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Glycodelin-A Protein Interacts with Siglec-6 Protein to Suppress Trophoblast Invasiveness by Down-regulating Extracellular Signal-regulated Kinase (ERK)/c-Jun Signaling Pathway*[S]

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During placentation, the cytotrophoblast differentiates into the villous cytotrophoblast and the extravillous cytotrophoblast. The latter invades the decidualized endometrium. Glycodelin-A (GdA) is abundantly synthesized by the decidua but not the trophoblast. Previous data indicate that GdA suppresses the invasion of trophoblast cell lines by down-regulating proteinase expression and activities. This study addresses the signaling pathway involved in the above phenomenon. GdA was found to suppress phosphorylation of ERKs and expression of their downstream effector c-Jun, a component of the transcription factor activator protein-1 (AP-1). The involvement of ERKs and c-Jun in suppressing trophoblast invasion and biosynthesis of proteinases was confirmed by using siRNA knockdown and pharmacological inhibitors. Desialylation reduced binding affinity of GdA toward and invasion suppressive activities on the trophoblast. Co-immunoprecipitation showed that Siglec-6 on the trophoblast was the binding protein of GdA. The binding of GdA to Siglec-6 was sialic acid-dependent. Treatment with anti-Siglec-6 antibody abolished the invasion suppressive activities of GdA. These results show that GdA interacts with Siglec-6 to suppress trophoblast invasiveness by down-regulating the ERK/c-Jun signaling pathway.

Placental development involves complex coordinated action between the maternal decidua and the fetal trophoblast. Successful placentation results in the formation of two distinct types of chorionic villi, namely the floating villi and the anchoring villi (1). A population of proliferative cytotrophoblast cells from the anchoring villi attached to the basement membrane differentiates into invasive extravillous cytotrophoblast that invades the decidua and spiral arteries, one of the key events in placentation.

Glycodelin comprises a group of secretory glycoproteins with four well defined glycoforms, each with a distinct glycosylation pattern (2, 3). Glycodelin-A (GdA)[3] is a uterine isoform that contributes to feto-maternal defense (4–6). It is synthesized in the secretory endometrial glands and the decidua, but not the trophoblast (4). In the first trimester of pregnancy, there is a coincidental rise of GdA concentration in the decidua with the active invasion of trophoblast (4, 7). Recent data show that GdA is a paracrine regulator of trophoblast invasion suppressing the invasion of the first trimester extravillous cytotrophoblast cell line (TEV-1) through down-regulation of matrix metalloproteinases (MMP)-2, MMP9, and urokinase plasminogen activator (uPA) activities (8). However, the molecular events involved in this process are not known.

Mitogen-activated protein kinases (MAPKs) are involved in a wide range of biological processes. Among the three MAPK subfamilies, the extracellular signal-regulated kinases (ERKs) are the major modulators of trophoblast invasion and migration (9, 10). The first objective of the present study was to investigate the action of GdA on ERKs.

ERKs modulate the expression of several transcription factors known to affect the MMP/uPA components. The transcription factors expressed in extravillous cytotrophoblasts (10–12) and involved in these systems include AP-1, p53, and v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS-1) (13–15). The second objective of the present study was to examine expression of these transcription factors after GdA treatment and their importance on trophoblast invasion.

The receptor of GdA on the trophoblast is unknown. As GdA is the most heavily sialylated glycodelin isoform (2) and sialic acid contributes to the apoptosis-inducing action of GdA on T-cells and binding of GdA to its potential receptor on T-cells, CD45 (16), we hypothesized that the glycodelin receptor on the trophoblast also interacts with sialic acids. In immune cells, a family of sialic acid binding immunoglobulin-like lectins, known as Siglecs, plays an essential role in cell-to-cell interac-

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[3] The abbreviations used are: GdA, glycodelin-A; Siglec, sialic acid binding immunoglobulin-like lectin; AP-1, activator protein 1; MMP, matrix metalloproteinase; uPA, urokinase plasminogen activator; ITIM, immunoreceptor tyrosine-based inhibitory motif; SHIP, SH2-containing inositol polyphosphate 5-phosphatase; DMSO, dimethyl sulfoxide; qPCR, quantitative PCR.

tion and regulation of cell functions (17, 18). Therefore, the third objective of the present study tested a hypothesis that Siglec-6, a trophoblast-specific Siglec (19), is the binding protein of GdA. The importance of sialic acid residues of GdA on binding to and anti-invasive action on trophoblast cells was also investigated.

EXPERIMENTAL PROCEDURES

This study protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster.

Cell Culture—Two cell lines were used. One was an immortalized first trimester extravillous cytotrophoblast cell line TEV-1 (20). This expresses extravillous cytotrophoblast-specific markers (21), including cytokeratin 7, human leukocyte antigen G1, and CD9. The other cell line was JEG-3 (HTB36) human choriocarcinoma cell line derived from first trimester trophoblast (ATCC). Both cell lines were cultured in DMEM/F12 medium (Invitrogen) containing 10% heat-inactivated FBS (Invitrogen), 1% penicillin and streptomycin in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Purification, Desialylation, and Fluorescence Labeling of GdA—GdA was purified as described using our established protocol (2, 8, 22) from amniotic fluid collected during amniocentesis from patients attending the prenatal diagnosis clinics at Tsan Yuk Hospital, Hong Kong, China. Diluted amniotic fluid was loaded onto a monoclonal anti-glycodelin antibody (clone F43-7F9) Sepharose column and washed successively by Tris-buffered saline (TBS), 1 M NaCl with 1% isopropyl alcohol, 10 mM ammonium acetate with 0.1% isopropyl alcohol (pH 5), and TBS. The bound glycodelin was eluted with 0.1% trifluoroacetic acid. The purified GdA showed a single band in 12% SDS-PAGE gel (supplemental Fig. S1). Its identity was confirmed by mass spectrometry analysis (2) (supplemental Fig. S1).

Desialylation of GdA was performed using neuraminidase-coated agarose beads (Sigma-Aldrich) as described (2). Desialylated GdA was dialyzed against PBS. GdA and desialylated GdA were labeled with Alexa Fluor 488 fluorescent dye using the Alexa Fluor 488 microscale protein labeling kit (Molecular Probes, Carlsbad, CA) according to the manufacturer's instructions.

Effect of Normal GdA and Desialylated GdA on Activated ERK/c-Jun N-terminal Kinase (JNK) Levels—TEV-1 or JEG-3 cells were cultured with or without GdA for 24 h. The cells were lysed by the Cytobuster protein extraction reagent (Merck, Darmstadt, Germany) to obtain soluble proteins. An equal amount of protein was resolved in 10% polyacrylamide gel and transferred to PVDF membrane for Western blot analysis using monoclonal antibodies (1:1000) against phosphorylated ERK, ERK, phosphorylated JNK, or JNK (all from Cell Signaling, Danvers, MA) or antibody against α-tubulin (1:10,000; Sigma-Aldrich) for normalization.

