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Effects of Global *O*-GlcNAcylation on Galectin Gene-expression Profiles in Human Cancer Cell Lines

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Abstract. *Background/Aim: The effects of O-linked β-N-acetyl-D-glucosamine (O-GlcNAc) transferase (OGT) and O-GlcNAcase (OGA) inhibitors on galectin gene expression profiles were examined in MCF7, HT-29, and HL-60 cancer cell lines. Materials and Methods: Cell cultures were treated for 24 h with OGA inhibitor thiamet G or OGT inhibitor 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-5-thio-α-D-glucopyranose, and global O-GlcNAc levels and expression of galectin genes were determined using an immunodot blot assay and real-time quantitative polymerase chain reaction. Results: Two galectin genes, LGALS3 in MCF7 cells and LGALS12 in HL-60 cells, were up-regulated by O-GlcNAc, whereas other cell-specific galectins were unresponsive to changes in O-GlcNAc level. Of interest, basal levels of O-GlcNAc in resting HL-60 and HT-29 cells were significantly higher than those in cells differentiated into neutrophilic or enterocytic lineages, respectively. Conclusion: O-GlcNAc-mediated signaling pathways may be involved in regulating the expression of only a limited number of galectin genes. Additional O-GlcNAc-dependent mechanisms may work at the protein level (galectin secretion and intracellular localization) and warrant further investigation.*

Galectins are multifunctional, soluble β-galactoside-binding proteins that have emerged as cancer biomarkers and as targets for anti-cancer therapy (1). Galectins contribute to regulating the processes of cell growth and death, and also play a role in assisting tumor cells in avoiding immune surveillance (2, 3). Galectin expression profiling has revealed that the transcript abundance of specific galectin genes varies significantly between normal and cancerous cells and tissues (1, 4-6). Despite their relevance to cancer biology, the development of practical biomedical applications has been hampered by the complexity of regulation of the 12 human galectin genes (*LGALS*; lectin, galactoside-binding, soluble) (7, 8) and diverse glycan-dependent and glycan-independent functions of galectins outside and inside cells (9-11).

There is evidence (12-14) suggesting that the expression of certain galectins is associated with specific glycosylation of intracellular regulatory proteins by addition of the single sugar *O*-linked β-*N*-acetyl-D-glucosamine (*O*-GlcNAc), in a process called *O*-GlcNAcylation (15, 16). This post-translational protein modification is directly controlled by the coordinated action of only two enzymes *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), which add and remove *O*-GlcNAc residues, respectively. Enhanced *O*-GlcNAcylation of intracellular proteins is a common feature of cells treated with a variety of stress stimuli (17). Furthermore, the levels of *O*-GlcNAc are elevated in tumor tissues, while reduced *O*-GlcNAcylation has been reported to inhibit oncogenesis (18). Although *O*-GlcNAcylation is known to change the functional activities of many regulatory molecules, including transcription factors, and govern protein localization within cells (15, 16), these mechanisms have never been evaluated for galectins at either the protein and transcript levels as far as we are aware of.

In this study, we investigated the effects of OGT and OGA inhibitors on the expression of galectin genes in three human cancer cell lines: MCF7 (breast carcinoma), HT-29 (colorectal carcinoma), and HL-60 (acute promyelocytic leukemia).

Materials and Methods

Cell cultures. All human cancer cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). The MCF7 human breast cancer cells, HT-29 colorectal carcinoma cells, and HL-60 promyelocytic leukemia cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY, USA), McCoy's 5A (modified) medium (Life Technologies), and Iscove's Modification of DMEM (Mediatech, Manassas, VA, USA), respectively, in a humidified atmosphere at 37˚C with 5% CO_2. The cell culture media were

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supplemented with 10% fetal bovine serum (Wisent, St-Bruno, QC, Canada) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) (Life Technologies). Fetal bovine serum was charcoal-stripped in the case of HL-60 cells and 50 μg/ml human recombinant insulin (Wisent) were added to the cell culture of MCF7 cells. Cell concentration and viability were determined using a hemocytometer and the trypan blue (0.4%) exclusion test.

