Dynamic Sialylation in Transforming Growth Factor-β (TGF-β)-induced Epithelial to Mesenchymal Transition

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Background: Changes in cell surface sialylation have recently been implicated in mediating epithelial-mesenchymal transition (EMT). Results: Cell surface sialylation was first down-regulated but then reverted and up-regulated during EMT, and inhibition of sialylation promoted EMT.

Conclusion: Sialylation dynamics plays an important role in regulating EMT.

Significance: Sialylation may have important functions in EMT-related diseases.

Epithelial-mesenchymal transition (EMT) is a fundamental process in embryonic development and organ formation. Aberrant regulation of EMT often leads to tumor progression. Changes in cell surface sialylation have recently been implicated in mediating EMT. Herein we report the visualization of dynamic changes of sialylation and glycoproteomic analysis of newly synthesized sialylated proteins in EMT by metabolic labeling of sialylated glycans with azides, followed by click labeling with fluorophores or affinity tags. We discovered that sialylation was down-regulated during EMT but then reverted and up-regulated in the mesenchymal state after EMT, accompanied by mRNA expression level changes of genes involved in the sialic acid biosynthesis. Quantitative proteomic analysis identified a list of sialylated proteins whose biosynthesis was dynamically regulated during EMT. Sialylation of cell surface adherent receptor integrin β4 was found to be down-regulated, which may regulate integrin functions during EMT. Furthermore, a global sialylation inhibitor was used to probe the functional role of sialylation during EMT. We found that inhibition of sialylation promoted EMT. Taken together, our findings suggest the important role of sialylation in regulating EMT and imply its possible function in related pathophysiological events, such as cancer metastasis.

The epithelial-mesenchymal transition (EMT) of adherent epithelial cells to a migratory mesenchymal state is a fundamental process during embryonic development and organ formation (1, 2). Transforming growth factor-β (TGF-β) signaling plays an important role in EMT, and TGF-β is the best characterized inducer of EMT (3, 4). TGF-β suppresses the expression of epithelial marker genes, such as E-cadherin, while enhancing mesenchymal markers, such as N-cadherin, fibronectin 1 (FN1), and matrix metalloproteinases (MMPs). Morphologically, the cobblestone-like epithelial appearance is converted to the elongated, spindle-like mesenchymal shape upon EMT. Aberrant activation of EMT has been implicated in disease progression, such as cancer metastasis (5). The exploration of EMT as a biomarker for diagnosis and as a drug target in cancer therapy has attracted increasing interest (6, 7).

On the other hand, cancer progression is often associated with abnormal glycosylation (8). In particular, sialic acids, a family of acidic nine-carbon monosaccharides that are usually attached at the outmost end of glycans, play essential roles in mediating cell adhesion and migration (9, 10). Hypersialylation of EMT-related proteins may regulate integrin functions during EMT.

The abbreviations used are: EMT, epithelial-mesenchymal transition; Ac2ManNAZ, peracetylated N-acetylmannosaminosamine; CMAS, cytidine monophosphate N-acetylmannosaminic acid synthetase; CMP-Neu5Ac, CMP-N-acetylneuraminic acid; FN1, fibronectin 1; GNE, glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosaminosamine kinase; HPAEC-PAD, high pH anion exchange chromatography followed by pulsed amperometric detection; MannAc, N-acetylmannosaminosamine; MMP, matrix metalloproteinases; NAPN, N-acetylneuraminic acid phosphatase; NANS, N-acetylneuraminic acid synthase; SiaNAz, N-acetylgalactosamine; ST3GAL1, ST3-β-galactoside α-2,3-sialyltransferase 1; ST3GAL2, ST3-β-galactoside α-2,3-sialyltransferase 2; ST3GAL4, ST3-β-galactoside α-2,3-sialyltransferase 4; ST3GAL5, ST3-β-galactoside α-2,3-sialyltransferase 5; ST3GAL6, ST3-β-galactoside α-2,3-sialyltransferase 6; ST6GAL1, ST6-β-galactosaminide α-2,6-sialyltransferase 1; ST6GALNAC1, ST6-α(N-acetylneuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminide α-2,6-sialyltransferase 1; ST6GALNAC4, ST6-α(N-acetylneuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminide α-2,6-sialyltransferase 2; ST6GALNAC6, ST6-α(N-acetylneuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminide α-2,8-sialyltransferase 5.
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has been reported to increase the mobility of cancer cells, thus contributing to tumor progression (11–13). Correlatively, as a complex molecular and cellular program, EMT is characterized by changes in cell morphology, adhesiveness, and motility (14). The facts that EMT promotes cancer progression and that sialylation up-regulation has been observed in a number of metastatic cancer types suggest a correlative link between hypersialylation and EMT. However, important work by the Hakomori group showed that certain glycosphingolipids, including the sialylated ganglioside GM2, were down-regulated during the TGF-β-induced EMT, and inhibition of the synthesis of glycosphingolipids promoted EMT (15, 16). Furthermore, some genes involved in sialic acid biosynthesis were found to be differentially regulated at the mRNA level during EMT: some up-regulated and others down-regulated (16–18). In addition, sialylation can be dynamically regulated on the cell surfaces (19). These results implicate a complex regulatory mechanism of sialylation in EMT.