Transwell Invasion Assay—A Matrigel-precoated cell invasion assay with 10% FBS as chemoattractant was used to measure the invasiveness of TEV-1 and JEG-3 cell as described (8). Cells suspended in serum-free medium at a density of 5×10^5 cells/ml were seeded into the culture inserts and treated with GdA, desialylated GdA, PD98059, U0126, SP600125, or DMSO (control). PD98059 and U0126 (Sigma-Aldrich) are specific MEK1/2 (MAPK kinase) inhibitors (23, 24), whereas SP600125 (Sigma-Aldrich) is a JNK inhibitor that down-regulates phosphorylated JNK and c-Jun specifically (25, 26). The cells were allowed to invade the matrix for 24 h. Those that had invaded through the membrane were either stained with cell stain (Millipore, Billerica, CA) or quantified by the CyQUANT GR dye (Cell Biolabs, San Diego, CA) using a fluorescence plate reader with excitation at 480 nm and emission at 520 nm. The results were expressed as the percentage of fluorescence intensity relative to the DMSO-treated or no treatment control. The fluorometric CyQUANT NF cell proliferation assay kit (Invitrogen) was used to study the effect of MEK and AP-1 inhibitors on cell viability, presented as the percentage of suppression relative to the DMSO-treated control.

Gelatin Zymography—The effects of MEK inhibitors and SP600125 on MMP2 proteinase activities were measured by gelatin zymography. In brief, TEV-1 or JEG-3 cells were grown to confluence in a culture plate. The culture medium was replaced by serum-free DMEM/F12 supplemented with 10 μM PD98058, 1 μM U0126, 10 μM SP600125, or DMSO (control), and the cells were cultured for a further 24 h. The serum-free conditioned media were then collected and resolved in 8% polyacrylamide gel containing 1 mg/ml gelatin (Sigma-Aldrich) under non-reducing conditions. After electrophoresis, the gel was rinsed twice in the renaturing buffer (50 mM Tris-HCl with 2.5% Triton X-100), incubated in the developing buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, at pH 7.5) at 37 °C for 24 h, stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 (Sigma-Aldrich) for 30 min, and destained in methanol:acetic acid:water (1:3:6). MMP2 proteolytic activities were identified as the band with reduced protein staining and with the molecular size of MMP2 (62 kDa).

Reverse Transcription and Quantitative PCR (qPCR) Analysis of mRNA Expression—TEV-1 or JEG-3 cells were cultured either with or without GdA, PD98059, U0126 or SP600125 treatment. The QuickPrep RNA extraction kit (GE Healthcare) was used to extract total RNA, reverse-transcribed with the use of the TaqMan reverse transcription reagent kit (Applied Biosystems, Foster City, CA) and multiscript reverse transcriptase. The resulting cDNA was subjected to qPCR analysis (TaqMan gene expression assays; Applied Biosystems) of MMP2 (Hs01548724_m1), uPA (Hs00170182_m1), ETS-1 (Hs00901425_m1), c-Jun (Hs00277190_s1), and p53 (Hs99999147_m1) using an ABI 7500 sequence detector (Applied Biosystems) as described (8). 18 S rRNA (Hs99999901_s1) was used as an internal control for sample loading. Water was used as the no template control. The relative gene expression levels were determined using the threshold cycle (C_T) method with reference to the endogenous 18 S control.

c-Jun siRNA Transfection—JEG-3 cells were seeded at a density of 5×10^5 cells/ml in a 24-well plate and were transfected with 10 nM c-Jun siRNA (S7658) or control siRNA (Ambion, Foster City, CA) by Lipofectamine 2000 reagent mix in Opti-MEM medium (Invitrogen) according to the manufacturer's instructions. After 8 h of incubation, the transfection mix was replaced by DMEM/F12 medium for another 64 h. The cells

were lysed by radioimmune precipitation assay lysis buffer for 3 h at 4 °C. The soluble proteins were collected and analyzed by Western blotting using a monoclonal anti-c-Jun antibody (Chemicon). Total RNA was also extracted to study the mRNA expression of MMP2 and uPA using qPCR analyses. The invasiveness of the transfected cells was determined 48 h after the addition of the siRNAs using the Transwell invasion assay as described above.

Flow Cytometric Analysis of Binding of GdA and Desialylated GdA to Trophoblast—GdA and desialylated GdA were labeled with Alexa Fluor 488 fluorescence dye as mentioned (Invitrogen). JEG-3 cells were cultured in the presence of 33 nM of labeled glycodelins for 3 h. The cells were then washed with PBS twice to remove unbound glycodelin, trypsinized, and resuspended in PBS before analysis with a BD FACSCanto II flow cytometer (BD Biosciences) equipped with an argon laser of 488 nm. Fluorescence signals from labeled glycodelins were measured using the 525-nm band pass filters. The results were analyzed by the WinMDI 2.9 (The Scripps Research Institute Cytometry Software, San Diego, CA) or FlowJo 7.6.3 (Tree Star Inc., Ashland, OR). Alexa Fluor 488 fluorescence dye-conjugated mouse IgG antibody (Invitrogen) was used as a control.

Detection of Siglec-6—Total RNA from JEG-3, TEV-1, and HeLa (CCL-2; ATCC) cells were extracted and reverse-transcribed using the QuickPrep RNA extraction kit and first-strand cDNA synthesis kit (both from GE Healthcare), respectively. The PCR primers for Siglec-6 were designed using the Primer 3 software (Whitehead Institute for Biomedical Research, Boston, MA): forward, 5′-ACGACCCAGACGAAGAAGTG-3′ and reverse, 5′-GCACAGAGAGCTTGGAAGATG-3′. The size of the amplicon was 183 bp. Endogenous GAPDH mRNA was used for normalization. PCR was performed at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s with a final extension step of 72 °C for 10 min after the 35th cycle. The amplified fragments were resolved by 1.5% agarose gel electrophoresis and visualized using the AlphaImager HP gel documentation system equipped with the AlphaEase FC software (Alpha Innotech, San Leandro, CA).

Immunofluorescence staining of Siglec-6 was performed as follows. JEG-3, TEV-1, or HeLa cells were grown to semiconfluence in a 24-well culture plate before fixing with 2% paraformaldehyde in PBS for 20 min at 4 °C. After washing three times with PBS, the cells were incubated with a polyclonal anti-Siglec-6 antibody (R&D Systems, Minneapolis, MN) (1:50). The bound antibody was detected by Alexa Fluor 555 fluorescence dye-conjugated anti-goat IgG (Invitrogen). Total cell lysates of JEG-3 or TEV-1 cells were also subjected to Western blot analysis using a monoclonal anti-Siglec-6 antibody (Sigma-Aldrich).

