Reduction of the ST6 β-Galactosamide α-2,6-Sialyltransferase 1 (ST6GAL1)-catalyzed Sialylation of Nectin-like Molecule 2/Cell Adhesion Molecule 1 and Enhancement of ErbB2/ErbB3 Signaling by MicroRNA-199a*§

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Background: Nectin-like molecule 2 (Necl-2) is a tumor suppressor and suppresses ErbB2/ErbB3 signaling.

Results: MicroRNA-199a (miR-199a) targets the glycosylation enzyme ST6GAL1, reduces the sialylation of Necl-2, indirectly reduces the protein level of Necl-2, and enhances ErbB2/ErbB3 signaling.

Conclusion: miR-199a indirectly regulates Necl-2 and enhances ErbB2/ErbB3 signaling.

Significance: MicroRNAs regulate the glycosylation of plasma membrane proteins.

Nectin-like molecule 2 (Necl-2)/cell adhesion molecule 1 (CADM1) is shown to be down-regulated by the promoter hypermethylation and/or loss of heterozygosity at chromosome 11q23.2 in many types of cancers, including lung and breast cancers, and is proposed to serve as a tumor suppressor. However, the incidence of these epigenetic and genetic abnormalities of Necl-2 is 30–60% in these cancers, and other mechanisms for the suppression of Necl-2 are presumed to be present. We previously showed that Necl-2 interacts in cis with ErbB3 and suppresses the heregulin (HRG)-induced ErbB2/ErbB3 signaling for cell movement and death. We studied here the relationship between Necl-2 and microRNA-199a (miR-199a) that is up-regulated or down-regulated in a variety of cancers. miR-199a did not directly target the Necl-2 mRNA or affect its mRNA level in human lung cancer A549 cells and human embryonic kidney HEK293 cells. Necl-2 was at least sialylated by the sialyltransferase ST6 β-galactosamide α-2,6-sialyltransferase 1 (ST6GAL1). miR-199a targeted ST6GAL1 and reduced both the sialylation and the protein level of Necl-2. In addition, miR-199a enhanced the HRG-induced ErbB2/ErbB3 signaling. These results indicate that the suppressive role of Necl-2 in the HRG-induced ErbB2/ErbB3 signaling is regulated by miR-199a at least through the reduction of the ST6GAL1-catalyzed sialylation of Necl-2 and/or through the reduction of the protein level of Necl-2 presumably by the protein degradation.

Nectin-like molecule-2 (Necl-2)2/cell adhesion molecule 1 (CADM1) is a member of the Necl family, consisting of five members, Necl-1, -2, -3, -4, and -5 (1). This family comprises a superfamily with the nectin family, which consists of four members, nectin-1, -2, -3, and, -4 (1). All members of this superfamily have similar domain structures: one extracellular region with three immunoglobulin (Ig)-like loops, one transmembrane segment, and one cytoplasmic region. Necl-2 has many names: IgSF4a, RA175, SglGSF, TSLC1, SynCAM, and CADM1 (2–6). In this study, we use “Necl-2,” because it was first reported. Necl-2 shows Ca2+-independent homophilic and heterophilic cell-cell adhesion activities with other members of the nectin and Necl families, such as nectin-3, Necl-1 and -3, and class I-restricted T-cell-associated molecule (7–10).

Necl-2 is down-regulated by the hypermethylation of the Necl-2 gene promoter and/or loss of heterozygosity at chromosome 11q23.2 in many types of cancers, such as lung and breast cancers, and is proposed to serve as a tumor suppressor (2, 3, 11). However, the incidence of these epigenetic and genetic abnormalities of Necl-2 is about 30–60% in these cancers, and other mechanisms of the suppression of Necl-2 are presumed to be present. Necl-2 is abundantly expressed in epithelial cells (1), but its role in epithelial cells remained to be elucidated. We previously showed that Necl-2 interacts

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2 The abbreviations used are: Necl, nectin-like molecule; CADM1, cell adhesion molecule 1; HRG, heregulin; miRNA, microRNA; Ab, antibody; pAb, polyclonal antibody; PNGase F, peptide-N-glycosidase F; ST6GAL1, ST6 β-galactosamide α-2,6-sialyltransferase 1.
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in cis with ErbB3, but not with ErbB2, through their extracellular regions and inhibits the heregulin (HRG)-induced, ErbB2-catalyzed tyrosine phosphorylation of ErbB3 and ErbB3-mediated activation of Rac small G protein and Akt protein kinase, resulting in the inhibition of cell movement and death. These inhibitory effects of Necl-2 require both the extracellular and cytoplasmic regions and the binding of the cytoplasmic region with protein-tyrosine phosphatase PTPN13, also known as a tumor suppressor (12).