Based on these considerations, we set out to probe the dynamic changes of sialylation during TGF-β-induced EMT, with the aim to better understand how glycosylation contributes to regulating a cell’s morphogenesis and pathophysiology. We employed a chemical reporter strategy, which metabolically labels sialylated glycans with azides in live cells (20). Coupling with click chemistry, the azido group serves as a chemical handle that can be conjugated with fluorophores for visualizing the dynamic changes of sialylation during EMT and with affinity tags for glycoproteomic analysis. In this study, we found a dynamic regulation of sialylation (i.e. down-regulation during EMT and up-regulation after the completion of EMT). Glycoproteomic analysis revealed a list of sialylated proteins whose biosynthesis was dynamically regulated during EMT, including cell surface adherent receptor integrin β4. Furthermore, by employing a chemical inhibitor of sialylation, we showed that suppression of cellular sialylation promoted EMT. These results suggest the important role of sialylation in EMT and imply its possible function in related pathophysiological events, such as cancer metastasis.

EXPERIMENTAL PROCEDURES

Compounds and Reagents—Peracylated N-azidoacetylmannosamine (Ac4ManNAz) (21), methyl 5-acetamido-2,4,7,8,9-penta-O-acetyl-3,5-dideoxy-3-fluoro-erythro-β-L-manno-2-nonalulopyranosylamine (3Fax-Neu5Ac) (22), and 2-(4-((bis((1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid (BTTAA) (23) were synthesized as described previously. Alkyne-PEG₄-biotin was obtained from Click Chemistry Tools (Scottsdale, AZ). Rhodamine phalloidin and streptavidin–Alexa Fluor 488 were purchased from Invitrogen. SB431542 and TGF-β1 were obtained from R&D Systems. Streptavidin–agarose beads were purchased from Thermo Fisher Scientific. Antibodies were purchased from Thermo Fisher Scientific (anti-biotin), BD Biosciences (anti-N-cadherin), and Santa Cruz Biotechnology, Inc. (anti-ITGB4 and anti-tubulin). Neuraminidase (sialidase) from Clostridium perfringens was obtained from Sigma.

Metabolic Labeling of Cell Surface Sialylated Glycans—Human keratinocyte HaCaT cells were cultured in DMEM containing 50 μM Ac₄ManNAz or Ac₄ManNAc as a control for 48 h. For investigating sialylation in EMT, the cells were further treated with 100 μM TGF-β1 or vehicle for up to 84 h.

Flow Cytometry Analysis—After metabolic incorporation, the cells were transferred and distributed into a 96-well tissue culture plate, and washed three times with PBS containing 1% FBS. Cells were then resuspended in PBS containing 0.5% FBS, 50 μM alkylne-PEG₄-biotin, 2.5 mM sodium ascorbate, and BTTAA-CuSO₄ complex (50 μM CuSO₄, BTTAA/CuSO₄ in a 6:1 molar ratio) at room temperature. After 5 min, the reactions were quenched by adding 2 μg of copper chelator bathocuproine disulfonate (50 μM). The cells were then pelleted (800 × g, 5 min), washed three times with PBS containing 1% FBS, and resuspended in the same buffer containing 2 μg/ml Alexa Fluor 488-streptavidin. After incubation on ice in the dark for 30 min, the cells were washed three times and then resuspended in 400 μl of FACS buffer (PBS buffer containing 1% BSA and 0.1% NaN₃) for flow cytometry analysis using a BD C6 flow cytometer.

Confocal Fluorescence Microscopy—The cells were seeded on the Lab-Tek™ 8-well chamber slides. After metabolic incorporation, the cells were washed three times with PBS containing 1% FBS, followed by incubation for 5 min in PBS containing 0.5% FBS, 50 μM alkylne-PEG₄-biotin, 2.5 mM sodium ascorbate, and BTTAA-CuSO₄ complex. After the reaction was quenched with bathocuproine disulfonate, the cells were washed three times, followed by incubation with 2 μg/ml Alexa Fluor 488-streptavidin for 30 min and staining for 10 min with 5 μg/ml Hoechst 33342 at 4°C. The cells were then washed three times and imaged using a Zeiss LSM 700 laser-scanning confocal microscope.

For fluorescence imaging of F-actin, the cells were fixed with 4% paraformaldehyde in PBS. After washing for three times, the cells were incubated with rhodamine phalloidin (4 units/ml) for 30 min.

Immunoprecipitation and Immunoblot Analysis—HaCaT cells were lysed in TNE buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA) for 30 min on ice. After centrifugation at 12,000 rpm at 4°C, the supernatants were incubated with primary antibody and protein A-agarose beads (Invitrogen) at 4°C overnight. Then the beads were washed three times with TNE buffer at 4°C. Proteins bound to the beads were eluted with SDS-loading buffer (0.25 mM Tris, pH 6.8, 40% glycerol, 0.4 M DTT, 8% SDS, 0.04% bromphenol blue) at 98°C for 3 min and then subjected to SDS-PAGE and immunoblot analysis.

