NCAM and attached polysialic acid affect behaviors of breast epithelial cells through differential signaling pathways

Lin Cao
Provincial Key Laboratory of Biotechnology, Joint International Research Laboratory of Glycobiology and Medicinal Chemistry, College of Life Sciences, Northwest University

Xin Wang
Shandong University of Arts

Juhong Yang
Joint International Research Laboratory of Glycobiology and Medicinal Chemistry, College of Life Science, Northwest University

Jia Guo
Institute of Biomedical Engineering and Health Sciences, Changzhou University

Xiang Li
Provincial Key Laboratory of Biotechnology, Joint International Research Laboratory of Glycobiology and Medicinal Chemistry, College of Life Sciences, Northwest University

Xiaomin Yang
Department of Surgical Oncology, The First Affiliated Hospital, Medical School, Xi'an Jiaotong University

Zengqi Tan (zengqtan@nwu.edu.cn)
Provincial Key Laboratory of Biotechnology, Joint International Research Laboratory of Glycobiology and Medicinal chemistry, College of Life Sciences, Northwest University https://orcid.org/0000-0002-2268-6248

Feng Guan
Provincial Key laboratory of Biotechnology, Joint International Research Laboratory of Glycobiology and Medicinal Chemistry, College of Life Sciences, Northwest University

---

Research article

Keywords: neural cell adhesion molecule, polysialic acid, epithelial-mesenchymal transition, signaling pathway, β-catenin/slug, EGFR/STAT3

Posted Date: April 27th, 2020

DOI: https://doi.org/10.21203/rs.2.24259/v3

Abstract

Background Neural cell adhesion molecule (NCAM), a common mammalian cell surface glycoprotein, is the major substrate of polysialic acid (polySia). Polysialylated NCAM occurs in many types of cancer, but rarely in normal adult tissues. Epithelial-mesenchymal transition (EMT) is an important process contributing to tumor metastasis. The functional role of NCAM hypersialylation in EMT is unclear.

Method Expression of NCAM and polysialylated NCAM in breast cancer progression were evaluated by western blot (WB), immunohistochemistry, semi-quantitative PCR and immunoprecipitation (IP). Overexpression of NCAM-140 and ST8SiaII were performed to assess the functional role of NCAM hypersialylation by liposome transfection. Cell proliferation ability was investigated with MTT assay. Transwell and wound closure assay were conducted to evaluate cell migratory ability. Phagokinetic gold sol assay and cell adhesion assay were performed to assess cell motility ability and cell adhesion ability, respectively. Furthermore, WB and IP were used to reveal the activated signaling pathway.

Results The present study indicates that NCAM and attached polysialic acid affect behaviors of breast epithelial cells through differential signaling pathways, based on the following observations: (i) NCAM and polysialylated NCAM were aberrantly regulated in breast cancer cells; (ii) NCAM and polysialylated NCAM expression were upregulated in normal breast epithelial cells undergoing EMT; (iii) NCAM overexpression induced EMT in breast epithelial cells; (iv) NCAM promoted cell proliferation and migration through activation of a β-catenin/slug signaling pathway; (v) modification of polySia attached to NCAM inhibited cell adhesion and promoted cell motility through activation of an EGFR/STAT3 pathway.

Conclusion This study demonstrates that NCAM and polysialylated NCAM facilitate different signaling pathway and affect different cell behaviors. Switching between the NCAM-mediated pathways appeared to depend on polySia decoration.

Background

Sialic acids are structures having a nine-carbon backbone, often found on terminal glycan chains of glycolipids and glycoproteins[1]. Elevated expression of sialoglycans has been correlated with tumor aggressiveness, tumor invasion, and poor prognosis in cancer patients[2, 3]. Aberrant sialylation is due primarily to abnormal regulation of sialyltransferases, enzymes that catalyze the linkage of sialic acid to other carbohydrates[4, 5]. For example, overexpression of ST3Gal I in breast cancer cells promoted tumorigenesis in a murine model[6], and upregulation of ST6Gal I was essential for maintenance of cancer stem cells[7]. Polysialyltransferases II (ST8SiaII) and IV (ST8SiaIV), another type of sialyltransferase, transfer sialic acid from CMP-sialic acid to sialic acid residues of other sialoglycans to yield 2,8-linked polysialic acid (polySia)[8]. PolySia is expressed in cancers of neuroendocrine-origin[9], including lung cancer[10] and many others[11, 12]. It has been reported that polySia is expressed in breast cancer MCF7 cells[13], but a few studies focussed on the biological function role of polySia in

breast cancer progression. Among many polySia-bearing glycoproteins, neural cell adhesion molecule (NCAM) is the major polySia substrate[14]. Polysialylated NCAM is of particular interest because it is present in many types of cancer cells but rarely expressed in most healthy adult tissues[15, 16].

NCAM is a member of the immunoglobulin superfamily of adhesion molecules and has three isoforms (NCAM-120, NCAM-140, NCAM-180) based on alternative splicing[17]. NCAM-140 and -180 are transmembrane proteins; NCAM-120 is a glycosylphosphatidylinositol (GPI)-anchored protein. The extracellular region of NCAM comprises five immunoglobulin (Ig1-5) and two fibronectin type III (FN1-2) domains and carries six N-glycosylation sites in Ig domains. Elevated NCAM expression has been reported in pancreatic cancer[18], neuroblastoma[19], small cell lung cancer[20], and other cancers. Polysialylated NCAM level was correlated with tumor metastasis[21]. Altered sialoglycan expression has been associated with the process of epithelial-mesenchymal transition (EMT), which plays a key role in development of invasive and metastatic cell phenotype[22]. NCAM has been shown to be important for promotion of EMT, and is upregulated during EMT[23, 24], but polySia was not specifically evaluated in that context. The effects of hypersialylation or hyposialylation of NCAM on various cell behaviors remain unclear.

In present study, the NCAM expression in clinical samples from breast cancer (BC) patients were assessed. Breast epithelial cell lines were used to investigate the role of polySia, and its major substrate NCAM, in modulation of various cell behaviors during EMT. NCAM and polysialylated NCAM were shown to facilitate different signaling pathway and thereby affect different cell behaviors. Switching between the NCAM-mediated pathways appeared to depend polySia decoration.

Methods

Antibodies and reagents

Antibodies used were mouse anti-E-cad IgG2a (part #610181), mouse anti-β-catenin IgG1 (610153), mouse anti-CD56 (NCAM13; 556324), anti-CD56 (NCAM 12F8; 556325), anti-STAT3 (610189) from BD Biosciences (San Jose, CA, USA); mouse anti-N-cad IgG1 (sc59987), anti-CK1α (sc6477), anti-c-Myc (sc40) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse anti-VM IgG1 (V5255), mouse anti-polysialylated NCAM antibody IgM 5A5 from Developmental Studies Hybridoma Bank (University of Iowa, IA, USA); anti-β-tubulin I IgG1 (T7816), anti-FN (F3648) from Sigma-Aldrich (St. Louis, MO, USA); anti-EGFR (D38B1), anti-p-EGFR (Tyr 1068; D7A5), anti-p-STAT3 (Tyr 705; D3A7), anti-GSK-3β (27C10), anti-slug (C19G7), anti-histone H3 (D1H2) from Cell Signaling Technology (Beverly, MA, USA); horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (A0216), HRP-labeled goat anti-rabbit IgG (A0208), FITC-labeled goat anti-mouse IgG (A0568), Cy3-labeled goat anti-mouse IgG (A0521), donkey anti-goat IgG (A0181), anti-His-tag mAb (AH367) from Beyotime Institute of Biotechnology (Haimen, China); FITC-labeled goat anti-rabbit IgG (CW0114), goat anti-rat IgG (CW0166) from CWBIO (Beijing, China).

FN, laminin, collagen IV, puromycin, and hygromycin were from Sigma-Aldrich. Matrigel was from Corning Life Sciences (Tewksbury, MA, USA). Other reagents were from Sigma unless described otherwise.

Cell lines and cell culture

Mouse mammary epithelial cell line NMuMG and human breast epithelial cell line MCF10A were from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in DMEM (Hyclone; Logan, UT, USA) supplemented with 10% FBS (Hyclone), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco; Carlsbad, CA, USA) in a humidified 5% CO_2 atmosphere at 37 °C. For NMuMG, medium was supplemented with 10 μg/mL insulin (Sigma). For MCF10A, medium was supplemented with 1% sodium pyruvate (Solarbio; Beijing).

Patients and tissue samples

BC tissues and normal breast tissues were obtained from the First Affiliated Hospital of Xi'an Jiaotong University. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Experiments using human tissues were approved by the Research Ethics Committee of Northwest University.

Gene amplification and transfection

Mouse genes NCAM-120, NCAM-140, and ST8SiaII were amplified by PCR as previously described[25]. Briefly, primers were as follows: for NCAM-120: sense 5'-CCCAAGCTTGCCACCATGCTGCGAACTAAGGATC, antisense 5'-CCGCTCGAGTCAGAGCAGAAGAAGAGTCAC; for NCAM-140: sense 5'-CCCAAGCTTGCCACCATGCTGCGAACTAAGGATC, antisense 5'-CCGCTCGAGCGGTCATGCTTTGCTCTCATTC; for ST8SiaII: sense 5'-GAAGGCCTGCCACCATGCAGCTGCAGTTCCG; antisense (containing the codons for 6His residues) 5'-CTAGCTAGCTTAGTGGTGGTGGTGGTGGTGCGTAGCCCCATCACACT. PCR products were ligated into vector pcDNA3.1 (Invitrogen; Carlsbad, CA, USA) or pIREShyg3 (Clontech; Mountain View, CA, USA), respectively. Stably transfected NMuMG cells were selected with G418 or hygromycin.

