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Cellular O-Glycome Reporter/Amplification to Explore O-Glycans of Living Cells

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Abstract

Protein O-glycosylation plays key roles in many biological processes, but the repertoire of O-glycans synthesized by cells is difficult to determine. Here we describe a new approach termed Cellular O-Glycome Reporter/Amplification (CORA), a sensitive method to amplify and profile mucin-type O-glycans synthesized by living cells. Cells incubated with peracetylated benzyl-α-N-acetylgalactosamine (GalNAc-α-Benzyl) convert it to a large variety of modified O-glycan derivatives that are secreted from cells, allowing easy purification for analysis by HPLC and mass spectrometry (MS). CORA results in ~100–1000-fold increase in sensitivity over conventional O-glycan analyses and identifies a more complex repertoire of O-glycans in more than a dozen cell types from *Homo sapiens* and *Mus musculus*. Furthermore, CORA coupled with computational modeling allows predictions on the diversity of the human O-glycome and offers new opportunities to identify novel glycan biomarkers for human diseases.

Introduction

Protein glycosylation is a common post-translational modification in all animals that helps to create post-genomic diversity[1]. Although systems-level approaches to evaluate genomes and proteomes have revolutionized our understanding of physiology and disease, similar approaches for glycomics are lacking in most biological settings. A primary need in glycomics is simple and sensitive technologies to analyze all glycans synthesized by cells (the cellular glycome)[2]. This has been challenging due to the diversity and complexity of

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CONTRIBUTIONS

M.R.K., R.D.C., and T.J. conceived of the project. M.R.K., Y.W., N.T.S., A.D., S.M.H., R.D.C., and T.J. designed experiments. M.R.K, A.A., Y.W., D.M.D., and X.S. performed experiments. M.R.K., A.A., Y.W., A.D., S.M.H., R.D.C, and T.J. analyzed the data. M.R.K., R.D.C, and T.J. wrote the manuscript. All authors edited the manuscript.

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glycans, low abundance of certain glycan species, poor sensitivity of existing glycomics approaches, and lack of efficient and unbiased strategies to release glycans from complex samples. Current technologies to evaluate glycans in biological samples require their release from glycoconjugates by chemo-enzymatic methods, followed by analyses by mass spectrometry (MS) and other technologies[2]. Relatively large amounts of biological samples are often needed for detailed structural analyses, limiting the widespread application of glycomics. One way to at least partly overcome these challenges would be to "amplify" the glycome, similar to PCR-hybridization-based technologies for the genome[3]. Such amplification technology would allow analysis of microscale samples of biological material, facilitating clinical and biological discovery.

One of the most common types of protein glycosylation is mucin-type O-glycosylation (R-GalNAcα1-O-Ser or Thr where R represents extended structures). O-glycans are present on >80% of proteins that traverse the secretory apparatus and are important in many normal and pathologic settings[4-7]. Nonetheless, little is known about either the repertoire of O-glycans or how specific O-glycan structures regulate biology, largely due to a lack of effective technologies for O-glycomics. In contrast to N-glycans, which can be released enzymatically, O-glycans require chemical strategies – primarily alkaline β-elimination, which is inefficient, potentially biased, may result in O-glycan degradation via the peeling reaction, and requires extensive expertise not available in most laboratories[8].

To meet these challenges, here we describe a novel method for profiling and amplifying mucin-type O-glycans from living cells, termed Cellular O-Glycome Reporter/Amplification (CORA). To assess the repertoire of glycosyltransferases and glycosylation reactions in the secretory pathway for the O-glycome, we developed a chemical O-glycan precursor, peracetylated Benzyl-α-GalNAc (Ac_3GalNAc-α-Benzyl, Bn), which is taken up by living cells, de-acetylated, modified by native glycosyltransferases in the presence of nucleotide sugar-donors in the secretory pathway, and then secreted into media (Fig. 1). Benzyl-α-GalNAc structurally mimics the precursor GalNAcα1-O-Ser or Thr (Tn antigen) in O-glycoproteins[9] and its peracetylation promotes transport across the plasma membrane[10]. Cells incubated with Ac_3GalNAc-α-Benzyl secrete a diversity of Benzyl-α-O-glycans that represent the cellular O-glycome. Using CORA, we identified 76 O-glycans in a variety of cell types, including those observed by traditional alkaline β-elimination/MS. Importantly, CORA provided ~100–1000-fold enhanced sensitivity, much cleaner MS profiles of O-glycans, and revealed novel O-glycans in a variety of cancer and primary cells not seen by classic approaches. This simple, sensitive, and versatile method for amplifying and profiling the cellular glycome could be a transformational technology in the biomedical sciences and allow dynamic studies of O-glycosylation in living cells.

Results

Cells uptake Ac_3GalNAc-α-O-Bn and secrete Bn-O-glycans

Mucin-type O-glycan biosynthesis begins with transfer of GalNAc to Ser or Thr residues in glycoproteins to generate GalNAcα1-O-Ser or Thr (Tn antigen), which T-synthase extends to the dominant Core 1 O-glycan Galβ3GalNAcα1-O-Ser or Thr. Bn-α-GalNAc structurally mimics GalNAcα1-O-Ser or Thr and has been used *in vitro* as an acceptor for T-synthase

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and Core 3 GnT[11, 12], which is present in primary GI tissues but not cell lines[11, 12]. Prior studies have shown that monosaccharides linked to hydrophobic aglycones can prime glycan biosynthesis[13, 14] and that high concentrations of Bn-α-GalNAc (2~20 mM) can inhibit extension of Core 1 O-glycans in glycoproteins of cultured cells[15], apparently by acting as a surrogate acceptor for T-synthase[16]. However, we hypothesized that Bn-α-GalNAc at low concentrations, if available to cells, might not inhibit biosynthesis, but could be used as a surrogate acceptor by the T-synthase to allow formation of free Bn-O-glycans representing the cellular O-glycome. We utilized Ac_3GalNAc-α-Bn, a more hydrophobic derivative of GalNAc-α-Bn (Supplementary Fig. 1a), to enhance cellular uptake as shown for other peracetylated carbohydrate compounds[10, 14] and predicted that upon entry into cells Ac_3GalNAc-α-Bn would become activated by cytosolic esterases to regenerate Bn-α-GalNAc. Bn-α-GalNAc would then be transported into the secretory pathway, modified by glycosyltransferases, and secreted into media as biosynthetic Bn-O-glycans that could be easily purified and analyzed by MS. We termed this technology Cellular O-Glycome Reporter/Amplification or CORA and the workflow of the technology is described Fig. 1.