ErbB2 and ErbB3 comprise the epidermal growth factor (EGF) receptor/ErbB family with ErbB1 and ErbB4 (13). No ligand has been identified for ErbB2 yet, whereas HRG-α (also called neuregulin-1) and HRG-β (also called neuregulin-2) have been identified as the ligands for ErbB3. ErbB2 has tyrosine kinase activity, but ErbB3 lacks it. The ErbB2/ErbB3 heterodimer formed by the binding of HRG to ErbB3 induces the phosphorylation of nine tyrosine residues of ErbB3, causing the recruitment and activation of PI3K and the subsequent activation of Rac and Akt (14). The activation of Rac enhances cell movement, and the activation of Akt prevents cell death (15). Amplification of the ErbB2 gene is observed in many types of cancers, including lung and breast cancers, and ErbB2 serves as an oncogenic protein (16, 17). Amplification or mutation of the gene causes enhanced signaling for cell movement and survival, eventually resulting in tumorigenesis, invasion, and metastasis. ErbB3 is expressed in a number of human cancers, including breast and ovarian tumors, and has a crucial role in tumorigenesis (18).

MicroRNAs (miRNAs) are short non-coding RNAs that regulate protein expression from targeted genes by pairing complementary sequences in the 3′-UTR (19). miRNAs regulate various cellular processes, such as differentiation, proliferation, apoptosis, and angiogenesis. In addition, alterations in miRNAs and other short or long non-coding RNA are involved in the initiation, progression, and metastasis of human cancer. Functional analyses of miRNAs have revealed their involvement in the regulation of various signal transduction pathways, such as Hippo, transforming growth factor-β/Nodal, and receptor tyrosine kinase signaling pathways (20, 21). miR-372 and miR-373 repress the LAT2, a kinase component of the Hippo pathway, and are implicated in testicular germ cell oncogenesis (22). *Xenopus* miRNAs miR-15 and miR-16 that target the Nodal type II receptor regulate Nodal signaling and the formation of the Spermann’s organizer (23). miR-7 targets ErbB1 and its downstream signaling molecules, including Raf1, and miR-125 coordinately regulates both ErbB2 and ErbB3 expression and suppresses their downstream signaling (24). miRNAs, including miR-214, miR-21, and miR-17–92, suppress PTEN expression and induce the activation of Akt (25).

miR-199a regulates the PI3K/Akt and ERK/MAPK signaling pathways, the oxidative stress signaling pathway, and prostatespecific antigen synthesis (26). Two mature miRNA products, miR-199a-5p and miR-199a-3p, are expressed from the miR-199a precursors that map to chromosome 19 (miR-199a-1) and chromosome 1 (miR-199a-2) in humans. Among them, miR-199a-2 is clustered with miR-214 at the intron of the human Dmm3os gene, and transcription of this miRNA cluster is activated by TWIST1, a transcription factor involved in the epithelial mesenchymal transition (27). miR-199a is either up-regulated or down-regulated in a variety of cancers. It is either up-regulated or down-regulated in ovarian cancers (27, 28); it is up-regulated in breast cancer stem cells and promotes proliferation and survival of breast cancer cells (29, 30); it is up-regulated in advanced lung cancers (31); and it is down-regulated in advanced hepatocellular carcinoma and small cell carcinoma of the cervix (32–34). In addition, miR-199a suppresses the expression of GSK-3β or CD44 that are involved in the proliferation and survival of cancer cells (35, 36).

Protein glycosylation, such as N-glycosylation, O-glycosylation, and sialylation, attaches glycan chains to the specific amino acid residues and is one of the most common posttranslational modifications during or after protein synthesis (37, 38). Nearly all proteins that pass through the endoplasmic reticulum-Golgi complex are N-glycosylated, and this modification can determine or influence protein folding, stability, trafficking, localization, and oligomerization, with important implications for cell–cell interactions and intracellular signaling. In addition, deregulation of glycosylation is associated with phenotypes of a variety of cancer cells (39). Necl-2 has six predicted N-glycosylation sites in the extracellular Ig-like loops (7), and some of the Necl-2 splicing variants can contain either no or up to 21 predicted O-glycosylation sites (41).