Semi-quantitative PCR and quantitative real-time PCR

Semi-quantitative PCR and quantitative real-time PCR was performed as previously described[25]. Briefly, total RNA was isolated using a RNApure Tissue Kit (CWBIO) and reverse-transcripted to cDNA using a ReverTra Ace-α Kit (Toyobo; Osaka, Japan). Primers were designed as shown in Suppl. Table 1. Quantitative real-time PCR (RT-qPCR) was performed with UltraSYBR Mixture (CWBIO) using a CFX96 PCR detection system (Bio-Rad; Richmond, CA, USA). Gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method[26] and expressed as mean ± SD from triplicate experiments.

Migration assay

Migration assay was performed as previously described[25]. Cells (5×10^4) were plated in an upper transwell insert without coating matrix (8-μm pore size, Corning) in serum-free medium, and complete-medium was added to the bottom chamber. After 24 h culture, cells migrating across the membrane were stained with 0.1% crystal violet, and photographed under microscope (magnification of 100×).

Wound closure assay

NMuMG cells were plated at a high density in 6-well plates and incubated until a confluent monolayer was achieved. A scratch was made with 100 µL pipette tips in each well and cultures were washed with PBS to remove any cell debris. Cells were incubated in DMEM supplementd with 10% FBS and 5 µg/mL mitomycin C (Sigma-Aldrich) for 24 h. Cell migration between the scratch areas was monitored at 0 h and 24 h, using optical microscope. Migration distance was measured with Image Pro Plus 6.0 (Media Cybemetics, MD, USA)

Proliferation (MTT) assay

Cell proliferation was determined by MTT assay as described previously[27]. Briefly, cells (4×10^3/well) in 96-well plates were incubated 4 h with 4 µL MTT solution (Cers, Yantai city, China). The reaction was terminated by addition of 100 µL DMSO, and absorbance at 595 nm was determined.

Cell motility assay

Cell motility was determined by phagokinetic gold sol assay as described previously[28]. Cells (2×10^3) in complete culture medium were seeded onto gold sol-coated wells, incubated for 12-18 h, and photographed under an inverted microscope. Tracking areas of 50 cells were measured using the ToupView imaging system and expressed as square pixels.

Cell adhesion assay

Adhesion assays were performed as described previously[29]. In brief, 96-well plates were coated overnight at 37 °C with FN (1 µg/well), collagen IV (1.5 µg/well), Matrigel (80 µg/well), or laminin (1 µg/well). Wells were rinsed and blocked for 1 h with 1% BSA in Hank's balanced salt solution (HBSS) at 37 °C. Cells were harvested with trypsin, plated (40,000 cells per coated well), and incubated 30 min at 37 °C. Wells were washed gently with HBSS to remove unattached cells. Adherent cells were fixed with 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet (in 20% methanol) for 10 min, dissolved in 100 µL of 10% acetic acid after removal of excess dye with PBS, and absorbance was measured at 595 nm.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described[25]. Cells (2×10^4) coated on glass cover slips in 24-well plates, were washed with PBS, fixed with 4% fresh paraformaldehyde, blocked with 1% BSA, incubated with appropriate antibody and Hoechst 33342 (Invitrogen; Paisley, UK), mounted with Glycergel (Dako; Carpinteria, CA, USA), and observed by fluorescence microscopy (model Eclipse Ti-U, Nikon; Tokyo, Japan) at 600× magnification.

Western blotting

Western blotting was performed as previously described[25]. Equal amounts of proteins were loaded on SDS-PAGE gels and transferred onto a PVDF membrane. The membrane was blocked with 5% BSA, incubated with primary antibody and appropriate HRP-conjugated secondary antibody, visualized by Pro-Light HRP (Tiangen Biotech; Beijing), and photographed using a Molecular Imager ChemiDoc XRS+ system (Bio-Rad).

Flow cytometry

Cells were plated in triplicate on 24-well plates (2×10^5 cells/well) as described previously[25], detached, incubated with primary and secondary FITC-conjugated antibody. Signals from cells were detected by flow cytometry (FACSCalibur, BD; San Jose, CA, USA), with data acquisition and analysis by the FlowJo software program (Tree Star; San Carlos, CA, USA).

Gene silencing with small interfering RNA (siRNA)

Duplexes of 21 nucleotides of mouse ST8SiaII siRNA target sequence and negative control siRNA (NC), having no homology to other known mouse genes, were designed and synthesized by Invitrogen; for mouse ST8SiaIIi, 5′-GCCUGGAGAUAUUAUUCAUTT (sense). siRNA was transfected using Lipofectamine 2000 reagent, and cells were examined after 24 h. Suppression of ST8SiaII expression was verified by semi-quantitative and quantitative RT-PCR.

Luciferase reporter assay

β-Catenin transcription was assessed using TOP FLASH/ FOP FLASH reporter luciferase assay[30]. Cells were seeded onto 24-well plates and transfected using Lipofectamine 2000 (Invitrogen) with 1 μg M50 Super 8X TOP Flash (Plasmid 12456) or M51 Super 8X FOP Flash (Plasmid 12457) reporter vector (Addgene), together with 0.05 μg internal pRL-TK Renilla plasmid (Promega). Cells were processed 48 h after co-transfection for luciferase reporter activity using a Dual Luciferase Reporter System (Promega). Firefly luciferase activity was normalized against Renilla luciferase activity. Reporter assay results were presented as relative luciferase activity (averaged ratio of firefly/Renilla luciferase ± S.E.) from three or more independent experiments.

Immunoprecipitation analysis

Cells were cultured on 60-mm diameter plates, washed with PBS, and added with IP lysis buffer (Beyotime), incubated on ice for 30 min, and centrifuged at 14,000 g for 15 min at 4 °C. Supernatants were collected. Proteins (500 μg) was incubated with 2 μg primary antibody for 1 h at 4°C, incubated with 20 μL of Protein A/G PLUS-Agarose (sc-2003, Santa Cruz) at 4°C for overnight, centrifuged at 2,000 g for 5 min at 4°C, washed to remove nonspecific binding with PBS. SDS-PAGE loading buffer was added, and the samples were boiled at 100°C for 15 min. Immunoprecipitated proteins were then subjected to western blot as described above.

Immunohistochemistry

Tissue slides were dewaxed, rehydrated, antigen retrieval, and incubated with 3% hydrogen peroxide for 30 min and blocked in 10% normal mouse serum for 30 min. The slides were then incubated with primary antibodies against NCAM (1:1000; Santa Cruze) at 4°C for overnight. The slides were rinsed with PBS, incubated with HRP-conjugated secondary antibody, visualized with DAB (Sigma-Aldrich).

Data analysis

Data were statistically analyzed using the GraphPad Prism (GraphPad software; San Diego, CA, USA). Differences between means were evaluated by Student's t-test, and p-values <0.05 were considered significant.

Results

Expression of NCAM in clinical BC samples

In previous study, we observed significantly increased polySia and ST8SiaIV in BC samples compared to normal tissues[31]. Since NCAM is the major polySia substrate[32], NCAM expression at the mRNA level was evaluated by PCR and RT-PCR in 24 clinical BC tissue samples in this study. NCAM expression was higher in BC tissues than in normal tissues (Fig. 1a,b; Supplementary Tables 2 and 3). Immunohistochemical staining showed positive NCAM signals in BC tissues but not in normal tissues (Fig. 1c). In Kaplan-Meier survival estimate, the mean survival time of the BC patients with high expression of NCAM was shorter than which with low expression of NCAM (Fig. 1d), suggesting that NCAM play essential role in BC tumorigenesis and progression.

Aberrant regulation of NCAM and polysialylated NCAM during EMT

EMT is a basic and highly conserved process that plays crucial roles in embryogenesis, cancer invasion and metastasis[33] . Expression of NCAM and polysialylated NCAM was studied in an in vitro EMT model established by TGF-β1 induction in MCF10A and NMuMG cells. Typical morphology shift from epithelial to mesenchymal was observed in the two cell lines (Suppl. Fig. 1a). Changes of protein levels associated with EMT were observed, including increase of N-cadherin (N-cad) ("cadherin switch"), and decreases of E-cadherin (E-cad) and the tumor markers vimentin (VM) and fibronectin (FN) (Suppl. Fig. 1b). Expression at the mRNA level of various NCAM isotypes was elevated in NMuMG cells undergoing EMT (Fig. 2a). NCAM-140 expression at the protein level was maximal in MCF10A and NMuMG cells undergoing EMT (Fig. 2b). It is known that NCAM can be posttranslationally polysialylated by the polysialyltransferases ST8SiaII and ST8SiaIV, resulting in polysialylated NCAM glycoforms. We therefore analyzed ST8SiaII and ST8SiaIV expression in the two model cell lines. Increased ST8SiaII expression and reduced ST8SiaIV expression were observed at the mRNA level (Fig. 2c). Polysialylated NCAM expression was much higher in cells undergoing EMT (Fig. 2d), which was validated by immunoprecipitation assay (Fig. 2e), indicating that upregulation of NCAM and polysialylated NCAM are involved in EMT process.