Cell treatments. Cells were cultured in small (35×10 mm) or medium (60×15 mm) Falcon tissue culture dishes to isolate total RNA for gene-expression analysis, or to prepare cell lysates for *O*-GlcNAc immunoassays. To modulate the level of *O*-GlcNAc, cells in complete media were treated for 24 h with micromolar concentrations of the OGA inhibitor thiamet G (Sigma-Aldrich, Oakville, ON, Canada), or the OGT inhibitor 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-5-thio-α-D-glucopyranose (Ac-5SGlcNAc) (kindly provided by Dr. David Vocadlo, Simon Fraser University, Burnaby, BC, Canada) (19). Control cell cultures were exposed to equal volumes of dimethyl sulfoxide vehicle.

Total RNA isolation and cDNA synthesis. Total mRNA was isolated using Ambion TRIzol® reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol and quantified with a Thermo Scientific™ Nanodrop 2000c UV-Vis spectrophotometer (Wilmington, DE, USA). The Maxima First Strand cDNA Synthesis Kit from Thermo Scientific (Waltham, MA, USA) or SensiFAST cDNA Synthesis Kit from FroggaBio (Toronto, ON, Canada) were used to synthesize cDNA from 1 μg RNA.

Real-time quantitative polymerase chain reaction (qPCR). Galectin gene expression analysis was performed by real-time qPCR using a CFX Connect™ Thermocycler (Bio-Rad, Mississauga, ON, Canada) and PCR oligonucleotide primers described elsewhere (20). Briefly, qPCR reaction mixes were prepared in 20 μl volumes containing 10 μl SensiFAST™ SYBR® No-ROX Kit from Bioline (London, UK), 1.6 μl primer mix of forward and reverse primers (10 μM), 1 μl of 10-fold diluted template cDNA, and 7.4 μl nuclease-free water. Specificity of qPCR amplification was verified by the presence of a single melt peak at a specific temperature for each amplicon. Relative transcript levels were quantified by the Livak method ($2^{-\Delta\Delta CT}$) using β-actin (*ACTB*) as a reference gene.

Cell lysis and protein quantification. Following treatment, cells were rinsed twice with ice-cold PBS and lysed in 200 μl of RIPA buffer (10 mM Tris–HCl, pH 7.6, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5 mM ethylene glycol-*bis*(2-aminoethylether)-*N,N,N',N'*-tetra-acetic acid, 0.1% sodium deoxycholate, and 140 mM NaCl) supplemented with 100 μM phenylmethylsulfonyl fluoride, 100 μM Na_3VO_4, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 5 mM ethylenediaminetetra acetic acid, 50 μM leupeptin, and 1 μM pepstatin. The cell lysates were incubated on ice for 10 min before being passed three times through a 23G needle and centrifuged at 10,000 × *g* for 15 min at 4˚C. Total protein concentration was quantified spectrophotometrically using DC™ Protein Assay Kit II (Bio-Rad). Absorbance was measured at 655 nm using a Model 3550 Microplate Reader (Bio-Rad).

Immunodot blot assay. The *O*-GlcNAc status of cells was evaluated using a Bio-Dot® Microfiltration apparatus (Bio-Rad). Nitrocellulose membranes (GE Healthcare, Chicago, IL, USA) were pre-wetted for 10 min with Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) before placement into the apparatus. Protein extracts (4 μg in 200 μl PBS) were loaded into wells and transferred to the membrane by gravity filtration for 90 min. Thereafter, the membrane was blocked with 3% bovine serum albumin and 1% skim milk in Tris-buffered saline with Tween (TBS-T) (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween 20) for 60 min at room temperature. The membrane was then incubated overnight at 4˚C with mouse monoclonal pan-specific primary antibody to *O*-GlcNAc (RL2) (Thermo Scientific) diluted 1:1,000 in TBS-T with 5% BSA and 0.1% NaN_3. Following treatment with primary antibody, the membrane was washed with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:10,000 in TBS-T with 3% BSA and 1% non-fat dry milk at room temperature for 1 h. To visualize immunodots, membranes were exposed to Luminata™ Classico Western HRP Substrate (Millipore, Etobicoke, ON, Canada) or Clarity™ Western ECL substrate (Bio-Rad) and imaged with the ChemiDoc® XRS system (Bio-Rad). Densitometry was performed using ImageLab Software, version 5.2 (Bio-Rad).