Interaction between Siglec-6 and GdA—Co-immunoprecipitation experiments were performed to study the interaction between GdA and Siglec-6. In brief, GdA was incubated with IgG-fused recombinant human Siglec-6 chimeric protein (R & D System; molar ratio = 1:1) at 4 °C in PBS. After overnight incubation with gentle shaking, protein-G-Sepharose beads (GE Healthcare) was used to precipitate the GdA-Siglec-6 complex. The captured complex was washed with PBS three times, resolved in 12% SDS-PAGE, and analyzed by Western blot using anti-glycodelin (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Siglec-6 antibody (R&D Systems). To examine the importance of sialic acid on the interaction between Siglec-6 and GdA, the above experiment was repeated by replacing GdA with desialylated GdA. Co-immunoprecipitation of the GdA-Siglec-6 complex in JEG-3 cell lysate (4×10^6 cells/ml) was also performed as described (27) to confirm the results obtained with the recombinant protein.

Competition Binding Assay—The binding of 33 nM Alexa Flour 488 fluorescence dye-labeled GdA to JEG-3 cells was determined in the presence of 330 nM recombinant human leptin (R&D Systems), which is a reported ligand of Siglec-6 (19). The cell-bound fluorescence was measured by flow cytometer as described after incubation for 2 h at culture conditions. Cells cultured with fluorescently labeled GdA only served as control.

Biological Significance of Siglec-6 on the Invasion Suppressive Activity of GdA—JEG-3 cells were treated with 165 nM anti-Siglec-6 antibody, 33 nM GdA, or 165 nM anti-Siglec-6 antibody together with 33 nM GdA (molar ratio of anti-Siglec-6 antibody: GdA = 5:1), and their invasiveness was analyzed by the Transwell invasion assay. Cells without any treatment were used as control. The specificity of the antibody was checked using Western blot analysis on the total cell lysate of JEG-3 cells.

Statistical Analyses—All the data were analyzed by SigmaPlot 10.0 and SigmaStat 2.03 (Jandel Scientific, San Rafael, CA) and expressed as mean and S.E. The results were analyzed by non-parametric rank sum test for comparison between groups. Parametric Student's *t* test was used as the post test if the data were normally distributed. A probability value <0.05 was considered to be statistically significant.

RESULTS

GdA Suppressed the Phosphorylated ERK1/2 Level of Trophoblast Cells—At 33 nM, GdA significantly suppressed the phosphorylated ERK1/2 levels, but not the total ERK levels, in TEV-1 and JEG-3 cells (Fig. 1*A*). The treatment did not affect the total and phosphorylated JNK levels (Fig. 1*A*), cell proliferation, or cell viability when compared with the control (8).

MEK Inhibitor Suppressed the Invasiveness and Proteinase Expression of the Trophoblast Cells—To correlate the degree of cell invasiveness with the phosphorylated ERK levels, invasiveness of the PD98059- and U0126-treated cells were analyzed by the Transwell invasion assays. PD98059 at concentrations of 1 and 10 μM and U0126 at concentrations of 0.1 and 1 μM significantly ($p < 0.05$) reduced the invasion of TEV-1 cells when compared with the no treatment control (Fig. 1*B*). This effect was not due to actions of the MEK inhibitors on cell viability (supplemental Table 1).

Both inhibitors significantly reduced the mRNA expression level of MMP2 and uPA (Fig. 1*C*), similar to the action of GdA on expression of these genes (8). At 10 μM PD98059, the MMP2 and uPA RNA expression levels were significantly inhibited by 31.7 ± 5.5 and 88.1 ± 4.4%, respectively, when compared with the DMSO-treated control ($p < 0.05$). The corresponding values with 1 μM U0126 were 60.2 ± 4.0 and 46.9 ± 19.8%, respectively. In addition, both inhibitors suppressed MMP2 protein-

