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Short communication

A living cell-based fluorescent reporter for high-throughput screening of anti-tumor drugs

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ABSTRACT

Suppression of cellular O-linked β-*N*-acetylglucosaminylation (O-GlcNAcylation) can repress proliferation and migration of various cancer cells, which opens a new avenue for cancer therapy. Based on the regulation of insulin gene transcription, we designed a cell-based fluorescent reporter capable of sensing cellular O-GlcNAcylation in HEK293T cells. The fluorescent reporter mainly consists of a reporter (green fluorescent protein (GFP)), an internal reference (red fluorescent protein), and an operator (neuronal differentiation 1), which serves as a "sweet switch" to control GFP expression in response to cellular O-GlcNAcylation changes. The fluorescent reporter can efficiently sense reduced levels of cellular O-GlcNAcylation in several cell lines. Using the fluorescent reporter, we screened 120 natural products and obtained one compound, sesamin, which could markedly inhibit protein O-GlcNAcylation in HeLa and human colorectal carcinoma-116 cells and repress their migration in vitro. Altogether, the present study demonstrated the development of a novel strategy for anti-tumor drug screening, as well as for conducting gene transcription studies.

1. Introduction

O-linked β-*N*-acetylglucosaminylation (O-GlcNAcylation) is a dynamic process that results in the modification of several nucleocytoplasmic proteins. The reversible O-GlcNAc balance mainly depends on the activity of a pair of highly conserved enzymes, namely O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), which catalyze the attachment of the monosaccharide (GlcNAc) or removal of the sugar moeity [1]. As the unique sugar donor, the amount of uridine diphosphate (UDP)-GlcNAc affects the control of the modification process [2], which can also modulate OGT activity through unknown mechanisms [3]. UDP-GlcNAc is an end product of the hexosamine biosynthesis pathway, which is controlled by a rate-limiting enzyme, glutamine-fructose-6-phosphate aminotransferase (GFAT) [4]. Therefore, various methods can be used to change the cellular O-GlcNAcylation status, including the expression of OGT, inhibition of OGA, treatment with glucosamine (GlcN) to increase cellular O-GlcNAcylation, inhibition of OGT and GFAT to reduce cellular O-GlcNAcylation [5,6].

Dysregulation of O-GlcNAcylation is involved in the development of various diseases, such as type 2 diabetes, neurodegenerative diseases, and cancer [7,8]. O-GlcNAcylation level is relatively high in a majority of cancer cells, such as in breast cancer [9], ovarian cancer, cervical cancer [10], lung cancer and colon cancer [11], and this is attributable to an increase in OGT and/or GFAT activity [12]. The elevated steady-state levels of O-GlcNAcylated targets observed in many cancers provide these cells with a selective advantage for their growth, metastasis, and immune evasion [13]. Pharmacological or biological inhibition of OGT/GFAT can notably repress cancer cell proliferation and migration [14–17]. Therefore, inhibition of OGT/GFAT with subsequent suppression of cellular O-GlcNAcylation is a new direction for cancer therapy.

Cells constantly sense and respond to extracellular/cellular changes through dynamic protein modification, such as phosphorylation and O-GlcNAcylation [18]. Notably, only a few transcription factors are modified by O-GlcNAc and exhibit different roles in downstream gene transcription [19]. For example, neuronal differentiation 1 (NeuroD1) and pancreatic and duodenal

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homeobox factor-1 (PDX-1) represent the two important transcription factors that control insulin gene transcription in pancreatic beta cells [20]. In O-GlcNAcylation states, the activity of NeuroD1 and PDX-1 toward insulin gene transcription is markedly increased [21,22]. We hypothesized that cellular O-GlcNAcylation might be reflected in insulin gene transcription.

The dual luciferase reporter assay is widely used in gene regulation studies. The routine dual luciferase reporter assay requires the transfection of two plasmids (reporter plasmid and reference plasmid) into target cells and the detection of chemiluminescence after cell disruption. Therefore, the routine method does not meet the requirements necessary for the conduction of a living cell-based assay or assays that require the use of repeatedly cultured cells. Thus so far, a more convenient nanoLuc reporter (Promega Inc.) has been developed based on the expression of a secretory enzyme, enabling the performance of living cell-based assays, but the inclusion of a specific substrate compound is necessary.