NCAM-140 overexpression induces EMT in NMuMG cells

To evaluate the effects of two isoforms of NCAM on cell behaviors, we cloned the genes encoding NCAM-120 and NCAM-140, and transfected them separately into NMuMG cells. In contrast to a previous finding that ectopic expression of NCAM caused cell death of NMuMG[34], we obtained stable transfectants of the two isoforms, termed NG/120 and NCAM-140 cells (Suppl. Fig. 1c). NG/140 had single, motile mesenchymal cell morphology, whereas NG/120 retained epithelial morphology similar to that of non-transfected cells ("NG") (Suppl. Fig. 1d). N-cad and VM were upregulated in NG/120 and NG/140, but not in NG or NG/3.1. Compared to NG/120, NG/140 showed nearly complete loss of E-cad expression, significantly enhanced FN expression (Fig. 3a,b), increased proliferation and migration (Fig. 3c,d,e). However, motility of NG/140 was similar to that of the other NG cell lines (Fig. 3f). These findings suggest that NCAM-140 overexpression switches cells to an EMT-like process, with consequent alteration of proliferation and migration.

Differential effects of polysialylated NCAM and NCAM on cell behaviors

We observed previously that polySia, catalyzed by ST8SiaII, facilitated NCAM-mediated cell migration in a polysialyltransferase-specific manner[25]. To assess the role of polySia in modulating NCAM-mediated cell behaviors, we cloned the ST8SiaII gene into NG, and the resulting cell lines were termed NG/ST8SiaII (Suppl. Fig. 2a,b). In comparison with NG and NG/140, polysialylated NCAM level was elevated in NG/ST8SiaII (Fig. 4a). Proliferation of NG/140 was significantly higher after 36 h and 48 h culture, and after 60 h NG/140 cells were almost completely confluent (Fig. 4b). These findings suggest that cell proliferation was increased by NCAM overexpression.

The effects of polysialylated NCAM and NCAM on cell motility and migration were examined. Cell motility was slightly increased in NG/140 and significantly increased in polySia-overexpressing NG/ST8SiaII (Fig. 4c), consistent with results shown in Fig. 3f. Cell migration was increased in NG/140 but not in NG/ST8SiaII, indicating that this cell behavior was affected by NCAM, but not by PSA (Fig. 4d,e). Because NCAM is a type of adhesion molecule, we examined the effects of polysialylated NCAM and NCAM on cell adhesion, based on attachment of our experimental cell lines to extracellular matrix (ECM) components (FN, laminin, collagen IV, Matrigel) (Suppl. Fig. 3). PolySia-overexpressing NG/ST8SiaII showed reduced attachment to collagen IV and Matrigel, which is consistent with previous study showing the reduced attachment of ST8SiaII overexpressed NIH-3T3 cells to Matrigel[35]. Whereas NCAM-overexpressing NG/140 showed reduced attachment to LN, increased attachment to FN and similar attachment to collagen IV compared to NG cells (Fig. 4f). Thus, polysialylated NCAM and NCAM had differing effects on cell adhesion to ECM components.

Polysialylated NCAM-mediated EGFR/STAT3 signaling pathway

The EGFR/STAT3 signaling pathway plays an important role in human BC[36, 37]. We examined the possible effects of polysialylated NCAM and NCAM on this pathway. EGFR expression differed significantly between NG/ST8SiaII and the other NG cell lines (Fig. 5a). TGF-β1-induced EMT increased total EGFR (tEGFR) in NG/ST8SiaII but had no effect on β-catenin expression (Fig. 5a). EGFR phosphorylation in NG/ST8SiaII differed before vs. after EMT (Fig. 5b). EGFR phosphorylation was

reduced in NG/140 undergoing EMT, suggesting that the EGFR/STAT3 signaling pathway was activated by polySia but not by NCAM. When polySia was knocked down by silencing ST8SiaII (ST8SiaIIi) (Suppl. Figs. 2c), phosphorylated EGFR and STAT3 were downregulated in each of the cell lines (Fig. 5c). Expression of β-catenin and FN, which were upregulated by NCAM, were notably reduced by ST8SiaII knockdown (Fig. 5c). These findings indicate that polySia is involved in activation of the EGFR/STAT3 signaling pathway.

NCAM-mediated β-catenin/slug signaling pathway

NCAM-140 overexpression in NG caused reduction of E-cad level (Fig. 3a,b). Dissociation of E-cad/β-catenin complex is a key step in EMT, and alterations in localization and expression levels of β-catenin have been observed in various types of cancers. The well-known EMT regulator/ transcription factor slug has been shown to inhibit E-cad expression and promote cell metastasis[38, 39]. The Wnt signaling pathway associated with β-catenin, a key downstream effector, is involved in BC development[40]. We examined the possible role of NCAM-140 in regulation of the β-catenin signaling pathway. β-catenin expression was increased in NG/140 nuclei, but reduced in NG/ST8SiaII nuclei (Fig. 6a). These findings suggest that NCAM induces translocation of β-catenin from cytoplasm into the nucleus, and that such translocation is inhibited by polySia overexpression. Slug was accumulated in nuclei of NCAM-overexpressing cells. β-catenin transcription was enhanced in NG/140 but reduced in NG/ST8SiaII (Fig. 6b). Expression of genes targeted by β-catenin (axin 2, c-myc, CCND1) was significantly upregulated (Fig. 6c). It thus appears that upregulation of the β-catenin/slug signaling pathway leads to increased expression of axin 2, slug, c-myc, and CCND1 genes in NG/140. In contrast, expression of receptor genes (fzd 7, wnt 3a) of the Wnt signaling pathway was not notably altered in association with the NCAM-mediated β-catenin/slug signaling pathway (Fig. 6c).

In the absence of Wnt ligands, β-catenin is usually phosphorylated by GSK-3 at Ser33 and Ser37, leading to its ubiquitin-dependent degradation through incorporation of APC and CK1α[41]. We measured APC, GSK-3β, and CK1α mRNA levels in our NG cell lines. CK1α level was notably reduced in NG/140 whereas GSK-3β was increased in polySia-overexpressing NG/ST8SiaII, suggesting that CK1α is downregulated in NG/140 to block β-catenin degradation, whereas GSK-3β is upregulated in NG/ST8SiaII to promote β-catenin degradation (Fig. 6c,d).

Discussion

Modulation of tumor cell adhesion molecules is crucial in control of the metastatic cascade. NCAM, the major substrate of polySia, has been associated with tumor invasion and formation of metastatic deposits in many types of cancer[42, 43]. PolySia molecules are able to affect NCAM function, and are associated with malignant and metastatic phenotype[11, 44]. Because of the large negative charge of polySia, its presence inhibits the adhesive properties of NCAM. We previously examined the role of polySia in NCAM function using cell line ldlD-14, an essential model for functional study of glycoproteins and glycolipids. NCAM-140 strongly enhanced cell adhesion to FN and reduced adhesion to Matrigel, and

these effects were reversed by the presence of polySia, indicating that polySia modulates NCAM-mediated cell behaviors[25]. We very recently reported correlation of polySia expression with disease stage in BC patients, and high polySia expression in TGF-β1-treated NMuMG and MCF10A cells[31]. The roles of NCAM and polySia alterations in determining or modulating various cell behaviors remain unknown.

EMT is a process whereby epithelial cells are transformed into cells with mesenchymal phenotypes, characterized by loss of cellular polarity and adhesion, and enhancement of invasiveness and migration. TGF-β1-induced EMT provides a useful in vitro model for studies of cancer cell responses to the tumor microenvironment. In the present study, we found that NCAM was overexpressed in clinical human BC tissues, and that expression of both NCAM and polysialylated NCAM was greatly increased in MCF10A and NMuMG cells undergoing EMT. Lehembre et al. showed previously that loss of E-cad function upregulated the expression of NCAM and created a subset of NCAM that was translocated into lipid rafts, where it stimulated focal adhesion[23]. We found that overexpression of NCAM-140, one of the three NCAM isotypes, reduced E-cad expression, greatly enhanced FN expression, and promoted cell proliferation and migration. Although the Lehembre et al. study revealed mechanistic links among loss of E-cad expression, NCAM function, focal adhesion assembly, and cell migration and invasion, it did not address the possible functional role of polySia in mediating the EMT process. We found that polySia overexpression caused a significant increase of cell motility but had negligible effect on cell migration, indicating that NCAM (but not polySia) affected migration. PolySia-overexpressing cells showed low adhesion to collagen IV and Matrigel, while NCAM overexpression had no effect on attachment to collagen IV, indicating that polysialylated NCAM and NCAM have differing effects on adhesion to various ECM components.

PolySia and NCAM are closely associated with cell adhesion, migration, and invasion, and help mediate tumor development and progression[23]. Our findings suggest that polySia overexpression stimulates the EGFR/STAT3 signaling pathway. NCAM induced translocation of β-catenin from cytoplasm into the nucleus, and promoted the β-catenin/slug signaling pathway. Such translocation was inhibited by polySia overexpression, indicating that polySia and NCAM affect cell adhesive properties through different signaling pathways. PolySia decoration of NCAM appears to play crucial roles in its altered expression during EMT, and its modulating effects on cell behaviors.