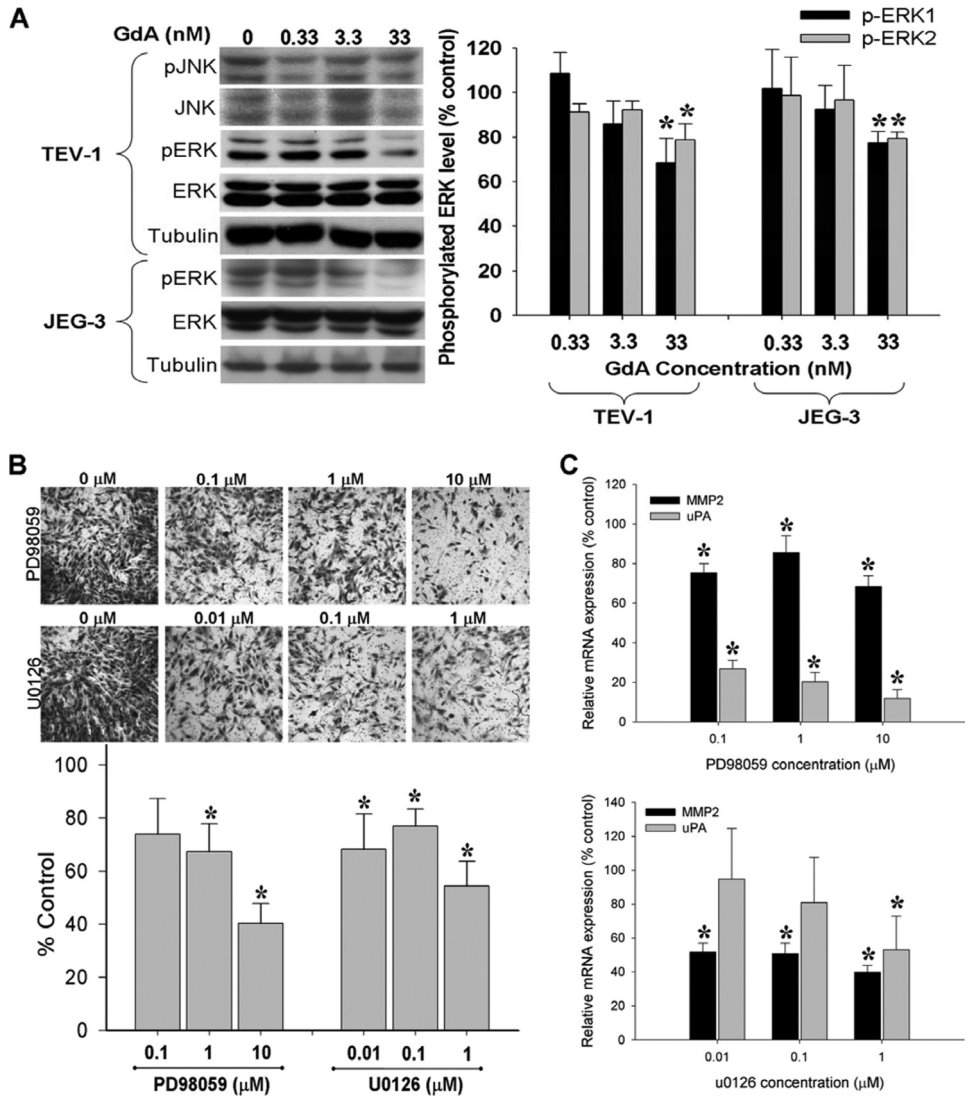

FIGURE 1. **Effects of GdA on ERK and trophoblast invasiveness.** *A*, effects of GdA treatment on activation of JNK and ERK in TEV-1 and JEG-3 cells. *Left*, representative Western blots for protein expression from five individual experiments. *Right*, the phosphorylated ERK (*pERK*) protein bands were quantified by densitometry ($n = 5$). *pJNK*, phosphorylated JNK. *B*, quantitative determination of TEV-1 cell invasion under PD98059 or U0126 treatment ($n = 5$). Representative photographs showing the invasion of TEV-1 cells after GdA treatment are shown. *C*, real time qPCR analysis of the mRNA expression of MMP2 and uPA after PD98059 (*upper*) or U0126 (*lower*) treatment in TEV-1 cells. All values are presented as the percentage of changes relative to the no treatment control (*A*) or DMSO control (*B* and *C*). *, $p < 0.05$ when compared with the no treatment control or DMSO control.

ase activities in TEV-1 and JEG-3 cells in gelatin zymography (supplemental Fig. S2).

GdA Suppressed the Expression Level of c-Jun—Real time qPCR analysis showed that GdA significantly suppressed the transcript level of c-Jun, but not of ETS-1 and p53 (Fig. 2*A*). At 33 nM GdA, the mRNA expression of c-Jun was significantly ($p < 0.05$) reduced by 78.0 ± 6.6 and 76.5 ± 15.2% in TEV-1 and JEG-3 cells, respectively. The c-Jun protein expression level was also reduced, as demonstrated by Western blot analysis (Fig. 2*B*).

JNK Inhibitor SP600125 and c-Jun siRNA Suppressed Trophoblast Invasion—To investigate the importance of c-Jun in trophoblast invasion, SP600125-treated cells were studied by the Transwell invasion assay. At 10 μM, SP600125 suppressed the invasion of TEV-1 and JEG-3 cells by 29.2 ± 4.2 and 67.6 ± 10.2%, respectively (Fig. 3*A*), and reduced the transcript levels of MMP2 and uPA by 56.5 ± 6.5 and 83.7 ± 3.6%, respectively (Fig. 3*B*). SP600125 at all the concentrations studied had no effect on cell viability (supplemental Table 1). The suppressive effect of SP600125 on MMP2 is confirmed by gelatin zymography (supplemental Fig. S2).

The above observation was confirmed by a c-Jun siRNA knockdown experiment. Western blot analysis indicated that the c-Jun protein level after knockdown was 65.6 ± 10.8% of the control siRNA transfected cells. The invasion of the c-Jun siRNA transfected JEG-3 cells was also significantly ($p < 0.05$) reduced by 28.4 ± 5.8% when compared with the control siRNA transfected cells (Fig. 3*D*). Lipofectamine treatment or transfection of control siRNA did not affect the c-Jun protein expression level and trophoblast invasion when compared with the no treatment control (data not shown). Real time qPCR analysis showed that uPA mRNA expression level was reduced by 55.9 ± 15.5% when compared with the control siRNA transfected cells ($p < 0.05$) (Fig. 3*E*). The treatment did not affect the mRNA level of MMP2.

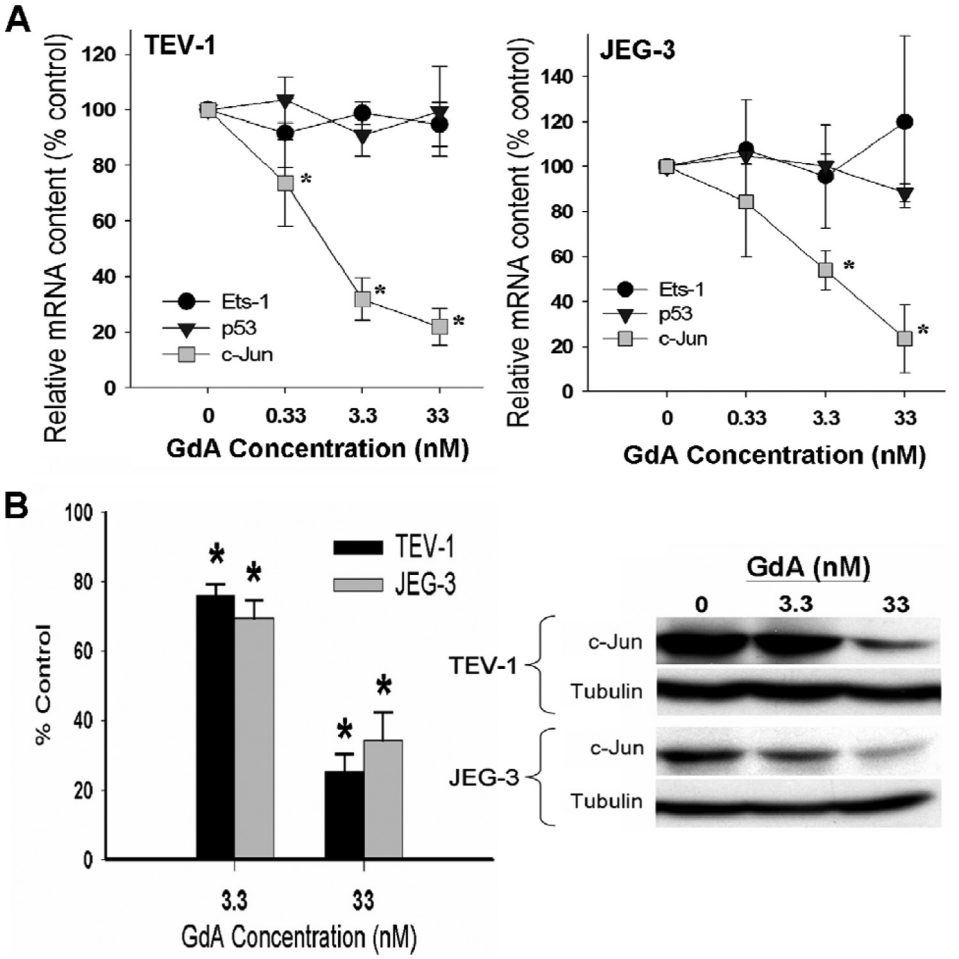

FIGURE 2. **Effects of GdA on c-Jun expression.** *A*, real time qPCR analysis ($n = 5$) of the mRNA expression level of ETS-1, p53, and c-Jun in TEV-1 cells (*left*) and JEG-3 cells (*right*) under GdA treatment. *B*, Western blot analysis of c-Jun protein expression after GdA treatment. The c-Jun protein bands were quantified by densitometry ($n = 3$). All values are presented as the percentage of changes relative to the no treatment control. *, $p < 0.05$ when compared with the no treatment control.

