Sialylation and fucosylation of epidermal growth factor receptor suppress its dimerization and activation in lung cancer cells

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Protein glycosylation is an important posttranslational process, which regulates protein folding and functional expression. Studies have shown that abnormal glycosylation in tumor cells affects cancer progression and malignancy. In the current study, we have identified sialylated proteins using an alkynyl sugar probe in two different lung cancer cell lines, CL1-0 and CL1-5 with distinct invasiveness derived from the same parental cell line. Among the identified sialylated proteins, epidermal growth factor receptor (EGFR) was chosen to understand the effect of sialylation on its function. We have determined the differences in glycan sequences of EGFR in both cells and observed higher sialylation and fucosylation of EGFR in CL1-5 than in CL1-0. Further study suggested that overexpression of sialyltransferases in CL1-5 and α1,3-fucosyltransferases (FUT4 or FUT6) in CL1-5 and A549 cells would suppress EGFR dimerization and phosphorylation upon EGF treatment, as compared to the control and CL1-0 cells. Such modulating effects on EGFR dimerization were further confirmed by sialidase or fucosidase treatment. Thus, increasing sialylation and fucosylation could attenuate EGFR-mediated invasion of lung cancer cells. However, incorporation of the core fucose by α1,6-fucosyltransferase (FUT8) would promote EGFR dimerization and phosphorylation.

Sialic acid | glycoproteomics | glycan sequencing | click chemistry | mass spectrometry

Protein glycosylation accounts for the vast majority of posttranslational processes to affect protein folding, stability, solubility, and function. Abnormal glycosylation has been reported to associate with malignant transformation of tumor cells. In particular, the terminal position of glycans with sugars such as sialic acid and fucose is usually exhibited at a high level in tumor cells (1). These two major terminal sugars may participate in important cell–cell interactions and cell migrations in cancer metastasis and angiogenesis (2). However, little evidence has been shown to support the causative roles of sialylation and fucosylation in malignant transformation. Because sialylation and fucosylation occur globally, their exact roles in the growth and metastatic behavior of tumors, especially at the molecular level, remain to be investigated.

Among the membrane glycoproteins, epidermal growth factor receptor (EGFR) with tyrosine kinase activity has been the target for drug discovery as its activity correlates with tumor progression. EGFR is overexpressed in various kinds of cancer cells and participates in the regulation of cancer invasion, metastasis, and angiogenesis (3). It belongs to the EGFR family and transduces cell signaling through ligand (EGF)-induced receptor dimerization (4), which then initiates its tyrosine kinase activity to direct the malignant behaviors of cancers (5). The extracellular domains of the EGFR family members are divided into four subdomains, with domains I and III participating in ligand binding (6) and domains II and IV for dimerization (7). In the state without ligand stimulation, an intramolecular interaction between domains II and IV maintains EGFR in a tethered conformation to inhibit dimerization (8), and this conformation is opened up by EGF binding to expose the dimerization interface for receptor activation (9, 10). After autophosphorylation, dimeric EGFR recruits and activates various downstream signaling proteins to direct cell proliferation, migration, differentiation, and apoptosis (11).

Twelve N-linked glycosylation sites were reported to exist in the extracellular region of EGFR (12). The major glycoforms of EGFR in an epidermoid carcinoma cell line, A431, have been identified (13). Some previous studies have indicated that the glycans could participate in regulating certain functions of EGFR (14). For example, mutagenesis to remove the Asn 420 and 579-linked glycans of EGFR and Asn 418-linked glycans of ErbB3 causes ligand-independent spontaneous dimerization (15–17). Besides, overexpression of sialidase in A431 cells would enhance EGFR activity, implying that sialylation of EGFR might inhibit its activation (18). On the other hand, core fucosylation was reported to aid EGFR activation: knocking down fucosyltransferase (FUT) 8 attenuates EGFR phosphorylation (19), and knocking out FUT8 reduces the EGF-binding ability of EGFR and down-regulates EGFR-mediated MAPK signaling (20). Further studies have indicated that overexpressing FUT8 could increase the susceptibility of EGFR to specific tyrosine kinase inhibitors, whereas knockdown of FUT8 decreases the susceptibility (21). Thus, delineating the roles of glycosylation on EGFR or other proteins may provide previously undescribed insights into disease biology and therapeutic strategy.

