while knockdown of OGA performed an opposite function. Future studies will focus on the mechanism of OGA and OGT, and may provide a rationale for exploring the role of O-GlcNAcylation in cancer development and progression.

Supplementary information is available in the online version of the paper.

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O-GlcNAcylation promotes malignant phenotypes of bladder cancer cells

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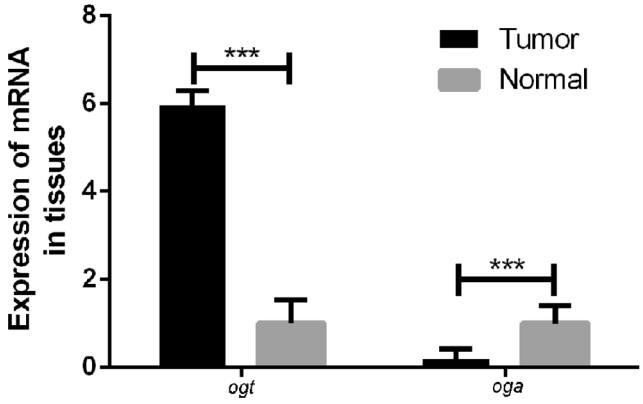
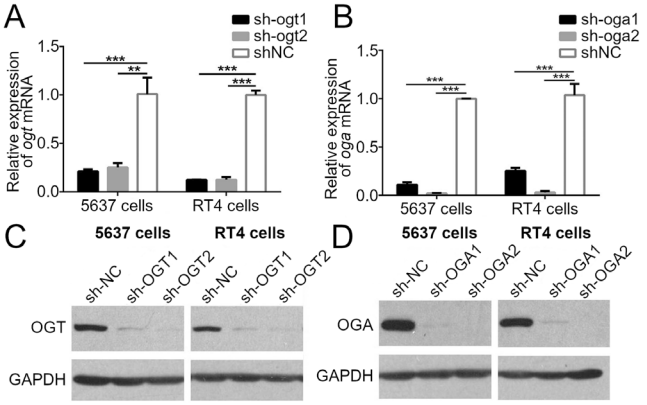
Supplementary Information



Supplementary Figure S1. Expression of *ogt* and *oga* mRNA in tissues. A) Results of RT-qPCR showed that expression of *ogt* mRNA in bladder cancer tissues was higher than that in paired normal tissues. B) Expression of *oga* mRNA in bladder cancer tissues is lower than that of normal tissues (*p<0.001)**



Supplementary Figure S2. Validation of knockdown of targeted genes. A, B) Expression of *ogt* or *oga* mRNA was significantly lower in cells transfected with shRNA (sh-ogt1/ sh-ogt2/ sh-oga1/ sh-oga2) compared with cells transfected NC. C, D) Results of western blot indicated similar results, OGT and OGA protein could be inhibited with transfection of shRNA (p<0.01, ***p<0.001)**