In this work, inspired by extensive studies conducted on insulin gene transcription, dual luciferase assay, and widespread use of fluorescent proteins in biological experiments, we designed a cell-based fluorescent reporter to indicate the cellular O-GlcNAcylation status. The fluorescent reporter mainly consisted of a reporter (green fluorescent protein (GFP)), an internal reference (red fluorescent protein (RFP)), and an operator (NeuroD1) (Scheme 1). The reporter was controlled by an insulin promoter, while the reference and operator were controlled by a human cytomegalovirus (CMV) promoter, with a self-cleaving "2A" peptide (P2A) linker between the two. Here, the operator served as a “sweet switch” to control GFP expression in response to cellular O-GlcNAcylation changes. Theoretically, the fluorescence intensity ratio of GFP/RFP (G/R) value would positively reflect the relative amount of O-GlcNAc NeuroD1 and the cellular O-GlcNAcylation status in specific cells.

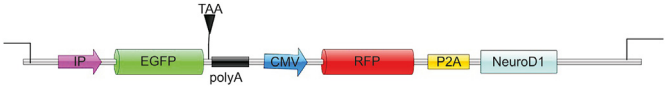
2. Materials and methods

2.1. Plasmid construction

The human *ogt* promoter (long promoter: −2000 to +200 bp; short promoter: −150 to +200 bp; referred to as transcript XM_017029908.1) was amplified from the genomic DNA of HEK293T cells using PCR; the promoter was cloned into the kpnI- and BglII-linearized PGL3-basic vector, or the fluorescent reporter using the ClonExpress II One Step Cloning Kit (C112-01, Vazyme, Nanjing, China). The human insulin promoter (−499 to +101 bp, referred to as transcript NM_000207.3), termination codon (TAA)/polyA, CMV/RFP, and P2A in the fluorescent reporter were sequentially used for construction of the pEGFP-c1 plasmid using the ClonExpress II One Step Cloning Kit. *NeuroD1* and *Pdx-1* genes were synthesized by Suzhou Jinweizhi Biotech Co., Ltd. (Suzhou, Jiangxi, China) and inserted into the pCMV-Tag 3 B vector using the ClonExpress II One Step Cloning Kit. All the primers used are listed in Table S1.

2.2. Fluorescent reporter assay and screening of natural products

HEK293T cells (4×10^4 cells/well) transfected with the fluorescent reporter (before or after optimization) were seeded in 96-well plates. After 12 h, the cells were treated with (αR)-α-[[(1,2-dihydro-2-oxo-6-quinolinyl)sulfonyl]amino]-N-(2-furanylmethyl)-2-methoxy-N-(2-thienylmethyl)-benzeneacetamide (OSMI-1), 6-diazo-5-oxo-L-norleucine (DON), Thiamet-G (TMG), O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc), or GlcN for 24 h. The fluorescence of GFP and RFP was measured using the Varioskan Flash multimode reader (Thermo Fisher Scientific, Waltham, MA, USA). The decrease in the fluorescence ratio of GFP/RFP (relative to the negative control) was used to indicate reduction in cellular O-GlcNAc. The excitation/emission wavelengths in the fluorescence assay were 485 nm/518 nm for GFP and 555 nm/584 nm for RFP, respectively.



Scheme 1. The structure of the cell-based fluorescent reporter. IP: insulin promoter; EGFP: enhanced green fluorescent protein; TAA: termination codon; polyA: polyadenylation; CMV: cytomegalovirus; RFP: red fluorescent protein; P2A: 2A peptides; NeuroD1: neuronal differentiation 1.

For screening of a natural product library, HEK293T cells were transfected with the optimized reporter in a 10-cm dish, and 24 h later, the cells were re-seeded in 96-well plates at a density of 4×10^4 cells/well. After a duration of 12 h, the cells were treated with various compounds (120 compounds) for 24 h, and the fluorescence (GFP/RFP) was measured as per methods described above.

2.3. Knockdown of ogt

We transfected HEK293T cells with scramble-short hairpin RNA (shRNA) (control) or OGT-shRNA (shOGT) [23] 12 h prior to the transfection with the reporter plasmid. After 36 h, the cells were examined with fluorescence microscopy and detected using the Varioskan Flash multimode reader or were subjected to disruption for conducting Western blotting analysis.

2.4. Transwell migration assay

Cell migration ability was detected using a 24-well plate and transwell chamber (0.4 μm pore size, Corning, Corning, NY, USA) with inserts precoated. Briefly, human colorectal carcinoma (HCT)-116 and HeLa cells (3×10^4) were seeded into the upper chamber in a serum-free medium with an inhibitor. Then, a complete medium (containing 20% fetal bovine serum and an equivalent concentration of inhibitor) was added to the lower chamber. After subjection to 24 h of treatment, cells on the upper surface of the membranes were removed using a cotton swab. Cells migrating to the pores of the underlying membrane were subjected to fixation with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of cells was counted under a fluorescence microscope with the selection of five random fields for each well.