Statistical analysis. Statistical analysis was performed using IBM SPSS Statistics v.25 (IBM, Armonk, NY, USA) and Prism 7 (GraphPad Software, La Jolla, CA, USA) software. Student *t*-tests or one-way analysis of variance (ANOVA) and *post-hoc* Tukey's honestly significant difference tests were used to determine significant differences across treatment means depending on the data set. At least three biological replicates were examined for each treatment and data were presented as mean±SEM. Differences were considered significant at $p<0.05$.

Results

Dose-dependent effects of OGA/OGT inhibitors on global O-GlcNAc levels in human cancer cell lines. Thiamet G and Ac-5SGlcNAc are currently the most potent and selective inhibitors of OGA and OGT, respectively (19, 21). To broadly determine the effectiveness of these inhibitors, we treated adherent (MCF7) and suspended (HL-60) cell lines with micromolar non-toxic concentrations of the drugs and monitored global *O*-GlcNAc levels 24 h post-treatment. Immunodot blot analysis revealed a dose-dependent increase in global in *O*-GlcNAc levels following thiamet G exposure, whereas a dose-dependent decrease in *O*-GlcNAc level was observed after Ac-5SGlcNAc treatment in both cell lines (Figure 1). These results establish the biological effectiveness of OGA/OGT inhibitors in these cancer cell types.

The effects of OGA/OGT inhibitors on galectin gene expression in MCF7 human breast cancer cells. The galectin expression profile of the MCF7 cell line is limited to three galectins, *LGALS1, LGALS3* and *LGALS8*, as reported elsewhere (5). Considering these findings, qPCR was used to quantify changes in the galectin mRNA expression profile of MCF7 cells treated with inhibitors of OGA (10 μM thiamet G) or OGT (50 μM Ac-5SGlcNAc). These treatments did not change the expression of *LGALS1* and