To test this, we cultured adherent (HEK293) and suspension (Molt-4) cells in complete media containing 50 μM Ac_3GalNAc-α-Bn or vehicle (DMSO) for 3 days. Putative Bn-O-glycans from media were separated from larger material using a cut-off membrane, purified by C18 chromatography, permethylated, and analyzed by MALDI-TOF-MS. Here as elsewhere we only analyzed the permethylated non-sulfated/non-phosphorylated glycans. We observed clean MALDI-TOF profiles with Bn-O-glycan compositions corresponding to Core 1 and 2-based structures (Supplementary Fig. 2) from cells cultured with Ac_3GalNAc-Bn but not vehicle, indicating efficient uptake and modification of the O-glycan precursor by glycosyltransferases *in vivo*.

To assess whether peracetylation of Bn-α-GalNAc enhanced uptake and subsequent sensitivity, we incubated breast cancer cells (MDA-MB-231) with 0–250 μM of Ac_3GalNAc-Bn or Bn-α-GalNAc for 3 days. We observed Bn-O-glycans with predicted sialylated Core 1 structure at a concentration as low as 25 μM for Ac_3GalNAc-Bn, but only at the highest concentration of 250 μM for Bn-α-GalNAc (Supplementary Fig. 3). Further, peracetylation of Bn-α-GalNAc was stable in complete media for at least 3 days (Supplementary Fig. 4). Thus, peracetylation improved the sensitivity, and at such low concentrations should limit potential side effects, as explored below.

To optimize conditions, we cultured MDA-MB-231 cells with 0–250 μM of Ac_3GalNAc-Bn for 2–4 days. Bn-O-glycans in culture media were seen at all time points and concentrations down to 5 μM of Ac_3GalNAc-Bn for 2 days (Supplementary Fig. 5), and their profiles were stable over time. Remarkably, increasing Ac_3GalNAc-Bn concentration shifted abundance from disialylated to monosialylated Core 2 (Supplementary Fig. 5d), supporting observations that glycosyltransferase:substrate ratios drive glycan microheterogeneity[17]. Thus, CORA should be performed at low concentrations of Ac_3GalNAc-Bn. For most subsequent studies, we incubated cells with 50 μM Ac_3GalNAc-α-Bn for 3 days. At these conditions, Ac_3GalNAc-α-Bn was not toxic to cells (Supplementary Fig. 6), did not alter cellular morphology or granularity (Supplementary Fig. 7), did not alter cell surface O- or

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N-glycosylation (Supplementary Fig. 8,9), and Bn-O-glycans were stable after secretion from cultured cells (Supplementary Fig. 10).

Synthesis of Core 1-based Bn-O-glycans requires T-synthase

The above results show that cells cultured with Ac_3GalNAc-α-Bn produce and secrete free Bn-O-glycans containing the Core 1-based structures, presumably requiring T-synthase. It is known that biosynthesis of active T-synthase requires a specific molecular chaperone Cosmc and genetic or epigenetic disruption of *Cosmc* results in inactive T-synthase and expression of Tn and sialylTn antigens[18-20].

To confirm that Bn-α-GalNAc can only be utilized by glycosyltransferases involved in mucin-type O-glycan biosynthesis, but not other irrelevant or unknown pathway(s), we performed CORA on cells with mutant or wild-type *Cosmc*. Only cells with functional *Cosmc* and active T-synthase secreted Bn-O-glycans when administered Ac_3GalNAc-Bn (Fig. 2). Furthermore, cells incubated with the isomer Ac_3GlcNAc-β-Bn (Supplementary Fig. 1b) secreted no Bn-O-glycans or only the simple trisaccharide Neu5Ac-Gal-GlcNAc-Bn (Supplementary Fig. 11), indicating that Ac_3GalNAc-α-Bn is specific for mucin-type O-glycans. These demonstrate that CORA faithfully reports the O-glycome and that modification in cells requires active T-synthase.

Accuracy of CORA

We compared O-glycome profiles from CORA to β-elimination, available through the CFG (http://www.functionalglycomics.org). WEHI-3 and HL-60 cells were analyzed because they have complex O-glycomes with unique structures, such as Cad, and extended poly-N-acetyllactosamines $[3Gal\beta4GlcNAc\beta-]_n$ that are challenging to detect by β-elimination of lysates but observed on purified glycoproteins[21, 22].

MALDI-TOF-MS and MS/MS profiles (Fig. 3, Supplementary Fig. 12,13) show that HL-60 and WEHI-3 cells produced 11 and 40 glycan structures, respectively, including sialylated Core 1 and 2-based glycans for both cells and Cad antigen in WEHI-3 (Fig. 3a,b). CORA detected most of the compositions seen in β-eliminated samples (12 of 13 for WEHI-3, 4 of 7 for HL-60) and many additional compositions (16 for WEHI-3, 6 for HL-60) (Fig. 3c–e), which were generally the most complex, including poly-N-acetyllactosamines with ~3 repeats and I antigen. The 4 masses observed by β-elimination but not CORA (Fig. 3c,d) are Core 1 and 2-based glycans, lacking terminal sialylation, and therefore are likely biosynthetic intermediates, such as from glycoproteins within an intermediate Golgi compartment prior to secretion.

To confirm our results, we repeated the experiments twice and obtained nearly identical O-glycome profiles (Supplementary Fig. 14) and performed ESI-MS and obtained similar results to MALDI-MS (Supplementary Fig. 15). We also evaluated a range of cell types (Supplementary Table 1) with diverse glycosyltransferases (as exemplified by C2GnT1–3, Supplementary Fig. 16) and obtained O-glycome profiles from all the cells indicating that Bn-GalNAc can access most if not all of the O-glycan machinery. Thus, CORA reflects the cellular O-glycome, which is relatively stable under optimal culture conditions.

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Sensitivity of CORA

Beta-elimination often requires $\geq 10^7$ cells and produces many unassignable peaks[23]. To determine how many cells are needed to get clean, interpretable profiles with CORA, we profiled four cell lines each seeded at 5×10^5, 10^5, or 2×10^4 cells. We obtained O-glycomes from all lines seeded at $\geq 10^5$ cells and 3 of 4 lines seeded at 2×10^4 cells (Fig. 4). Notably, profiles did not change with different cell numbers. The detection of Bn-O-glycans from 2×10^4 cells cultured for 3 days (to a total of ~8×10^4 cells assuming ~24 hour doubling times) represents a ~100–1000-fold increase in sensitivity compared to β-elimination.