2.5. Western blotting

Total proteins were extracted from HEK293T, HCT-116, or HeLa cells using a whole cell lysis assay kit (KGP2100, KeyGEN, Nanjing, China) and quantified using the BCA Protein Assay Kit (KGP902, KeyGEN, Nanjing, China).

All protein samples (25 μg) were subjected to separation using an 8% SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes (0.22 μM, Roche, Basel, Switzerland). The membranes were blocked using 5% skim milk and incubated with the primary antibody against O-GlcNAc (RL2, dilution of 1:5000, Abcam, Cambridge, MA, USA) or β-actin (dilution of 1:5000, Proteintech, Rosemont, IL, USA) overnight at 4 °C, followed by incubation with the secondary anti-mouse antibody (dilution of 1:5000, Sungene, Tianjin, China). The blots were visualized using an enhanced chemiluminescence detection system (Millipore, Burlington, MA, USA) on the Chemidoc XRS Gel Imaging System (Bio-Rad, Hercules, CA, USA).

2.6. Statistical analysis

All reporter assays were performed in triplicate (in parallel). Statistical significance was assessed using OriginPro 8.6, or the GraphPad Prism 5 software. The GraphPad Prism 5 software was used to calculate the EC_{50} value. A two-sample *t*-test was used to compare the differences between the groups. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Confirmation of the development of the dual fluorescent protein reporter

To test the fidelity of the conceptual dual fluorescent protein reporter, we compared its activity with that of a routine dual luciferase reporter assay. We have previously investigated *ogt* gene transcription in HEK293T cells using a routine method, which can report variant lengths of *ogt* promoters [24]. We assembled a long *ogt* promoter (−2000 to +200 bp) and a short promoter (−150 to +200 bp) before conducting assembly using the GFP reporter, and examined the reporter activity in HEK293T cells. As expected, the G/R value with the long promoter (−2000 to +200 bp) was markedly higher than that with the short promoter (−150 to +200 bp), and this was in line with the results obtained from the dual luciferase reporter assay (Fig. S1). As opposed to the tedious efforts engaged for conduction of a luciferase reporter assay, transfection of only one plasmid is necessary for the design and activity of the dual fluorescent protein reporter and the reporter could be tested in living cells with convenience and repeatability.

3.2. Examination of the cellular O-GlcNAc reporter system

We constructed an "insulin transcription reporter" by performing replacement of the *ogt* promoter with a human insulin promoter (Fig. 1A). The reporter was co-expressed with NeuroD1 or PDX-1 in HEK293T cells and the GFP/RFP fluorescence was determined 24 h after transfection. In contrast to the negative control co-expressed with an empty vector, co-expression with NeuroD1 or PDX-1 led to a significant increase in the G/R value, especially the NeuroD1 (Fig. 1B). These findings demonstrated the effectiveness of the insulin promoter, which exhibited better results with NeuroD1 than those observed with PDX-1 in HEK293T cells.

Thereafter, we examined the activity of the reporter in response to different cellular O-GlcNAcylation statuses. Co-expression of the insulin transcription reporter and the transcription factor (NeuroD1 or PDX-1) or empty vector in HEK293T cells was conducted, and treatment of cells with TMG (OGA inhibitor) in the presence of 3 mM glucose (Glc) or with OSMI-1 (OGT inhibitor) in the presence of 25 mM Glc was performed. Fig. 1C shows that TMG treatment markedly increased the levels of protein O-GlcNAcylation, while OSMI-1 caused a slight decrease. The reporter did not respond to