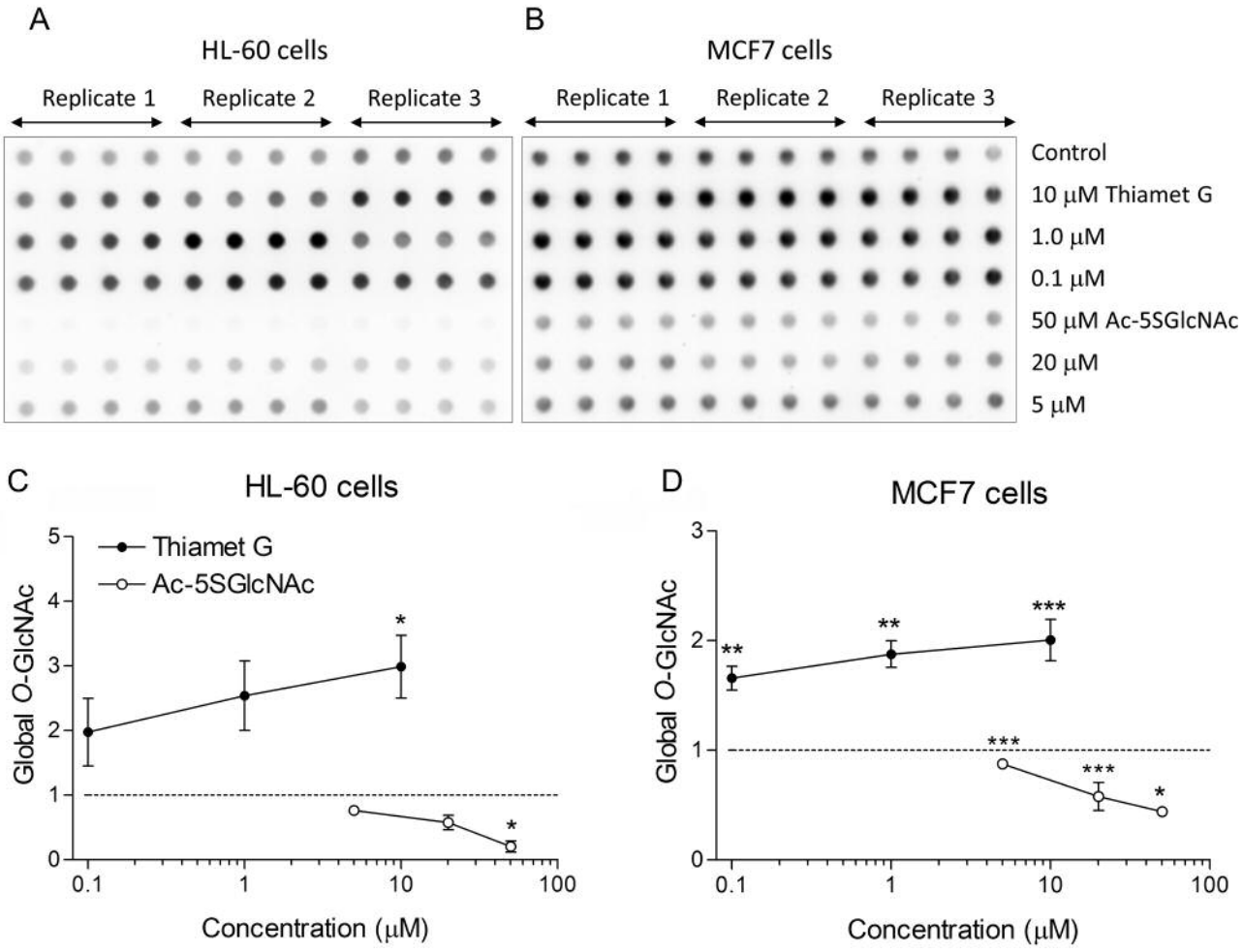

Figure 1. *O-Linked β-N-acetyl-D-glucosamine (O-GlcNAc) transferase (OGT) and O-GlcNAcase (OGA) inhibitors modify the global O-GlcNAc level in human cancer cell lines. A and B: HL-60 and MCF7 cells were treated for 24 h with three different concentrations of OGA inhibitor thiamet G or OGT inhibitor 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-5-thio-α-D-glucopyranose (Ac-5SGlcNAc) followed by immunodot blot analyses of O-GlcNAc in quadruplicate for three biological replicates. C and D: Quantification of the immunodot blots of O-GlcNAc for MCF7 and HL-60 cells. The intensity of each dot was normalized to the average integral intensity of all dots. Opposing dose-dependent changes in O-GlcNAc levels induced by thiamet G versus Ac-5SGlcNAc are evident. Data are presented as means±SEM (n=3). Significantly different at *p<0.05, **p<0.01, and ***p<0.001 versus control (normalized to 1, dotted lines) by one-sample t-test.*

LGALS8 compared to vehicle control-treated cells, however the expression of *LGALS3* was significantly ($p<0.05$) higher in cells after OGA inhibition with thiamet G treatment compared to OGT inhibition with Ac-5SGlcNAc treatment (Figure 2).

The effects of OGA/OGT inhibitors on galectin expression in HL-60 cells. Undifferentiated HL-60 cells robustly express five galectins, *LGALS1, LGALS3, LGALS8, LGALS9* and *LGALS12* (20, 22). To examine the sensitivity of this galectin gene network to *O*-GlcNAc regulation, the cells were treated for 24 h with 10 μM thiamet G and 50 μM Ac-5SGlcNAc. Thiamet G treatment (high *O*-GlcNAc) significantly ($p<0.05$) up-regulated the expression of *LGALS12* compared to Ac-5SGlcNAc (low *O*-GlcNAc), however, neither treatment significantly altered the expression of other galectin genes (Figure 3A). This finding was intriguing, as we had previously shown that only *LGALS12* was specifically down-regulated in HL-60 cells upon DMSO-induced differentiation into neutrophil-like cells (20). We were, thus, interested to determine whether there were differences in the global *O*-GlcNAc level between undifferentiated and differentiated HL-60 cells. A 25-fold decrease of *O*-GlcNAc level was observed in HL-60 cells treated for 3 days with 1.3% DMSO (neutrophilic differentiation) in comparison with the control cell culture ($p<0.001$, independent sample *t*-test) (Figure 3B).