Profiling the O-glycome of mouse and human primary cells

Because primary cells differ metabolically from cancer cells[24], we validated CORA on primary human and mouse cells. First we profiled immortalized murine pulmonary endothelial cells (mPECs) with or without *Cosmc* from Tie2-*Cre*$^{+}$;*Cosmc*$^{F/+}$ mice[25] to determine if Bn-O-glycan synthesis requires functional *Cosmc*. Only mPECs with a functional *Cosmc* secreted Bn-O-glycans (Supplementary Fig. 17a,b). Next we isolated Tn(−) mPECs from mice (Tie2-*Cre*$^{-}$;*Cosmc*$^{F/y}$) and performed CORA (Supplementary Fig. 17c,d). Glycan structures were similar in immortalized and primary mPECs, except for a glycan with the disialyl motif, only found in the primary cells. However, the ratio of O-glycans differed in these two mPECs, suggesting that transformation may alter glycan biosynthesis, as in human tumors[26].

Next, we evaluated primary human dermal fibroblasts and umbilical vein endothelial cells (HUVECs) (Fig. 5, Supplementary Fig. 18,19). HUVECs produced 43 O-glycan structures, including those containing poly-N-acetyllactosamine, Lewis structures, blood group antigens, and I antigen, as confirmed by MS/MS (Fig. 5a, Supplementary Fig. 18a,19). Fibroblasts also produced at least 18 glycans (unique masses), including poly-N-acetyllactosamine, Lewis structures, and blood group antigens (Fig. 5b, Supplementary Fig. 18b). The remarkable diversity of O-glycans in these cells indicates their potential importance.

CORA promotes the discovery of novel glycans

MS/MS sequencing is needed for definitive determination of glycan structure. However, this is often not possible with β-elimination because of insufficient material. Using CORA, we performed MS/MS sequencing on HL-60, WEHI-3, and HUVEC cells and observed many diverse glycan structures (11 for HL-60, 40 for WEHI-3, 43 for HUVEC) as well as novel and unexpected O-glycans, including extended Core 1 (HL-60), Vim-2 (HL-60, HUVEC), disialic acid (WEHI-3), and I antigen (WEHI-3, HUVEC) (Fig. 3,5, Supplementary Fig. 12,13,19). Although this is the first report of extended Core 1 on HL-60 cells and of Vim-2 and disialic acid on O-glycans from myelocytic cells, these epitopes have been observed in related contexts or structures, indicating that they are biosynthetically reasonable products[27-29].

The I antigen replaces i antigen on RBCs after embryogenesis, but had not been identified on O-glycans from other cell lineages, except in secretions[30]. The discovery of I antigen on

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O-glycans from 2 distinct non-RBC cell lineages suggests that this may be a common, yet unappreciated structure. We have no explanation as to why I antigen was not previously observed on O-glycans from these cell lines, except that in general, O-glycans released by β-elimination are present in insufficient quantities to identify larger O-glycans in the high mass range and when larger O-glycans are identified, MS/MS usually is not feasible. CORA permits analyses of small numbers of cells since they continuously produce more Bn-O-glycans, thus amplifying their O-glycomes.

CORA can evaluate the complexity of the cellular O-glycome

The complete repertoire of O-glycans is not known. Here we analyzed 18 cell lines including primary cells and *Cosmc*-deficient cells and identified 57 unique O-glycan compositions (Supplementary Table 1, 2). Detailed MS/MS structural characterization on 3 of these cells (HUVEC, HL-60, WEHI-3) revealed 76 unique structures, derived from 48 unique compositions (#structures:#compositions = 1.6) (Supplementary Table 3). From our 18 cell lines, we evaluated the frequency of cell lines expressing a given glycan composition (Supplementary Fig. 20a,b, Supplementary Table 2) and determined that a few outstanding cell lines encompassed most of the complexity of all 18 glycomes (Supplementary Fig. 23c). We developed a computational model to determine the number of unique O-glycan compositions we would observe on average from a given number of cell lines and predicted that the estimated 200 cell types in the human body produce 235 unique compositions (Supplementary Fig. 21). Using the observation above that the number of glycan structures may be 1.6-fold more than the number of compositions, we could thus predict that there are 1.6 × 235 = 376 non-sulfated/phosphorylated structurally different O-glycans in the human body. While purely speculative to a degree, this is the first estimate of the size of the human cellular O-glycome based on comparative cellular O-glycomics and provides a roadmap to comprehensively sequence the human cellular O-glycome.

Discussion

Here we show that CORA provides a sensitive new approach to both amplify and profile the mucin-type O-glycome in living cells. O-glycans are present on most secreted and cell surface proteins, but previous strategies for O-glycomics have limited their investigation. Compared to traditional methods for O-glycan analysis, CORA 1) amplifies the O-glycome, 2) bypasses glycan release, 3) avoids O-glycan degradation, 4) enhances sensitivity ~100–1000-fold, 5) increases signal-to-noise, 6) detects novel complex O-glycans, 7) provides a biosynthetic process for real-time studies of dynamic changes in the O-glycome of living cells, and 8) is technically simple with potential for wide-spread and high-throughput application.

Several novel O-glycans were identified in cells by CORA, but not seen in the β-elimination released samples, indicating that β-elimination may not provide enough material for MS or MS/MS evaluation of these low abundance species. For example, MS analyses of β-eliminated O-glycans from WEHI-3 cells have not identified Sialyl-Le^X on core 2 O-glycans, a critical component on PSGL-1 recognized by P-selectin, which binds WEHI-3 cells. However, this minor structure was previously confirmed by an enrichment analysis[22],

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and now detected by CORA (Fig. 3, glycan with a Mass of 1939), directly demonstrating the improved sensitivity of CORA over conventional methods. Thus, the novel structures we identified are natural O-glycans, mostly present on glycoproteins at low abundance. Interestingly, CORA using Ac_3GalNAc-α-Bn is specific for O-glycans, since *Cosmc*-deficient cells do not produce Bn-O-glycans and culturing cells with the isomer Ac_3GlcNAc-β-Bn produced no glycans or a trisaccharide (Sia-Gal-GlcNAc-Bn) only. The latter result suggests that the enzymes responsible for poly-N-acetyllactosamine production, I antigen synthesis, and α3-fucosylation are relatively specific to extended O-glycans in the cell lines examined.