Sialylation Is Important for Binding and Biological Activity of GdA—The binding of fluorescently labeled GdA and desialylated GdA on JEG-3 cells was analyzed by flow cytometry (Fig. 4*A*). The results indicated that GdA bound to 75.1 ± 3.3% of the cells, which was significantly ($p < 0.05$) higher than that of desialylated GdA (30.2 ± 1.3%). Both GdA and desialylated GdA significantly ($p < 0.05$) suppressed the invasion of JEG-3 cells when compared with the no treatment control (GdA, 66.5 ± 3.3%; desialylated GdA, 84.8 ± 3.4% of control). The extent of suppression was significantly ($p < 0.05$) greater after GdA than desialylated GdA treatment (Fig. 4*B*), indicating a reduction in the invasion-suppressive activities of GdA after desialylation, associated with a reduced inhibition on phosphorylated ERK levels when compared with that of GdA (Fig. 4*C*).

Siglec-6 Is Present on the Trophoblast—RT-PCR analysis showed the presence of Siglec-6 mRNA in the JEG-3 cells and the TEV-1 cells but not the HeLa cells (Fig. 5*A*). Western blotting demonstrated the presence of an immunoreactive band consistent with the predicted molecular size (49 kDa) of Siglec-6 in the total cell lysate of JEG-3 and TEV-1 (Fig. 5*A*). Siglec-6 immunoreactivity was also detected in the cultured JEG-3 and TEV-1 cells, but not in the HeLa cells (Fig. 5*B*).

Siglec-6 Interacts with GdA—The interaction between GdA and Siglec-6 recombinant chimeric protein was studied by co-immunoprecipitation followed by Western blot analysis. Glycodelin immunoreactivity was found in the positive control containing only GdA (Fig. 6*A*, *left panel*, *lane 1*) and in the interacting complex (Fig. 6*A*, *left panel*, *lane 4*), indicating interaction between GdA and the recombinant Siglec-6 chimeric protein. The analysis also showed that protein-G did not interact with GdA and that the anti-glycodelin antibody did not cross-react with Siglec-6. Reduced interaction (62.1 ± 2.2% of normal GdA) between desialylated GdA and Siglec-6 chimera protein was observed, as indicated by the weaker immunoreactive band corresponding to desialylated GdA (Fig. 6*A*, *middle panel*, *lane 2*) when compared with that of normal GdA (Fig. 6*A*, *middle panel*, *lane 1*). The interaction between GdA and Siglec-6 was confirmed by co-immunoprecipitation using JEG-3 cell lysates (Fig. 6*B*). No Siglec-6 immunoreactivity was observed when GdA was omitted during co-immunoprecipitation. Leptin, a Siglec-6 ligand, significantly ($p < 0.05$) reduced the binding of GdA to JEG-3 cells by 18.2% (Fig. 6*C*).

Siglec-6 Is Involved in the Invasion-suppressive Activity of GdA—The effect of polyclonal anti-Siglec-6 antibody on JEG-3 cell invasion was investigated by the Transwell invasion assay

