miRNA proxy approach reveals hidden functions of glycosylation

Tomasz Kurcon¹, Zhongyin Liu¹, Anika V. Paradkar, Christopher A. Vaiana, Sujeethraj Koppolu, Praveen Agrawal, and Lara K. Mahal²

Biomedical Chemistry Institute, Department of Chemistry, New York University, New York, NY 10003

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Glycosylation, the most abundant posttranslational modification, holds an unprecedented capacity for altering biological function. Our ability to harness glycosylation as a means to control biological systems is hampered by our inability to pinpoint the specific glycans and corresponding biosynthetic enzymes underlying a biological process. Herein we identify glycosylation enzymes acting as regulatory elements within a pathway using microRNA (miRNA) as a proxy. Leveraging the target network of the miRNA-200 family (miR-200f), regulators of epithelial-to-mesenchymal transition (EMT), we pinpoint genes encoding multiple promesenchymal glycosylation enzymes (glycogenes). We focus on three enzymes, beta-1,3-glucosyltransferase (B3GLCT), beta-galactoside alpha-2,3-sialyltransferase 5 (ST3GAL5), and (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 (ST6GALNA5), encoding glycans that are difficult to analyze by traditional methods. Silencing these glycogenes phenocopied the effect of miR-200f, inducing mesenchymal-to-epithelial transition. In addition, all three are up-regulated in TGF-β-induced EMT, suggesting tight integration within the EMT-signaling network. Our work indicates that miRNA can act as a relatively simple proxy to decrypt which glycogenes, including those encoding difficult-to-analyze structures (e.g., proteoglycans, glycolipids), are functionally important in a biological pathway, setting the stage for the rapid identification of glycosylation enzymes driving disease states.

Significance

Carbohydrates hold an unprecedented capacity for altering biological function, but determining which glycans and underlying enzymes are crucial for a specific biological pathway is a major impediment to our understanding of this posttranslational modification. Here we demonstrate that the miRNA target networks of microRNA (miRNA), small noncoding RNA, identify glycosylation enzymes acting as regulatory elements within a biological pathway. Leveraging the miRNA-200 family (miR-200f), regulators of epithelial-to-mesenchymal transition (EMT), we identify multiple promesenchymal glycosylation enzymes. Silencing miR-200f-targeted glycogenes phenocopies the effect of miR-200f, inducing mesenchymal-to-epithelial transition. These enzymes are upregulated in TGF-β-induced EMT, suggesting tight integration within the signaling network. Our work indicates that miRNA networks can be used to identify crucial glycosylation enzymes driving disease states.

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¹T.K. and Z.L. contributed equally to this work.

²To whom correspondence should be addressed. Email: lkmahal@nyu.edu.