Lung cancer cells are known to express various sialylated or fucosylated glycan epitopes on the surface including sLeα, sLeβ, sLeγ, Globo H, polysialic acid, fucosyl GM1, GM2, etc. (1). To uncover the differences of protein glycosylation and further link to protein functions, we labeled glycosylated proteins with alkynyl-sugar probes, followed by Cu [I]-catalyzed alkyne-azide click chemistry (22) to identify the sialylated and fucosylated proteins from lung cancer cell lines CL1-0 and CL1-5 (23), two subpopulations that are derived from the same parental cell line with distinct invasion capabilities. Here, we showed that the more invasive cell line CL1-5 exhibited higher sialylation and fucosylation levels and expressed more sialylated proteins. Among these identified sialylated proteins, we chose...
EGFR to further study the effect of sialylation and fucosylation on its functions, especially dimerization and phosphorylation.

Results

Identification of Sialyl Glycoproteins in CL1 Lung Cancer Cells. By employing alkyl sulfars as probes for metabolically labeling and enriching cellular glycoproteins (22, 24), we compared the sialyl glycoproteins expressed on the lung cancer cells CL1-0 and CL1-5. Fig. S1A shows the flowchart of the glycoproteomic approach. Fig. S1B shows the patterns and labeling intensity of the protein extracts derived from these two cell lines labeling with alkyl ManNAc [ManNAcye, the precursor to CMP-alkynyl sialic acid (CMP-NeuAcye)], and azido biotin probe. Consistently, more sialyl proteins were identified in CL1-5 cells (Fig. S1C and Table S1). Again, the merit of this labeling method was demonstrated by the results that >95% of the enriched peptides bore NXS/T sequon, and most of them were derived from membrane (72%) or secreted (13%) proteins. The MALDI-TOF MS profiles of permethylated N glycans from CL1-0 and CL1-5 also showed different sialylation levels between CL1-0 and CL1-5 cells and a higher level of fucosylation in CL1-5 cells (Fig. S1D–F), although the branching level of N glycans in CL1-0 and CL1-5 were similar (biantennary: 56.1% vs. 60.3%; triantennary: 28.5% vs. 26.7%; tetraantennary: 15.4% vs. 13.0%).

Sialylation of EGFR in CL1 Cells. Among these CL1-5 uniquely expressed sialyl proteins (Table S1), EGFR was chosen for further investigation for its important role in promoting tumor growth and metastasis, and the link of EGFR N-linked glycosylation to its function (14, 16, 17). To verify if EGFR in CL1-5 was oversialylated, we next examined the expression and sialylation of EGFR in CL1-0 and CL1-5 cells. As shown in Fig. S2A, anti-EGFR antibody immunoprecipitated similar amounts of EGFR from very different quantities of CL1-0 and CL1-5 protein lysates (8:1), indicating that EGFR was up-regulated in CL1-5 cells. Nevertheless, the sialylation level of EGFR as demonstrated in ManNAcye-treated cells was higher in CL1-5. To further confirm the sialylation status, CL1-0/EGFR, an EGFR stable clone of CL1-0 that expressed an equal level of EGFR as CL1-5 cells, and CL1-5 cells were subjected to lectin pull-down experiment by SNA (Sambucus nigra lectin, binds 2,3-linked sialic acid) and MALII (Maackia amurensis lectin II, binds 2,6-linked sialic acid) and most of them were derived from membrane glycan from CL1-0 and CL1-5 also showed –Fuc–), although non-reducing glycan pull-downs revealed that EGFR was up-regulated in CL1-5 cells. Nevertheless, the sialylation level of EGFR as demonstrated in ManNAcye-treated cells was higher in CL1-5. To further confirm the sialylation status, CL1-0/EGFR, an EGFR stable clone of CL1-0 that expressed an equal level of EGFR as CL1-5 cells, and CL1-5 cells were subjected to lectin pull-down experiment by SNA (Sambucus nigra lectin, binds 2,6-linked sialic acid) and MALII (Maackia amurensis lectin II, binds 2,3-linked sialic acid). Fig. S2B showed that SNA and MALII pulled down more EGFR from CL1-5 lysates. Accordingly, the relative percentages of sialylated glycans in CL1-5 were also higher than in CL1-0 (Figs. S2C and S1E and F). These experiments confirmed that the EGFR in CL1-5 had higher sialylation than in CL1-0 cells.

