Proteomic Analysis Reveals Aberrant *O*-GlcNAcylation of Extracellular Proteins from Breast Cancer Cell Secretion

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Abstract. *Background: O-GlcNAcylation is a unique intracellular protein modification; however, few extracellular O-GlcNAc-modified proteins have been discovered. We have previously demonstrated that many cellular proteins were aberrant in O-GlcNAcylation in breast cancer tissues. In the present study, therefore, we investigated whether O-GlcNAc-modified proteins were abnormally secreted from breast cancer cells. Materials and Methods: Intracellular and extracellular proteins were prepared from cell lysates of breast cancer cells (MCF-7 and MDA-MB-231) and normal breast cells (HMEC) and from their serum-free media (SFM), respectively. O-GlcNAcylation level was examined by immunoblotting. O-GlcNAc-Modified proteins were identified using two-dimensional gel electrophoresis and Liquid Chromatography-tandem Mass Spectrometry. Results: O-GlcNAcylation level was significantly increased in the extracellular compartment of both types of cancer cells compared to normal cells. Interestingly, O-GlcNAc patterns differed between intracellular and extracellular proteins. Proteomic analysis revealed that many O-GlcNAc spots in MCF-7 secretions were abnormally increased in comparison to those in HMEC secretions. Among these, transitional endoplasmic reticulum ATPase (TER ATPase) and heat-shock 70 kDa (HSP70) were confirmed to be O-GlcNAc-modified. The levels of O-GlcNAc-HSP70 and O-GlcNAc-TER ATPase were higher in SFM from MCF-7 cells than in that from HMEC. Conclusion: O-GlcNAcomic study of the extracellular compartments reveals aberrant O-GlcNAc-secreted proteins, which may be of interest as potential biomarkers in breast cancer.*

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Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women. Moreover, breast cancer ranks fifth as cause of death from cancer overall (522,000 deaths) (1). Breast cancer is curable, but most patients with breast cancer are not diagnosed until the disease has progressed to advanced stages, thus reducing overall survival rates. Identification of biomarkers of early-stage disease and molecular targets for effective drug treatment are, therefore, important. Samples from patients with cancer and cancer cell lines have been studied extensively for this purpose.

O-GlcNAcylation is a post-translational modification where a single β-D-N acetylglucosamine (*O*-GlcNAc) is added to serine or threonine residues of nuclear, cytoplasmic and mitochondrial proteins. This glycosylation is dynamically regulated by two key enzymes: *O*-GlcNAc transferase (OGT) (2) and *O*-GlcNAcase (OGA) (3), that are responsible for the addition and removal of a single GlcNAc residue from target proteins, respectively. This glycosylation is implicated in a wide range of effects on cellular function and signaling in metabolic diseases and cancer. Emerging evidence reveals that certain cellular proteins are abnormally *O*-GlcNAc-modified in breast cancer, indicating *O*-GlcNAcylation is associated with malignancy (4-6). Many *O*-GlcNAc-modified proteins in breast cancer were reported (7). They include proteins involved in cytoskeleton and structure formation, glycolytic enzymes, chaperones, nucleotide metabolism, mitochondria, nuclear proteins including heterogeneous nuclear ribonucleoproteins and proteins related to transcription and translation, as well as transcription factors. *O*-GlcNAcylation is generally a unique intracellular modification. However, *O*-GlcNAc modification has also been reported to occur in extracellular proteins (8). In addition, an atypical, epidermal growth factor (EGF) domain-specific OGT, responsible for modification of extracellular *O*-GlcNAc was identified (8, 9). This atypical *O*-GlcNAcylation may take place in the lumen of the endoplasmic reticulum, and the *O*-GlcNAc-modified proteins exported extracellularly through the secretory pathway. Recently, it was reported that defects in this atypical modification may cause human diseases, such as Adam-Oliver syndrome (10).

Previously, we demonstrated that the *O*-GlcNAcylation level was increased in primary breast and colorectal cancer tissues, with many proteins being selectively modified by *O*-GlcNAc in both types of cancer (6, 11). As *O*-GlcNAcylation takes place mainly in intracellular compartments, in this study, we investigated whether *O*-GlcNAc-modified proteins can be secreted or released from the cells. Breast cancer cell lines (MDA-MB-231 and MCF-7) and normal human mammary epithelial cells (HMEC) were cultured, and cell lysates collected as the source of intracellular proteins, while cell culture supernatants were collected as the source of extracellular proteins. A proteomic approach was used with *O*-GlcNAc immunodetection to determine and validate extracellular *O*-GlcNAc-modified proteins.

Materials and Methods

Cell lines. Normal human mammary epithelial cell (HMEC) and Mammary Epithelial Cell Growth Medium (MEGM®) were purchased from LONZA (Lonza Walkersville, Inc., Walkersville, MD, USA) and cultured as per the manufacturer's recommendation. Breast cancer cells, MDA-MB- 231 and MCF-7 were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen).