The immunoblot analysis of azide-labeled sialylated glycoproteins was performed as described previously (24). For other immunoblot detections, the blocked membrane was incubated for 1 h at 4°C with a primary antibody (1:1,000 to 1:3,000 dilution) in blocking buffer. After washing three times, the membrane was incubated with HRP-conjugated second antibody (1:1,000 dilution) for 1 h at room temperature, washed three times, and developed using Super ECL Plus chemiluminescent substrate.
Measurement of Cellular CMP-N-acetylneuraminic acid (CMP-Neu5Ac)—HaCaT cells were harvested and lysed using ultrasonication in deionized water. The supernatants were filtered through 10,000 molecular weight cut-off membranes (Millipore) and then analyzed with high pH anion exchange chromatography, followed by pulsed amperometric detection (HPAEC-PAD/UV) as reported previously (25). For the quantitative analysis of CMP-Neu5Ac concentration in cells, the HPAEC-PAD picks for the CMP-Neu5Ac were integrated and normalized with the total protein weight of each sample.

Profiling N-Linked Glycans with Mass Spectrometry (MS)—After treated with vehicle or 100 pM TGF-β1 for 24 h, HaCaT cells (∼5 × 10^6 cells) were harvested and then treated as described previously (22). Briefly, all samples were dissolved in ice-cold radioimmunoprecipitation assay lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM triethanolamine, pH 7.4, 150 mM NaCl, EDTA-free Pierce Halt™ protease inhibitor mixture). Then the samples were subjected to DTT reduction, carboxymethylation, and trypsin digestion. After treated with vehicle or 100 pM TGF-β1 for 24 h, HaCaT cells were harvested and lysed using ultrasonication in deionized water. The supernatants were incubated with streptavidin-agarose beads (Sigma). Then the beads were washed three times with TNE buffer at 4 °C. Proteins bound to the beads were eluted with SDS-loading buffer (0.25 mM Tris, pH 6.8, 40% glycerol, 0.4M EDTA) and 50 mM iodoacetamide. Then in gel digestion was carried out with the sequenc- ing grade modified trypsin (Promega) in 50 mM ammonium bicarbonate at 37 °C overnight. The peptides were extracted twice with 1% trifluoroacetic acid in 50% acetonitrile aqueous solution for 30 min. The extractions were then centrifuged in a SpeedVac to reduce the volume.

LC-MS/MS analysis was performed as described previously (26). Briefly, the samples obtained above were subjected to an Easy nLC 1000 coupled to an LTQ Velos Pro-Orbitrap Elite mass spectrometer (Thermo Fisher). Peptides were pressure-loaded onto a 100-μm diameter, 2-cm C18 precolumn and separated on a 75-μm diameter, 20-cm C18 capillary column with a gradient running from 95% buffer A (HPLC water with 0.1% (v/v) formic acid) and 5% buffer B (HPLC grade CH₃CN with 0.1% (v/v) formic acid) to 35% B over 60 min at 300 nl/min, next ramping to 75% B over 2 min and holding at 75% B for 10 min. Both precolumn and separation column were packed in house. One full MS scan (375–1,600 m/z) was followed by 10 data-de
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To elucidate the correlation between sialylation and EMT, we assayed the level of cell surface sialylated glycans in response to TGF-β treatment. HaCaT cells were preincubated with 50 μM Ac4ManNAz for 48 h to metabolically label cell surface sialic acids with azides. While maintaining the Ac4ManNAz supply in the culture media, the cells were further treated with TGF-β1 or vehicle for varying durations of time (Fig. 2A). The level of cell surface SiaNAz, which reflects the level of sialylated glycans, was measured by click labeling followed by flow cytometry analysis and confocal fluorescence microscopy. In the absence of TGF-β, the level of sialylated glycans remained steady. However, upon TGF-β treatment, the amount of newly synthesized cell surface sialic acids gradually decreased over the period of 24-h incubation (Fig. 2B). A maximum reduction of ~30% was observed at 24 h. TGF-β also induces EMT in other epithelial cells, such as canine kidney epithelial MDCK cells and human lung carcinoma A549 cells (3, 27). Similar reduction of cell surface sialic acids was also observed in MDCK cells and A549 cells upon TGF-β treatment for 24 h (Fig. 2, C and D). Furthermore, the reduction in sialylation was abolished in the presence of SB431542, a selective inhibitor for TGF-β type I receptor (Fig. 2B). Confocal fluorescence microscopy showed that cell surface sialoglycoconjugates were mainly distributed on cell-cell junctions before EMT and dramatically decreased after TGF-β1 treatment for 24 h, along with loss of cell-cell contacts and formation of actin stress fibers (Fig. 2E). Immunoblotting confirmed that sialylated proteins were reduced upon TGF-β treatment for 24 h (Fig. 2F).