Conclusion

Collectively, we propose that NCAM-140 overexpression in NMuMG cells inhibits CK1α expression and β-catenin phosphorylation, and promotes translocation of released β-catenin into nuclei, with consequent downregulation of E-cad expression and upregulation of FN, VM, and slug expression. Consistent with this hypothesis, β-catenin downstream genes axin 2, c-myc, and CCND1 were activated in the Wnt-independent β-catenin/slug signaling pathway (Fig. 6c and Fig. 7). As polySia accumulated on NCAM, it stimulated of EGFR/STAT3 signaling pathway, reduction of cell adhesion, and increased cell motility (Fig. 7). The degree of polySia attachment on NCAM molecules is a crucial factor in mediation of various

pathways by NCAM. NG/ST8SiaII cells contain relatively few NCAM molecules, each with a high amount of attached polySia. As a result, polySia dominates the pathway that mediates cell adhesion and motility.

Abbreviations

NCAM: Neural cell adhesion molecule

PolySia: Polysialic acid

EMT: Epithelial-mesenchymal transition

ST8SiaII: Polysialyltransferases II

ST8SiaIV: Polysialyltransferases IV

BC: Breast cancer

NG: NMuMG cells

NG/3.1: NMuMG cells transfected with vector pcDNA3.1

NG/120: NMuMG cells transfected with NCAM-120

NG/140: NMuMG cells transfected with NCAM-140

NG/180: NMuMG cells transfected with NCAM-180

NG/ST8SiaII: NMuMG cells transfected with Polysialyltransferases II

EGFR: Epidermal growth factor receptor

STAT3: Signal transducer and activator of transcription 3

N-cad: N-cadherin

E-cad: E-cadherin

FN: Fibronectin

VM: Vimentin

Declarations

Ethics approval and consent to participate

The research involving human subjects, including human specimens, data, cell lines and the animal experiments reported in the manuscript were approved by the Research Ethics Committee of Northwest University, China. We observed the privacy rights of human subjects. All the participants in the study confirmed and written the consent about the forms of personally identifiable data including biomedical, clinical, and biometric data. Our research was in compliance with the Helsinki Declaration (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/) and also in line with the recommendations for the conduct, reporting, editing and publication of scholarly work in medical journals.

Consent for publication

Not applicable.

Availability of data and meterials

Data presented in this manuscript are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflict of interests regarding the publication of this paper.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81802654 and No. 81672537), the Youth Innovation Team of Shaanxi Universities, Natural Science Basic Research Plan in Shaanxi Province of China (No. 2018JQ3051), and the Natural Science Foundation of Jiangsu Province, China (No. BK20161132). The Grants-in-Aid just supported this study financially, and had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' Contributions

XL, ZT and FG conceived and designed the project. LC, XW, JY, JG, and ZT performed acquisition, analysis, and interpretation of data. XY provided samples. LC, XW, ZT and FG made drafting of the manuscript.

Acknowledgments

The authors are grateful to Dr. S. Anderson for English editing of the manuscript.

Authors’ Information

Lin Cao and Xin Wang contributed equally to this work.

Affiliations

Provincial Key Laboratory of Biotechnology, Joint International Research Laboratory of Glycobiology and Medicinal Chemistry, College of Life Sciences, Northwest University, Xi'an, Shaanxi 710069, China.

Lin Cao, Juhong Yang, Xiang Li, Zengqi Tan & Feng Guan

Shandong University of Arts, Ji'nan,Shandong 250000,China.

Xin Wang

Institute of Biomedical Engineering and Health Sciences, Changzhou University, Jiangsu 213164, China.

Jia Guo

Department of Surgical Oncology, The First Affiliated Hospital, Medical School, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China.

Xiaomin Yang

Corresponding author

Correspondence to Feng Guan or Zengqi Tan

References

1. Varki NM, Varki A. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest. 2007;87(9):851-7.
2. Varki A, Kannagi R, Toole BP: Glycosylation Changes in Cancer. In: Essentials of Glycobiology. 2nd edn. Edited by Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME. New York: Cold Spring Harbor; 2009.
3. Li F, Ding J. Sialylation is involved in cell fate decision during development, reprogramming and cancer progression. Protein Cell. 2019;10(8):550-65.
4. Astronomo RD, Burton DR. Carbohydrate vaccines: developing sweet solutions to sticky situations? Nat Rev Drug Disco. 2010;9(4):308-24.
5. Wu X, Zhao J, Ruan Y, Sun L, Xu C, Jiang H. Sialyltransferase ST3Gal1 promotes cell migration, invasion, and TGF-β1-induced EMT and confers paclitaxel resistance in ovarian cancer. Cell Death Dis. 2018;9(11):1102.
6. Picco G, Julien S, Brockhausen I, Beatson R, Antonopoulos A, Haslam S, et al. Over-expression of ST3Gal-I promotes mammary tumorigenesis. Glycobiology. 2010;20(10):1241-50.
7. Yang X, Pursell B, Lu S, Chang T-K, Mercurio AM. Regulation of β4-integrin expression by epigenetic modifications in the mammary gland and during the epithelial-to-mesenchymal transition. J Cell Sci. 2009;122(14):2473-80.

8. Berger RP, Sun YH, Kulik M, Lee JK, Nairn AV, Moremen KW, et al. ST8Sia4-dependent polysialylation is part of a developmental program required for germ layer formation from human pluripotent stem cells. Stem Cells. 2016;34(7):1742-52.
9. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. Nat Rev Cancer. 2015;15(9):540-55.
10. Tanaka F, Otake Y, Nakagawa T, Kawano Y, Miyahara R, Li M, et al. Prognostic significance of polysialic acid expression in resected non-small cell lung cancer. Cancer Res. 2001;61(4):1666-70.
11. Falconer RA, Errington RJ, Shnyder SD, Smith PJ, Patterson LH. Polysialyltransferase: a new target in metastatic cancer. Curr Cancer Drug Targets. 2012;12(8):925-39.
12. Figarella-Branger DF, Durbec PL, Rougon GN. Differential spectrum of expression of neural cell adhesion molecule isoforms and L1 adhesion molecules on human neuroectodermal tumors. Cancer Res. 1990;50(19):6364-70.
13. Martersteck CM, Kedersha NL, Drapp DA, Tsui TG, Colley KJ. Unique α2, 8-polysialylated glycoproteins in breast cancer and leukemia cells. Glycobiology. 1996;6(3):289-301.
14. Thompson MG, Foley DA, Colley KJ. The polysialyltransferases interact with sequences in two domains of the neural cell adhesion molecule to allow its polysialylation. J Biol Chem. 2013;288(10):7282-93.
15. Fuster MM, Esko JD. The sweet and sour of cancer: glycans as novel therapeutic targets. Nat Rev Cancer. 2005;5(7):526-42.
16. Hildebrandt H, Muhlenhoff M, Gerardy-Schahn R. Polysialylation of NCAM. Adv Exp Med Biol. 2010;663:95-109.
17. Soroka V, Kasper C, Poulsen FM. Structural biology of NCAM. Adv Exp Med Biol. 2010;663:3-22.
18. Tezel E, Kawase Y, Takeda S, Oshima K, Nakao A. Expression of neural cell adhesion molecule in pancreatic cancer. Pancreas. 2001;22(2):122-5.
19. Valentiner U, Muhlenhoff M, Lehmann U, Hildebrandt H, Schumacher U. Expression of the neural cell adhesion molecule and polysialic acid in human neuroblastoma cell lines. Int J Oncol. 2011;39(2):417-24.
20. Vander Borght A, Duysinx M, Broers JLV, Ummelen M, Falkenberg FW, Hahnel C, et al. The 180 splice variant of NCAM-containing exon 18-is specifically expressed in small cell lung cancer cells. Transl Lung Cancer Res. 2018;7(3):376-88.
21. Wai Wong C, Dye DE, Coombe DR. The role of immunoglobulin superfamily cell adhesion molecules in cancer metastasis. Int J Cell Biol. 2012:340296.
22. Bull C, Stoel MA, den Brok MH, Adema GJ. Sialic acids sweeten a tumor's life. Cancer Res. 2014;74(12):3199-204.
23. Lehembre F, Yilmaz M, Wicki A, Schomber T, Strittmatter K, Ziegler D, et al. NCAM-induced focal adhesion assembly: a functional switch upon loss of E-cadherin. EMBO J. 2008;27(19):2603-15.