Preparation of extracellular proteins from serum-free media and intracellular proteins from cell lysates. The total number of living cells was determined by the trypan blue exclusion test. To obtain the extracellular proteins and avoid contamination of the endogenous proteins from those of fetal bovine serum, serum-free media (SFM) was used as described previously (12). All cells were seeded in T-175 cm^2 cell culture flasks and cultured in a humidified incubator at 37˚C and 5% CO_2 to 80% confluency in the complete growth medium. Ten flasks of each cell line were cultured and cells were washed three times with 10 ml of SFM, replaced with 20 ml of SFM, and returned into the incubator. After culturing for 24 h, the SFM was harvested by centrifugation at 500 × *g*, 4˚C for 5 min to sediment non-adherent cells, and further centrifuged at 2,000 × *g*, 4˚C for 10 min to remove cellular debris. The SFM supernatants were then concentrated to ~1 ml by centrifugation at 4,000 × *g*, 4˚C for 35 min, using 3-kDa molecular mass cutoff concentrators (Millipore, Bedford, MA, USA). The SFM was then supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) plus an OGA inhibitor, Thiamet-G (Sigma-Aldrich). To extract proteins from cells, culture cells were lysed with 1× RIPA supplemented with 1% protease inhibitor cocktail and Thiamet-G on ice for 30 min. Supernatant was collected as the whole-cell lysate by centrifugation at 10,000 × *g*, 4˚C for 15 min. Protein concentration was determined using Bradford assay (Bio-Rad, Hercules, CA, USA). All samples were stored at −80˚C for further analysis.

Assessment of the levels of O-GlcNAcylation and O-GlcNAc cycling enzymes. *O*-GlcNAcylation and *O*-GlcNAc cycling enzymes were determined by immunoblotting using *O*-GlcNAc antibodies, RL2 (1:1,000) (Abcam, Cambridge, UK) and CTD110.6 (1:5,000) (Sigma-Aldrich), OGT (1:1,000) (Sigma-Aldrich), and OGA (1:10,000) (Abcam), respectively. Protein loading was performed using α-tubulin antibody (1:10,000) (Cell Signalling Technology, Boston, MA, USA) for cell lysate samples and Coomassie brilliant blue R-250 (CBB) for SFM samples, respectively. The protocol was performed as described previously (11). Band intensity was measured using Image Quant TL 1D version 7.0 (GE Healthcare, Buckinghamshire, UK).

Confirmation of O-GlcNAc-modified proteins from SFM. Three experiments were performed to confirm whether extracellular proteins from the SFM were *O*-GlcNAc-modified proteins (6). Firstly, protein (20 μg) was digested with PNGase F (New England Biolabs, Beverly, MA, USA) at 37˚C for 1 h to remove the innermost GlcNAc of *N*-linked oligosaccharides at the asparagine residues. Secondly, protein (20 μg) was digested with *O*-GlcNAcase (R&D Systems, Minneapolis, MN, USA) to remove *O*-GlcNAc from serine or threonine residues at 37˚C for 24 h. After treatment, samples were separated on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane, and immunoblotted with RL2 antibody to determine the *O*-GlcNAc level. For on-blot β-elimination, once protein (20 μg) was transferred to the PVDF membrane, it was soaked in 55 mM NaOH, overnight at 40˚C to release *O*-linked glycans prior to RL2 immunoblotting.

Two-dimensional gel electrophoresis (2-DE). The concentrated protein samples from SFM were replaced with 2D lysis buffer using Bio-Spin® 6 chromatography columns (Bio-Rad). The 2-DE of *O*-GlcNAc protocol was performed as described previously (6). Once proteins were transferred to PVDF membranes, they were stained with SYPRO Ruby Protein Blot Stain (Molecular Probes, Eugene, OR, USA) prior to probing with RL2 antibody. Duplicate gels were also stained with 0.1% CBB for protein identification using Liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Identification of proteins by LC-MS/MS. *O*-GlcNAc-modified proteins signals present in X-ray films were aligned with total protein spots on membranes and CBB gels using Master 2D Platinum 7.0 software (GE Healthcare). Proteins spots from CBB gels were excised, destained and enzymatically digested using trypsin (Promega, Fitchburg, Wisconsin, USA) as described previously (13). Parent mass peaks with a range from 50 to 3,000 m/z were selected for MS/MS analysis and proteins were then identified using MASCOT with similar search parameters as in a previous study (14). Proteins with molecular weight and pI consistent with the gel region, having at least one peptide exceeding the score threshold ($p<0.05$) were considered as being positively identified.