The Rate of Sialic Acid Biosynthesis, but Not the Degradation, Is Reduced during TGF-β-induced EMT—The level of sialic acids on cell surfaces is dependent on both the sialic acid synthesis and degradation. We therefore investigated how these two factors contribute to the observed decrease of sialic acids on the cell surface during EMT. To detect the rate of sialic acid biosynthesis, we monitored the cellular level of CMP-Neu5Ac, the nucleotide sugar donor for sialylation. HaCaT cells treated with TGF-β1 for 24 h were lysed, and the cell lysates were analyzed by HPAEC-PAD. We observed that the TGF-β treatment reduced the cellular CMP-Neu5Ac level (Fig. 3, A and B).

Next, we performed a pulse-chase experiment using Ac4ManNAz to monitor the degradation of cell surface sialylated glycans during EMT. HaCaT cells were pulse-labeled with Ac4ManNAz for 48 h, followed by adding TGF-β1 and simultaneously chasing with Ac4ManNAc for up to 24 h (Fig. 3C). Similar decay rates were observed in the presence and absence of TGF-β treatment, suggesting that the decay of cell surface sialylated glycans is not influenced during TGF-β-induced EMT (Fig. 3D). Taken together, these results indicate that the biosynthesis rate but not the degradation rate of cell surface sialylated glycans is reduced during TGF-β1-induced EMT.

We also examined whether the decrease of sialic acid biosynthesis was accompanied with structural changes of sialylated
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A

AcManNAz
metabolic incorporation
fluorescent probe
Cu(I), BTTAA
click labeling

B

Mean Fluorescence Intensity (arb. unit)

|          | AcManNAz | AcManNAz | Sialidase |
|----------|----------|----------|-----------|
| +        | -        | -        |           |
| -        | +        | +        |           |

C

D

AcManNAz + -
AcManNAz - +

Biotin
-175kDa
-80kDa
-58kDa
-46kDa

Tubulin
-46kDa

E

- TGF-β
+ TGF-β

AcManNAz
AcManNAz

F

TGF-β
AcManNAz
N-cadherin
-175kDa

Tubulin
-46kDa

G

Relative MMP14 mRNA level

|          | AcManNAz | AcManNAz |
|----------|----------|----------|
| - TGF-β | -        | 0.5      |
| + TGF-β | 4.5      | 4.5      |

H

Relative FN1 mRNA level

|          | AcManNAz | AcManNAz |
|----------|----------|----------|
| - TGF-β | -        | 0.5      |
| + TGF-β | 160      | 160      |
sialylation of cell surfaces. N-Linked glycans were released from HaCaT cells treated with TGF-β for 0 and 24 h and analyzed by MALDI-TOF MS (Fig. 3, E and F). No significant loss of the classes of the sialylated epitopes was observed in 24 h, indicating that the reduction of cell surface sialic acids does not prefer specific glycan structures.

Regulated Expression of Enzymes Involved in Sialic Acid Biosynthesis during EMT—Generation of cell surface sialylated glycans is regulated by a series of enzymes, including enzymes converting ManNAc to CMP-Neu5Ac, and sialyltransferases adding sialic acids onto substrates in the Golgi apparatus (Fig. 4A). Because the cellular CMP-Neu5Ac level was found to decrease during the TGF-β-induced EMT process, we first checked the mRNA level of enzymes involved in CMP-Neu5Ac synthesis by quantitative RT-PCR. Among these genes, GNE and NANP were found to be down-regulated quickly after adding TGF-β, and the down-regulation at the mRNA level was recovered gradually in a time-dependent manner (Fig. 4B). GNE is thought to be the rate-limiting enzyme and the node for regulation in the sialic acid biosynthetic pathway (28). These results indicate that the biosynthesis of sialic acid is down-regulated by TGF-β during EMT. In addition, we characterized the expression of sialyltransferases, which were reported to correlate with the cell surface sialic acid level (29). At 4 h after adding TGF-β1, seven of the 10 sialyltransferases (ST3GAL1, ST3GAL4, ST3GAL5, ST3GAL6, ST6GAL1, ST6GALNAC1, and ST6GALNAC4) were down-regulated (Fig. 4C). Only ST3GAL6 and ST6GALNAC1 maintained down-regulation during the whole 24 h period of EMT. In contrast, ST3GAL1, ST3GAL4, ST3GAL5, ST6GAL1, and ST6GALNAC4 were gradually recovered and then up-regulated at 24 h. The mRNA level of ST3GAL2 and ST8SIA5 was significantly up-regulated at 4 h and kept increasing up to 24 h (Fig. 4C).