24. Yang AH, Chen JY, Lee CH, Chen JY. Expression of NCAM and OCIAD1 in well-differentiated thyroid carcinoma: correlation with the risk of distant metastasis. J Clin Pathol. 2012;65(3):206-12.
25. Guan F, Wang X, He F. Promotion of cell migration by neural cell adhesion molecule (NCAM) is enhanced by PSA in a polysialyltransferase-specific manner. 2015;10(4):e0124237.
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25(4):402-8.
27. Chen Z, Chen X, Chen P, Yu S, Nie F, Lu B, et al. Long non-coding RNA SNHG20 promotes non-small cell lung cancer cell proliferation and migration by epigenetically silencing of P21 expression. Cell Death Dis. 2017;8(10):e3092.
28. Sousa-Squiavinato ACM, Rocha MR, Barcellos-de-Souza P, de Souza WF, Morgado-Diaz JA. Cofilin-1 signaling mediates epithelial-mesenchymal transition by promoting actin cytoskeleton reorganization and cell-cell adhesion regulation in colorectal cancer cells. Biochim Biophys Acta Mol Cell Res. 2019;1866(3):418-29.
29. Yu Q, Xue Y, Liu J, Xi Z, Li Z, Liu Y. Fibronectin promotes the malignancy of glioma stem-like cells via modulation of cell adhesion, differentiation, proliferation and chemoresistance. Front Mol Neurosci. 2018;11:130.
30. Kolesnikoff N, Attema JL, Roslan S, Bert AG, Schwarz QP, Gregory PA, et al. Specificity protein 1 (Sp1) maintains basal epithelial expression of the miR-200 family: implications for epithelial-mesenchymal transition. J Biol Chem. 2014;289(16):11194-205.
31. Wang X, Li X, Zeng YN, He F, Yang XM, Guan F. Enhanced expression of polysialic acid correlates with malignant phenotype in breast cancer cell lines and clinical tissue samples. Int J Mol Med. 2016;37(1):197-206.
32. Simon P, Baumner S, Busch O, Rohrich R, Kaese M, Richterich P, et al. Polysialic acid is present in mammalian semen as a post-translational modification of the neural cell adhesion molecule NCAM and the polysialyltransferase ST8SiaII. J Biol Chem. 2013;288(26):18825-33.
33. Chen T, You Y, Jiang H, Wang ZZ. Epithelial-mesenchymal transition (EMT): A biological process in the development, stem cell differentiation, and tumorigenesis. J Cell Physiol. 2017;232(12):3261-72.
34. Mani SA, Yang J, Brooks M, Schwaninger G, Zhou A, Miura N, et al. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. Proc Natl Acad Sci U S A. 2007;104(24):10069-74.
35. Li J, Dai G, Cheng YB, Qi X, Geng MY. Polysialylation promotes neural cell adhesion molecule-mediated cell migration in a fibroblast growth factor receptor-dependent manner, but independent of adhesion capability. Glycobiology. 2011;21(8):1010-8.
36. Yang J, Liao D, Chen C, Liu Y, Chuang TH, Xiang R, et al. Tumor-associated macrophages regulate murine breast cancer stem cells through a novel paracrine EGFR/Stat3/Sox-2 signaling pathway. Stem Cells. 2013;31(2):248-58.
37. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res Tr. 2012;136(2):331-45.

38. Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, et al. Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. Oncogene. 2011;30(12):1436-48.
39. Knowles LM, Gurski LA, Engel C, Gnarra JR, Maranchie JK, Pilch J. Integrin αvβ3 and fibronectin upregulate Slug in cancer cells to promote clot invasion and metastasis. Cancer Res. 2013;73(20):6175-84.
40. Wang Z, Li B, Zhou L, Yu S, Su Z, Song J, et al. Prodigiosin inhibits Wnt/β-catenin signaling and exerts anticancer activity in breast cancer cells. Proc Natl Acad Sci U S A. 2016;113(46):13150-5.
41. Kimelman D, Xu W. β-Catenin destruction complex: insights and questions from a structural perspective. Oncogene. 2006;25(57):7482.
42. Zecchini S, Bombardelli L, Decio A, Bianchi M, Mazzarol G, Sanguineti F, et al. The adhesion molecule NCAM promotes ovarian cancer progression via FGFR signalling. EMBO Mol Med. 2011;3(8):480-94.
43. Bataille R, Jego G, Robillard N, Barille-Nion S, Harousseau JL, Moreau P, et al. The phenotype of normal, reactive and malignant plasma cells. Identification of "many and multiple myelomas" and of new targets for myeloma therapy. Haematologica. 2006;91(9):1234-40.
44. Suzuki M, Suzuki M, Nakayama J, Suzuki A, Angata K, Chen S, et al. Polysialic acid facilitates tumor invasion by glioma cells. Glycobiology. 2005;15(9):887-94.

Supplementary Information

Supplementary Table 1. Primers used in gene amplification

Supplementary Table 2. NCAM levels in normal and malignant breast tissue samples

Supplementary Table 3. Association of disease characteristics with NCAM gene expression in malignant BC patients

Supplementary Figure 1 Verification of EMT cell model and NCAM overexpressing cell lines.

Supplementary Figure 2 Verification of polysialylated NCAM overexpressing cell lines.

Supplementary Figure 2 Cell adhesion assay.

Figures

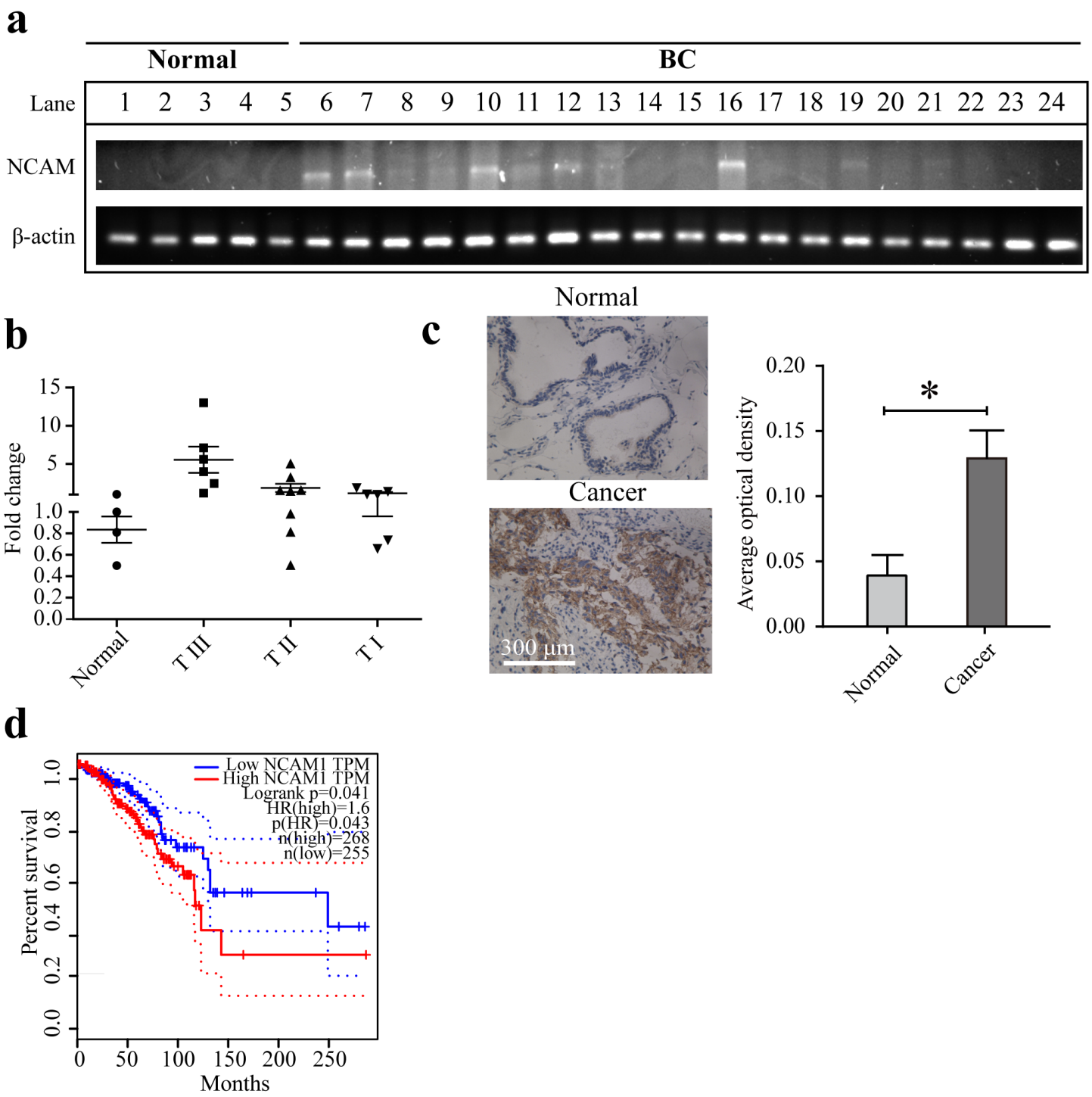

Figure 1

Expression of NCAM in human BC samples. (a and b) mRNA levels of NCAM in malignant tissues (n=20) were compared with those in normal tissues (n=4) by PCR (a) and RT-qPCR (b). Results are shown as mean ± SD from triplicate experiments. β-actin: loading control. Lanes 1-4: normal. Lanes 5-10: TNM III. Lanes 11-18: TNM II. Lanes 19-24: TNM I. (c) Immunohistochemical staining of NCAM in BC samples. Malignant tissues and matching normal tissues were obtained from BC patients. One typical sample pair is shown (c, left), histogram is shown (c, right) Scale bars: 300 μm. (d) Kaplan-Meier overall survival (OS) curves according to NCAM expression levels analyzed using GEPIA tool (http://gepia.cancer-pku.cn).