Validation of O-GlcNAc-modified proteins. The potential *O*-GlcNAc-modified proteins identified from LC-MS/MS analysis were confirmed using immunoprecipitation (IP). Antibodies (Abcam) including those against heat-shock 70 kDa (HSP70) and transitional endoplasmic reticulum ATPase (TER ATPase) were used for coupling with the slurry protein A/G agarose beads (Pierce Biotechnology, Rockford, IL, USA) to enrich HSP70 and TER ATPase proteins from SFM. Briefly, proteins (1000 μg) in low-salt lysis buffer were incubated with antibodies to HSP70 (1:250) and TER ATPase (1:100). The suspensions were mixed gently by shaking in an end-over-end manner at 4˚C for overnight. After that, the immune complexes were incubated with protein A/G agarose for

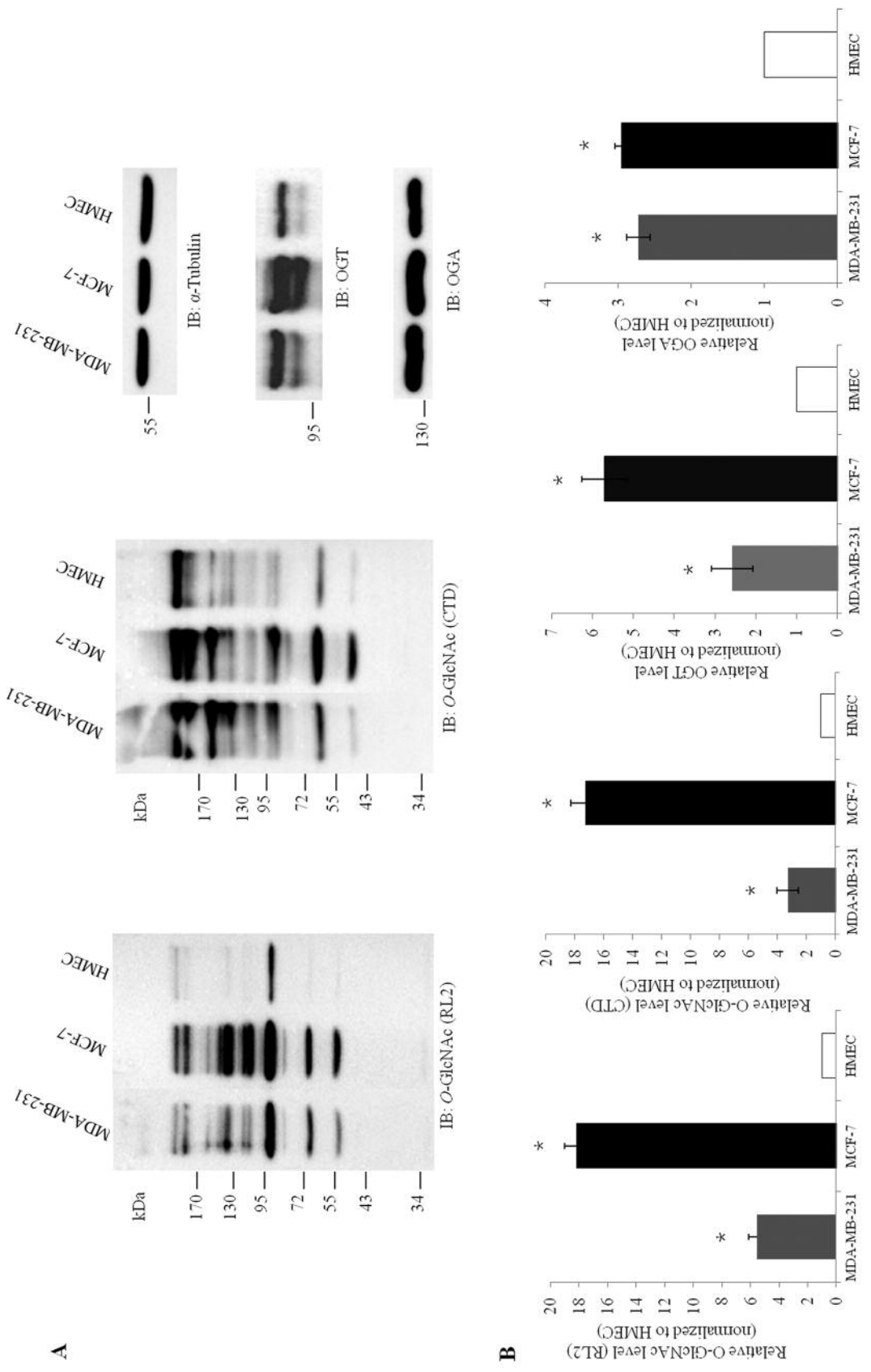


Figure 1. *O-GlcNAcylation, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) levels in intracellular compartments of normal (HMEC) and breast cancer cell lines (MDA-MB-231 and MCF-7). A: Representative immunoblots of O-GlcNAc (RL2 and CTD110.6), OGT, OGA and α-tubulin (loading control). B: Relative band intensity of RL2, CTD110.6, OGT and OGA determined by densitometric analysis with levels normalized to that of normal HMEC cells. Protein samples (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with antibodies, as indicated. Data are presented as means±SD of three independent experiments. *p<0.05 vs. normal HMEC.*