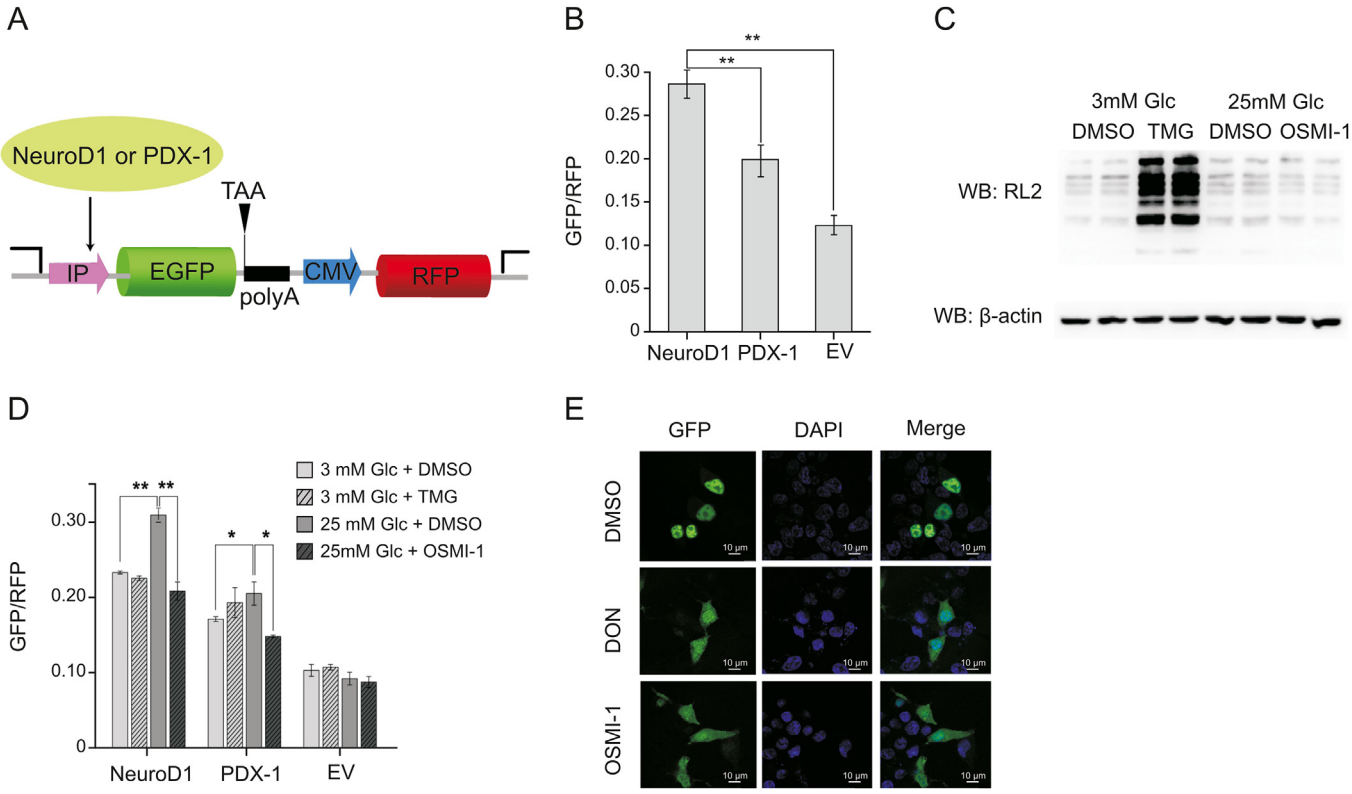


Fig. 1. (A) Diagram of the insulin reporter. (B) The insulin reporter was subjected to co-expression with NeuroD1 or PDX-1 in HEK293T cells. GFP/RFP fluorescence was measured and the influence of NeuroD1 or PDX-1 was compared to the vector control (EV) (n=3, $^{**}P < 0.01$). (C) The cells were treated as indicated, and cellular O-GlcNAcylation (Glc) level was detected via Western blotting. (D) The insulin reporter was subjected to co-expression with NeuroD1 or PDX-1 in HEK293T cells, and the cells were treated as indicated. GFP/RFP fluorescence was measured, and the influence of O-GlcNAcylation was depicted (EV) (n=3, $^{*}P < 0.05$, $^{**}P < 0.01$). (E) GFP-NeuroD1 was expressed in HEK293T cells and the cells were treated with 50 μM DON or 15 μM OSMI-1. Subcellular distribution of NeuroD1 was visualized via laser confocal scanning microscopy. PDX-1: pancreatic and duodenal homeobox factor-1; GFP: green fluorescent protein; DON: 6-diazo-5-oxo-L-norleucine; OSMI-1: (αR)-α-[[(1,2-dihydro-2-oxo-6-quinolinyl)sulfonyl]amino]-N-(2-furanylmethyl)-2-methoxy-N-(2-thienylmethyl)-benzeneacetamide; EV: empty vector; WB: Western blotting; DMSO: dimethyl sulfoxide; TMG: Thiamet-G; RL2: recombinant lactaptin; GlcN: glucosamine; DAPI: 4',6-diamidino-2-phenylindole.

cellular O-GlcNAcylation changes when the reporter was co-expressed with the vector. In the presence of NeuroD1 or PDX-1, the G/R value showed a marked decrease after OSMI-1 treatment, but the reporter did not respond to an increase in O-GlcNAcylation after TMG treatment (Fig. 1D).

To address the decrease in the G/R value in response to OSMI-1 treatment, we detected the subcellular localization of GFP-NeuroD1 in HEK293T cells. When the cells were treated with OSMI-1 or DON (GFAT inhibitor), NeuroD1 markedly reduced its nuclear entry (Fig. 1E), and this was in line with the previously reported findings based on studies conducted using MIN6 cells [21].