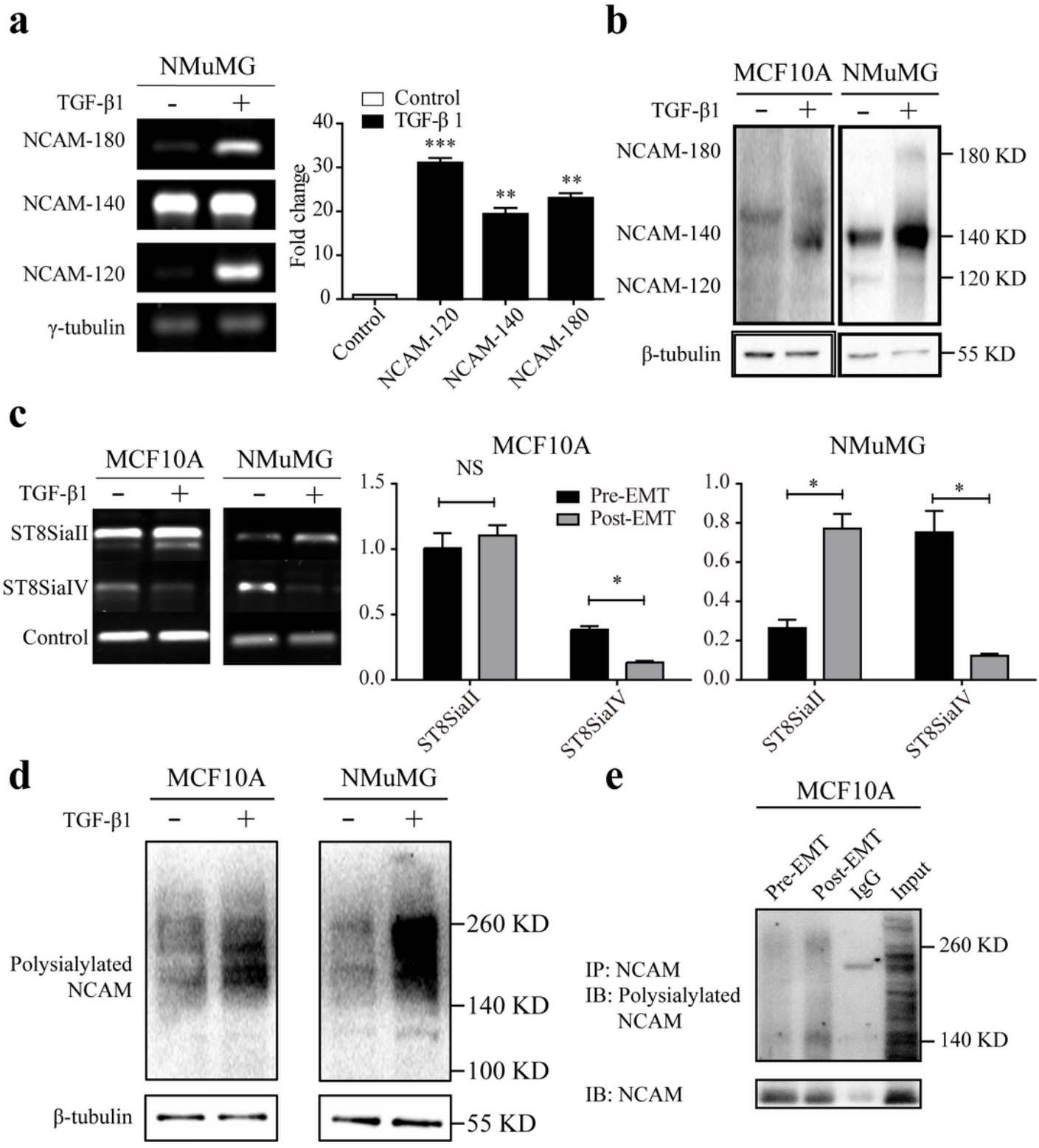

Figure 2

Altered polysialylated NCAM expression during EMT. (a) mRNA levels of three NCAM isoforms in NMuMG cells during EMT. Semiquantitative PCR (left panel) and RT-qPCR (right panel) were performed. γ-tubulin: loading control. **, p< 0.01. ***, p< 0.001. (b) Western blotting of NCAM in control and TGF-β1-treated MCF10A and NMuMG cells. (c) Expression of ST8SiaII and ST8SiaIV at the mRNA level in MCF10A and NMuMG cells during EMT. Cells were treated (+) or not (-) with TGF-β1 (5 ng/ml) for 48 h, and

semiquantitative PCR was performed. β-actin and γ-tubulin: loading controls. (d) Western blotting of polysialylated NCAM during EMT. (e) Immunoprecipitation and western blotting of polysialylated NCAM in MCF10A during EMT.

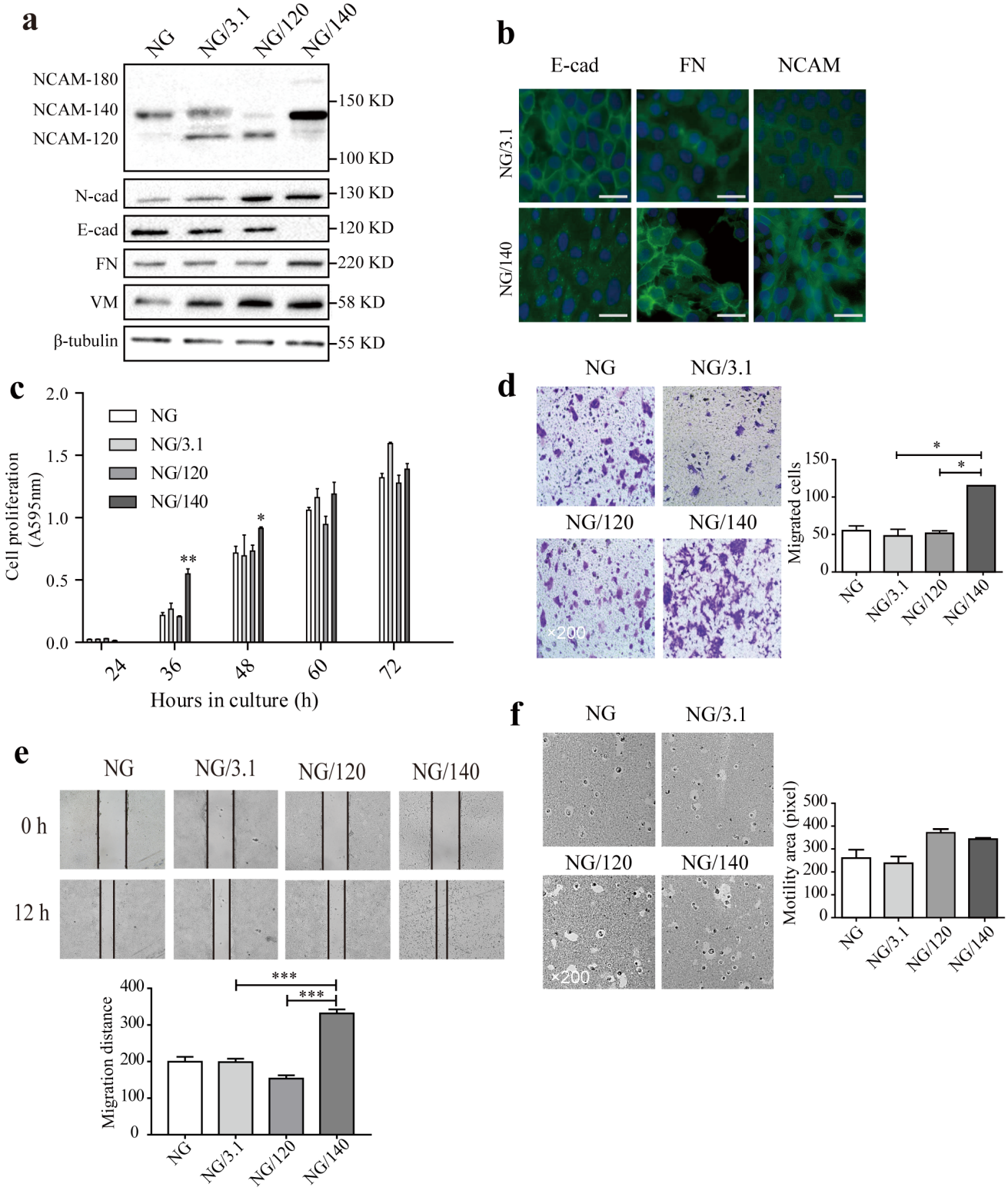

Figure 3

Differential effects of NCAM-120 and NCAM-140 on cell behaviors. (a) Western blotting of EMT markers in NCAM-overexpressing NMuMG cells. β-tubulin: loading control. (b) Immunofluorescence staining of E-

cad, FN, and NCAM in NCAM-140-overexpressing cells. NG/3.1 and NG/140 cells were cultured, and nuclei were visualized by Hoechst staining. Scale bars: 20 μm. (c) Proliferation assay. Transfected cells were cultured for 24, 36, 48, 60, or 72 h and proliferation was assessed by MTT assay. *, $p< 0.05$. **, $p< 0.01$. (d) Migration assay. Cells were cultured for 48 h as described above, and migration was assessed as described in M&M. Migrating cells were quantified, and values are shown as mean ± SD. Two independent experiments gave similar results. Magnification: 200×. *, $p< 0.05$. (e) Wound assay, performed as described in M&M. Confluent cells were wounded, incubated with 5 μg/mL mitomycin for 24 h, and wounds were photographed and marked using ImagePro Plus software. Results are presented as average of migration distance (0 h-24 h) ± SD from three independent experiments. ***, $p< 0.001$. (f) Motility assay, performed as described in M&M. Cleared areas on gold sol were measured as square pixels using the ToupView Image program. Values shown are mean ± SD from three independent experiments. NG: NMuMG cells; NG/120: NMuMG-120 overexpressing cells; NG/140: NMuMG-140 overexpressing cells; NG/3.1: NMuMG cells transfected with vector pcDNA3.1.