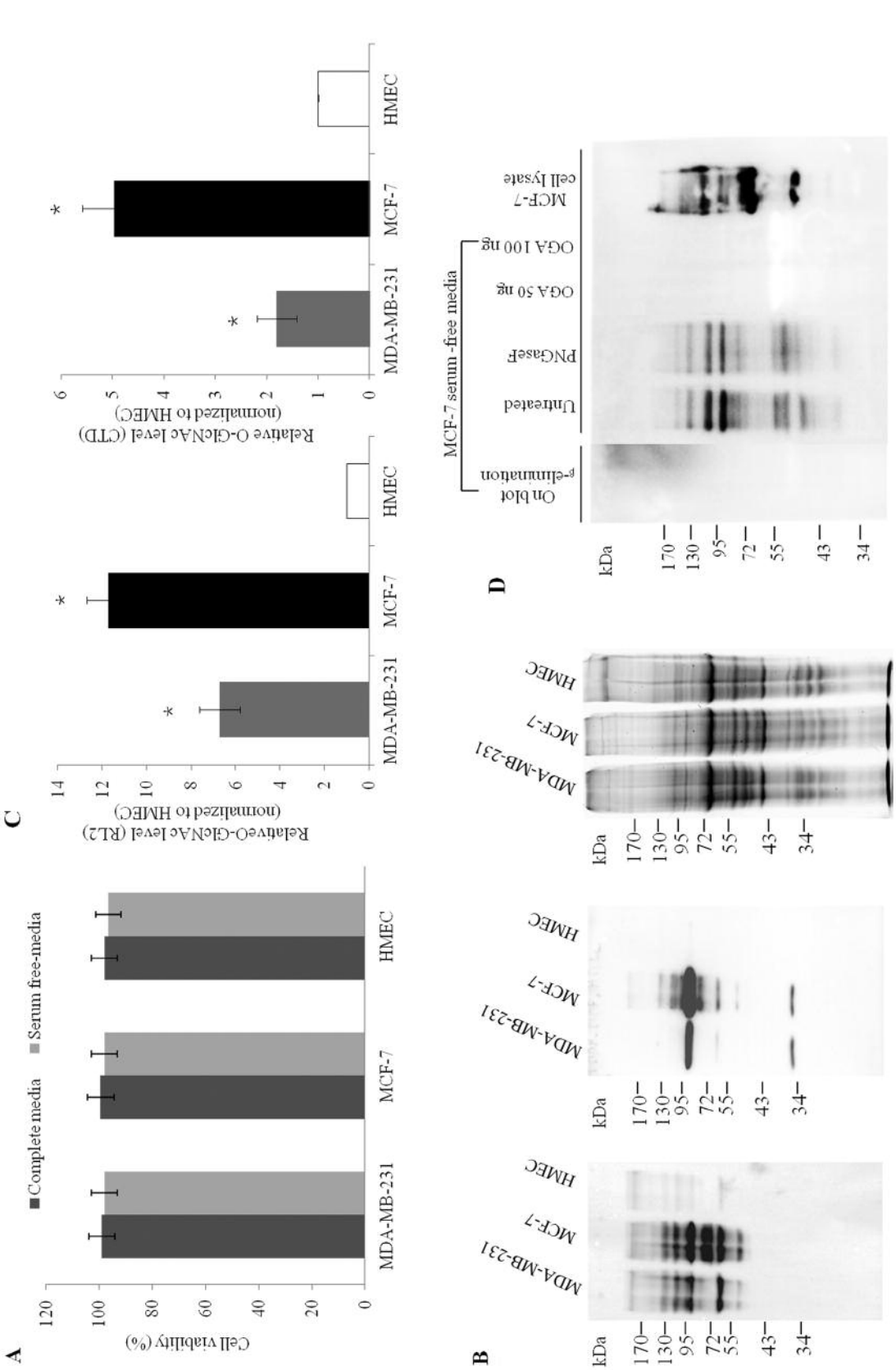
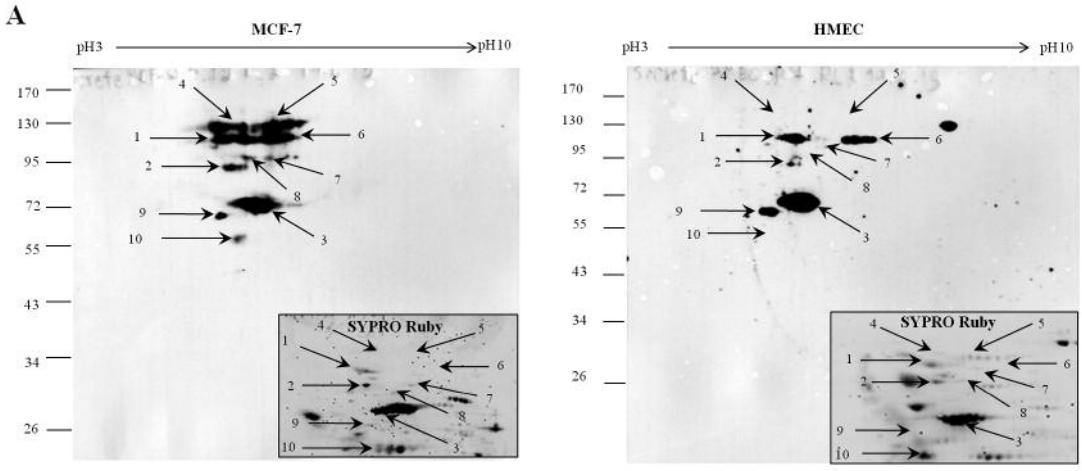