On the basis of these earlier observations, we studied here the relationship between Necl-2 and miR-199a using human lung cancer A549 cells as a model. We found that miR-199a did not target the Necl-2 mRNA directly but targeted the sialyltransferase ST6 β-galactosamide α,2,6-sialyltransferase 1 (ST6GAL1), reduced the sialylation of Necl-2, and enhanced the HRG-induced Erb2/ErbB3 signaling. We describe here a novel mechanism for the suppression of Necl-2 by miR-199a in the HRG-induced Erb2/ErbB3 signaling.

**EXPERIMENTAL PROCEDURES**

*Cell Culture*—A549 cells were purchased from the American Type Culture Collection (Manassas, VA). A549 and human embryonic kidney HEK293 cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin and cultured at 5% CO₂ at 37 °C.

*Plasmid Vectors*—Total RNA was extracted from A549 or Caco-2 cells and reverse transcribed using Superscript III (Invitrogen) according to the manufacturer’s instructions. The partial and the full-length 3′-UTR sequences of the putative miR-199a target genes were amplified by PCR using the cDNA or pLightSwitch CADM1 3′-UTR plasmid (S811807, Switch-Gear) as a template and cloned at the 3′-end of the luciferase gene of the firefly luciferase reporter pGL3-MC plasmid (30). The following primers with restriction enzyme sites were used for cloning of the 3′-UTR sequences of Necl-2, ST6GAL1, or IKBKB: Necl-2: partial 3′-UTR, forward (5′-CCGGCCCTTTAGTTAGGAAGGAGTGG-3′) and reverse (5′-CCTAGTGTTAAGCTTCCCTTACC-3′); ST6GAL1: WT 3′-UTR, forward (5′-AAAGGGCACTAGCCTTGTGTTTGTTC-3′) and reverse (5′-AAACTAGTCACTTTGTTAACATTTTTTTTTA-3′); IKBKB: 3′-UTR.
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A549 cells were transfected with the miR-199a or negative control precursors were cultured at 37 °C for 72 h. The cells were lysed with a collection buffer (20 mM NaH2PO4/NaHPO4, pH 7.5, 1% Nonidet P-40, 10% glycerol, 1 µg/ml leupeptin, and 1 µg/ml aprotinin) containing 2-mercaptoethanol and boiled at 95 °C for 5 min. The lysates were then incubated with neuraminidase at 37 °C for 2 h, incubated with peptide-N-glycosidase F (PNGase F) at 37 °C overnight, or co-incubated with O-glycosidase and neuraminidase at 37 °C overnight. The lysates were boiled in an SDS sample buffer (60 mM Tris-HCl, pH 6.7, 3% SDS, 2% 2-mercaptoethanol, and 5% glycerol) for 5 min and subjected to SDS-PAGE, followed by Western blotting using the indicated Abs. Neuraminidase, PNGase F, and O-glycosidase were purchased from Roche Applied Science.

Serum Starvation and HRG Stimulation—A549 cells were transfected with the miR-199a or negative control precursor using Lipofectamine RNAiMAX reagent, plated in 6-well plates at a density of 2.5 × 10^5/cm^2, and cultured for 48 h. The cells were starved of serum with DMEM containing 0.5% fatty acid-free BSA (Sigma) for 20 h, followed by stimulation with 10 ng/ml HRG (Sigma) in DMEM containing 0.5% fatty acid-free BSA. The cells were washed with ice-cold PBS twice and lysed with a radioimmunoprecipitation assay buffer (20 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 137 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 50 mM NaF, 1 mM Na2VO4, 10 µg/ml leupeptin, 2 µg/ml aprotinin, and 10 µM phosphatase inhibitor mixture 3 (Sigma)). The lysates were then boiled in the SDS sample buffer for 5 min and subjected to SDS-PAGE, followed by Western blotting using the indicated Abs.