Reversion and Up-regulation of Sialylation in the Mesenchymal State after EMT—During the 24 h period of time following the TGF-β treatment, the HaCaT cells acquired the most important major mesenchymal features, including the spindle-like morphology and stress fiber formation (Figs. 1E and 2E). TGF-β-induced EMT can be accomplished in 24 h, and then the cells adjust to maintain the mesenchymal state (27). Because hypersialylation was reported to be accompanied by high metastasis of cancer cells (11–13), which are considered to be in the mesenchymal state (30), we asked whether the suppression of sialylation during EMT would be reverted after the completion of EMT. To address this question, we maintained the cells in the mesenchymal state by sustaining TGF-β in the culture medium after 24 h and continued to monitor the sialylation dynamics for up to 84 h. We observed a gradual increase of azide labeling after 24 h and saturation at 72 h, indicating that cell surface sialylation was up-regulated after the completion of the epithelial to mesenchymal transition (Fig. 2, B–D). The down-regulation of sialylation during EMT, followed by reversion and up-regulation in the mesenchymal state, was similarly observed in MDCK and A549 cells, indicating the generality of this phenomenon among different cell types undergoing EMT (Fig. 2, C and D). These results indicate that sialylation is dynamically down-regulated during the process of EMT, followed by reversion and up-regulation in the mesenchymal state. Hypersialylation in the mesenchymal state is in agreement with the previously reported function of sialic acids in enhancing cell motility (12).

Proteomic Analysis of Sialylated Glycoproteins during EMT—The global sialylation level formed a valley curve during the TGF-β-induced EMT process; on the other hand, the expression of sialyltransferases is differentially regulated during the process. These observations prompted us to assess the possibility that the biosynthesis of sialylated glycoproteins could be regulated in a protein-dependent manner during EMT. We employed a glycoproteomic approach to enrich and identify the sialylated proteins newly synthesized during the TGF-β-induced EMT (Fig. 5A). The Ac4ManNAz-treated HaCaT cells that were induced by TGF-β1 for 0, 24, and 72 h were reacted with alkyne-biotin and then lysed. The cell lysates were enriched with streptavidin beads (Fig. 5B), followed by gel-based proteomic identification by tandem mass spectrometry. Using the vehicle-treated HaCaT cells as negative controls, we selected proteins with ≥5-fold increases of the spectra counts in the Ac4ManNAz-treated samples above the control samples. 424, 424, and 409 glycoproteins were selected at 0, 24, and 72 h, respectively (supplemental Tables S1–S3). Most of the identified glycoproteins contain the consensus sequence of N-linked glycosylation (NX(S/T), where X is any amino acid except proline). There are 282 sialylglycoproteins commonly identified in all three stages (Fig. 5C). Gene ontology analysis of identified proteins showed several enriched biological processes, such as cell adhesion, cell motion, cell migration, and glycosylation (Fig. 5D). These processes are essential in EMT (2, 3).

Furthermore, we compared the relative abundance of individual proteins according to their spectral counts at different stages of EMT (Fig. 5E). Proteins with significant changes (≥2-fold) and high confidence (≥100 spectral counts) were selected for further analysis. The abundance of the newly synthesized sialylated glycoproteins could be either up-regulated or down-regulated (Fig. 5F).

**FIGURE 1.** Metabolic incorporation of Ac₄ManNAz in HaCaT cells. A, the cell surface sialylated glycans are metabolically labeled with Ac₄ManNAz. The incorporated azides serve as a chemical reporter to chemoselectively react with an alkyne-bearing fluorescent probe via the BTAAs-assisted copper(i)-catalyzed azide-alkyne cycloaddition (click chemistry) for visualization. B, flow cytometry analysis of cell surface azide-incorporated glycans. The cells were treated with 50 μM Ac₄ManNAz or Ac₄ManNAc for 48 h, followed by conjugation with alkyne-biotin and streptavidin-Alexa Fluor 488, and analyzed by flow cytometry. For sialidase treatment, cells were incubated with 0.4 unit/ml neuraminidase (sialidase) for 30 min in 37°C before they were harvested. Error bars, S.D. from three replicate experiments. ***, Student’s t test (p < 0.001); **, Student’s t test (p < 0.01). arb. unit, arbitrary unit. C, confocal fluorescence microscopy of labeled HaCaT cells. The nuclei were counterstained with Hoechst 33342. Scale bars, 20 μm. D, immunoblot analysis of azide-incorporated cell surface glycoproteins in HaCaT cells. The treated cells were reacted with alkyne-biotin and lysed. Immunoblotting was performed using standard procedures with detection by HRP-conjugated anti-biotin. Tubulin was used as a loading control. E–H, metabolic labeling does not affect TGF-β-induced EMT process. HaCaT cells treated with 100 μM TGF-β1 for 24 h in the absence and presence of 50 μM Ac₄ManNAz were sent for phase-contrast microscope observation (E), immunoblotting (F), and quantitative real-time RT-PCR analysis (G and H). Scale bars, 20 μm. Error bars, S.D. from three replicate experiments.
FIGURE 2. Cell surface sialylation dynamics during and after EMT. A, schematic of the experimental procedures for metabolic labeling of sialylated glycans during and after EMT induced by TGF-β1. The cells were preincubated with 50 μM Ac₄ManNAz for 48 h. While maintaining Ac₄ManNAz in the culture media, the cells were treated with 100 pM TGF-β1, 100 pM TGF-β1, and 10 μM SB431542 or with vehicle for up to 84 h. The cell surface azides were reacted with biotin-alkyne, followed by staining with Alexa Fluor 488-streptavidin for analysis.