Figure 2. *Extracellular O-GlcNAc-modified proteins from serum-free media. A: Cell viability detected by the trypan blue exclusion under serum-free and complete media conditions of breast cancer cell lines (MDA-MB-231 and MCF-7) and human mammary epithelial cells (HMEC). B: Representative O-GlcNAc immunoblots (RL2 and CTD110.6) and SYPRO Ruby-stained membranes of proteins from the serum-free media of MDA-MB-231, MCF-7, and HMEC. C: Relative band intensity of RL2 and CTD110.6 was determined by densitometric analysis with levels normalized to that of normal HMEC cells. D: An O-GlcNAc immunoblot (RL2) to confirm O-GlcNAc-modified proteins from MCF-7 serum-free media treated with (i) PNGase F, (ii) O-GlcNAcase (OGA) at 50 μg and 100 μg, and (iii) on-blot β-elimination. MCF-7 cell lysate was used as a positive control for intracellular O-GlcNAc-modified proteins. Data are presented as means±SD of three independent experiments. *p<0.05 vs. normal HMEC.*



B

Spot ID	Protein name	Accession number	MW/pI (kDa/pI)	Peptide matches	Mascot score	References for *O*-GlcNAcylation
1	CST1	CSTN1_HUMAN	111.0/4.81	12	254	-
2	TER ATPase	TERA_HUMAN	89.8/5.14	6	161	(28, 29, 30)
3	HSP70	HSP71_HUMAN	70.3/5.48	10	262	(23, 24)

Figure 3. *Two-dimensional gel electrophoresis and mass spectrometric analysis of extracellular O-GlcNAc-modified proteins of MCF-7 and human mammary epithelial cell (HMEC) secretions. A: Representative O-GlcNAc immunoblots and SYPRO Ruby-stained polyvinylidene difluoride membranes (insets) of MCF-7 and HMEC secretions. B: List of extracellular O-GlcNAc-modified proteins using Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Arrows indicate O-GlcNAc spots which matched to those on SYPRO Ruby-stained membranes and identified using LC-MS/MS as calsyntenin 1 (1), transitional endoplasmic reticulum ATPase (TER ATPase) (2) and heat shock protein of 70 kDa (HSP70) (3) while arrows (4-10) indicate O-GlcNAc spots which did not match any protein spot on stained membranes and were not identified using LC-MS/MS analysis. Protein samples (100 μg) were separated by immobilized pH gradient strips (pI 3-10) followed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were stained with SYPRO Ruby prior to probing with RL2 antibody. LC-MS/MS analysis was performed as described in the Materials and Methods.*

2 hours to perform coupling reaction. After three washes, 50 μl of 2× sampling buffer was added to the column and heated at 95-100˚C for 10 minutes. The eluted samples (25 μl) were loaded onto 10% SDS-PAGE and immunoblotted with HSP70, TER ATPase and RL2 antibodies to determine the levels of total HSP70, total TER ATPase, *O*-GlcNAc-HSP70 and *O*-GlcNAc-TER ATPase, respectively. Band intensity was measured using Image Quant TL 1D version 7.0 (GE Healthcare).

Statistical analysis. The statistical analysis was analyzed using unpaired Student's *t*-test to test for the difference between two groups. The statistical significance was defined at $p<0.05$.

Results

Levels of intracellular O-GlcNAcylation and O-GlcNAc cycling enzymes in breast normal and cancer cells. To determine whether *O*-GlcNAcylation level was up-regulated in breast cancer cell line; we performed *O*-GlcNAc immunoblots using two commercial antibodies, RL2 and CTD110.6. The level of *O*-GlcNAcylation was significantly increased in breast cancer cells, MDA-MB-231 and MCF-7, in comparison to normal mammary epithelial cells (HMEC) (Figure 1). Moreover, the expression levels of OGT and OGA were determined in order to observe whether alteration of *O*-GlcNAcylation was related to their *O*-GlcNAc cycling enzymes. The result showed that both OGT and OGA levels were overexpressed in both breast cancer cell lines when compared to those in normal cells (Figure 1).