RESULTS

No Effect of miR-199a on the Translation or the Expression Level of the Necl-2 mRNA—We further examined whether miR-199a directly regulates the translation of Necl-2 because Necl-2 was predicted by TargetScan to have a miR-199a-targeted site (42) (Fig. 1A). We tested the ability of miR-199a to regulate the 3′-UTR of the Necl-2 mRNA fused to the 3′-end of a luciferase minigene using HEK293 and A549 cells, neither of which expressed miR-199a. The 3′-UTR of the Necl-2 mRNA, which contains a predicted target site for miR-199a, was cloned into the pGL3-Necl-2 partial downstream of a luciferase minigene (pGL3-Necl-2 partial) (Fig. 1A). HEK293 cells were co-transfected with the pGL3-Necl-2 3′-UTR plasmid or the control luciferase plasmid, together with pRL-TK Renilla luciferase plasmid and miRNA precursors. Co-transfection of the miR-199a precursor did not affect the luciferase activity of the pGL3-
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Necl-2 3′-UTR plasmid but suppressed the activity of the positive control luciferase plasmid with the 3′-UTR of the IKBKB mRNA (43) (Fig. 1B). We confirmed that co-transfection of the miR-199a precursor did not affect the luciferase activity of the pGL3-Necl-2-full-length 3′-UTR plasmid in which the full-length 3′-UTR of the Necl-2 mRNA was cloned into the pGL3-MC plasmid (Fig. 1, A and B). Consistent with the results in HEK293 cells, miR-199a did not target the 3′-UTR of the Necl-2 mRNA in A549 cells (data not shown). These results indicate that miR-199a does not target the 3′-UTR of the Necl-2 mRNA.

We then tested whether miR-199a affects the expression level of the Necl-2 mRNA using A549 cells, which endogenously expressed the Necl-2 protein but did not express miR-199a. miR-199a did not change the expression level of the Necl-2 mRNA, suggesting that miR-199a did not cause degradation of the Necl-2 mRNA and/or indirect transcriptional repression of Necl-2 (Fig. 1C). Taken together, these results indicate that 1) miR-199a does not target the 3′-UTR of the Necl-2 mRNA, 2) miR-199a does not affect the translation of Necl-2, 3) the Necl-2 mRNA is not degraded in the miR-199 precursor-transfected cells, and 4) miR-199 does not affect the transcription of the Necl-2 gene.

Impairment of Posttranslational Modification(s) of Necl-2 by miR-199a—We then examined the effect(s) of miR-199a on the Necl-2 protein in A549 cells by Western blotting. The immunoreactive bands of Necl-2, which were about 90 kDa with several additional bands in the control precursor-transfected cells,

shifted to give many bands from 75 to 90 kDa (mainly located at 75 kDa) in the miR-199a precursor-transfected cells (Fig. 2A). These results suggest that miR-199a affects the posttranslational modification(s) of Necl-2.

We then measured the intensity of the immunoreactive bands of Necl-2. They were reduced by about 42% in the miR-199a precursor-transfected cells, compared with those in the control precursor-transfected cells (Fig. 2B). The reduction of the intensity and the shift of the immunoreactive bands of Necl-2 were also observed in the A549 cells transfected with the plasmid expressing FLAG-Necl-2 without 3′-UTR (data not shown). These results indicate that miR-199a reduces the protein level of Necl-2, but we further investigated the effect of miR-199a on the possible posttranslational modification(s) of Necl-2.

Reduction of the Sialylation of Necl-2 by miR-199a—Necl-2 has predicted N-glycosylation sites, and some of its splicing variants have predicted O-glycosylation sites (7, 41). We therefore analyzed the glycosylation status of Necl-2 in A549 cells (Fig. 3A). The total cell lysates were collected, and then the same amounts of the total proteins were subjected to digestion with the indicated deglycosidase enzymes. The position of the immunoreactive bands of Necl-2 markedly shifted to 60 kDa when digested with PNGase F, which cleaves asparagine-linked oligosaccharides from glycoproteins (Fig. 3A). The position of the immunoreactive bands of EGFR and ErbB3 shifted when digested with PNGase F. The shifts of the immunoreactive bands of EGFR and ErbB3 after digestion by PNGase F were consistent with the previous observations (44, 45) (Fig. 3A). These results indicate that Necl-2 is an N-glycosylated protein and suggest that miR-199a does not regulate the N-glycosylation processes of Necl-2.