Role of Sialylation on EGFR Activation. To understand how different EGFRs might act in CL1-0 and CL1-5 cells and whether the sialylation status could influence EGFR activation, we next analyzed EGFR dimerization and phosphorylation upon EGF stimulation in both cells. Fig. 1A revealed that EGFR dimerization occurred in CL1-0/EGFR without EGF treatment, and EGF induced less EGFR dimers in CL1-5 than in CL1-0/EGFR cells. To evaluate the phosphorylation status of these two cells, cells were treated with EGF at various concentrations (Fig. 1B), or for different periods of time (Fig. 1C), and then examined for EGFR tyrosine phosphorylation. Consistent with the dimerization results, CL1-5 cells showed weaker EGFR tyrosine phosphorylation compared to CL1-0/EGFR. Because CL1-5 showed higher sialylation than CL1-0 and CL1-0-EGFR cells, we subsequently tested if EGF-induced EGFR dimerization would be intensified in CL1-5 cells after sialidase treatment. Accordingly, CL1-5 cells treated with α2,3/6,8-linked sialidase showed stronger EGFR dimerization (Fig. S3A) and phosphorylation (Fig. S3B), suggesting that sialylation suppressed ligand-induced EGFR dimerization and the downstream signaling.

Role of Fucosylation on EGFR Activation. Our results that sialylation on EGFR could attenuate EGFR dimerization and phosphorylation prompted us to inspect the effect of terminal fucosylation, another kind of glycosylation associated with inflammation and cancers, in CL1 cells. We performed quantitative-PCR to examine the expression of FUT transcripts in CL1 cells. Comparing to CL1-0, CL1-5 showed a higher expression of FUT2 (2.5 folds), FUT4 (2.4 folds), FUT5 (2.4 folds), FUT6 (2.5 folds), and FUT11 (4.3 folds) (Fig. S4A), suggesting that CL1-5 cells displayed higher α1,3- and α1,6-linked fucosylation (core fucosylation). To understand the significance of α1,3-linked fucosylation on EGFR, we established FUT4 and FUT6 stable clones in lung adenocarcinoma A549 cells (A549-FUT4 and A549-FUT6) with Tet-off inducible protein expression system. Equal expression of EGFR in/on stable clones was confirmed by anti-EGFR immunoblotting and flow cytometric analysis (Fig. S4B and C), and the increased fucosylation of EGFR in A549-FUT4/6 cells was detected by alkynyl fucose (Fucyne) labeling (Fig. S4D). The results from lectin blotting confirmed that the increased fucosylation of EGFR in these cells was mainly α1,3-linked, because AAL (Aleuria aurantia lectin, recognizes α1,2, α1,3/4, and α1,6-linked fucose), but not Lens culinaris agglutinin-A (recognizes α1,6-linked fucose) or Ulex europaeus agglutinin (recognizes α1,2-linked fucose), showed a stronger EGFR-binding signal in lectin blotting (Fig. S4E).