It is interesting that CORA is effective at low concentrations (<250 μM) and incubation times with no observed impact on cellular properties or glycosylation. Prior studies had shown that treatment of cells with high concentrations of Bn-α-GalNAc produced mainly small Galβ3GalNAcα1-O-benzyl and sialyl-Galβ3GalNAcα1-O-benzyl derivatives[31, 32]. However, the success of CORA using low concentrations of this new Ac_3GalNAc-Bn derivative as a precursor indicates that it is readily taken up by live cells, converted to Bn-α-GalNAc, efficiently utilized by the T-synthase, and further accessed by a wide range of enzymes in the secretory pathway, including the most terminal types of glycan modifications and extensions. It is worth noting that this approach defines the global ability of cells to make O-glycans, but may not always reflect their natural relative abundances, which may be influenced by the concentration of protein substrates or expression of O-glycosylated polypeptide cores.

This approach may allow assessment of the total diversity and repertoire of O-glycans in an animal O-glycome. Recent studies using transfected CHO cells engineered to express all major O-glycan core structures, along with chemical release techniques, identified ~70 different glycan structures[33]. The repertoire of O-glycans is likely much larger, as mucin-type O-glycan determinants have been estimated to be nearly 1,000, with probably <500 non-sulfated O-glycans[34]. In this regard, our computational modeling allowed us to predict the size of the non-sulfated/phosphorylated animal O-glycome to be in the range of ~376 unique glycan structures. What drives this diversity? Amplification of an entire O-glycome from a simple chemical precursor suggests that the biosynthetic machinery and not diverse protein substrates largely drives O-glycan heterogeneity.

An advantage of CORA is that live cells are used to generate the O-glycome, but the cells afterward can also be analyzed by conventional techniques if desired for comparative analyses. Advances in cell and organoid culture, have enabled culture of many normal and diseased tissues[35-38], yet glycan release is often not sufficiently sensitive to analyze such precious specimens. CORA could address this challenge by amplifying the glycome of these cells in addition to analyzing tumor cells in a high-throughput manner. Although we have limited our analyses to mucin-type O-glycans, this may be a general strategy for amplifying and profiling many classes of glycosylation, with appropriate precursors. While we used small numbers of cells for glycan analysis, large numbers of cells in continuous culture could also be used as glycan biosynthesis factories to prepare any and all natural O-glycans, even those difficult to synthesize chemically. Bn-O-glycans or their derivatives could be isolated to generate therapeutic glycans, test the roles of unusual glycans in cell recognition

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or growth, or for display in glycan microarrays. Amplifying the O-glycome by CORA offers a new paradigm in cellular glycomics that will enable new types of investigations in a wide range of basic and clinical settings to give new insights into O-glycans in physiology and disease.

Methods

Analyses

Sample size—At least two different cell lines were selected for most experiments in order to validate across cell types. More cell lines were used in some instances when we thought this could improve the generalizability of the result. To provide a global view of the O-glycome and validate CORA across cell lines and types, we analyzed a total of eighteen cell lines from distinct organs (skin, kidney, breast, colon, stomach) and tissues (epithelia and connective, including blood cells, fibroblasts, and endothelia) from multiple species (*H. sapiens*, *M. musculus*).

Randomization and blinding—Cell lines and primary cells were selected to capture a diversity of tissues and analyzed at different time points throughout the course of the experiments. Randomization and blinding were not performed for glycan analyses, as this would not have changed the interpretation of mass spectrometry data.

Exclusion criteria—No data were excluded. Data shown were randomly selected from replicate experiments.

Compounds

Benzyl-α-D-GalNAc was purchased from Sigma. Benzyl-β-D-GlcNAcAc$_3$ and 4MU-α-D-GalNAc were purchased from Carbosynth. Benzyl-α-D-GalNAcAc$_3$ was generated by addition of 2:1 pyridine:acetic anhydride to Benzyl-α-D-GalNAc for 1 hr at 65°C in molar excess, dried by centrivap, resuspended in 1 ml water, and lyophilized.

Cell culture

Molt-4, Jurkat (clone E6-1), LS174T, HL-60, WEHI-3, HUVEC, and human dermal fibroblasts were purchased from ATCC. Lox were a kind gift from Dr. Oystein Fodstad (Oslo University Hospital). MKN45, Colo205, MDA-MB-231, and MCF7 were a kind gift from Dr. Henrik Clausen (University of Copenhagen). Tn(−) and Tn(+) LS174T were subcloned from a mixed population as previously described[19]. Lox and Jurkat were transfected with full length *Cosmc* or empty vector (pcDNA3.1+) and selected by G418. Tn(−) cells were further sorted by FACS. Molt-4, Jurkat, Lox, HL-60, MKN45, and Colo205 were cultured in RPMI (Corning) supplemented with 10% FBS and 2% P/S. LS174T, MDA-MB-231, and MCF7 were cultured in DMEM (Corning) supplemented with 10% FBS and 2% P/S. MCF7 were further supplemented with 0.01 mg/ml insulin. WEHI-3 were cultured in Iscove's (Corning) supplemented with 10% FBS, 0.05 mM 2-ME, and 2% P/S. HUVEC and human dermal fibroblasts were cultured with endothelial cell growth kit-VEGF (ATCC) and fibroblast growth kit- low serum (ATCC) as instructed. All cells were cultured on

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plastic, except HUVECs, which were cultured on plastic pre-coated with 0.1% gelatin. We did not perform independent verification of cell lines or testing for mycoplasma.

Administration of compound

Ac_3GalNAc-α-Bn or Bn-α-GalNAc was dissolved to 50 or 100 mM in DMSO and further diluted to 5–250 μM in complete media with 5% FBS, except for HUVECs and human dermal fibroblasts which were incubated in ATCC pre-formulated media. Media with compound was administered 1 day (most cells) or 2 days (HUVEC, human dermal fibroblasts) after seeding. Cells were then incubated 2 – 4 days with compound before collecting media.

Glycan purification from media

Compound was added to complete media and collected after incubation with cells. Media was run over 10kDa centrifugal filter (Amicon – Ultra 4, Millipore) for ~30 minutes at 2465 × g and flow through was collected. Bn-O-glycans were subsequently purified from flow through by Sep-Pak 3 cc C18 cartridge (Waters) by gravity chromatography. The column was equilibrated with 2 × 2 ml acetonitrile, then 4 x 2 ml 0.1% TFA. Media was applied and then column washed with 4 x 2 ml 0.1% TFA. Bn-O-glycans were then eluted with 2 × 1.5 ml 50% acetonitrile/0.1% TFA. Eluent was divided into 3 fractions, centrivapped to remove organic solvents, and lyophilized.