Digestion of Necl-2 with neuraminidase, which releases the terminal sialic acids, resulted in the shift of the immunoreactive bands of Necl-2 mainly to 75 kDa, which was comparable with the position of the densest band of Necl-2 in the miR-199a precursor-transfected cells (Fig. 3A). The position of the immunoreactive band of EGFR shifted slightly when digested with neuraminidase, which is consistent with the previous observation (46). In contrast, the shift of the immunoreactive band of ErbB3 after neuraminidase treatment was undetectable. Co-digestion of Necl-2 with neuraminidase and O-glycosidase, which cleaves the Galβ1–3 N-acetylgalactosamine (GalNAc) (core 1).
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and/or the N-acetylgalcosamine (GlcNAc) β1–3GalNAc (core 3) structure(s), resulted in the further shift of the position of the immunoreactive bands of Necl-2, suggesting that Necl-2 was O-glycosylated (Fig. 3A). Taken together, these results suggest that miR-199a regulates the sialylation processes of Necl-2.

We then digested the lysates of the miR-199a or control precursor-transfected cells with PNGase F. The multiple immunoreactive bands of Necl-2 in the miR-199a- or control precursor-transfected cells shifted to give a single immunoreactive band at 60 kDa (Fig. 3B). The intensity of the immunoreactive bands of Necl-2 in the miR-199a precursor-transfected cells after PNGase F digestion was reduced by about 29% as compared with that of the control precursor-transfected cells (Fig. 3B), which is comparable with the result shown in Fig. 2B. The reduction of the intensity of the immunoreactive bands of Necl-2 was also observed after other deglycosylation treatments, consistent with the result of the PNGase F treatment (Fig. 3B). These results indicate that miR-199a regulates the sialylation processes of Necl-2 and reduces the protein level of Necl-2.

Reduction of the Sialyltransferase ST6GAL1 by miR-199a—
The results of the glycosylation analyses raised the possibility that miR-199a targeted a sialyltransferase(s) and indirectly affected the posttranslational modification(s) of Necl-2. We tested a series of glycosylation enzymes that were predicted by TargetScan to be targeted by miR-199a. The 3′-UTR sequences that contained a predicted target site for miR-199a were mutated in the ST6GAL1 mutant plasmid. The number indicates the position of the nucleotides in the reference wild-type sequence of ST6GAL1 (NM_003032). C, activity of luciferase gene linked to the 3′-UTR of the ST6GAL1 mRNA. The mean of the results from the cells transfected with the control precursor was set at 1.0. The data are mean ± S.D. of three separate transfections (*, p < 0.05).

FIGURE 3. Characterization of the glycosylation status of Necl-2. A, the same amounts of the A549 cell lysates were subjected to digestion with various deglycosylation enzymes. The lysates of the A549 cells transfected with the control or miR-199a precursors were also analyzed. B, the lysates of the A549 cells transfected with the control or miR-199a precursors were digested with the indicated deglycosylation enzymes. The samples were subjected to SDS-PAGE, followed by Western blotting using the indicated Abs.

FIGURE 4. Targeting of the sialylation enzyme ST6GAL1 by miR-199a. A, screening of glycosylation enzymes regulated by miR-199a. The putative target sites of miR-199a within the 3′-UTR of the glycosylation enzymes were cloned into the pG3 luciferase expression plasmid. The luciferase activities were measured after 48 h. The mean of the results from the cells transfected with the control precursor was set at 1.0. The data are mean ± S.D. (error bars) of three separate transfections (*, p < 0.05). B, schematic representation of the pG3 luciferase plasmid linked to the predicted miR-199a target site within the 3′-UTR of the ST6GAL1 mRNA. Three nucleotides complementary to the seed sequence for miR-199a were mutated in the ST6GAL1 mutant plasmid. The number indicates the position of the nucleotides in the reference wild-type sequence of ST6GAL1 (NM_003032). C, activity of luciferase gene linked to the 3′-UTR of the ST6GAL1 mRNA. The mean of the results from the cells transfected with the control precursor was set at 1.0. The data are mean ± S.D. of three separate transfections (*, p < 0.05).
miR-199a targeted the 3'-UTR of ST6GAL1 mRNA with a predicted target site in the 3'-UTR wild-type plasmid, its mutant plasmid, or the control luciferase plasmid, when co-transfected with the pGL3-ST6GAL1 3'-UTR wild-type plasmid, the miR-199a precursor suppressed the luciferase activity by 35%, whereas mutation of the predicted miR-199a target site in the 3'-UTR of the ST6GAL1 mRNA abrogated the repressive ability of the miRNA, demonstrating the specificity of the target sequence within the 3'-UTR of the ST6GAL1 mRNA (data not shown).