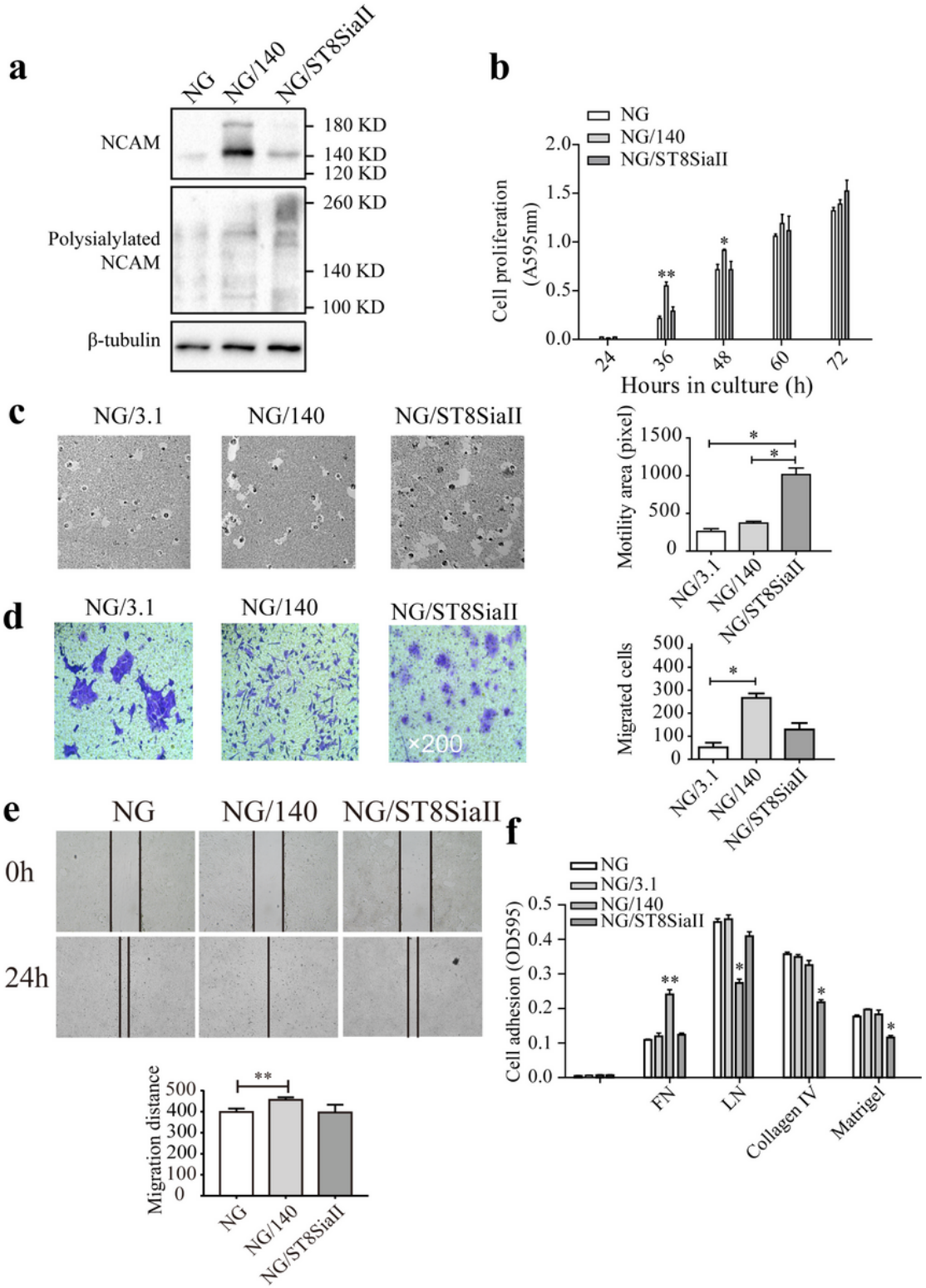

Figure 4

Effects of polySia and NCAM on cell behaviors. (a) Expression of NCAM and polysialylated NCAM in polySia-overexpressing cell lines, by western blotting. (b) Proliferation assay. Transfected cells were cultured for 24, 36, 48, 60, or 72 h, and proliferation was assessed by MTT assay. (c) Motility assay, performed as described in M&M. Data presentation as in Fig. 3f. *, p< 0.05. **, p< 0.01. (d) Migration assay, and data presentation, as described for Fig. 3d. *, p< 0.05. **, p< 0.01. (e) Wound assay, performed

as described in M&M. Confluent cells were wounded, incubated with 5 µg/mL mitomycin for 24 h, and wounds were photographed and marked using ImagePro Plus software. Results are presented as average of migration distance (0 h-24 h) ± SD from three independent experiments. **, p< 0.01. (f) Adhesion assay. Transfected cells were cultured for 48 h, and cell adhesion to FN, laminin, collagen IV, Matrigel, and BSA solution was determined as described in M&M. Absorbance of crystal violet-stained cells was recorded at 595 nm. Four independent experiments gave similar results. *, p< 0.05. **, p< 0.01. * vs. NG/3.1.

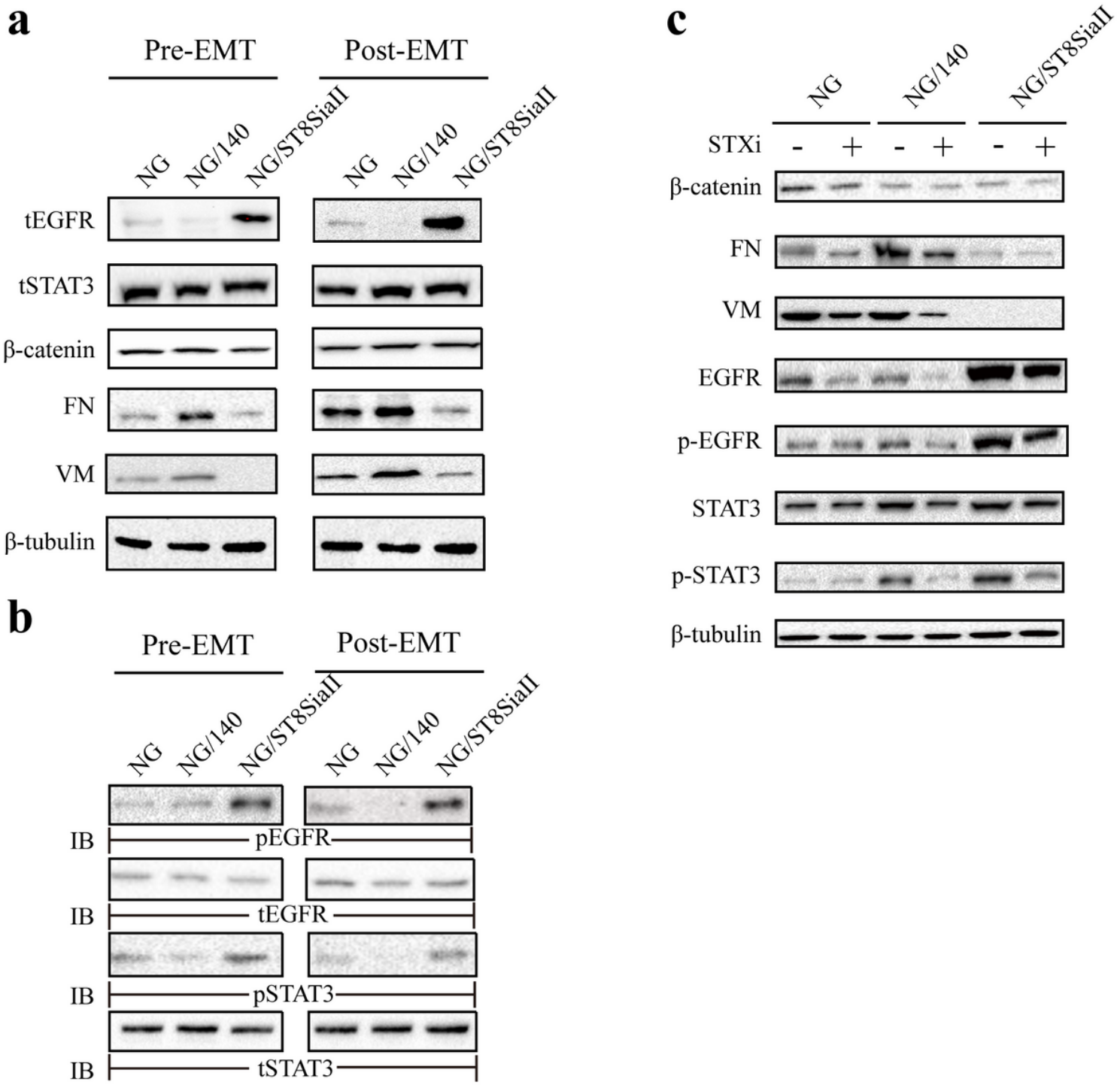

Figure 5

PolySia-mediated EGFR/STAT3 signaling pathway. (a) Western blotting of transfected cells during EMT. tEGFR: total EGFR. tSTAT3: total STAT3. β-tubulin: loading control. (b) Western blotting analysis of p-EGFR and p-STAT3. Equal amounts of tEGFR and tSTST3 were subjected to immunoblotting (IB), and p-EGFR (Tyr1068) and p-STAT3 (Tyr705) were detected. (c) Transfected or nontransfected NMuMG cells were further transfected with negative control RNA (-) or siRNA-targeting mouse ST8SiaII (+). Protein lysates were collected after 24 h and subjected to immunoblotting analysis. β-tubulin: loading control.