Permethylation and glycan analysis

Dried samples were permethylated by standard procedures[39]. Two hundred μl NaOH/DMSO slurry was added to samples followed by 200 μl methyl iodide. Samples were shaken for 30 minutes and then spun down at 5000 × g for 5 minutes. Supernatant was collected and chloroform extraction performed to isolate permethylated glycans. Five hundred μl chloroform and 500 μl water were added to supernatant, mixed, and centrifuged 5000 × g for 1 minute. Two more washes with 500 μl water were performed before evaporating chloroform by centrivap for 30 minutes. Bn-O-glycans were then resuspended in 25 or 50 μl 50% methanol. 0.5 μl matrix (10 mg/ml 2,5-dihydrobenzoic acid (Sigma), 50% acetonitrile, 0.1% TFA) and 0.5 μl sample were spotted on an Anchorchip target plate, air dried, and analyzed by MALDI-TOF mass spectrometry using Ultraflex-II TOF-TOF system (Bruker Daltonics). Peak masses were identified and structures assigned by composition and knowledge of glycan biosynthetic pathways, or MS/MS where indicated. This procedure only detects non-sulfated/phosphorylated structures; however, these modifications have not been previously reported from cells in our study.

Cell and enzyme assays

T-synthase and mannosidase assays were performed as previously described[9]. Briefly, 4MU-GalNAc was incubated with cell lysate and reaction mix containing O-glycanase and UDP-Gal in triplicate for 45 minutes at 37°C to assay T-synthase. O-glycanase releases 4MU from 4MU-Core 1, which can be assessed in a fluorimeter. Reaction mix without UDP-Gal is used as control. To assess mannosidase activity, 4MU-mannoside was added to lysate in reaction mix in triplicate for 45 minutes at 37°C. Boiled lysate was used as control. Mannosidase

releases 4MU from 4MU-mannoside. Fluorescence was converted to specific activities for T-synthase and mannosidase as described.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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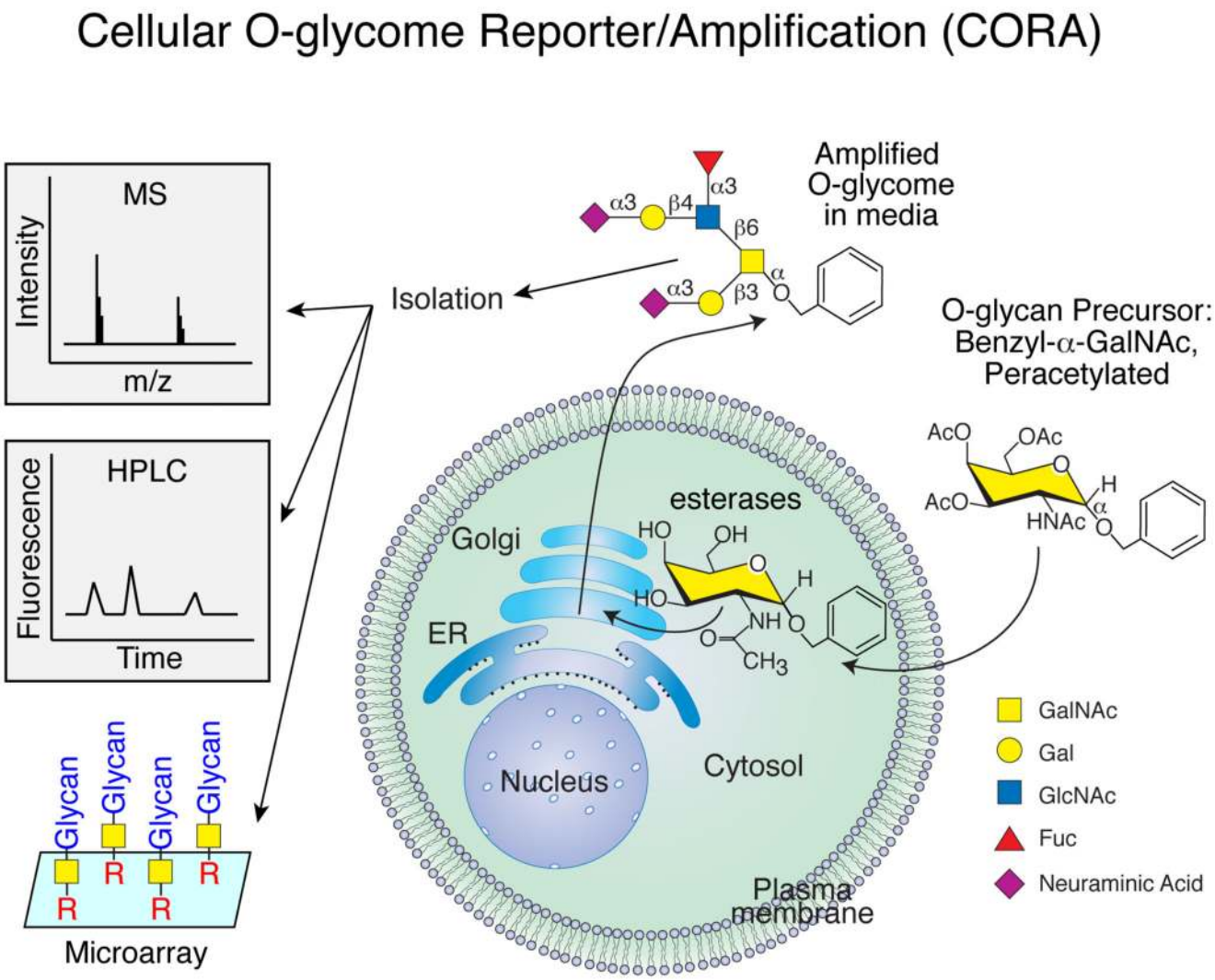

Figure 1.

Overview of Cellular O-glycome Reporter/Amplification (CORA). Cells are incubated with chemical O-glycan precursor or primer (Benzyl-α-D-GalNAc), which is peracetylated (Ac_3GalNAc-Bn) to facilitate passive transport across the plasma membrane. Cytosolic esterases generate Bn-GalNAc, which is taken up in the Golgi and modified by native glycosyltransferases during anterograde transport. Elaborated Bn-O-glycans are secreted into the media, purified, and analyzed by MS (reported here), HPLC, or printed on glycan microarray for interrogation by GBPs.