To confirm the findings after TMG treatment, we treated the cells with GlcN or PUGNAc. GlcN can increase cellular O-GlcNAcylation levels by increasing the UDP-GlcNAc content, while PUGNAc is another OGA inhibitor that efficiently enhances insulin transcription in MIN6 cells [25]. Although both treatments considerably elevated cellular O-GlcNAcylation levels, no definite influence was observed in the fluorescence readout (Fig. S2). Since the transcription of insulin was markedly elevated in MIN6 cells after PUGNAc treatment, we inferred that the upregulation might depend on the presence of other transcription factors or cofactors.

The above-mentioned findings indicated that the co-expression of the insulin transcription reporter and NeuroD1 in HEK293T cells might prove to be useful in reporting the reduction in cellular O-GlcNAcylation levels.

3.3. Optimization and verification of the cellular O-GlcNAc reporter

"2A" peptide is a class of 18–22 amino acids-long peptide derived from the genome of virus, which is widely used in multi-gene expression systems [26]. Herein, we aimed to utilize the peptide to achieve transfection of the insulin transcription reporter and NeuroD1 into a single plasmid, to simplify the transfection process. After a preliminary attempt with two "2A" peptides (P2A or T2A), we found that P2A could efficiently separate GFP from NeuroD1 in the expression of GFP-2A-NeuroD1 (data not shown). To render sensing capability to an inducible NeuroD1 for the detection of slight changes in the level of O-GlcNAcylation, NeuroD1 was initially assembled after GFP, with the insertion of the P2A between them. However, this design resulted in less remarkable GFP fluorescence (data not shown), probably due to the lack of NeuroD1, which is necessary to initiate the transcription of GFP. Thereafter, we placed NeuroD1 after the RFP. In this case, the RFP gene and NeuroD1 were controlled by the CMV promoter. Adequate NeuroD1 expression was observed using P2A (Fig. S3). To this end, we developed an optimized reporter (Scheme 1).

Next, we performed *ogt* knockdown to reduce cellular O-GlcNAcylation levels to validate the optimized reporter. Fig. 2A illustrates that shOGT remarkably decreased the amount of OGT, as well as cellular O-GlcNAcylation levels. Accordingly, less GFP fluorescence was visualized with fluorescence microscopy (Fig. 2B), and lower G/R values were obtained from the fluorescence readout (Fig. 2C), which confirmed the effectiveness of the optimized reporter.

Thereafter, we examined the OGT and GFAT inhibitors mentioned above using the optimized reporter. We transfected the reporter into HEK293T cells and treated the cells with either OSMI-1 or DON for 24 h. As shown in Fig. 2D, OSMI-1 or DON treatment led to a significant decrease in G/R values, which was in line with the decrease in cellular O-GlcNAcylation levels (Fig. 2E). To calculate the EC_{50} values, we treated the cells with varying concentrations of OSMI-1 or DON. The G/R value gradually decreased with increasing concentrations of OSMI-1 or DON, but was more pronounced than that revealed by Western blotting (Fig. S4). Eventually, the EC_{50} value of OSMI-1 was estimated to be 5.93 μM (Fig. 2F), which was comparable to its in vitro IC_{50} value (2.7 μM) reported previously [27], and the EC_{50} value of DON was estimated to be 33.08 μM (Fig. 2G). These findings suggested the versatility and utility of the reporter for the detection of variant inhibitors.

We also tested the utility of the reporter in other cells, including HepG2 and N2a cell lines. We transfected the reporter into these cells and treated them with DON or OSMI-1. We found that the reporter could respond to the treatments in both cell lines, although the signal difference before and after the conduction of treatments was more pronounced in HepG2 cells (Fig. 2H) than in N2a cells (Fig. 2I), indicating the wide adaptability of the reporter.

3.4. Application of the reporter for antitumor drug screening

Next, we used a reporter in HEK293T cells for the study of natural products. Using OSMI-1 as a positive control, we screened our in-house natural product library (120 compounds). Most compounds (103 of 120) in the library exhibited no detectable influence on the G/R value (data not shown). Seventeen compounds (Fig. 3A, Table S2) led to a decrease in the G/R value, especially ZQN-17, suggesting its potential to reduce cellular O-GlcNAcylation levels.

We then treated HeLa and HCT-116 cells with 20 μM ZQN-17 and examined its influence on cellular O-GlcNAcylation levels. Fig. 3B shows that ZQN-17 markedly reduced cellular O-GlcNAcylation levels in both cell lines, substantially supporting the findings of the reporter assay.