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Mir-200f Regulates the Glycome in MDA-MB-231 Cells Undergoing MET. MiR-200f levels are high in epithelial cells, which express high levels of E-cadherin, form tight junctions, and have low motility. Conversely, miR-200f is low in mesenchymal cells, which lose cell-cell contacts and are characterized by low levels of E-cadherin and high motility. The highly metastatic breast cancer cell line MDA-MB-231 is considered mesenchymal and is commonly used to examine MET (12, 17). MiR-200f is known to cause MET within this cell line (12, 17, 19). Previous reports have not distinguished clearly between the effects of the two miR-200f groups, often using combinations of miRNAs from both sets. Our luciferase data suggest that the two miR-200f groups have disparate effects on the glycome. Transformation of MDA-MB-231 cells with either miR-200a or -200b mimics induced changes in morphology consistent with MET, but these changes were more apparent in miR-200b–treated cells (Fig. 2 A). This observation was confirmed by examination of MET markers. The expected increase in E-cadherin and loss of vimentin and ZEB1 expression were observed in both miRNA treatments but were more pronounced for cells treated with miR-200b mimics (Fig. 2 B and C and Fig. S2 A). We next examined the effects of miR-200a or -200b treatment on the expression of predicted miR-200f glycome targets by quantitative RT-PCR (qRT-PCR) (Table S3). Overall, we found a larger number of predicted glycome targets altered in the miR-200b–treated samples (miR-200a: 8/33; miR-200b: 15/33), indicating that miR-200b has a more profound effect on the glycome in this system. In examining the data, we noticed that none of the glycome targets exclusive to the N-linked pathway was affected significantly by miR-200b, in line with our mRNA expression (Table S3). We examined the effects of miR-200a or -200b treatment on the expression of predicted miR-200f glycome targets by quantitative RT-PCR (qRT-PCR) (Table S3). Overall, we found a larger number of predicted glycome targets altered in the miR-200b–treated samples (miR-200a: 8/33; miR-200b: 15/33), indicating that miR-200b has a more profound effect on the glycome in this system. In examining the data, we noticed that none of the glycome targets exclusive to the N-linked pathway was affected significantly by either miRNA. Lectin microarray analysis of miR-200a– and -200b–treated cells showed no changes in N-linked–specific lectin binding (Fig. S3), in line with our mRNA expression (Table S3) and luciferase (Fig. 1 B) data. In general, few changes were observed on our lectin microarrays, which have limited analytical capacity for glycopolysaccharides and glycosaminoglycans, and noncanonical O-linked (i.e., the majority of miR-200f targets). In light of these data, we focused our attention on three specific glycome targets in the miR-200b network from the glycopid and noncanonical O-linked pathways: B3GLCT, which catalyzes the addition of a glucose to O-fucosylated serines and threonines; ST3GAL5, a sialyltransferase that modifies the...
Silencing B3GLCT, ST3GAL5, or ST6GALNAC5 Phenocopies the Effects of miR-200b in MDA-MB-231 Cells. To examine whether glycosyltransferases targeted by miR-200b are directly involved in EMT, we silenced B3GLCT, ST3GAL5, or ST6GALNAC5 in MDA-MB-231 cells using shRNA. If these enzymes are involved in the EMT pathway, silencing them should phenocopy the effects of miR-200b, driving MET forward in the mesenchymal cell line. Morphological changes indicative of MET were observed as early as 5 d following lentiviral vector transduction and selection for shRNA-expressing cells (Fig. 3A). We evaluated E-cadherin expression in B3GLCT- and ST3GAL5-silenced cells at day 5 and, because of slower growth rates, in ST6GALNAC5-silenced cells at day 10 (Fig. 3 and Fig. S4). Gene silencing resulted in increased expression of E-cadherin at both the protein and transcript levels, in line with the observed morphological changes. ZEB1 levels also were increased in all three knockdowns, indicating that the increase in E-cadherin is not caused by repression of this transcriptional regulator (Fig. S2B).

miR-200f regulates not only the morphology but also the migratory capacity of cells (12, 19). Previous studies have shown that miR-200f expression in MDA-MB-231 cells inhibits the migration of this cell line in wound-healing assays (12, 19). To test glycosylipid lactosylceramide to form GM3; and ST6GALNAC5, which transfers a sialic acid to the N-acetylgalactosamine on the glycolipid GM1b to form GD1α (Fig. 2 D–F, respectively) (20).

Our candidate genes were chosen for three reasons: (i) all three displayed >40% down-regulation by miR-200b in our luciferase assays (Fig. 1B); (ii) all three were associated with human disease (3, 21–24); (iii) all three glycosyltransferases showed significant changes in mRNA expression levels in miR-200b-treated MDA-MB-231 cells undergoing miRNA-induced MET ($P \leq 0.01$) (Table S3). Unlike B3GLCT and ST3GAL5, the expression of ST6GALNAC5 mRNA increased in the miR-200b-treated samples (Fig. 2 G–I). This finding is in contrast to both the luciferase data for the ST6GALNAC5 3′-UTR construct (Fig. 1B) and protein expression levels, which were repressed for all three enzymes by miR-200b (Fig. 2 J–L). It is common for miRNA to repress translation in the absence of an mRNA degradation effect (6), as is observed here for ST6GALNAC5. To determine whether glycans synthesized by these glycosyltransferases are down-regulated by miR-200b, we used fluorescence microscopy to examine the levels of GM3, the glycolipid product of ST3GAL5 (Fig. 2E), in response to miR-200b treatment (Fig. 2M). We observed a clear loss of anti-GM3 antibody staining for miR-200b–treated MDA-MB-231 cells, as would be expected. Taken together, our data show that miR-200f alters the glycome by targeting glycosyltransferases in MDA-MB-231 cells undergoing miRNA-induced MET.
whether silencing B3GLCT, ST3GAL5, or ST6GALNAC5 mimics the effects of miR-200b on the migratory capacity of MDA-MB-231 cells, we performed in vitro scratch assays (25). Migration was evaluated after 24 h, which is less than the doubling time of MDA-MB-231 cells (26). As expected, transfection of MDA-MB-231 cells with miR-200b mimics inhibited gap closure in the scratch assay as compared with a scrambled control (46% of control, \( P < 0.01 \)) (Fig. 4 A and B). Silencing of B3GLCT, ST3GAL5, or ST6GALNAC5 recapitulated the inhibition of migration observed with miR-200b (\( P < 0.001 \) for B3GLCT and ST6GALNAC5; \( P = 0.02 \) for ST3GAL5) (Fig. 4 C and D). The strongest effect was observed in the B3GLCT knockdown (20% of control). Migration was reduced to 38% of control in ST6GALNAC5-silenced cells and to 67% of control in ST3GAL5-silenced cells. Thus, silencing glycosygenes targeted by miR-200b phenocopies the effects of the miRNA, suggesting that miR-200f regulates glycosylation enzymes that are important components of the biological pathways it controls.