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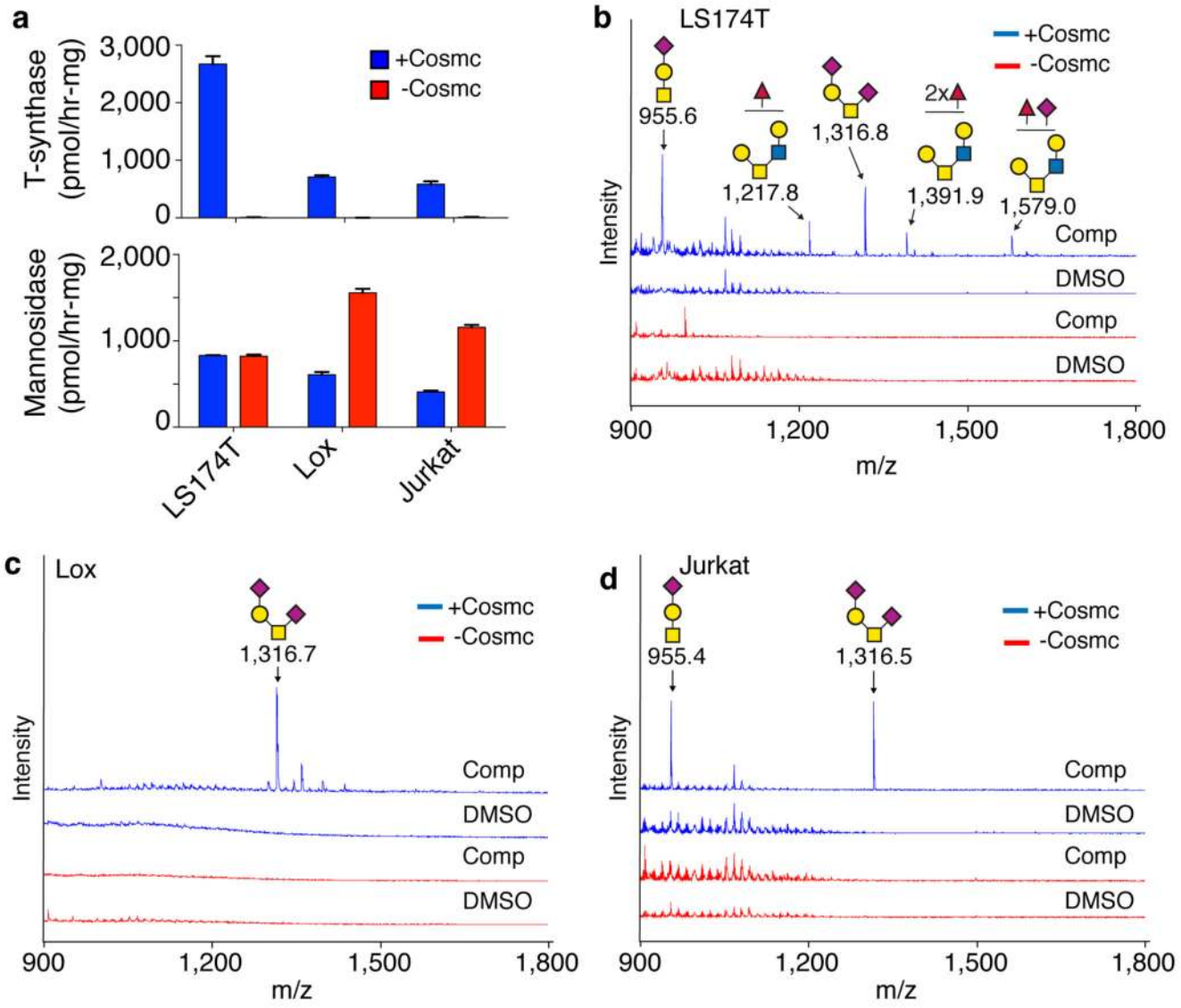

Figure 2.

The chaperone Cosmc and active T-synthase are required for production of Core 1-and 2-based Bn-O-glycans. (**a**) T-synthase activity was measured and reported relative to mannosidase control enzyme for LS174T colorectal cells, LOX melanoma cells, and Jurkat T cells with or without a functional *Cosmc*. Enzymes were assayed in triplicate (n = 2), and a representative experiment is shown, mean ± SD of triplicates. (**b**–**d**) The same cells were incubated with 50μM Ac_3GalNAc-Bn for 3 days and the media was analyzed for Bn-O-glycans for LS174T (**b**), LOX (**c**), and Jurkat (**d**) ± *Cosmc*. Only major glycans are annotated (composition) for LS174T (**b**) for clarity; highly fucosylated minor species as shown in Supplementary Fig. 17b were also observed. Spectra for each graph (**b**–**d**) are off-set, but scaled to same absolute intensity for each cell; representative profiles are shown (n = 2).