Thus, in both cases (treatment with OGT inhibitor or DMSO-induced cell differentiation), a lower level of *O*-GlcNAc was positively associated with a lower expression of *LGALS12* in HL-60 cells.

The expression of galectins in HT-29 cells. HT-29 cells express most of the human galectins including *LGALS1, LGALS2, LGALS3, LGALS4, LGALS7, LGALS8*, and *LGALS9* (4, 5). Considering the intricate galectin network present in HT-29 cells, we decided to examine *O*-GlcNAcylation not only *via* direct inhibition of OGA and OGT, but also *via* inducing enterocytic differentiation by cell culture over-confluency (cell crowding stress) (23). To select optimal conditions for cell-crowding stress, HT-29 cells were plated at a density of 0.1×10^6 cells/ml in 5 ml of McCoy's 5A medium in T25 flasks and their growth was monitored daily over 6 days (Figure 4A). Exponential growth was observed between days 1 and 4. Importantly, cell viability was not significantly compromised even after entry into stationary phase (completely confluent monolayer) after day 4. Based on these observations, HT-29 cell RNA was extracted on day 3 and day 6 to assess *O*-GlcNAc levels and galectin expression in the exponential (control) and stationary (cell-crowding stress) phases. As expected, thiamet G significantly increased the global level of *O*-GlcNAc in HT-29 cells, while Ac-5SGlcNAc and crowding stress significantly reduced it (Figure 4B).

We next examined the expression of specific galectin gene in response to thiamet G, Ac-5SGlcNAc, and crowding stress and found that these treatments induced only moderate changes in galectin gene expression in HT-29 cells (not exceeding 2-to 3-fold) (Figure 4C). There were no significant differences in the expression of any of the galectin genes upon thiamet G or Ac-5SGlcNAc treatment ($p>0.05$, Tukey's honestly significant difference test). In contrast, we did observe a significant increase in the expression of *LGALS3* ($p=0.011$), *LGALS4* ($p=0.007$), and *LGALS8* ($p=0.007$) in cells subject to crowding stress versus control cells (multivariate tests of between-subject effects). Importantly, although stressed cells had low levels of *O*-GlcNAc similarly to that of Ac-5SGlcNAc-treated cells, the expression of *LGALS1, LGALS3, LGALS4*, and *LGALS7* was found to be significantly different ($p<0.05$) between these two treatments (Figure 4C). Thus, while expression of certain galectin genes increases following crowding stress, the direct inhibition of *O*-GlcNAc does not appear to affect overall galectin gene expression in HT-29 cells.

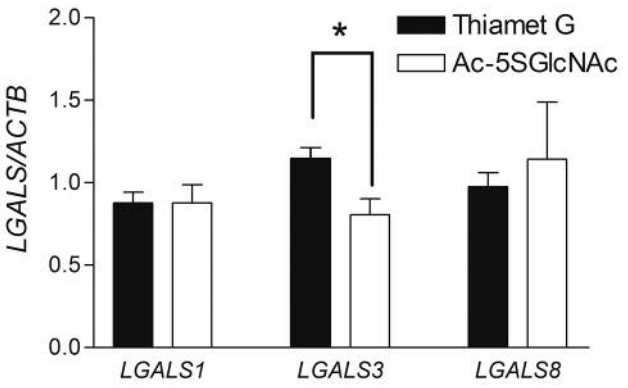

Figure 2. *Effects of O-linked β-N-acetyl-D-glucosamine (O-GlcNAc) transferase (OGT) and O-GlcNAcase (OGA) inhibitors on the expression of galectin genes (LGALS) in MCF7 cells. Cells were treated with either OGA inhibitor thiamet G (10 μM) or OGT inhibitor 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-5-thio-α-D-glucopyranose (Ac-5SGlcNAc) (50 μM) for 24 h. Quantification of real-time quantitative polymerase chain reaction results was performed using the Livak method with β-actin gene (ACTB) as a reference; fold expression differences compared to dimethyl sulfoxide vehicle are shown. Data are presented as means±SEM (n=3). Significantly different at *p<0.05 by unpaired t-test.*