B, time course analysis of cell surface SiaNAz during and after EMT using flow cytometry in HaCaT cells. The time point of 0 h on the x-axis corresponds to the time of adding TGF-β1. Error bars, S.D. from three replicate experiments. Arb. unit, arbitrary unit.

C and D, sialylation dynamics of MDCK and A549 cells during the TGF-β-induced EMT process. After fed with 50 μM Ac₄ManNAz for 48 h, MDCK or A549 cells were treated with 100 pM TGF-β1 for the indicated time and then labeled by azide-alkyne cycloaddition. Flow cytometry was then performed to analyze the sialylation status.

E, confocal fluorescence microscopy imaging of cell surface sialylated glycans (green) and F-actin (red) in HaCaT cells during and after EMT. The nuclei were visualized by staining with Hoechst 33342 (blue signal). Scale bars, 20 μm.

F, the HaCaT cells were incubated with Ac₄ManNAz or vehicle for 48 h, followed by treatment with TGF-β1 or vehicle for the time indicated, and then harvested for the reaction with biotin-alkyne. The sialylated proteins were analyzed with SDS-PAGE and detected with HRP-conjugated anti-biotin. Equal protein loading was confirmed by anti-tubulin blotting.
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Sialylation of Integrin β₄ Is Down-regulated during EMT—The decreasing spectral counts of integrin α₆β₄ during the EMT caught our interest. Integrin α₆β₄ is expressed on epithelial cells and binds to the extracellular matrix protein laminin, which regulates the adhesion of epithelial cells (31). Knockout of the β₄ gene in mice resulted in epithelial detachment (32). The activity of integrin α₆β₄ has been reported to be important for EMT and cancer metastasis (33). Interestingly, desialylation of integrin β₄ by sialidase NEU1 was shown to suppress metastasis of human colon cancer cells (34). Because the biotin tag was conjugated onto the metabolically incorporated SiaNAz on the newly synthesized β₄ protein, the decrease in spectral counts could be due to the down-regulation of protein synthesis and/or the down-regulation of β₄ sialylation. To test this, we...

![Graphical representation of sialylation dynamics and its function in TGF-β-induced EMT.](image)

**FIGURE 3.** The rate of sialic acid biosynthesis, but not the degradation, is reduced during TGF-β-induced EMT. A, sialic acid biosynthesis pathway. The nucleotide sugar donor for sialylation is CMP-Neu5Ac, which is used by sialyltransferases (STs) to produce sialoglycoconjugates in the Golgi apparatus. ManNAc is the sialic acid biosynthetic precursor. The conversion from ManNAc to CMP-Neu5Ac requires four enzymes, which are GNE, NANS, NANP, and CMAS. SLC35A1 (solute carrier family 35, member A1) is a transporter that is responsible for the transport of CMP-Neu5Ac to Golgi apparatus. B, upon TGF-β1 treatment, the mRNAs were measured by quantitative RT-PCR at 0, 4, 12, and 24 h in HaCaT cells. The expression of the genes related to the synthesis of CMP-Neu5Ac, including GNE, NANS, NANP, and CMAS, was down-regulated. C, mRNA levels of sialyltransferases upon TGF-β1 treatment. Error bars, S.D. from three replicate experiments.

**FIGURE 4.** Analysis of the mRNA expression of the genes involved in the sialic acid biosynthesis. A, sialic acid biosynthesis pathway. The nucleotide sugar donor for sialylation is CMP-Neu5Ac, which is used by sialyltransferases (STs) to produce sialoglycoconjugates in the Golgi apparatus. ManNAc is the sialic acid biosynthetic precursor. The conversion from ManNAc to CMP-Neu5Ac requires four enzymes, which are GNE, NANS, NANP, and CMAS. SLC35A1 (solute carrier family 35, member A1) is a transporter that is responsible for the transport of CMP-Neu5Ac to Golgi apparatus. B, upon TGF-β1 treatment, the mRNAs were measured by quantitative RT-PCR at 0, 4, 12, and 24 h in HaCaT cells. The expression of the genes related to the synthesis of CMP-Neu5Ac, including GNE, NANS, NANP, and CMAS, was down-regulated. C, mRNA levels of sialyltransferases upon TGF-β1 treatment. Error bars, S.D. from three replicate experiments.