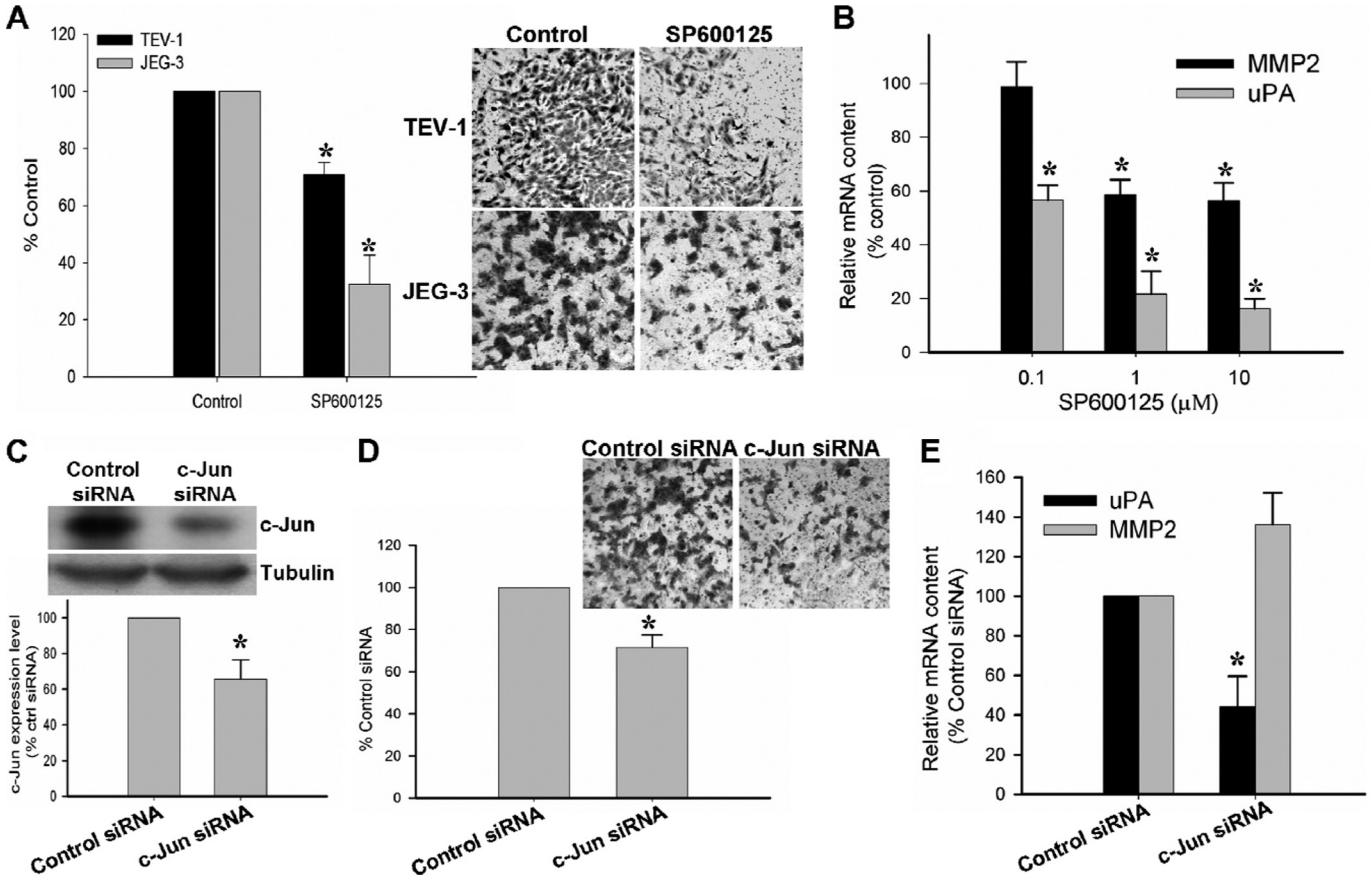

FIGURE 3. **Effects of c-Jun inhibition and knockdown on trophoblast invasiveness.** *A*, quantitative determination of TEV-1 or JEG-3 cell invasion under 10 μM SP600125 treatment ($n = 5$). Representative photographs showing the invasion of TEV-1 and JEG-3 cells after SP600125 treatment are shown. *B*, real time qPCR analysis of the mRNA expression of MMP2 and uPA in TEV-1 cells after SP600125 treatment ($n = 5$). *C*, Western blot analysis of c-Jun protein expression in c-Jun siRNA transfected JEG-3 cells. The c-Jun protein bands were quantified by densitometry ($n = 3$). *D*, quantitative determination of JEG-3 cell invasion ($n = 3$) in c-Jun siRNA transfected cells. Representative photographs showing the invasion of c-Jun siRNA transfected cells is shown. *E*, real time qPCR analysis of the mRNA expression of MMP2 and uPA of c-Jun siRNA transfected JEG-3 cells ($n = 5$). All values are presented as the percentage of changes relative to the DMSO control (*A* and *B*) or control siRNA transfected cells (*C–E*). *, $p < 0.05$ when compared with the DMSO control or control siRNA transfected cells.

(Fig. 7). Anti-Siglec-6 antibody did not affect JEG-3 cell invasion when compared with the no treatment control. In keeping with previous observations, GdA significantly ($p < 0.05$) reduced 29.9 ± 4.5% of JEG-3 invasiveness. Co-incubation of anti-Siglec-6 antibody with GdA abolished the suppressive action of GdA on invasion.

DISCUSSION

The present data show that ERK is a downstream effector of GdA in the studied cell lines. This is consistent with the action of GdA on ERK1/2 in human spermatozoa (28) and lymphocytes (22). Interestingly, the level of activated ERKs in the human cytotrophoblast decreases significantly after 11 weeks of gestation (29) when the decidual GdA concentration is at its peak (4), prompting a relationship between ERKs and GdA in early pregnancy. Indeed, MAPKs, especially ERK, have been suggested to be involved in controlling trophoblast migration and invasion. For example, epidermal growth factor modulates the invasion and migration of a trophoblast cell line, HTR-8/Svneo cells, through phosphorylation of ERK1/2; such effects are abolished by MEK inhibitors (30). When compared with normal pregnant women, the immunoreactivities of placental phosphorylated ERKs are reduced in preeclampsia patients with shallow trophoblast invasion (31). MAPKs/ERKs are believed to control the invasion of cancer cells as well (32).

GdA suppresses trophoblast invasion by reducing the expression of MMP2 and uPA (8). The present study demonstrates that the suppressive action is mediated by reduction of phosphorylation of ERKs, consistent with the inhibitory action of ERK inhibitors on mRNA expression of MMP2 and uPA in the trophoblast cells. In cancer cells, ERKs regulate the level of the membrane type 1 MMP (MT1-MMP or MMP14), one of the activators of MMP2, and treatment with PD98059 inhibited the activation of MMP2 (32).

GdA suppresses the mRNA expression level of c-Jun, and knockdown of c-Jun reduces invasion and uPA mRNA expression of trophoblast cells. c-Jun is a member of the Jun proteins, which also include JunB and JunD. They are the essential components of a family of dimeric transcription factors known as AP-1, in which the Jun proteins form either homodimers or heterodimers with the Fos protein family (33). The AP-1 complex plays a critical role in placentation, and the Jun protein family is frequently linked to implantation and cancer metastasis. Among the Jun proteins, JunB-deficient embryos are unable to establish adequate connections to the maternal circulation

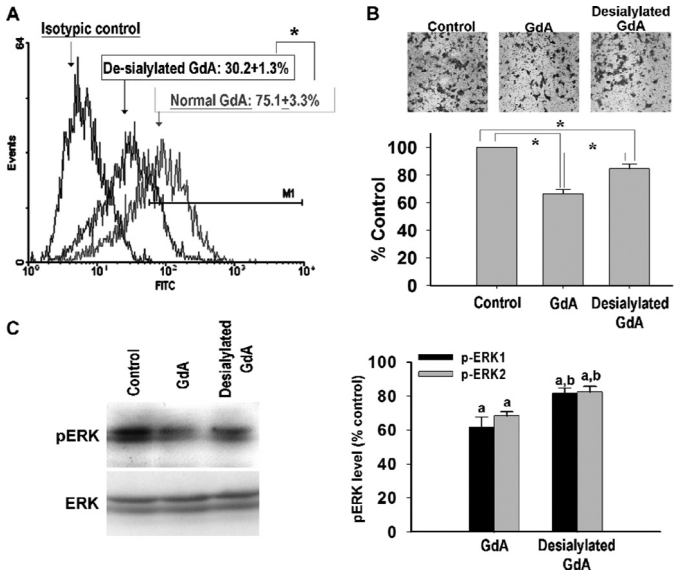

FIGURE 4. **Effects of desialylation on GdA activities and binding to JEG-3 cells.** *A*, flow cytometry analysis of the binding of normal or desialylated GdA to JEG-3 cells ($n = 5$). *, $p < 0.05$ when compared with normal GdA group. *B*, quantitative determination of JEG-3 cell invasion under normal or desialylated GdA treatment ($n = 5$). *, $p < 0.05$ when compared between groups. Representative photographs showing the invasion of JEG-3 cells after GdA treatment are shown. *C*, the effect of desialylated GdA on activation of ERK in JEG-3 cells. *Left*, representative Western blot for phosphorylated ERK (*pERK*) protein expressions; *right*, the protein bands were quantified by densitometry ($n = 3$). *a* indicates $p < 0.05$ when compared with the no treatment group; *b* indicates $p < 0.05$ when compared with the GdA-treated group. All values are presented as the percentage of changes relative to the no treatment control.