Discussion

Post-translational modification of intracellular proteins with *O*-GlcNAc represents a powerful signaling pathway that acts as an adaptive mechanism promoting cytoprotection, and is activated both in cancer cells and in cells treated with stress stimuli (17, 18). *O*-GlcNAcylation often competes with protein phosphorylation to control a variety of regulatory proteins, including transcription factors (15). In our study, we investigated galectin expression profiles in three different human cancer cell lines (MCF7, HL-60, and HT-29), examining their sensitivity to the inhibition and stimulation of *O*-GlcNAcylation using the highly selective drugs, Ac-5SGlcNAc and thiamet G (19, 21), respectively. As expected, these drugs induced robust and opposite changes in global *O*-GlcNAc levels in all tested cell lines, however, cell-specific changes in the expression of only limited number of galectin genes were noted. In particular, *O*-GlcNAc induced moderate increases not exceeding 2-fold in the expression of *LGALS3* in MCF7 cells and *LGALS12* in HL-60 cells. The expression of other galectin genes was not affected by *O*-GlcNAc in these cell lines. Surprisingly, the full network of seven galectin genes in HT-29 cells was unaffected by chemical modulation of *O*-GlcNAc. These data suggest that at the transcriptional level, *O*-GlcNAc signaling pathways have limited influence on galectin gene-expression profiles in human cancer cell lines, although cell-specific regulation of selected galectin genes (*LGALS3* and *LGALS12*) does occur in MCF7 and HL-60 cells. In the case of MCF7 cells, this regulation may be important to maintain high levels of intracellular galectin-3 which mediates protection of breast cancer cells from apoptosis (24) and maintains the stemness of cancer cells (25). The latter stemness option can also be applied to *O*-GlcNAc-dependent up-regulation of