**TGF-β1 Induces Both EMT and Increased Protein Expression of B3GLCT, ST3GAL5, and ST6GALNAC5 in A549 Cells.** We tested whether B3GLCT, ST3GAL5, and ST6GALNAC5 were involved in the more complex EMT networks induced by a natural stimulus of EMT, TGF-β1. TGF-β1 is a cytokine known to induce EMT, in part through up-regulation of the transcription factor Zeb1, a repressor of both miR-200f and E-cadherin (16, 27). We examined the effects of TGF-β1 on A549 cells, a human nonsmall cell lung carcinoma line that has been used previously to study TGF-β1-induced EMT (28). In brief, cells were treated with TGF-β1 (5 ng/mL) for 48 h. Changes in morphology consistent with EMT could be observed as soon as 24 h after treatment. By 48 h, the majority of cells displayed the spindle-shaped morphology characteristic of a mesenchymal phenotype (Fig. 5A). Analysis of E-cadherin, fibronectin (a mesenchymal marker), vimentin, and Zeb1 by qRT-PCR in these cells confirmed the induction of EMT (Fig. 5B). We analyzed the expression of B3GLCT, ST3GAL5, and ST6GALNAC5 transcripts in these cells (Fig. 5C). Because of the very low expression levels of the ST6GALNAC5 mRNA, we were not able to determine changes in this transcript by qRT-PCR. Both B3GLCT and ST3GAL5 showed significant increases in expression (3.5-fold and 1.5-fold, respectively) compared with untreated cells. We observed increases in protein expression levels consistent with the changes in glycogene mRNA (Fig. 5D). By loading threefold more protein than was used in the Western blot analysis of B3GLCT and ST3GAL5, we were able to observe an increase in ST6GALNAC5 protein expression because of TGF-β1-induced EMT. Overall our results suggest that glycogenes targeted by miR-200b are promesenchymal genes that are part of the broader EMT-signaling network induced by TGF-β1.

**Discussion**

Glycosylation can impact many aspects of the cell, from cell adhesion to receptor clustering and activation (Fig. 6). For example, glycosylation of E-cadherin, fibronectin, and other EMT-associated glycoproteins governs their roles in adhesion and motility, altering their stability and protein-interaction networks (29–31). A single glycosylation enzyme can regulate multiple components of a biological pathway, exerting a synergistic effect. Current approaches to identifying functionally relevant glycans are focused on glycan analysis to identify glycans that alter in disease and on glyco profiling to identify changes in mRNA that correlate with disease states. These approaches have several limitations. (i) High-throughput analysis of glycans typically is...
not comprehensive (5). The great majority of studies on glycans in disease focus on only the N-linked glycome. Glycolipids, O-linked glycans, and glycosaminoglycans often are ignored because of difficulties in sample preparation and annotation in mass spectrometry and a lack of specific binding reagents. (ii) Glycan transcripts often are at the limits of detection, where quantitation is inaccurate, complicating mRNA profiling (32, 33). (iii) The multiple glycosylation enzymes with overlapping catalytic specificities present a challenge in mapping changes in glycan structure to changes in specific enzyme levels (34). (iv) It is not clear that all changes in glycosylation correlating with a disease state or biological process are relevant. Some alterations may be bystanders of the expression of other functionally relevant glycans (35). Identifying the parts of the glycome that are meaningful in terms of biological function in a specific system is more complex than simply identifying changes in glycosylation.