Extracellular O-GlcNAc-modified proteins from serum-free media. To study extracellular *O*-GlcNAc-modified proteins, SFM was used. There was no significant difference in cell viability between normal breast and cancer cells cultured in the SFM and complete media (Figure 2A). Immunoblot

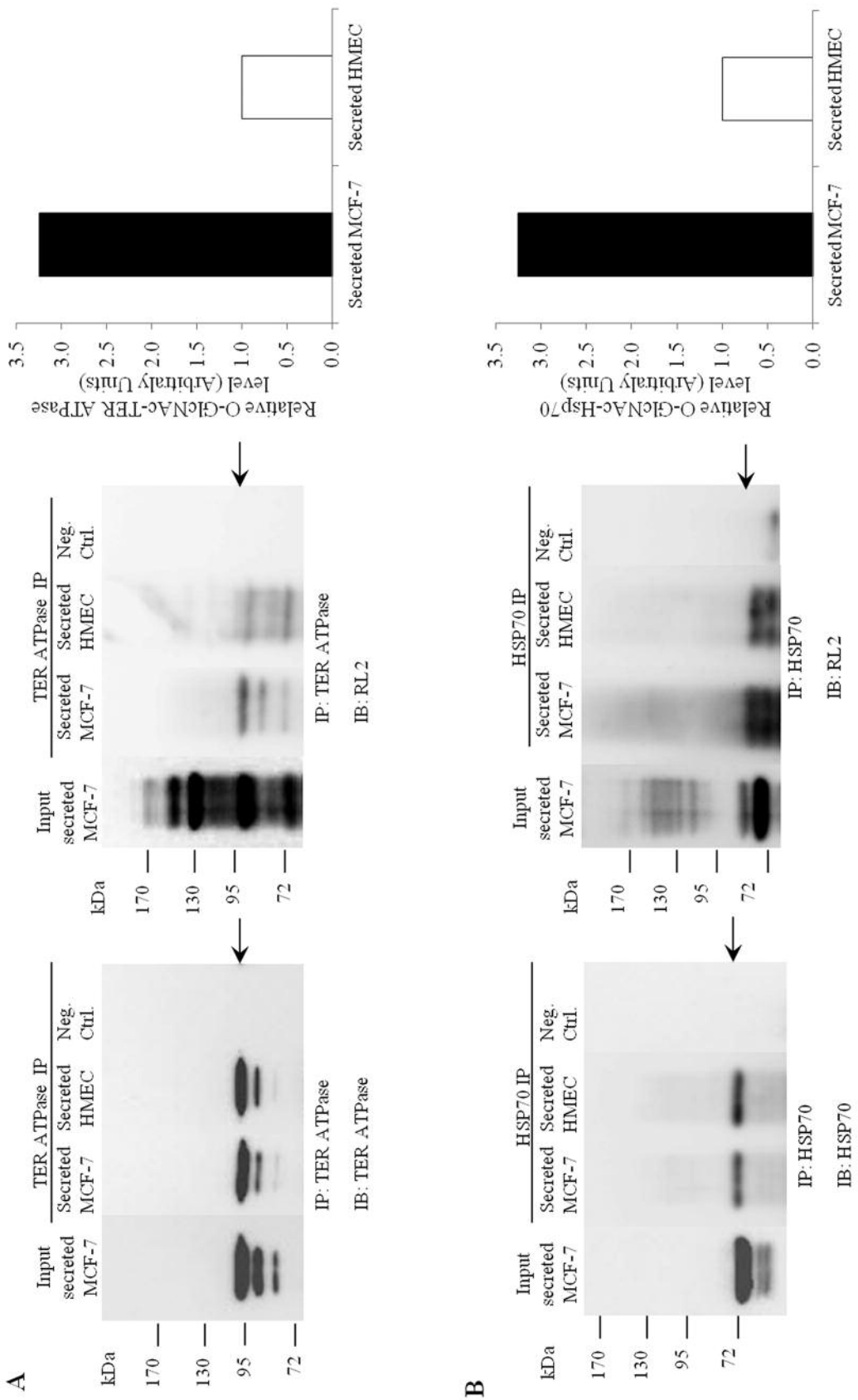


Figure 4. *Confirmation of O-GlcNAc-heat shock protein of 70 kDa (O-GlcNAc-HSP70) and O-GlcNAc-transitional endoplasmic reticulum ATPase (O-GlcNAc-TER ATPase) in the serum-free media (SFM) of MCF-7 and HMEC. A: Immunoblots of HSP70 and O-GlcNAc-HSP70 and relative band intensity of O-GlcNAc-HSP70 to total HSP70. B: Immunoblots of TER ATPase and O-GlcNAc-TER ATPase and relative band intensity of O-GlcNAc-TER ATPase to total TER ATPase. Extracellular proteins from SFM were immunoprecipitated (IP) with antibodies against HSP70 and TER ATPase and immunoblotted (IB) with HSP70, TER ATPase and RL2 antibodies, respectively. Arrows indicate HSP70 and TER ATPase.*

analysis revealed that *O*-GlcNAcylation level of extracellular proteins from the SFM of MDA-MB-231 and MCF-7 cells was significantly increased when compared to those of HMEC. In contrast, no differences were detected in the total proteins stained by SYPRO Ruby (Figure 2B and C).