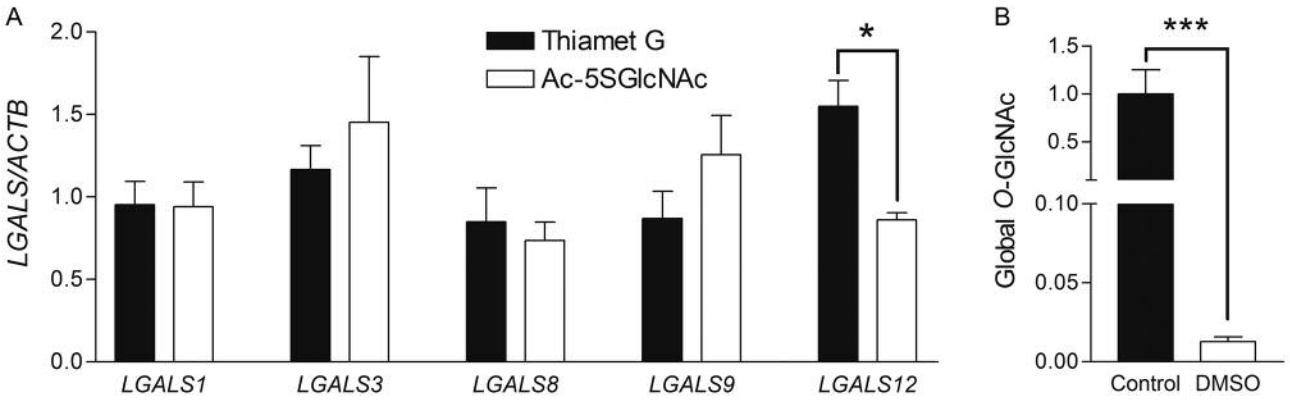

Figure 3. *Effects of O-linked β-N-acetyl-D-glucosamine (O-GlcNAc) transferase (OGT) and O-GlcNAcase (OGA) inhibitors on the expression of galectin genes in HL-60 cells. A, cells were incubated with OGA inhibitor thiamet G (10 μM) or OGT inhibitor 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-5-thio-α-D-glucopyranose (Ac-5SGlcNAc) (50 μM) for 24 h. Quantification of real-time quantitative polymerase chain reaction results was performed using the Livak method with β-actin gene (ACTB) as a reference; fold expression changes relative to vehicle exposed controls are shown. B: Cells were treated with 1.3% dimethyl sulfoxide (DMSO) to induce neutrophil-like differentiation (20, 22). O-GlcNAc decreased in differentiated HL-60 cells. Data are presented as means±SEM (n=3). Significantly different at *p<0.05, and ***p<0.001 by unpaired t-test.*

LGALS12 in HL-60 cells since galectin-12 is known to inhibit differentiation of promyelocytic progenitor cells into neutrophilic lineage (26). Indeed, in agreement with previous reports regarding the inhibition of *O*-GlcNAc in specific lineages of differentiated cells (27-30), we found a significant decrease of *O*-GlcNAc level in HL-60 cells differentiated into neutrophil-like cells through DMSO treatment, and in HT-29 cells under cell-crowding stress (spontaneous post-confluency induced differentiation into enterocytes). The low level of *O*-GlcNAc in differentiated cells was similar to that in Ac-5SGlcNAc-treated HL-60 and HT-29 cells. However, galectin expression profiles were significantly different between differentiated and undifferentiated cells, suggesting *O*-GlcNAc-independent regulation of galectin gene expression by alternative mechanisms.

It should be noted that even if *O*-GlcNAc has limited influence on expression of certain galectin genes, galectin protein abundance and localization may be significantly affected by this mechanism, especially between resting cells (high *O*-GlcNAc) and differentiated cells (low *O*-GlcNAc). Indeed, *O*-GlcNAc regulates trafficking of proteins between intracellular compartments and can inhibit protein secretion (31). Although some reports confirm requirements of galectins for cell differentiation (32, 33), little is known about the molecular mechanisms that drive the trafficking and secretion of these soluble proteins. If global *O*-GlcNAcylation plays a role in galectin trafficking rather than in gene expression, it would be reasonable to expect that galectin secretion will be stimulated in differentiated cells, while less galectins will be accumulated in the intracellular compartments due to low *O*-GlcNAc. This hypothesis remains to be tested in future studies.

In conclusion, our study revealed two different *O*-GlcNAc-sensitive galectin genes in breast cancer MCF7 cells (*LGALS3*) and acute leukemia HL-60 cells (*LGALS12*), whereas galectin expression was insensitive to *O*-GlcNAc manipulation in colorectal carcinoma HT-29 cells. Further studies are underway to determine the molecular mechanisms of how *O*-GlcNAc controls the expression of *LGALS3* and *LGALS12* genes and whether *O*-GlcNAc controls secretion and subcellular localization of galectin proteins in cancer cells.