We next analyzed EGF-induced EGFR dimerization in A549-FUT4/6 cells. Compared with mock control, A549-FUT4 and A549-FUT6 cells showed less EGFR dimerization (Fig. 2A) and lower EGFR tyrosine phosphorylation levels (Fig. 2B). After treating the cells with doxycycline to turn off FUT4 and FUT6 expression, EGFR in these cells showed lower AAL binding (Fig. S5A), but increasing dimerization and tyrosine phosphorylation levels (Fig. S5B and C) upon EGF stimulation. These results suggested that increasing α1,3-linked fucosylation on EGFR reduced receptor dimerization and activation. Because CL1-5 expressed a very high level of FUT8 mRNA compared to CL1-0 (Fig. S4F), we then tested if overexpressing...
or knocking down FUT8 could influence the behavior of EGFR. For this purpose, we established CL1-0-FUT8, A549-FUT8 stable lines (FUT8 overexpression), and CL1-5-FUT8 knockdown stable clones to examine EGF-induced EGFR dimerization and tyrosine phosphorylation (Fig. S6). Different from what we observed that α1,3-linked fucosylation suppressed EGF-induced receptor dimerization and phosphorylation, overexpressing FUT8 in CL1-0 and A549 did not influence (Fig. S6 A−F), whereas knocking down FUT8 in CL1-5 cells reduced (Fig. S6 G and H) EGFR dimerization and phosphorylation. Our results were consistent with the report that FUTX knockout cells are less sensitive to EGF treatment, and this is possibly due to the reduction of EGF-binding affinity when EGFR bears no core fucoses (20).

**Site-Specific Glycoform Mapping and Glycan Sequencing of EGFR in CL1-0 and CL1-5 Cells.** To profile the glycoforms of EGFR, the full-length EGFR was overexpressed and purified for analysis. The MALDI-TOF MS profiles (Fig. 3A) were detected from the EGFR samples derived from CL1-0 and CL1-5. Types of glycans: Man, high mannose; Bi, biantennary; Tri, triantennary; Tetra, tetraantennary; Penta, penta-antennary; Hexa, hexa-antennary; F, fucose; S, sialic acid; N, N-acetylhexosamine. Sialic acid index = (% with one sialic acid × 1) + (% with two sialic acids × 2) + (% with three sialic acids × 3) + (% with four sialic acids × 4) / 100; Fucose index = (% with one fucose × 1) + (% with two fucoses × 2) + (% with three fucoses × 3) / 100.

every individual EGFR glycopeptide derived from CL1-0 and CL1-5 cells was compositionally assigned and quantified in a site-specific manner.

Ten N-linked glycosylation sites on EGFR peptides were identified by Asn to Asp conversion after PNGase F treatment (Table S2). Within these 10 sites, 3 of them (Asn positions 328, 337, and 599) were identified to compose mostly high mannose-type N glycans (Man5 to Man9), 6 of them (Asn positions 32, 151, 389, 420, 504, and 579) were attached mainly with complex-type glycans, and Asn 544 was ligated with both high mannose-type and complex-type N glycans (Table 1 and Fig. S7). The glycosylation analysis revealed that EGFR from CL1-0 displayed more Man8 structure, and CL1-5 bore more bi- and triantennary glycans attached with at least one sialic acid and one fucose residue (Fig. 3B). The branching levels of complex-type N glycans
Together with the previous reports that Le^+ylation and fucosylation could suppress EGFR dimerization, EGF ligation (Fig. 2) of the fucosidase-treated sEGFR yielded higher dimerization upon in accordance with the observations from cell-based experiments, inactivated sialidase did not influence sEGFR dimerization. Also, compared to the untreated control, whereas treating with heat-(Fig. S4) the remaining sialic acids attached on sialidase-treated sEGFR were detected in EGFR of A431 cells (13, 25), our results indicated that sialylation and fucosylation on EGFR in tumor metastasis, we tested if sialidase treatment could understand the significance of sialylation and fucosylation on EGFR dimerization, the FLAG-tagged soluble EGFR (sEGFR) were shown in Fig. S8. To further test whether sialylation and fucosylation have a role in EGFR dimerization, the FLAG-tagged soluble EGFR (sEGFR) was overexpressed in 293F cells and treated with glycosidases (α2,3/6/8/9-sialidase and α1,3/4-fucosidase). The digestion of sEGFR with sialidase or fucosidase was confirmed by quantifying the remaining sialic acids attached on sialidase-treated sEGFR (Fig. S4F) or AAL lectin blotting (Fig. S4G), respectively. According to the analysis, the sialic acids on sEGFR were removed completely by sialidase treatment (from approximately 6 to approximately 0.2 sialic acids per sEGFR molecule). We then tested the in vitro dimerization of sialidase-treated sEGFR. As shown in Fig. 1D, more dimers were formed in sialidase-treated sEGFR compared to the untreated control, whereas treating with heat-inactivated sialidase did not influence sEGFR dimerization. Also, in accordance with the observations from cell-based experiments, the fucosidase-treated sEGFR yielded higher dimerization upon EGF ligation (Fig. 2C). All these results confirmed that sialylation and fucosylation could suppress EGFR dimerization. Together with the previous reports that Le^+ and sLe^+ epitopes were detected in EGFR of A431 cells (13, 25), our results indicated that Le^+ and sLe^+ could affect EGFR dimerization.