Since the elevation of cellular O-GlcNAcylation levels in certain cancer cells (including HeLa and HCT-116) contributes to their migration [10,11,14], we hypothesized that treatment with ZQN-17 might suppress their migration. To validate this hypothesis, we performed a transwell migration assay using HeLa and HCT-116 cells. We treated HeLa and HCT-116 cells with 20 μM ZQN-17. Figs. 3C and D show shows that ZQN-17 notably inhibited the migration of HeLa and HCT-116 cells (~46% and ~52%, respectively), which partly demonstrated its antitumor activity. Thereafter, we confirmed the identity of ZQN-17 through NMR and HRMS(ESI) analyses, and it was identified as sesamin (Fig. 3E, Figs. S5–S8).

Sesamin is a well-known antitumor molecule that has garnered increasing attention in recent years [28,29]. Sesamin can modulate cancer cell progression via the Janus kinase 2/signal transducers and activators of transcription (JAK2/STAT3) pathway in colorectal cancer [30]. In HeLa and SiHa cells, sesamin induced p53 phosphorylation at Ser-46 and Ser-15 and upregulated the levels of p53 upregulated modulator of apoptosis, Bax, and phosphatase and tensin homolog (PTEN), while inhibiting protein kinase B (AKT) phosphorylation at Ser-473 [31]. Sesamin can also trigger down-regulation of programmed death-ligand 1 expression through the inhibition of AKT, NF-κB, and JAK/STAT signaling in MDA-MB231 cells [32]. The antimetastatic effects of sesamin were also reported in human head and neck squamous cell carcinoma, which was mediated via the regulation of matrix metalloproteinase-2 [33]. These findings demonstrated that sesamin exerted its anti-cancer effects through various signaling pathways, including p53, AKT, STAT3, and PTEN.

The O-GlcNAcylation of a few proteins and the role of the modifications have been well documented. For example, STAT3 has been identified as an O-GlcNAc protein [34], and augmented cellular O-GlcNAcylation by GlcN can inhibit phosphorylation of STAT3 and exert anti-cancer effects on prostate cancer [35]. It is known that sesamin inhibits AKT phosphorylation at Ser-473 [31], while hyper-O-GlcNAcylation increases AKT phosphorylation at Ser-473 and stimulates proliferation in 8305C cells [36]. We demonstrated that sesamin repressed protein O-GlcNAcylation in HeLa and HCT-116 cells, which might reduce AKT phosphorylation at Ser-473 and suppress cell proliferation and/or migration.