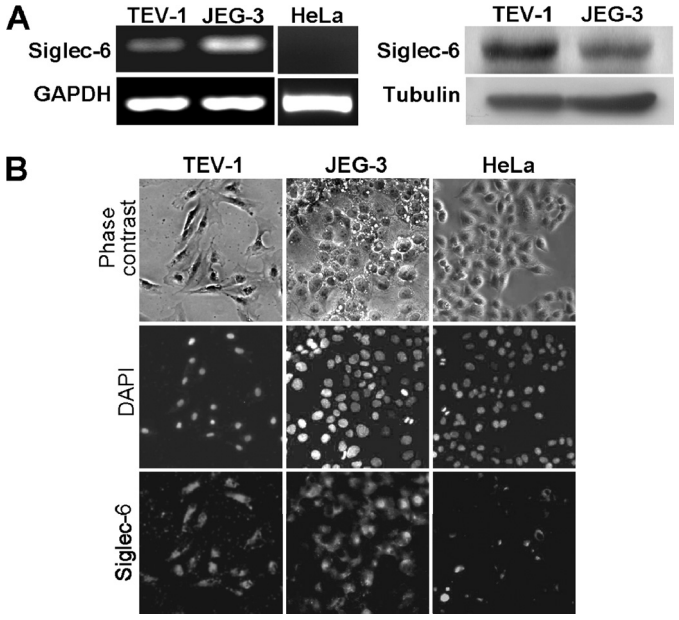

FIGURE 5. **Expression of Siglec-6 in TEV-1 and JEG-3 cells.** *A*, *left*, RT-PCR analysis using Siglec-6 primers. The housekeeping gene GAPDH was used for normalization. *Right*, Western blot analysis of cell lysate using anti-Siglec-6 antibody. *B*, immunofluorescence staining of Siglec-6. TEV-1, JEG-3, or HeLa cells were first incubated with anti-Siglec-6 antibody. They were then visualized using Alexa Fluor 555 fluorescence dye-conjugated anti-goat IgG.

(34), resulting in fetal growth restriction (35). c-Jun is required for the expression of uPA (36), and expression of dominant negative c-Jun mutant results in inhibition of invasion of human squamous cancer cells (37).

The fact that c-Jun mediates the action of GdA on uPA is understandable because the promoter of uPA contains an Ets/AP-1a binding site (38). On the other hand, the regulation of MMP2 gene expression by GdA is less apparent as the promoter of MMP2 lacks an AP-1 site. Therefore, c-Jun down-regulation may *not* be a prerequisite for the GdA-mediated MMP2 suppression. This is supported by the failure of reducing MMP2 expression by transfection of c-Jun siRNA.

The promoter of MMP2 contains an AP-2 element (11). The regulatory role of ERK on the expression of AP-2 and subsequently the expression of MMP2 have been demonstrated (39). Therefore, GdA may modulate MMP2 expression through AP-2 or other ERK-dependent signaling molecules.

GdA suppresses the phosphorylation of ERKs but not of JNKs. Both ERKs and JNKs phosphorylate c-Jun (40), leading to enhancement of the transcriptional activities of c-Jun protein (41, 42). Differential regulation of phosphorylation of c-Jun by ERKs and JNKs in the rat pheochromocytoma cell line PC12 has been reported (43). Experiments on the effect of GdA on the activities of the third MAPK family member, p38 MAPK, were not performed because it is not involved in the regulation of c-Jun gene expression (44).

GdA carries sialylated glycans (45). This study demonstrated the contribution of sialic acid residues in suppression by GdA of trophoblast invasion; the suppressive effects on trophoblast invasion and phosphorylated ERK levels were reduced after desialylation of GdA, probably resulting from lower binding affinity of desialylated GdA on trophoblast cells. Whether sialic acid is a requisite for the binding and/or biological activities of GdA on trophoblast invasion requires further investigation because the enzymatic desialylation used in this study is not likely to remove all the sialic acid from GdA. Moreover, it can only remove the terminal sialic acids from GdA, leaving its sialylated Sda (NeuAcα2–3(GalNAcβ1–4)Gal) glycans unaffected (2).

Sialic acid is the most common neuraminic acid derivative in the human glycome (46). It always appears in the exposed *N*-glycans chain terminal, and it contributes to the binding of a glycoprotein to its receptor (47). GdA is most sialylated among the glycodelin isoforms (2, 45, 48). GdA, but not the non-sialylated glycodelin-S (GdS), possesses immunosuppressive activities on lymphocytes, and such activities are abolished after desialylation (2, 49). Nevertheless, other sugar residues also contribute to the biological activities of glycodelins. Thus glycodelin-F (GdF), but not GdA, suppresses progesterone-induced acrosome reaction in spermatozoa, although the former contains fewer sialic acids (50, 51). It is likely that isoform-specific biological actions of glycodelin isoforms are exhibited through their specific sets of glycans.

Sialic acid-binding proteins, including selectins and Siglecs, are expressed in trophoblast (18, 52). We demonstrate, for the first time, that Siglec-6 serve as a GdA-binding protein on human trophoblast. The conclusion was drawn from four observations. First, GdA bound to recombinant and native Siglec-6, as demonstrated by co-immunoprecipitation. Second, anti-Siglec-6 antibody abolished the invasion-suppressive effect of GdA. Third, Siglec-6 transcripts were detected in trophoblast cells, and its protein was detected on the cell surface as