Role of Sialylation and Fucosylation in EGF-Mediated Cell Invasion. To understand the significance of sialylation and fucosylation on EGFR in tumor metastasis, we tested if sialidase treatment could influence EGF-mediated CL1-5 cell invasion. CL1-5 cells were first confirmed for the low expression level of surface sialic acid 6 h after sialidase treatment (Fig. S4F). EGF-mediated CL1-5 cell invasion was then evaluated by matrigel-coated transwell assay within 6 h. The results in Fig. A4 showed a higher invasion ability of CL1-5 cells when exposed to EGF. Notably, treating CL1-5 cells with sialidase enhanced EGF-mediated cell invasion. Nevertheless, A549-FUT4 and A549-FUT6 cells showed weaker invasion ability than mock control cells, whereas doxycycline treated A549-FUT4/6 and mock cells showed similar invasion ability (Fig. 4B). Here, our results suggested that sialylation and fucosylation could suppress the metastatic ability of cancer cells possibly via affecting EGF dimerization and activation.

In summary, we have identified sialylated and fucosylated proteins by metabolically labeling the glycoproteins of lung cancer cells with ManNAc and Fuc and compared the sialyl and fucosyl protein profiles. Our results revealed a regulatory mechanism of sialylation and fucosylation on EGF-mediated EGF behavior: Increasing sialylation and α1,3-linked fucosylation would suppress EGFR dimerization and phosphorylation, which could in turn affect the metastatic ability of cancer cells (Fig. 4C). On the other hand, the core fucosylation would promote EGFR dimerization and phosphorylation.

Discussion
A aberrant glycosylation has been discovered in many kinds of cancer cells, but the exact functions of altered glycans on glycoproteins or cellular tumorigenesis are still unclear. Many reports suggest that structural changes in cell surface carbohydrates may promote tumor transformation. For example, the α1,6-linked fucosylation of complex-type glycans may be an important feature of tumor progression related to increased metastasis (26), overexpression of N-acetylgalactosamintransferase V can enhance the invasion ability of glioma cells (27), and overexpression of FUT4 would promote the proliferation of human epidermoid carcino A431 cell by controlling the cell cycle machinery via MAPK.

Table 1. Site-specific representative glycans of EGFR

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In Vitro Dimerization of Sialidase- and Fucosidase-Modified EGFR. To further test whether sialylation and fucosylation have a role in EGFR dimerization, the FLAG-tagged soluble EGFR (sEGFR) was overexpressed in 293F cells and treated with glycosidases (α2,3/6/8/9-sialidase and α1,3/4-fucosidase). The digestion of sEGFR with sialidase or fucosidase was confirmed by quantifying the remaining sialic acids attached on sialidase-treated sEGFR (Fig. S4F) or AAL lectin blotting (Fig. S4G), respectively. According to the analysis, the sialic acids on sEGFR were removed completely by sialidase treatment (from approximately 6 to approximately 0.2 sialic acids per sEGFR molecule). We then tested the in vitro dimerization of sialidase-treated sEGFR, as shown in Fig. 1D. More dimers were formed in sialidase-treated sEGFR compared to the untreated control, whereas treating with heat-inactivated sialidase did not influence sEGFR dimerization. Also, in accordance with the observations from cell-based experiments, the fucosidase-treated sEGFR yielded higher dimerization upon EGF ligation (Fig. 2C). All these results confirmed that sialylation and fucosylation could suppress EGF dimerization. Together with the previous reports that Le^+ and sLe^+ epitopes were detected in EGFR of A431 cells (13, 25), our results indicated that Le^+ and sLe^+ could affect EGFR dimerization.
and PI3K/AKT signaling pathway (28). Thus it is proposed that specific inhibitors targeting the glycosyltransferases associated with numerous cancers may help defend against cancers. However, some other reports mention that glycosylation may have negative effects on protein functions or cellular behavior. For instance, α2,6-sialylated glycans may inhibit glioma malignancy because decreased α2,6-sialylation on glycoproteins (29, 30) or on gangliosides (31) enhances glioma formation and invasion; inhibition of N-linked glycosylation by tunicamycin can induce functional E-cadherin-mediated cell–cell adhesion (32); removal of sialic acids on cell surface by sialidase enhances connexin-43 gap junction functions (33). In virus infection, it has been observed that removal of glycans on influenza hemagglutinin enhances binding to cell surface receptors (34). Previous studies based on site-directed mutagenesis of EGFR also reveal that the glycans at positions 420 and 579 of EGFR pose an inhibitory effect on EGFR-independent dimerization (14, 17, 35).