**Putative structures are based on composition, tandem MS, and biosynthetic knowledge.** Structures that show sugars outside of a bracket have not been unequivocally defined. The intensity of the highest peak in each spectrum was normalized to 100%.
analyzed the expression of integrin $\beta_4$ at the protein level. The cell surface $\beta_4$ was labeled with anti-integrin $\beta_4$ antibody and analyzed by flow cytometry, which indicated that the expression level was steady upon treatment of TGF-$\beta_1$ for up to 72 h (Fig. 5G). Together with the proteomic data, these results suggest that the sialylation of integrin $\beta_4$ is down-regulated during EMT. To further confirm this conclusion, we purified integrin $\beta_4$ by immunoprecipitation. The presence of SiaNAz on the protein was detected by conjugating with biotin-alkyne, followed by anti-biotin Western blot analysis, which showed decreased sialylation upon TGF-$\beta_1$ treatment (Fig. 5H).
Global Inhibition of Sialylation Promotes EMT—The observed dynamic changes of sialylation during and after EMT then raised the question of whether the down-regulation of sialylation during EMT has functional significance or it is just the downstream effect of TGF-β treatment. To answer this question, we employed a fluorinverted analog of sialic acid, 3Fax-Neu5Ac, which globally inhibits sialylation through inhibiting sialyltransferase activities in live cells (22) and in mice (35). Inhibition of sialylation by 3Fax-Neu5Ac was confirmed in HaCaT cells both in the presence and in the absence of TGF-β1 treatment (Fig. 6A). The 3Fax-Neu5Ac treatment sensitized the TGF-β-induced formation of stress fiber during EMT (Fig. 6, B and C). We next assayed the effects of 3Fax-Neu5Ac on EMT induction by analyzing the expression of typical mesenchymal markers at both protein and mRNA levels. The expression of mesenchymal markers (MMP14, FN1, N-cadherin, Slug, and PAI-1) was significantly enhanced by 3Fax-Neu5Ac, either alone or together with TGF-β1 during the 24-h period of EMT (Fig. 6, D–I). 3Fax-Neu5Ac also enhanced the mobility of epithelial cells, an effect similar to what was observed in TGF-β induction (Fig. 6, J and K). Furthermore, we evaluated the effect of 3Fax-Neu5Ac when the cells were maintained in the mesenchymal state at 48 h. The expression level of mesenchymal markers was not significantly different regardless of the presence of 3Fax-Neu5Ac. These results demonstrate that global inhibition of sialylation promotes the EMT process, suggesting that down-regulation of sialylation may play an important functional role in EMT induction. On the other hand, sialylation has little effect on the maintenance of mesenchymal state.

DISCUSSION

By applying a chemical reporter strategy, we have herein visualized the time course of sialylation changes in TGF-β-induced EMT process. We discovered that cell surface sialylation undergoes a multistep change: a reduction in the first 24-h period upon EMT induction, followed by a reversion and an up-regulation when the cells are maintained in the mesenchymal state up to 72 h. A recent surprising analysis on the mRNA time course data from cancer cells showed that TGF-β-induced EMT is a multistep process, which correlates with changes in cell metabolism (27). The time scale of the EMT steps was defined as follows. The progression from the epithelial to the mesenchymal state lasts for ~24 h, followed by a second change into the mesenchymal state with the completion of the energy buildup by 72 h. An increased level of ATP was observed in correlation with the transition process and thereafter (27). Our results on the multistep changes of sialylation correlate with the multistep EMT reprogramming defined at the mRNA level, suggesting a correlation between glycosylation (e.g. sialylation) and the multistep progression through EMT.

Cell surface sialylated glycans are important in regulating a variety of physiological processes (36, 37). In particular, cell-cell interactions, cell adhesion, and cell migration, which are closely related to EMT, involve sialic acid-mediated recognition and signal transduction. Although the function of sialylation in EMT remained elusive, the sialylation dynamics had been investigated in cancer metastasis, which is closely related to EMT. Hypersialylation was implicated in regulating cancer progression. Our results revealed hypersialylation in the mesenchymal state, which is in correlation with what is found in metastatic cancer cells. On the other hand, the discovery that the biosynthesis of cell surface sialylated glycans was down-regulated during EMT was somewhat unexpected. This phenomenon may have important implications in cancer therapies. Efforts have been made to develop sialylation inhibitors for cancer treatment, based on the fact that hypersialylation promotes metastasis (38–40). In addition, inhibition of EMT has been evaluated as a potential cancer therapy. The results in this study showing that sialylation inhibition promotes EMT raise the possibility that sialylation inhibitor may have double-edged effects depending on the cellular stages of cancer cells.

The anti-biotin Western blot analysis on Ac4ManNAz-treated and biotin-alkyne-reacted cell lysates showed that the overall sialic acids on the newly synthesized proteins was decreased at 24 h and increased at 72 h (Fig. 2D), because the band intensity reflects the amount of incorporated azides. On the other hand, after the azide-incorporated sialylated proteins were pulled down, the silver staining indicated that the overall amount of the newly synthesized sialylated glycoproteins did not change significantly (Fig. 5B). This was further supported by similar total spectral counts of all identified proteins at three stages (supplemental Tables S1–S3). The proteomic results revealed the dynamics of individual sialylated proteins during