In this work we demonstrate a previously undescribed strategy for decrypting the functional glycode that leverages miRNA as a proxy for identifying glycosylation enzymes that are important for a specific biological process, EMT (Fig. 6). By leveraging miR-200f target networks (Fig. 1), we identified a set of relevant glycogenes from the cohort that biosynthesize glycans ignored in typical glycan analysis. MiR-200f strongly upon glycan expression. Inhibiting the expression of the glycosyltransferase enzyme ST6GALNAC5, and the downregulation of the related glycan biosynthesis (Table S3), which comprise 65% of predicted targets (Table S1), rather than the N-linked–associated glycogenes that are the focus of most research. Our miRNA strategy bypasses the analytical constraints attached to glycolipids, glycosaminoglycans, and O-linked glycans to identify specific glycosylation enzymes and, by extension, the epitopes they biosynthesize as modulators of the EMT pathway.

From the broader miR-200f network, we focused our studies on three targets: B3GLCT, ST6GALNAC5, and ST3GAL5, which biosynthesize noncanonical O-linked glycans and glycolipids (Fig. 2 D–F). Knocking down any of these three glycogenes phenocopied the known effects of miR-200f, reversing the mesenchymal phenotype, increasing E-cadherin, and slowing cell migration. These effects were not mediated by the transcription factor Zeb1, indicating that these glycans affect protein expression and/or stabilization of E-cadherin independent of the transcriptional repressor. Induction of EMT by TGF-β1 increased the expression of all three glycosylation enzymes. This finding is in line with the known function of TGF-β, which inhibits miR-200. Taken together, our data demonstrate that miR-200f controls glycosylation enzymes that are functionally relevant to EMT and that miRNA can be used as a proxy to place these enzymes into biological context.

Placing glycogenes into context within specific pathways using miRNA as a predictor may offer insight into the roles of glycosylation in human disease. MiR-200f is a regulatory component of the TGF-β EMT-signaling pathway, implying that B3GLCT, ST6GALNAC5, and ST3GAL5 also are components of that pathway. Increased levels of the glycolipid GM3, the product of ST3GAL5, have been shown to promote the dimerization and activation of TGF-β receptors (36). However, B3GLCT and ST6GALNAC5 are not known to be involved in EMT. Mutations that alter the enzymatic activity of B3GLCT, the β1,3-galactosyltransferase previously annotated as B3GALT, are the underlying cause of Peter’s Plus syndrome (21), a congenital disorder characterized by malformation of the eye (Peter’s anomaly), short stature, and craniofacial features commonly including cleft palate (37). TGF-β-induced EMT plays a predominant role in both the formation of the eye (38) and the closure of the palate during embryonic development (39), and recent work has implicated B3GLCT in the secretion of thrombospondin-1, a protein involved in TGF-β activation (40). Taken together, this report and our data suggest that Peter’s Plus is a TGF-β–related EMT dysregulation syndrome, similar to other congenital disorders with TGF-β–related mechanisms (41). Coronary artery disease (CA) caused by activating mutations in ST6GALNAC5 (3) also may be related to TGF-β–induced EMT. CAD has been linked to higher TGF-β levels (42) and fibrosis, an EMT process (43). Thus, higher ST6GALNAC5 activity may promote TGF-β signaling, causing CA. If these rare genetic mutations do impact TGF-β networks, drugs that target TGF-β signaling potentially could help patients with these syndromes, effectively deorphanizing these diseases by placing them in the larger context of TGF-β–related diseases.

Identifying functionally relevant glycosylation enzymes opens new avenues for therapeutics for virtually any disease (1). Glycosylation enzymes are overlooked as druggable targets because of the difficulties in pinpointing the critical enzymes in a disease state (34, 35, 44). However, glycans play a role in every major disease (1), and there are clinical examples of drugs targeting glycosylation enzymes (Zavesca in Gaucher’s disease (44), zanamivir (Relenza) and oseltamivir (Tamiflu) in influenza (35), and migalastat in Fabry’s disease (44)), suggesting that glycosylation enzymes may provide a fail-safe ground for drug development. Multiple biological pathways governed by the activity of miRNA often are involved in disease processes. Our approach enables these miRNA networks to be leveraged to yield the most critical enzymes to target in a disease state. Taking advantage of this concept will require a better map of glycogene–miRNA interactions (8). By using miRNA as a relatively simple proxy to decrypt which glycogenes, including encoding difficult-to-analyze structures (e.g., proteoglycans, glycolipids), are important in specific biological states, this work provides a decryption key for the functional glycode.