In our study, we found that EGFR in CL1-5 exhibited higher sialylation and fucosylation levels and resulted in lower dimerization and tyrosine phosphorylation than in CL1-0 during EGF stimulation. In addition, removal of sialic acids from EGFR by sialidase increased dimer formation of EGFR upon EGF treatment on cell and in vitro, and pretreating EGFR with fucosidase also resulted in a similar dimerization enhancement in vitro. Moreover, induction of FUT4 and FUT6 overexpression in lung cancer A549 cells with Tet-off system decreased EGFR dimerization and activation, and this FUT4/6-mediated suppression was reverted when treating the cells with doxycycline to turn off FUT4/6 overexpression (Fig. S5 and Fig. 4B). These results demonstrated that sialylation and terminal α1,3-fucosylation could inhibit EGFR activation in lung cancer cells, and these modifications could actually affect EGFR-mediated signaling and cellular behavior. Previous studies have reported the fractionation and identification of EGFR glycans in A431 cells and indicated that the blood group A antigen, as exemplified by the A431 cell, has enhanced EGFR dimerization and signaling. It is worthy of mentioning that the study also suggests a negative role of blood group type A antigen/terminal N acetylgalactosamine in EGFR affinity, tyrosine kinase activation, and receptor turnover (37).

One of the major locations that showed elevated sialylation and fucosylation of EGFR in CL1-5 was Asn position 420 (Fig. 3C), the N-glycosylation site that has been shown to prevent EGFR spontaneous dimerization (17). This implies that sialylation and fucosylation on Asn 420 can be critical in suppressing EGFR dimerization and activation. Regarding the core fucosylation, although CL1-5 expressed a much higher level of FUT8 than CL1-0, overexpressing FUT8 in CL1-0 did not affect EGFR dimerization and phosphorylation, indicating that the core fucosylation could be fulfilled in the cells with even a limited amount of FUT8 expression. On the other hand, consistent with the results reported previously, knocking down FUT8 expression in CL1-5 cells showed a slight reduction of EGFR activation, which could be contributed by a reduced affinity to EGFR (20).

Our study provides a clear correlation between sialylation/fucosylation and EGFR dimerization/activation, but the conclusion is contradictory to the malignant phenotypes of CL1-5 because CL1-5 cells display more sialylation/fucosylation and are still more invasive than CL1-0 (23). This is understandable because CL1-5 cells are selected based on the invasion ability instead of glycosylation patterns of the cells. This sorting system results in two distinct cell populations that express very different sets of proteins, especially those for cancer progression; for example, CL1-5 cells express more MMP-9 (23). Thus it is inappropriate to directly link the invasive ability of CL1-5 and CL1-0 to only EGFR glycosylation in this complex situation. Nevertheless, our results clearly showed that reducing the terminal sialylation and α1,3-fucosylation on EGFR enhanced its dimerization and activation, and according to our data, the sialic acid and fucose on Asn 420 glycans may be most effective to inhibit EGFR dimerization and phosphorylation.