FIGURE 5. Glycoproteomic analysis during TGF-β-induced EMT process. A, schematic of glycoproteomic analysis. HaCaT cells fed with 50 μM Ac4ManNAz for 48 h were treated with 100 pm TGF-β for 0, 24, and 72 h, and then the cell surface azides were reacted with biotin-alkyne. The cells were lysed, and the biotin-labeled sialylated proteins were pulled down and sent to MS analysis. B, SDS-PAGE image. Top channel, silver staining of enriched sialylated proteins. Bottom channel, immunoblot of tubulin was used as internal input control. C, overlap of identified sialylated proteins from three groups. D, biological processes enriched in the sialylated glycoproteins identified in all three groups. Gene ontology analysis was performed using the DAVID method (49). We submitted our gene list to the DAVID Web site and analyzed for the biological process categories following the instructions. The results included the significantly enriched biological processes and their significance, which is represented by the p value. A smaller p value represents greater significance. The eight most significant biological processes are shown, and the log p value was used to represent the significance. E, relative changes of identified sialylated proteins between the 72 and 0 h groups. Spectral counting was used to access the relative abundance of individual glycoproteins. Hits from two conditions were combined for this analysis. The proteins were sorted by the maximum spectral counts of the two groups (x-axis). F, heat map of the spectral counts of representative proteins with significant changes in relative abundance. The spectral counts of each glycoprotein identified in all of the samples were compared. The glycoproteins with significant increase or decrease in their spectral counts during EMT are listed. The color-coded scale represents the spectrum counts. CELSR2, cadherin EGF LAG seven-pass G-type receptor 2; CTSC, dipetidylpeptidase 1; FAT2, protocadherin fat 2; HLA, HLA class I histocompatibility antigen; IGF3, immunoglobulin superfamily member 3; JAG1, protein jagged-1; NT5E, 5'-nucleotidase; SEMG1, semenogelin 1; SEMG2, semenogelin 2; SERPINE1, plasminogen activator inhibitor 1 (PAI1); SERPINE2, glia-derived nexin; SLC3A2, 4F2 cell surface antigen heavy chain; SLC7A5, large neutral amino acid transporter small subunit 1; TMEM2, transmembrane protein 2; TPP1, tripeptidylpeptidase. G, HaCaT cells were treated with 100 pm TGF-β for the time indicated and then harvested for flow cytometry with anti-ITGB4 antibody. Student’s t test was used (p > 0.05). arb. unit, arbitrary unit. H, HaCaT cells fed with 50 μM Ac4ManNAz for 48 h were treated with 100 pm TGF-β for the time indicated and then harvested for immunoprecipitation (IP) and immunoblotting (IB). Protein expression was confirmed with whole cell lysate (WCL).
FIGURE 6. Inhibition of sialylation promotes EMT in early stage. A, 3F<sub>as</sub>-Neu5Ac significantly inhibited the sialylation in HaCaT cells. The cells were treated with 200 μM 3F<sub>as</sub>-Neu5Ac for 72 h, 50 μM Ac<sub>4</sub>ManNAz for 48 h, and then 100 pM TGF-β for the time indicated. The cell surface sialylated glycans were labeled by azide-alkyne cycloaddition and then analyzed by flow cytometry. arb. unit, arbitrary unit. B, confocal fluorescence microscopy images of F-actin in HaCaT cells treated with 3Fax-Neu5Ac for 72 h, followed by treatment with 100 pM TGF-β1 for 14 h. Scale bars, 20 μm. C, statistical results for confocal images in B. D–H, HaCaT cells treated with 200 μM 3F<sub>as</sub>-Neu5Ac for 72 h and then 100 pM TGF-β for the time indicated were harvested for quantitative real-time PCR analysis. I, HaCaT cells treated with 200 μM 3F<sub>as</sub>-Neu5Ac for 72 h and then 100 pM TGF-β for 24 h were harvested for immunoblot analysis of N-cadherin. J and K, HaCaT cells treated with 200 μM 3F<sub>as</sub>-Neu5Ac for 72 h and then 100 pM TGF-β for 24 h were used in the transmigration assays. The transmigrated cells were stained with crystal violet and counted. Error bars, S.D. from three replicate experiments. * Student’s t test (p < 0.05).
The functional importance of the dynamic changes of sialylation in EMT was further investigated using the global sialylation inhibitor 3F_{ax}-Neu5Ac. Chemical inhibition of sialylation has been proven valuable, given that there are more than 20 different sialyltransferases, which makes it difficult to suppress the overall sialylation using genetic knockdown. The chemical inhibition experiments demonstrate that sialylation inhibition has a significant effect on the EMT induction, and once the cells enter the mesenchymal state, the effect is no longer significant. It is quite possible that 3F_{ax}-Neu5Ac does not impose a significant functional perturbation when the cells are in the hypersialylation state. The exact mechanism by which sialylation is involved or regulates the EMT process needs further elucidation.

There are few methods to study the sialylation process. Traditional methods (e.g., lectin staining) suffer from low affinity, nonspecificity, and low efficiency for isolation and enrichment. The chemical reporter strategy allows visualization and enrichment of sialylated glycans. Notably, this methodology is well suited for investigating the newly synthesized glycans during the EMT process, due to its metabolic labeling nature. The strategy can be further explored for studying other types of glycosylation, such as mucin-type O-linked glycosylation (47) and fucosylation (48). In addition, other cellular processes can be studied in a similar manner. Notably, metabolic labeling of the glycans with a chemical reporter can also be used for enrichment and glycoproteomic identification, by bioorthogonal conjugation with an affinity tag. In summary, our work highlights the applications of chemical tools in probing dynamic glycosylation in important cellular processes and suggests a close relationship between the EMT process and glycosylation.

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