Furthermore, extracellular proteins from the SFM were confirmed to be *O*-GlcNAc-modified proteins, since there was no change in the pattern of *O*-GlcNAc-modified proteins when treated with PNGase F, indicating that the protein bands were not *N*-GlcNAc linked (Figure 2D). Moreover, when compared to the untreated sample, on blot β-elimination and OGA treatment (50 μg and 100 μg of OGA) demonstrated that most *O*-GlcNAc bands were eliminated, showing that proteins from the MCF-7 SFM present in the immunoblot were specifically modified by *O*-GlcNAc (Figure 2D).

Differential expression of O-GlcNAc-modified proteins secreted into the extracellular compartment of normal and breast cancer cells. MCF-7 cells had a high level of intracellular and extracellular *O*-GlcNAcylation compared to MDA-MB-231cells (Figures 1 and 2). Therefore, we identified the extracellular *O*-GlcNAc-modified proteins in MCF-7 using 2-DE and *O*-GlcNAc immunoblot analysis. Interestingly, more *O*-GlcNAc-modified protein spots were found in MCF-7 secretion than in HMEC secretion, with some spots being expressed only in cancer cell secretion (Figure 3A). However, only three *O*-GlcNAc spots matching CBB protein spots were successfully identified using LC-MS/MS. These were calsyntenin 1, TER ATPase, and HSP70 (Figure 3B).

Verification of O-GlcNAc-modified proteins secreted into the extracellular compartment. HSP70 and TER ATPase were confirmed to be modified by *O*-GlcNAc using IP and immunoblotting with specific antibodies against HSP70, TER ATPase and RL2, respectively. The results showed that HSP70 and TER ATPase were modified by *O*-GlcNAc. Both *O*-GlcNAc-HSP70 and *O*-GlcNAc-TER ATPase from the SFM of MCF-7 cells were increased in comparison to the equivalent proteins in the SFM of HMEC (Figure 4).

Discussion

Traditionally, *O*-GlcNAcylation has been considered a post-transitional protein modification occurring in intracellular compartments. Aberrant *O*-GlcNAcylation has been found to be associated with malignant phenotypes of many types of cancer (15). In this study, we explored this modification in proteins derived from intracellular and extracellular compartments using breast cell lines as models for study. We found that intracellular *O*-GlcNAc levels were increased in MDA-MB-231 and MCF-7 cells compared to those in normal breast cells, HMEC. Surprisingly, both OGT and OGA expression levels were up-regulated in breast cancer cells. Augmentation of the two *O*-GlcNAc cycling enzyme levels may be important and, at least in part, control the overall *O*-GlcNAc level in cancer cells. Consistent with these results, we and other groups have found that *O*-GlcNAcylation level is increased in breast cancer tissues (4-6). To study extracellular proteins from healthy cells, we optimized cell culture conditions with SFM. Interestingly, *O*-GlcNAc-modified proteins were found to be secreted into the extracellular compartment under these culture conditions. SFM from both cancer cell lines had high *O*-GlcNAcylation levels when compared to those of HMEC. Moreover, *O*-GlcNAc patterns differed between intracellular and extracellular compartments, representing a non-identical set of *O*-GlcNAc-modified proteins. Moreover, from treatment with PNGase F, *O*-GlcNAcase, and on-blot β-elimination, we demonstrated that the extracellular proteins from the SFM were *O*-GlcNAc-modified proteins. These data showed for the first time that *O*-GlcNAcylated proteins were secreted into the extracellular environment of breast cancer cells.

Currently, there is little information concerning *O*-GlcNAcylation of extracellular proteins. Matsuura *et al*. first reported that EGF domains on extracellular proteins such as NOTCH receptors were *O*-GlcNAc-modified in *Drosophila* cuticle (8). Later, the same group reported a novel EOGT in *Drosophila* and mouse, responsible for extracellular *O*-GlcNAcylation (8, 9). They propose that *O*-GlcNAcylation of secreted and membrane glycoproteins is required for cell–cell or cell–matrix interactions at the cell surface. Extracellular *O*-GlcNAc-modified proteins were also reported from releasate of thrombin-activated platelets (16), from cell surface of stem cells (17) and from pig nasal mucus (18).

There has been an increasing interest in cancer cell secretomes, which contain an important class of proteins that are secreted or shed from the cell surface, as well as intracellular proteins released into the extracellular compartment *via* classical or non-classical secretory or exosomal pathways (19). These proteins play vital roles in cancer cell differentiation, invasion and metastasis. In the present study, we applied 2-DE with *O*-GlcNAc immunoblotting to identify extracellular *O*-GlcNAc-modified proteins. We found that there were more *O*-GlcNAc-modified protein spots in MCF-7 secretion than those in HMEC secretion. However, we successfully identified three proteins using proteomic approaches: HSP70, TER ATPase, and calsyntenin-1 (CST1).