This study raises a question that whether inhibiting sialylation or fucosyltransferases is a practical way to counteract cancer, because EGFR, and possible other sialyl/fucosyl receptors, are widely involved in the malignancy of many types of tumors. If sialylation and fucosylation suppress receptor activation, inhibiting sialylation and fucosylation may create more aggressive cancer cells. Conversely, increasing sialylation or fucosylation in aggressive cancers may help counteract cancer progression; therefore, the enhancement of sialyl- or fucosyltransferase activity may be a unique therapeutic approach to tumors. Further attempts to examine how glycoforms alter protein conformation and protein–protein interaction, the events that closely link with signaling and cellular behaviors, will provide more evidence to understand the multifaceted roles of protein glycosylation.

Materials and Methods

EGFR Dimerization Assay. Purified FLAG-tagged sEGFR (1 μg), with or without glycosidase treatment, was incubated with 100 μg/mL human EGF in 100 mM NaCl/20 mM Hepes buffer (pH 7.4) for 2 h at room temperature (RT). Cross-linked BS3 (bis(sulfosuccinimidyl) suberate, Thermo Scientific; 0.25 mM) was added into the protein mixtures and allowed to react for 1 h at RT. The samples were analyzed by SDS-PAGE and immunoblotting with Peroxidase-conjugated anti-FLAG (M2) MAb (Sigma-Aldrich). For analyzing the EGFR dimerization on cell surface, cells were first starved in serum-free medium overnight, followed by stimulation with EGF (0–50 ng/mL) for 5 min. Cross-link reaction was conducted by the addition of 1 mM BS3 and the incubation at RT for 20 min, and terminated by adding 50 mM Tris-HCl (pH 7.4). The samples were analyzed by SDS-PAGE and Western blot with anti-EGFR Ab (Cell Signaling).

Detection of EGFR Tyrosine Phosphorylation. Cells were starved in serum-free medium overnight and stimulated with EGF (0–50 ng/mL) for 5 min or with 10 ng/mL EGF for 0–10 min. The cells were washed with cold PBS immediately and harvested with lysis buffer (1% NP-40, 25 mM Tris, 150 mM NaCl, 3 mM KCL, pH 7.4, 1 × EDTA-free protease inhibitor cocktail from Roche) with 1× phosphatase inhibitor (Roche). The EGFR in cell lysates was immunoprecipitated with EGFR antibody (Cell Signaling) on protein G agarose (Thermo Scientific). The immunoprecipitated samples were resolved SDS-PAGE and analyzed for the phospho-tyrosine signals by Western blot with anti-phosphotyrosine antibody (4G10, Millipore).

Glycosidase Treatment. For sialidase treatment on sEGFR, 1 μg sEGFR was mixed with 28.6 mU/μL sialidase (α2,3/6/9-linked, Prozyme) at 37 °C for overnight in 5 μL as instructed by the vendor. For sialidase treatment of CL1-5 cells, 106 cells were incubated with 0.04 U/mL sialidase (α2,3/6/8-linked, Roche) in 3 mL serum-free RPMI medium 1640 at 37 °C for 30 min. The reaction efficiency was further detected by sialic acid quantitation kit (Sigma-Aldrich). For cleaving fucoses on sEGFR, 1 μg sEGFR was mixed with 200 μU fucosidase (α1,3/4-fucosidase, from Calbiochem) in 10 μL of sodium phosphate buffer (pH 5.0) at 37 °C overnight.

EGF-Mediated Invasion Assay. The 24-well transwell plates with inserts (8-μm-pore polycarbonate membrane (Corning)) were set up for invasion assays. First, the membranes of the inserts were coated with 50 μg Matrigel (BD Bioscience) in 0.1 mL RPMI medium 1640 (Invitrogen). Cells were suspended in RPMI medium 1640 at the density of 105 cells/mL and seeded into the inserts (100 μL/insert) of transwell plates. The attractant human EGF (20 ng/mL, Millipore) was diluted in RPMI medium 1640 and dispensed into the lower chambers. Following the incubation for 6 h at 37 °C, the inserts were removed and the numbers of cells that migrated to the reverse side of the membrane were quantified by microscopy after nuclear staining with DAPI.

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