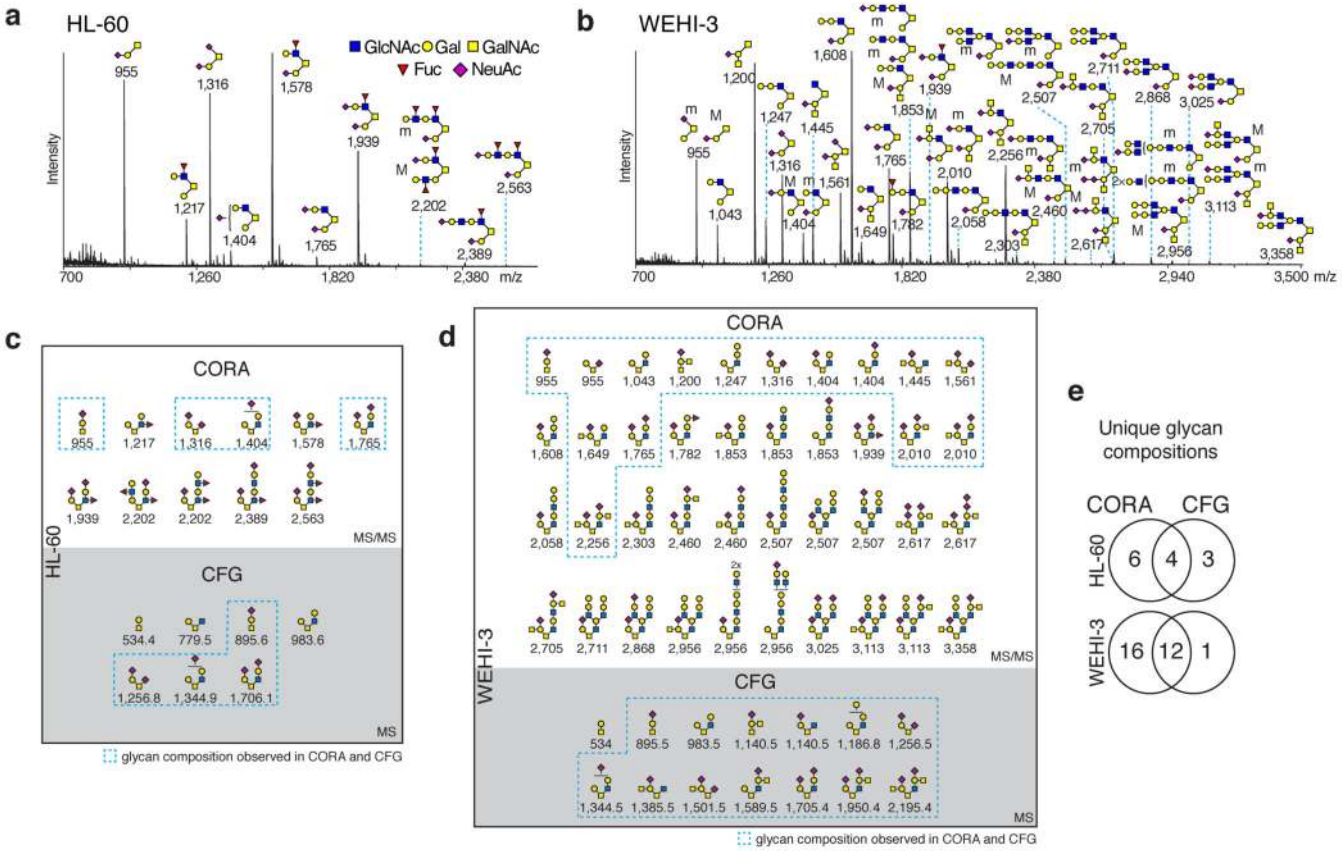

Figure 3.

Accuracy of CORA for profiling the O-glycome. (**a**,**b**) 50 μM Ac_3GalNAc-Bn was incubated with HL-60 (**a**) and WEHI-3 cells (**b**) for 3 days and Bn-O-glycans were purified, permethylated, and analyzed by MALDI-TOF-MS. Here as in all studies we only analyzed the non-sulfated/non-phosphorylated glycans. (**c**,**d**) Bn-O-glycans from CORA (MS/MS structures) were compared to profiles from alkaline β-elimination (MS compositions) (CFG) for HL-60 (**c**) and WEHI-3 (**d**) cells; blue boxes indicate glycan compositions observed in both CORA and β-elimination. Glycan amplification by CORA generated enough material for high quality MALDI-TOF/TOF-MS/MS analysis, which presumably was not possible with the CFG data (previously deposited at http://www.functionalglycomics.org) due to insufficient material from β-elimination. (**e**) The number of glycans (by composition) observed in CORA, β-elimination (CFG), or both are indicated for each cell line. All spectra of O-glycans were derived from the 75% acetonitrile fraction (see Methods). All molecular ions are $[M+Na]^+$. Putative structures are based on composition, tandem MS, and biosynthetic knowledge.

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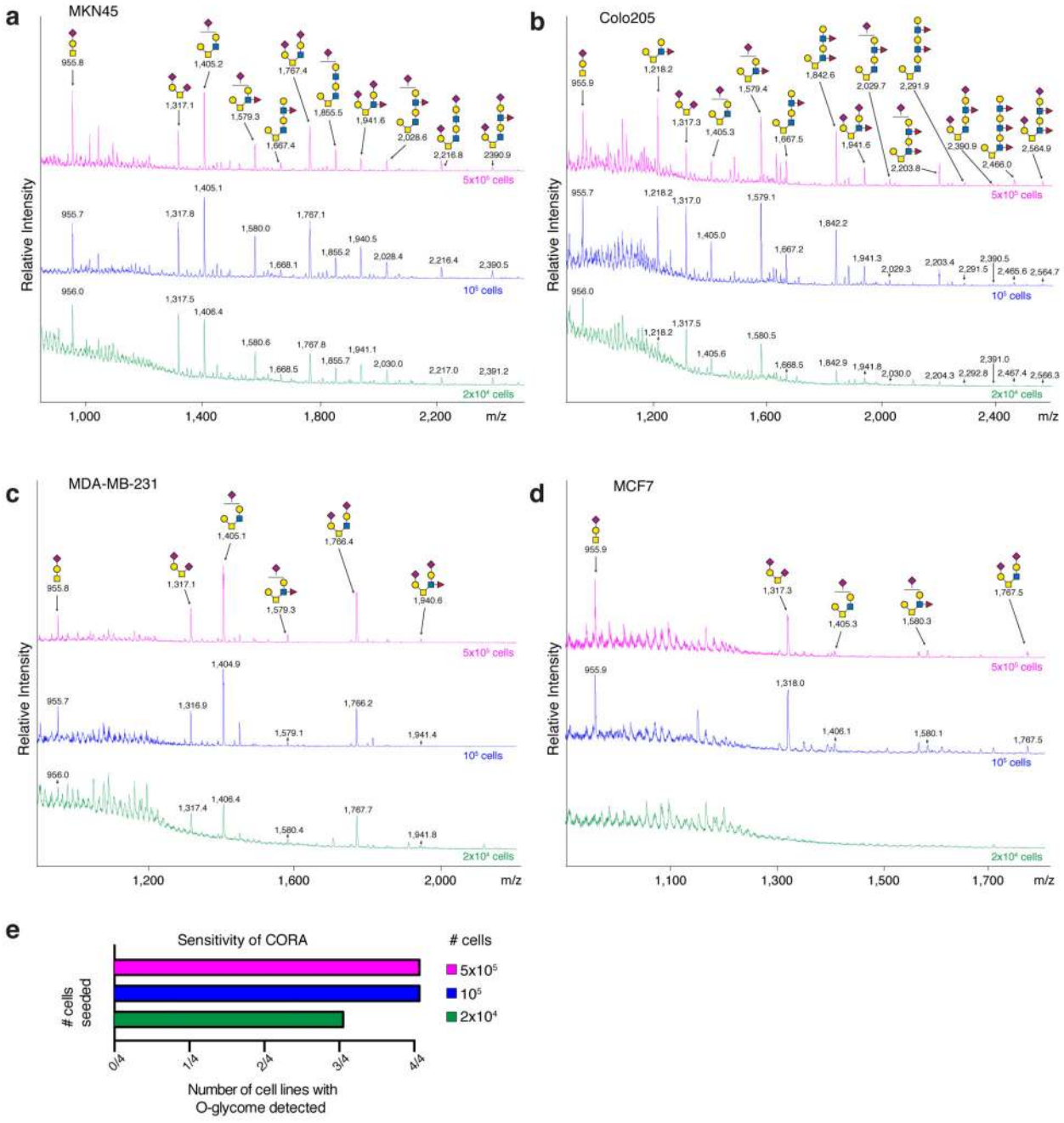

Figure 4.