HSP70 is a stress-inducible protein which is ubiquitously expressed in cellular and subcellular compartments and plays an important role in protein folding and protecting cells from stress. High levels of HSP70 are frequently detected in various cancer cells, and presumably enhances cell proliferation and confers resistance to stress-induced apoptosis (20). HSP70 can be secreted extracellularly in a free soluble form, complexed to antigenic peptides, or in exosomes (21, 22). HSP70 was identified as a GlcNAc-specific binding lectin and itself was

modified by *O*-GlcNAc (23). Several HSP70 isoforms have been shown to be *O*-GlcNAc-modified, with modification level being increased in high-glucose media (24). Our data show that HSP70 is secreted from breast cancer cells and that extracellular HSP70 was modified by *O*-GlcNAc. The level of extracellular *O*-GlcNAc-HSP70 from breast cancer cells was increased in comparison to that from normal cells. Currently, there is no report on the sites of *O*-GlcNAc modification in HSP70. However, online software *O*-GlcNAcScan (http://cbsb.lombardi.georgetown.edu/hulab/OGAP.html) suggests five potential *O*-GlcNAc sites on HSP70: Ser106, Thr140, Thr158, Ser400, and Ser563. *O*-GlcNAc-HSP70 may play roles in protecting cytoplasmic and nuclear proteins from degradation. In addition, extracellular *O*-GlcNAc-HSP70 may have other functions in cell–cell communication or interaction, which requires further study to understand the roles of extracellular *O*-GlcNAc-HSP70 in cancer biology.

TER ATPase, also called valosin-containing protein, is a member of the ATPase group associated with diverse cellular activities, including transport mechanisms, membrane fusion, protein degradation and cell cycle regulation (25). It is markedly elevated in many cancer types and associated with poor prognosis (26). Here, we found that TER ATPase was secreted into the extracellular compartment of breast cancer cells and was shown to be modified by *O*-GlcNAc. The level of extracellular *O*-GlcNAc-TER ATPase from breast cancer cells was increased compared to that from normal cells. TER ATPase was reported to be secreted into culture media during adipocyte differentiation (27) and was modified by *O*-GlcNAc in *Xenopus laevis* oocytes (28), β cells (29), and from the cell lysates of HeLa, 3T3, COS-1 and S2 cell lines (30). There has been no report on the *O*-GlcNAc modification sites of TER ATPase: however, online software *O*-GlcNAcScan suggests seven *O*-GlcNAc potential sites in TER ATPase, namely Ser3, Ser13, Ser40, Ser326, Ser416, Ser444 and Ser702. However, the role of this glycosylation on how extracellular TER ATPase affects cellular processes requires further study to elucidate the functional consequences of this modification in cancer.

CST1, also known as alcadein-α, is a transmembrane protein involved in vesicle transportation (31). CST1 is composed of two cadherin-like repeats in its *N*-terminal extracellular region. This *N*-terminal can be constitutively cleaved and its ecto-domain can be released into the extracellular space (32). CST1 was found on the cell surface of ovarian cancer cells and can be secreted extracellularly (33). However, the functional relevance of the constitutive cleavage of CST1 remains unclear. There has been no report on *O*-GlcNAc modification of CST1, but *O*-GlcNAcScan predicts 11 potential *O*-GlcNAc sites in TER ATPase: Thr149, Thr172, Thr248, Thr307, Thr387, Ser606, Thr624, Ser751, Ser858, Thr859 and Ser936. However, how this glycosylation affects the function of extracellular CST1 protein in cellular processes requires further investigation.

In conclusion, we demonstrated for the first time that extracellular proteins were modified by *O*-GlcNAc in the secreted products of breast cancer cells. *O*-GlcNAc immunoblots showed that patterns of *O*-GlcNAcylation between intracellular and extracellular proteins differed. Interestingly, extracellular *O*-GlcNAc level was dramatically increased in secretions from breast cancer cell lines (MDA-MB-231 and MCF-7) compared to those from normal breast cells (HMEC). Proteomic profiles revealed aberrant *O*-GlcNAc spots in secretion of MCF-7 cells in comparison to those in HMEC cells. Among them, TER ATPase and HSP70 were identified by MS and confirmed to be *O*-GlcNAc-modified by immunodetection. Both proteins had higher *O*-GlcNAc modification in secretion of breast cancer cells than in those of normal cells. These data indicate the need to study *O*-GlcNAcomics in the extracellular compartment of cells, and suggests that secreted *O*-GlcNAc-modified proteins may be of interest as potential biomarkers for breast cancer. Further studies are required to clarify the pathways and mechanisms of how these extracellular proteins are *O*-GlcNAc-modified, as well as their roles in breast cancer pathogenesis and disease progression.

Acknowledgements

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