Experimental Procedures

Glycogene Target Analysis of miR-200f. Identification and visualization of miR-200f glycome targets and construction of the regulatory network was performed as previously described (7) with the following exception: Only glycosyltransferases with an miR-SVR score of −0.1 or less were considered (18).

Luciferase Reporter Assay. Luciferase reporter assays were performed as previously described using pLightSwitch-MT vector constructs (SwitchGear Genomics) (8). Plasmids containing glycogene 3′ UTRs were obtained from SwitchGear Genomics (B3GLCT) or were cloned from cDNA using standard methods. Dataset S1 shows the primers and sequenced 3′ UTRs.

Cell Culture and TGF-β1 Treatment. The MDA-MB-231 and AS49 cell lines [Diagnosis Tumor Repository, National Cancer Institute (NCI)] were grown in RPMI-1640 medium (Lonza) supplemented with 10% (vol/vol) FBS (Innovative Research) and 2 mM β-glutamine (Lonza) at 37 °C in 5% CO2. For TGF-β1 treatment, AS49 cells were seeded at a density of 500,000 cells in a 10-cm dish; 24 h later the medium was replaced with reduced FBS (5%) growth medium supplemented with 5 ng/mL human recombinant TGF-β1 (R&D; 240-B-002) or vehicle, and cells were cultured for additional 48 h.

Transfection of miRNA Mics in MDA-MB-231 Cells. MDA-MB-231 cells were seeded onto 24-well plates at 30,000 cells per dish. Cells were treated for 24 h, and transfected with miRNA mimics (50 nM) using Lipofectamine 2000 (Life Technologies). Cells were transfected again 72 h after the initial treatment and 6–12 h later were split into 2 × 60 mm dishes. Samples were harvested for protein or RNA extraction on day 7 posttransfection.
Western Blotting. Lysates from cells lysed in cold RIPA buffer supplemented with protease inhibitors were subjected to standard Western Blot protocols (8). The primary antibodies were α-GAPDH (glyceraldehyde-3-phosphate dehydroge-
nase; 1:10,000), α-E-cadherin (1:1,000), α-ST6GALNAC1 (1:1,000), and α-Vimentin (1:10,000) from Abcam and α-B3GLCT (B3GALT1, 1:1,000) and α-NST3GALS1 (1:1,000) from Novus Biologicals. Secondary antibodies (α-mouse-HRP (GAPDH) or α-rabbit-HRP (all others) (Bio-Rad)) were used at 1:5,000. Blots were developed using SuperSignal West Pico (Thermo Scientific).

RNA Extraction and Real-Time qPCR. Total RNA was extracted from samples as previously described (8). Glycogene profiling summarized in Tables S3 and S4 was obtained by using RT² Profiler PCR Array Human Glycosylation (Qiagen) and Power SYBR Green (Life Technologies) using the manufacturers’ pro-
tocols. Additional primers were designed using PrimerSelect or NCBI primer blast and were purchased from Integrated DNA Technologies (Dataset S1). All cycle threshold values (Cq) were obtained by using the LightCycler Roche 480 Second Derivative Max algorithm.

Gene Silencing. MISSION shRNA clones (Sigma Aldrich) were obtained as viral stocks from the shRNA Core at New York University Langone Medical Center. MDA-MB-231 cells were seeded at 80,000 cells per well in a six-well format and were transduced 24 h in later with shRNA lentivectors (multiplicity of infection = 2) in medium containing 8 μg/mL Polybrene (Sigma Aldrich; see Dataset S1 for shRNA sequences). After 48 h, the medium was replaced with selection me-
dium (1 μg/mL puromycin). Cells were harvested for protein and RNA extraction as described in the text.

Scratch Assay. MDA-MB-231 cells were seeded at 80,000 cells per dish in 35-mm dishes. MI RNA treatment and gene silencing were performed as previously de-
scribed. Confluent monolayers of cells were scratched on day 6 (mif200t treatment) or day 5 (silencing) using a P200 tip, and the medium was replaced to re-
move floating cells. Images were acquired immediately after the scratch and 24 h later. Images were analyzed in ImageJ using the MitoBo plugin (www2.informatik.
uni-due.de/∼evans/mito/software/index.php#MitoBo_Plate) to quantify the wound area at time 0 and h 0.24. The percent of wound closure was calculated for a minimum of three regions per replicate for each of the scramble-treated biological replicates only one region was imaged) and was averaged for three biological replicates.

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