Acknowledgements

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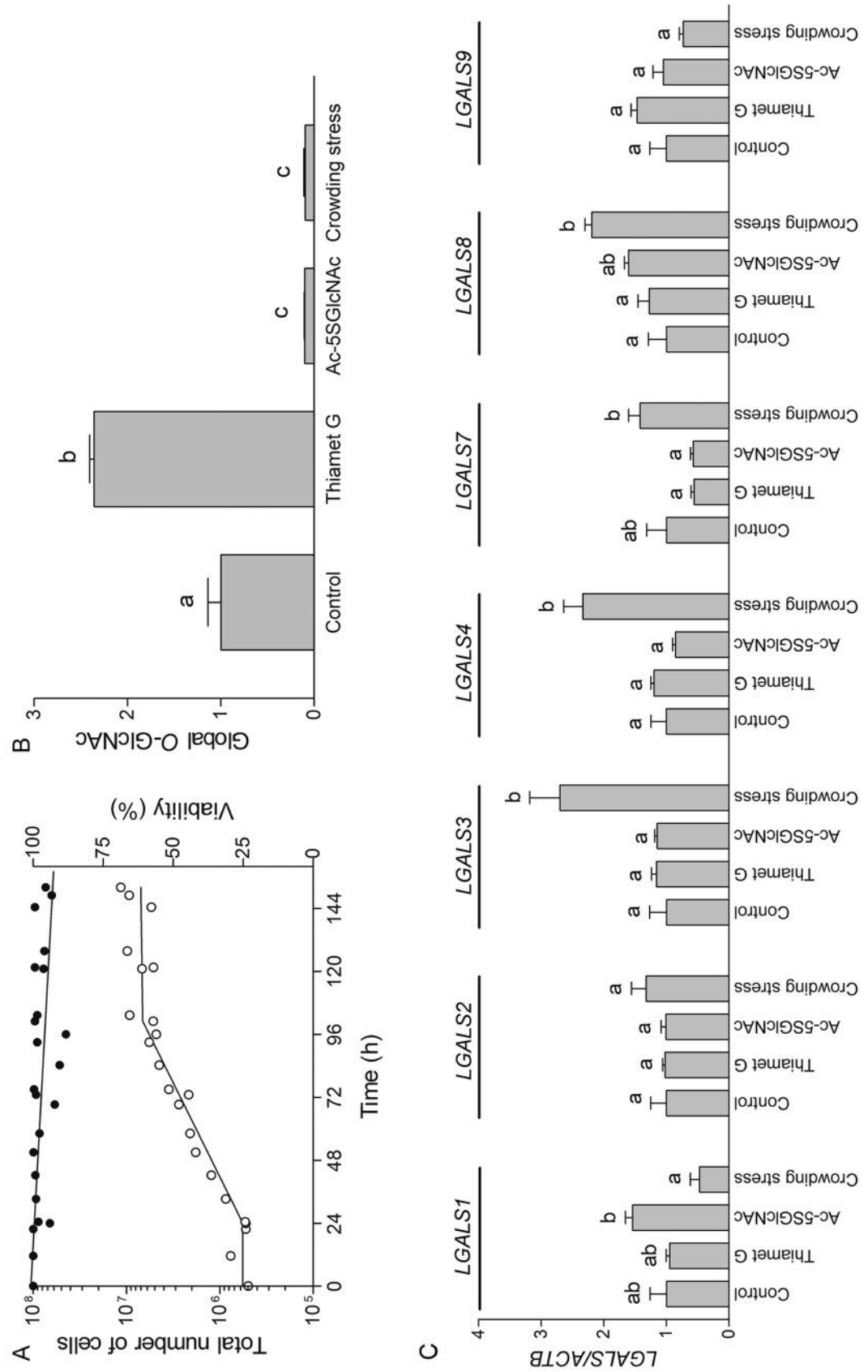

Figure 4. *Effects of O-linked β-N-acetyl-D-glucosamine (O-GlcNAc) transferase (OGT) and O-GlcNAcase (OGA) inhibitors and cell crowding stress of the expression of galectin genes in HT-29 cells. A: Growth curve (closed symbols) and viability (open symbols) of HT-29 cells in culture. Cells grew exponentially until reaching the stationary phase on day 6. Cell viability remained high throughout. B: Global O-GlcNAc levels in control HT-29 cell culture (day 3), cells treated for 24 h with OGA inhibitor thiamet G (10 μM), OGT inhibitor 2-acetamido-1,3,4,6-tetra-O-acetyl-2-deoxy-5-thio-α-D-glucopyranose (Ac-5SGlcNAc) (25 μM), and in post-confluent cell culture (cell crowding stress, day 6). C: Effects of the tested treatments on galectin gene-expression levels, as normalized to β-actin (ACTB) reference gene. Data are presented as means±SEM (n=3). Values with different letters are statistically significantly different ($p<0.05$) by one-way ANOVA followed by Tukey's difference test.*

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