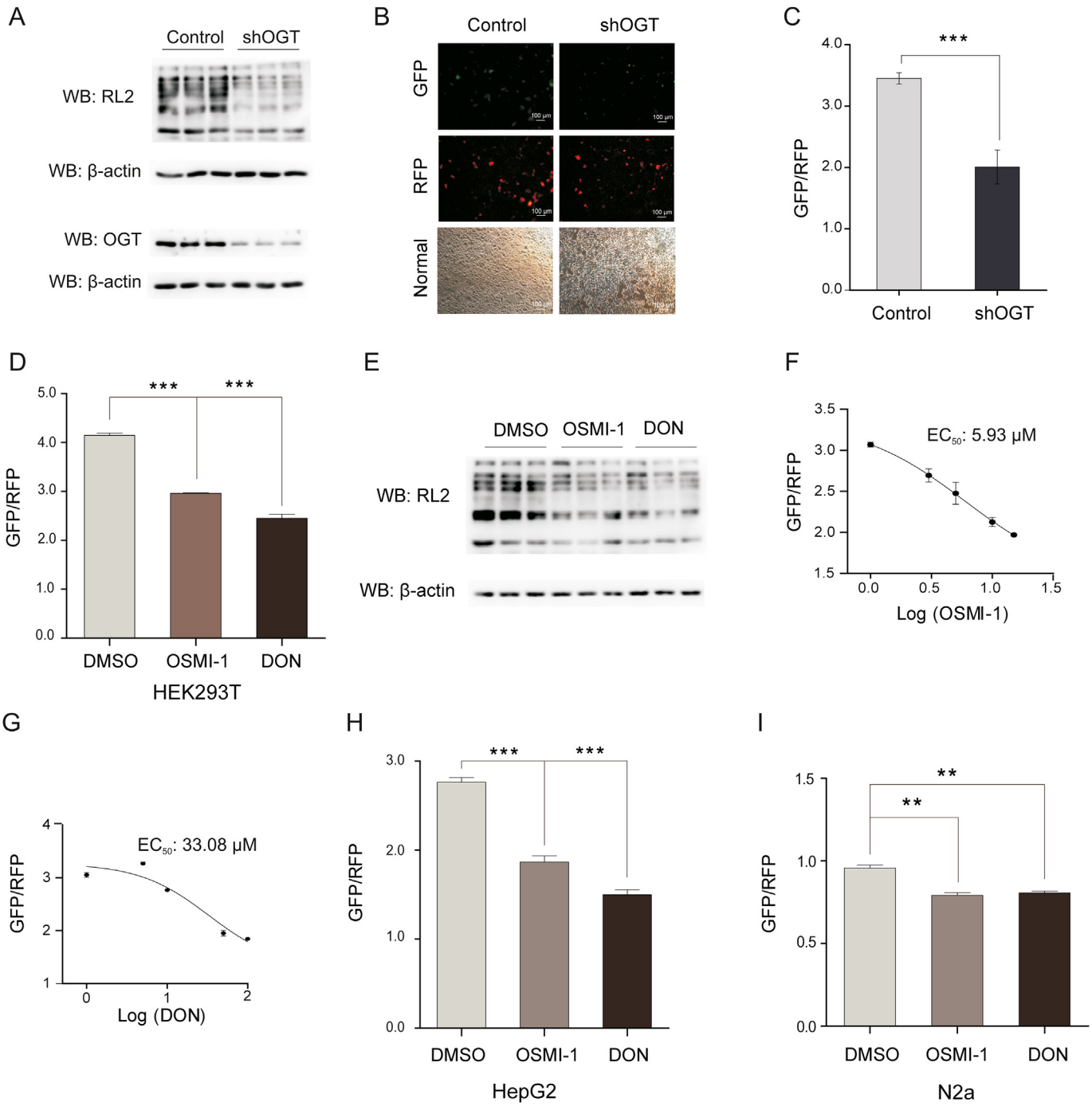


Fig. 2. (A) Knockdown of O-GlcNAc transferase (OGT) was performed in HEK293T cells, and the efficiency of short hairpin RNA targeting OGT (shOGT) was evaluated using Western blotting. (B) shOGT and the fluorescent reporter were co-transfected into HEK293T cells, and the influence of shOGT on GFP/RFP fluorescence was visualized using a fluorescence microscope. (C) Same as (B), GFP/RFP fluorescence was measured using the multimode reader as described in the methods (n=3, ***P < 0.001). The fluorescent reporter was expressed in HEK291T cells, and the cells were treated with 50 μM DON or 15 μM OSMI-1. (D) GFP/RFP fluorescence was measured, and the influence of DON/OSMI-1 has been presented (n=3, ***P < 0.001), and (E) cellular O-GlcNAcylation level was detected using Western blotting. The fluorescent reporter was expressed in HEK291T cells, and the cells were treated with varying concentrations of DON or OSMI-1. GFP/RFP fluorescence was measured, and EC_{50} of (F) OSMI-1 and (G) DON were calculated based on the fluorescent reporter data (cellular O-GlcNAcylation level was also detected using Western blotting, as shown in Fig. S4) (n=3, P < 0.05). (H) The reporter was transfected into HepG2 cells and the cells were treated with OSMI-1 or DON for 24 h; then, GFP/RFP fluorescence was measured and the influence of DON/OSMI-1 has been illustrated (n=3, ***P < 0.001). (I) The reporter was transfected into N2a cells, and the cells were treated with OSMI-1 or DON for 24 h; then, GFP/RFP fluorescence was measured and the influence of DON/OSMI-1 was shown (n=3, **P < 0.01).