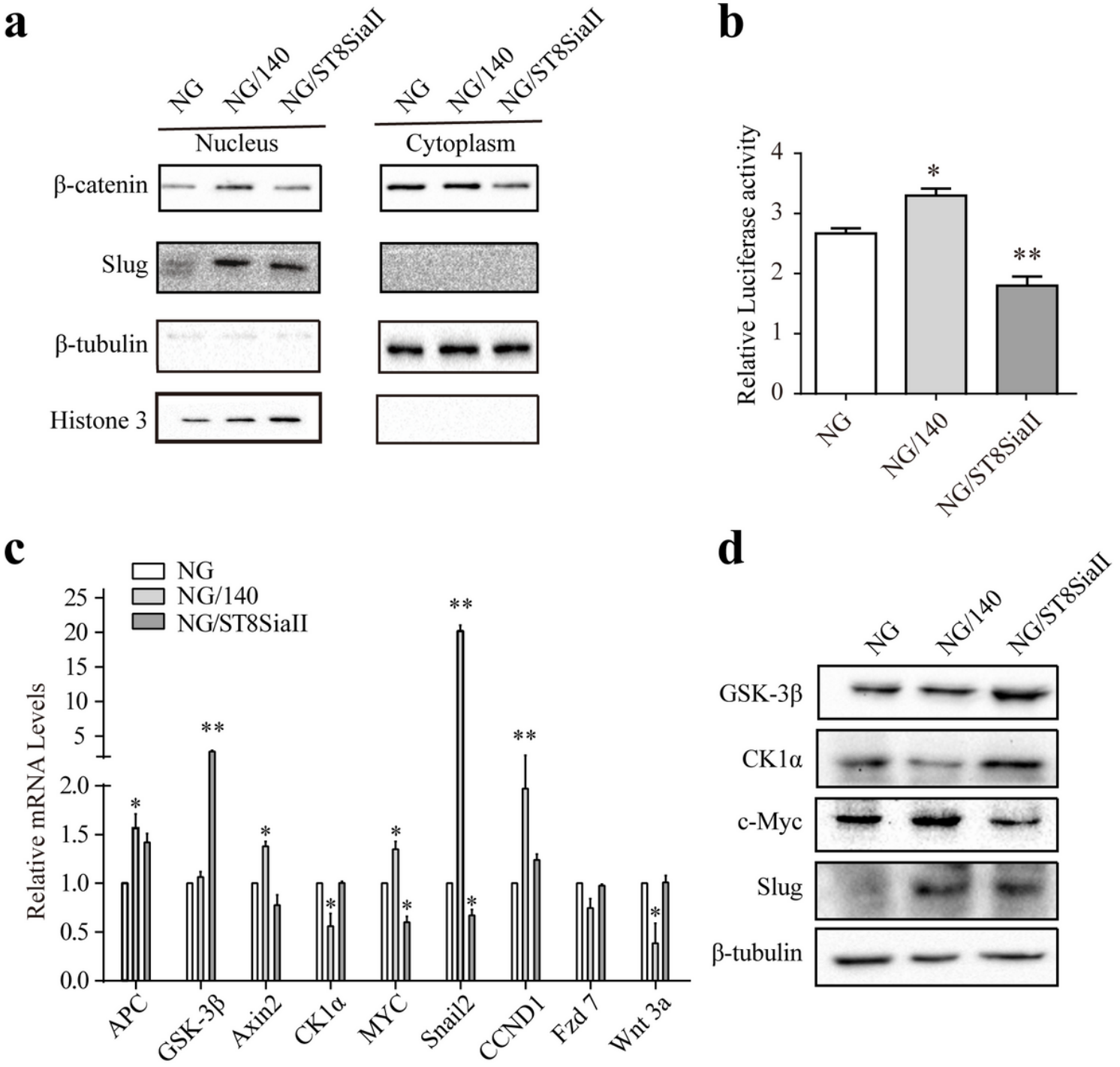

Figure 6

NCAM-mediated β-catenin/ slug pathway. (a) Western blotting. Lysates of transfected cells were fractionated as described in M&M, and subjected to SDS-PAGE. (b) Quantification of active β-catenin by luciferase gene reporter assay. Values of active β-catenin transcription were determined based on level of luciferase activity and normalized to internal control (Renilla luciferase plasmid). Values shown are mean ± SEM from three independent experiments, expressed as relative activation in comparison with cells transfected with β-catenin-LEF/TCF insensitive (FOP) reporter vector. *, p< 0.05. **, p< 0.01. (c) RT-qPCR. *, p< 0.05. **, p< 0.01. (d) Western blotting. β-tubulin: loading control.

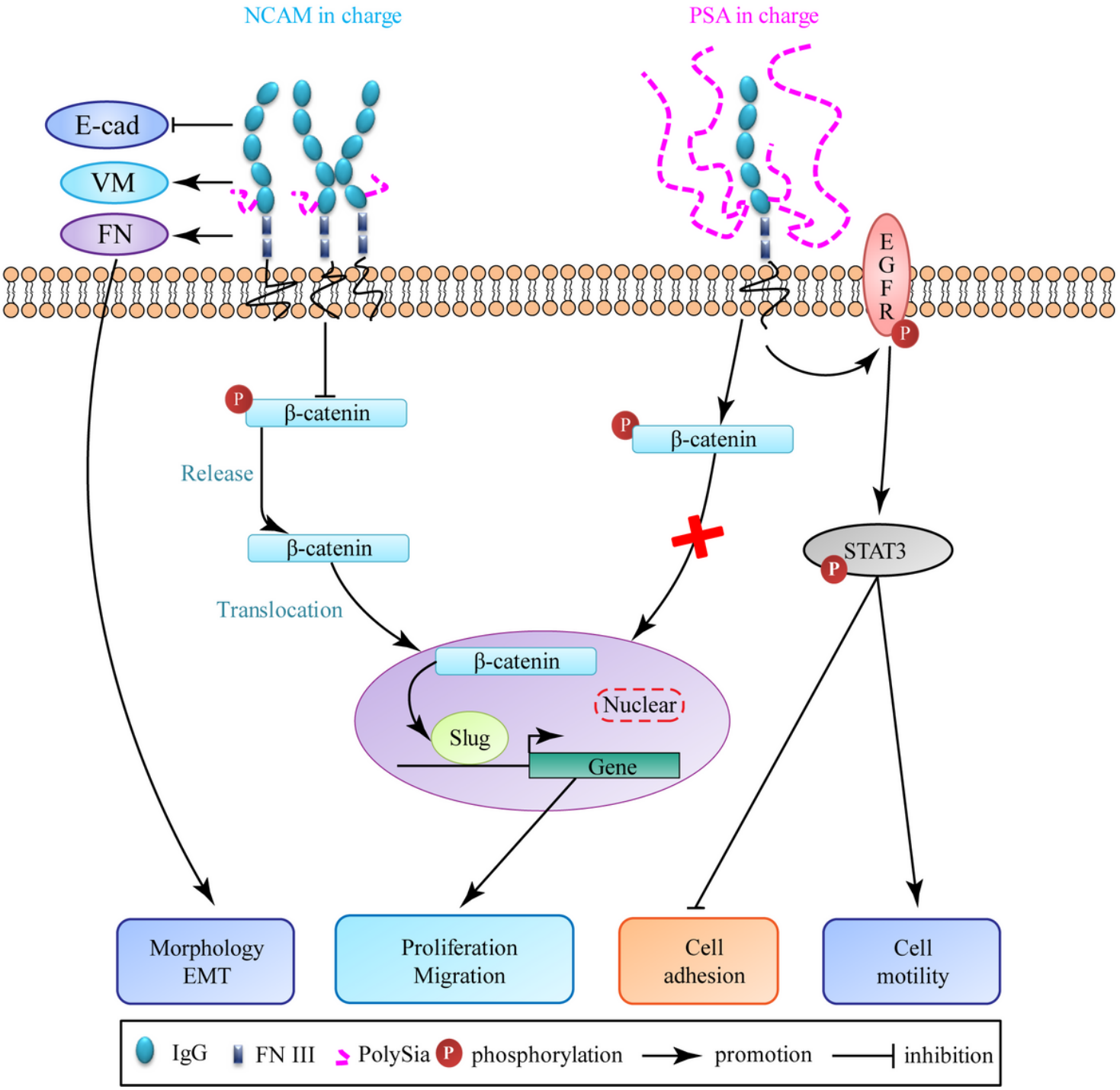

Figure 7

Possible transition pathway between NCAM-mediated β-catenin/slug signaling pathway and polysialylated NCAM-mediated EGFR/STAT3 signaling pathway.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- RevisedSupplementaryMaterialwithouthighlight.pdf
- orginalimagesforblots.docx