We then analyzed the effect of ST6GAL1 knockdown on the posttranslational modification of Necl-2 using an siRNA for ST6GAL1 (Fig. 5A). When transfected with siRNA against ST6GAL1, the immunoreactive bands of Necl-2 shifted to give several bands mainly located at 75 kDa, and the intensity of the immunoreactive bands of Necl-2 was reduced by about 34% as compared with that in the control siRNA-transfected cells, similar to the observations in the miR-199a precursor-transfected cells (Fig. 5B). These results indicate that miR-199a directly targets ST6GAL1 and thereby indirectly regulates the posttranslational modification of Necl-2.

Enhancement of the HRG-induced ErbB2/ErbB3 Signaling by miR-199a—In the last set of experiments, we examined the effect of miR-199a on the HRG-induced ErbB2/ErbB3 signaling by analyzing the phosphorylation of ErbB3 and Akt in A549 cells. We previously showed that this cell line expressed ErbB2, ErbB3, and Necl-2 and that Necl-2 inhibited the HRG-induced ErbB2/ErbB3 signaling (12). A549 cells were serum-starved and stimulated with HRG. The intensities of the immunoreactive bands of ErbB3, in which the tyrosine 1289 was phosphorylated, were increased in response to HRG in both miR-199a precursor-transfected and control precursor-transfected cells, but the intensity was much higher in the miR-199a precursor-transfected cells than in the control precursor-transfected cells (Fig. 6Aa). We confirmed that the intensities of the immunoreactive bands of the phosphorylated ErbB3 were significantly increased in the miR-199a precursor-transfected cells when they were normalized to those of the total ErbB3 (Fig. 6Ab). Consistently, the intensities of the immunoreactive bands of Akt, in which the threonine 308 was phosphorylated, were increased in response to HRG in both miR-199a precursor-transfected and control precursor-transfected cells, but the intensities in the miR-199a precursor-transfected cells were twice as high as that in the control precursor-transfected cells (Fig. 6B). These results indicate that miR-199a enhances the HRG-induced ErbB2/ErbB3 signaling.

**DISCUSSION**

Glycosylation is one of the important posttranslational modifications of proteins, and about 50% of all proteins are glycosylated (47). Protein glycosylation plays roles in a variety of biological functions, such as cell-cell and cell-substrate adhesions, membrane organization, cell immunogenicity, and protein targeting (48). Glycopeptide linkages, such as N- and O-glycosylation, C-mannosylation, phosphoglycation, and glypiaction, are formed by the enzymatic transfer of an activated monosaccharide directly to the substrate proteins at various intracellular sites, including the endoplasmic reticulum, the Golgi complex, the cytosol, and the nucleus (48). Sialic acids are terminal acidic monosaccharides that are involved in various functions, such as immune regulation, and bacterial and viral infections (49). It is considered that the human genome encodes more than 20 different sialyltransferases (50). The Galβ1–4GlcNAc disaccharide of N-linked oligosaccharide chains can be sialylated by α2,3- or α2,6-sialyltransferase (ST3 or ST6) (50). ST6GAL1 is expressed ubiquitously and catalyzes the α2,6-sialylation of a terminal Gal residue of Galβ1–4GlcNAc disaccharide that exists as a free disaccharide or as a terminal N-acetyllactosamine unit of N- or O-linked oligosaccharide chains. We showed here by use of various deglycosylation enzymes that Necl-2 is posttranslationally modified at least by the sialylation in addition to the N-glycosylation (7).

A few miRNAs that target glycosylation enzymes have been identified so far. miR-378 targets UDP-N-acetyl-α-D-galactosamine-polypeptide N-acetylgalactosaminyltransferase 7 and enhances the glycosylation of naphthoestin, a ligand for integrin α9β1 (51). miR-148 targets the enzyme core 1 β1,3-galactosyltransferase 1 and modulates IgA1 O-glycosylation and the level of secreted galactose-deficient IgA1 (52). We showed here that miR-199a targeted the sialyltransferase ST6GAL1 and reduced its expression and the sialylation of Necl-2. The ST6GAL1 sialyltransferase adds an α2,6-linked sialic acid to the termini of N-linked glycans on secretory and membrane glycoproteins (53). We were not able to confirm the reduction of the...
protein level of ST6GAL1 in miR-199a precursor-transfected cells because an anti-ST6GAL1 Ab failed to detect this protein in human cancer cell lines, such as MCF-7, MDA-MB-231, and Caco-2, by Western blotting (data not shown). It was also practically difficult to find cell lines that expressed both miR-199a and Necl-2 or to construct tagged ST6GAL1 expression plasmids with enzymatic activity. However, a series of deglycosylation enzyme digestion experiments and analysis of Necl-2 by SDS-PAGE using the ST6GAL1 knockdown cells further support that miR-199a reduced the sialylation of Necl-2.