Sensitivity of CORA. (**a**) MKN45, (**b**) Colo205, (**c**) MDA-MB-231, and (**d**) MCF-7 cells were seeded at 5×10^5, 10^5, and 2×10^4 cells/well in 6 well, 12 well, and 48 well flasks. 50 μM Ac_3GalNAc-Bn was added and Bn-O-glycans were purified and permethylated after 3 days. $1/150^{th}$ (6 well, 12 well) or $1/50^{th}$ (48 well) of total glycans were analyzed by MALDI-MS (composition). (**e**) O-glycomes were observed from all cells seeded at 5×10^5 and 10^5 cells/well and 3 of 4 cells seeded at 2×10^4 cells/well. Spectra are off-set for each

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seeding density and scaled relative to maximum intensity. Representative profiles are shown (n = 2).

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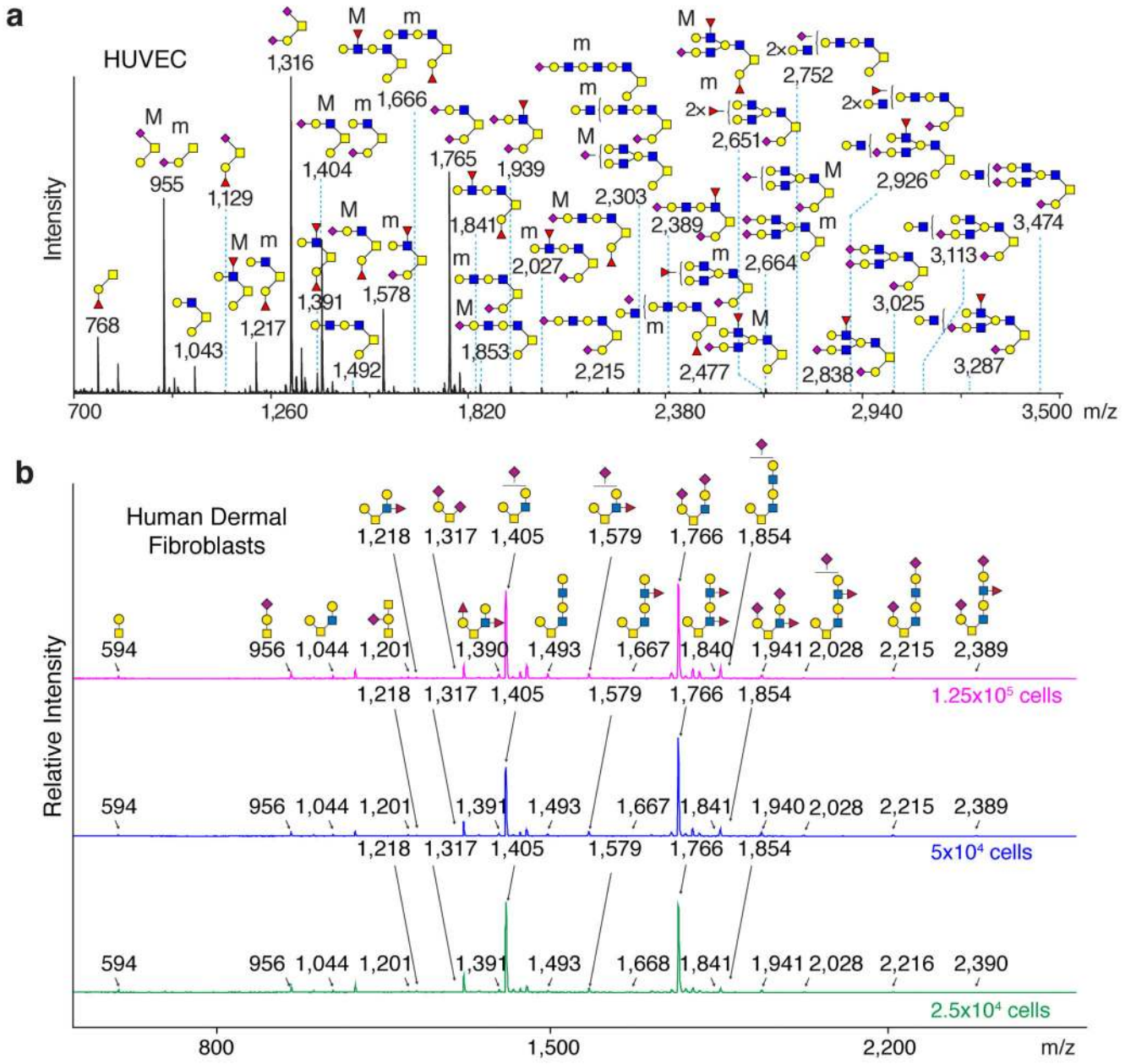

Figure 5.

MALDI-TOF-MS and MS/MS profiling of the O-glycome of primary cells. (**a**,**b**) CORA was used to profile the O-glycome of HUVECs (**a**) and primary human dermal fibroblasts (**b**). 12.5×10^4 (**a, b**), 5×10^4 (**b**), and 2.5×10^4 (**b**) cells were seeded in T25, 6 well, or 12 well flasks, respectively. 50 μM Ac_3GalNAc-Bn was added after 2 days, and Bn-O-glycans were purified, permethylated, and analyzed by MALDI after 3 more days. MALDI-TOF/TOF-MS/MS (structure) analysis was performed for HUVECs and MS (composition) was performed for fibroblasts. Spectra are off-set for each seeding density and scaled relative to

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maximum intensity. Putative structures are based on composition, tandem MS or MS, and biosynthetic knowledge.

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