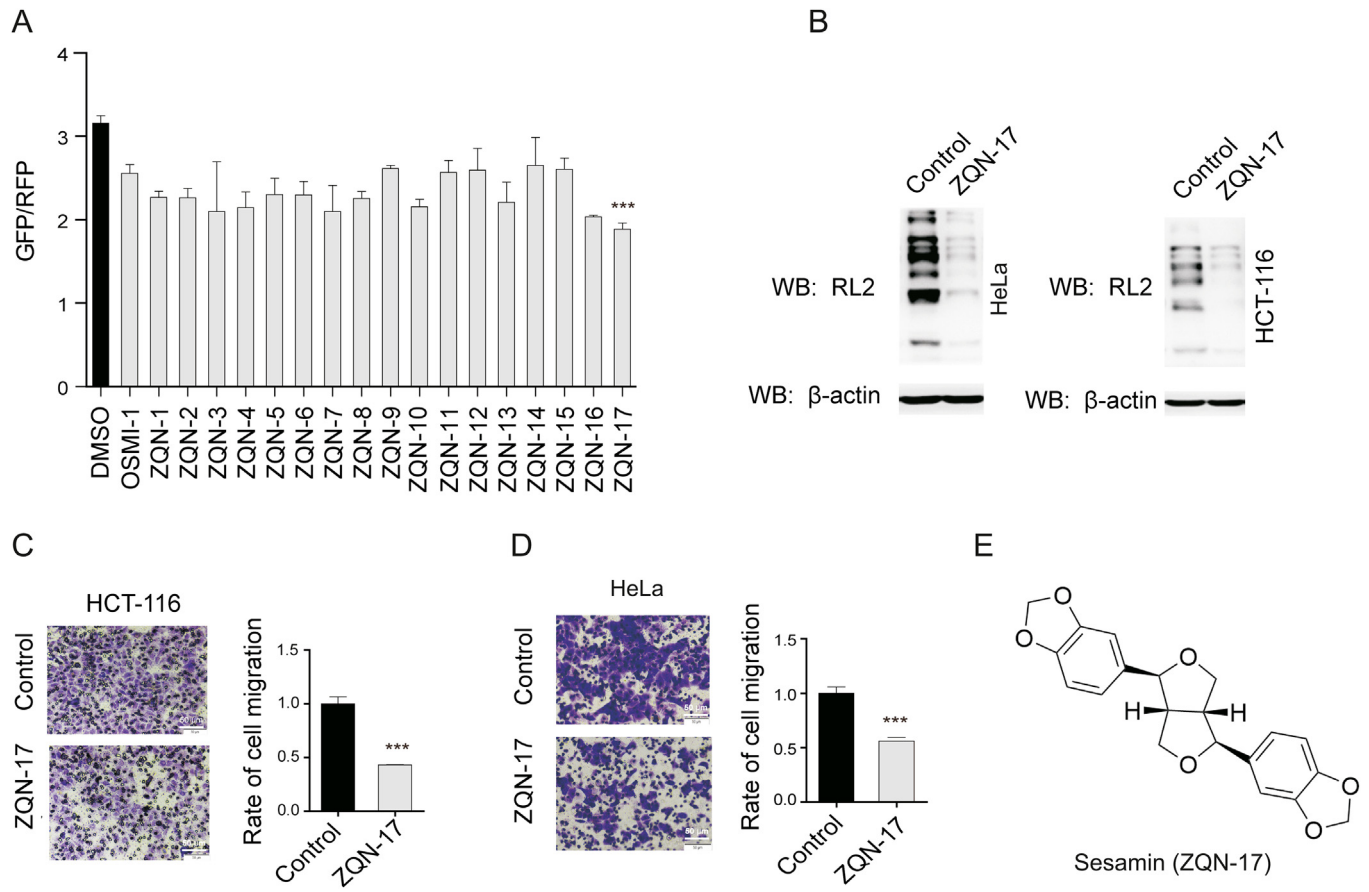


Fig. 3. (A) HEK293T cells transfected with the reporter were re-seeded into 96-well plates and the cells were treated with variant compounds for 24 h; GFP/RFP fluorescence was measured, and the influence of indicated compounds has been presented (n=3, ***P < 0.001). (B) Normally cultured HCT-116 and HeLa cells were treated with 20 μM ZQN-17, and cellular O-GlcNAcylation level was detected via Western blotting. Transwell migration assay was performed using (C) HCT-116 and (D) HeLa cells to evaluate the anti-tumor activity of ZQN-17 (n=3, ***P < 0.001). (E) Molecular structure of ZQN-17.

More recently, a Forster resonance energy transfer (FRET) method was developed to monitor cellular O-GlcNAcylation levels [37]. The FRET method is based on the modification of catenin, which is labeled by using GFP and an O-GlcNAcylation-dependent fluorophore. Such a method may be used to report cellular O-GlcNAcylation changes occurring due to the modification of catenin in a timely manner. However, the metabolic labeling of catenin with N-azidoacetyl-d-glucosamine and the subsequent chemical ligation of the fluorophore are necessary steps for the execution of this method.

4. Conclusions

In summary, we described a fluorescent reporter that could be readily used to monitor reduced but not increased cellular O-GlcNAcylation levels. The response to OGT/GFAT inhibitors and the identification of sesamin showed the utility of the high-throughput method, which represented a new strategy for the discovery of anti-tumor drugs. During the construction of the cell-based fluorescent reporter, we also validated the applicability of the dual fluorescent protein reporter in the conduction of gene regulation studies. We believe that the present study and its findings will contribute to the development of a methodology for the study of gene regulation or for future exploration of antitumor drugs.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2021.04.001.

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