In addition to the reduction of the sialylation of Necl-2, the protein level of Necl-2 was reduced in the miR-199a precursor-transfected cells because an anti-ST6GAL1 Ab failed to detect this protein in human cancer cell lines, such as MCF-7, MDA-MB-231, and Caco-2, by Western blotting (data not shown). It was also practically difficult to find cell lines that expressed both miR-199a and Necl-2 or to construct tagged ST6GAL1 expression plasmids with enzymatic activity. However, a series of deglycosylation enzyme digestion experiments and analysis of Necl-2 by SDS-PAGE using the ST6GAL1 knockdown cells further support that miR-199a reduced the sialylation of Necl-2.

In addition to the reduction of the sialylation of Necl-2, the protein level of Necl-2 was reduced in the miR-199a precursor-transfected cells (Figs. 2 and 3B). Because miR-199a did not directly target the 3′-UTR of the Necl-2 mRNA (Fig. 1), other mechanisms for the down-regulation of Necl-2 were presumed to exist. Glycosylation of secretory and membrane glycoproteins is generally thought to promote protein stability (54). Moreover, the covalent binding of glycans to the protein surface may inherently enhance the thermal and kinetic stability of proteins (55). In addition, several studies support a role of the sialylation in regulating the protein stability (56–58). Thus, it is possible that the reduction of the sialylation of Necl-2 enhances its protein degradation. However, the protein degradation of Necl-2 was not affected when the miR-199a precursor-transfected cells were treated with a proteasome inhibitor, epoxomicin, or a protease inhibitor, leupeptin. This issue will be studied in detail in future.

We finally showed here that miR-199a enhances HRG-induced ErbB2/ErbB3 signaling. We previously showed that Necl-2 interacts in cis with ErbB3 and inhibits the HRG-induced ErbB2/ErbB3 signaling (12). Therefore, miR-199a enhances the HRG-induced ErbB2/ErbB3 signaling by reducing the protein level of Necl-2. The reduction of the sialylation of Necl-2 may also be involved in the miR-199a effect on the HRG-induced ErbB2/ErbB3 signaling. It may be noted that miR-199a slightly increased the protein level of ErbB3 in A549 cells (Fig. 6A), but the miR-199a-induced enhancement of the ErbB2/ErbB3 signaling was not simply explained by this increase of the ErbB3 protein. It is also noted that the immunoreactive band of ErbB3 shifted from 185 to 155 kDa with PNGase F treatment, but the effects of neuraminidase or O-glycosidase treatment on ErbB3 were undetectable (Fig. 3A), indicating that ErbB3 is posttranslationally N-glycosylated. However, because miR-199a did not shift the position of the immunoreactive band of ErbB3, these results indicate that miR-199a does not modulate the glycosylation status of ErbB3.

It is established that the hypermethylation of the Necl-2 gene promoter and the loss of heterozygosity at chromosome 11q23.2 down-regulate Necl-2 in many types of cancers (11). In addition to these two mechanisms, we recently found that miR-214 targeted the 3′-UTR of the Necl-2 mRNA and reduced the protein level of Necl-2 (59). We further showed here that miR-199a reduced the sialylation of Necl-2 and the protein level of Necl-2, presumably through protein degradation. Furthermore, we recently found that hypoxia reduced the protein level of Necl-2 in human colon cancer Caco-2 cells in an unknown mechanism (59). Necl-2 is involved in a variety of cellular functions, such as cell polarization, proliferation, differentiation, survival, and sorting and plays roles in a wide range of events from normal development to tumorigenesis (40). Thus, it is speculated that these multiple regulatory mechanisms for Necl-2 have developed. Further studies are required to clarify roles of the respective regulatory mechanisms of Necl-2 under various physiological and pathological conditions.
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