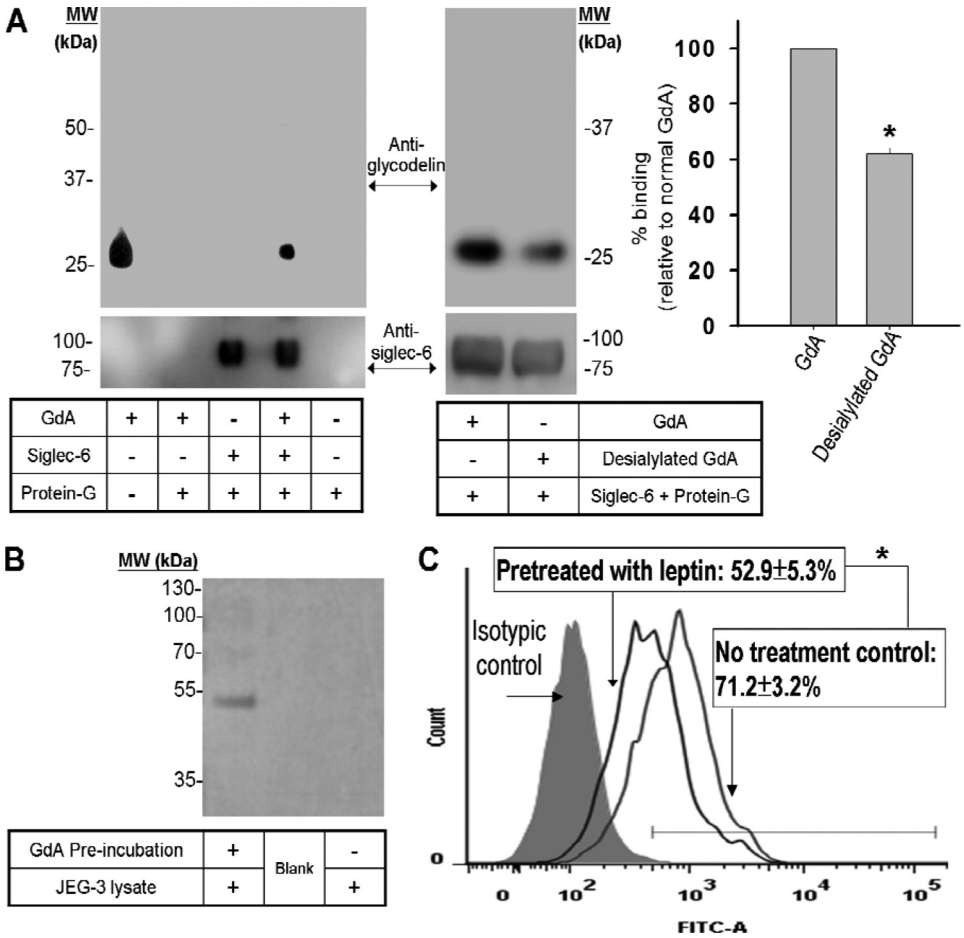

FIGURE 6. **Interaction between GdA and Siglec-6.** *A*, Western blot analysis of the interaction between recombinant Siglec-6 chimeric protein and either GdA (*left*) or desialylated GdA (*middle*). *Right*, the GdA protein bands were quantified by densitometry ($n = 3$). Data were expressed as the percentage of changes relative to the normal GdA group. *, $p < 0.05$ when compared with normal GdA group. *MW* indicates molecular mass markers. *B*, GdA-treated JEG-3 cell lysates were co-immunoprecipitated with an anti-glycodelin antibody Sepharose column. Immunoblotting was performed with anti-Siglec-6 antibody. JEG-3 cell lysate without GdA treatment served as control. *C*, competition binding of JEG-3 cells to Alexa Fluor 488 fluorescence dye-conjugated GdA in the presence of leptin. Flow cytometry analysis was done to quantify the binding of GdA ($n = 4$). Values are presented as the percentage of changes relative to the no treatment group. *, $p < 0.05$ when compared with the no treatment group.

detected by immunofluorescence using a non-cell permeation approach. Fourth, a reported Siglec-6 ligand, leptin, significantly reduced the binding of GdA to JEG-3 cells.

Siglecs are membrane proteins with an amino-terminal V-set immunoglobulin domain that binds sialic acids (18). They were first identified in macrophages (53). Research on Siglecs focuses on their role in regulating cell adhesion and activation of immune cells (54). Studies of Siglec function in the trophoblast are lacking. Although most Siglecs are found in cells of the immune and hematopoietic systems, Siglec-6 is the only member of the Siglec family present in the trophoblast (18, 55). The preferred ligands of Siglec-6 are predominantly glycoproteins, but not glycolipids (55). However, the natural ligand(s) of Siglec-6 are not known. The preferred ligand of Siglec-6 determined by using synthetic targets is terminal Neu5Acα2–6GalNAc (19). This structure is frequently present in the glycan terminals of GdA (2). The contribution of sialic acid residues of GdA to interaction with Siglec-6 is indicated by the reduced affinity of desialylated GdA toward recombinant Siglec-6 chimeric protein. The binding of Siglec-6 to frozen placental sections is also reduced after sialidase treatment of the sections (55).

Leptin reduced the binding of GdA to trophoblast; however, the reduction was not complete. One possible reason is that leptin binds more strongly to its own receptor than Siglec-6, thus reducing the amount of leptin available for binding to Siglec-6. Another possibility is the presence of more than one GdA receptor on trophoblast. This is supported by the presence of some biological activities of desialylated GdA on trophoblast.

The cytosolic part of Siglec-6 contains the immunoreceptor tyrosine-based inhibitory motif (ITIM) and the ITIM-like domains. Receptors with ITIM including inositol phosphatase SHIP (SH2-containing inositol polyphosphate 5-phosphatase) and tyrosine phosphatase SHP-1 (Src homology 2 domain phosphatase-1) (56, 57) function as inhibitory receptors through recruitment of cytoplasmic phosphatase with Src homology 2 (SH2) domain (58). These phosphatases in the trophoblast have not been studied. In macrophages, SHIP suppresses IL-1 production through down-regulation of ERK activities (59). In B-cells, SHIP suppresses B-cell antigen receptor to trigger proliferation through perturbation of the MAPK cascade (60).

Recently, Winn *et al.* (61) compared gene expression between placental tissue from patients with preeclampsia and

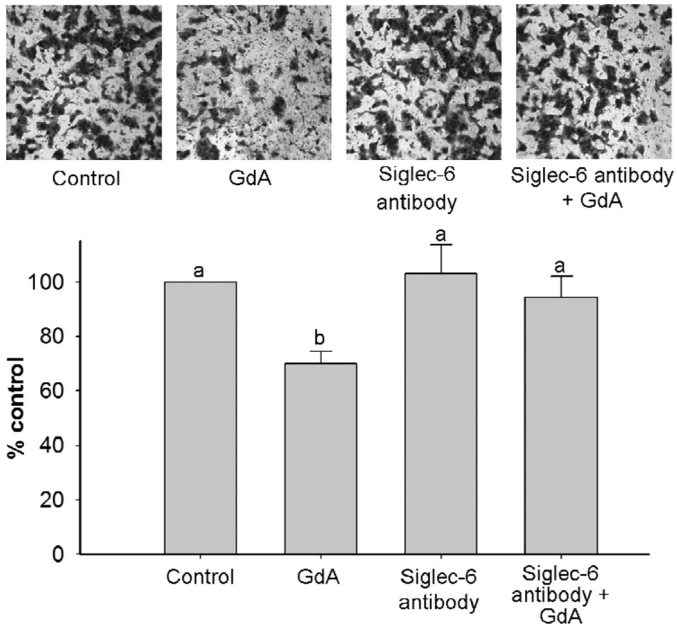

FIGURE 7. **Effect of Siglec-6 antibody on JEG-3 cell invasion.** Quantitative determination of JEG-3 cell invasion after treatment with GdA, Siglec-6 antibody, or both ($n = 5$) is shown. *a* and *b* indicate significant ($p < 0.05$) differences between groups. Values are presented as the percentage of changes relative to the no treatment group.

preterm labor from late second trimester to third trimester. They concluded that the expression of Siglec-6 protein and mRNA was up-regulated in the placenta of the preeclamptic patient. Although there is no difference in GdA expression between normal and preeclamptic serum and decidual samples (62, 63), the up-regulated trophoblastic Siglec-6 expression might increase the binding of GdA to trophoblast cells. Whether such an increase in GdA-Siglec-6 interaction could cause the characteristic shallow invasion in preeclamptic patients (64) is worth investigation. Interestingly, Siglec-6 is only expressed in human but not in other species including non-human primates (55), and preeclampsia is thought to be a human disease only (65). These observations further enhance the linkage between Siglec-6 and preeclampsia.

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