Siglec-6 is expressed in gestational trophoblastic disease and affects proliferation, apoptosis and invasion

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Abstract

Sialic acid immunoglobulin-like lectin (Siglec)-6 is a transmembrane receptor that binds leptin. Leptin is an obesity-associated peptide hormone overexpressed in gestational trophoblastic disease (GTD). GTD encompasses several placental abnormalities that range from benign to malignant. Among GTD, molar placentas are characterized by excess proliferation, whereas gestational trophoblastic neoplasias (GTN) have characteristically aggressive invasion. We hypothesized that in GTD, Siglec-6 expression would increase with disease severity and that Siglec-6 and leptin would promote proliferation, inhibit apoptosis and/or promote invasion. Siglec-6 expression patterns were evaluated with particular attention to the diagnostic utility of Siglec-6 in GTD (controls: normal placentas (n=32), hydropic abortus placentas (n=7), non-GTD reproductive tract cancers (n=2); GTD: partial moles (PM; n=11), complete moles (n=24), GTN (n=6)). In normal placentas, Siglec-6 expression dramatically decreased after 8 weeks gestation. Complete molar placentas had significantly higher Siglec-6 expression than controls, but expression was not significantly different from PM. In GTN, Siglec-6 expression was low. These data suggest that Siglec-6 may have diagnostic utility for distinguishing complete moles from normal and hydropic abortus placentas. Functional studies in choriocarcinoma-derived BeWO cells demonstrated a complex interplay between Siglec-6 expression and leptin exposure. In cells lacking Siglec-6, leptin treatment promoted invasion, likely through interaction with LepR leptin receptor, without affecting proliferation or apoptosis. Siglec-6 expression promoted proliferation in a leptin-dependent manner, but protected cells from apoptosis and promoted invasion in a leptin-independent manner. We propose that Siglec-6 and leptin play a role in the aberrant properties characteristic of GTD, namely excess proliferation and invasion.

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Introduction

Gestational trophoblastic disease (GTD) describes a compilation of disorders of abnormal placental development. GTD includes hydatidiform moles, which are characterized by edematous placental villi and excess proliferation of placental trophoblast cells that line the villi. Hydatidiform moles are divided into partial (PM) and complete hydatidiform moles (CM). Histologically, CM have more edematous changes and excess proliferation of trophoblasts compared with PM. After conventional treatment, typically dilation and curettage (D&C), 5% of PM persist and rarely become malignant. CM persist after conventional treatment ~20% of the time and develop into choriocarcinoma (CCA) 5% of the time (Benirschke & Kaufmann 2000). GTD also encompasses

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gestational trophoblastic neoplasias (GTN), such as CCA. CCA is a highly malignant form of GTN, which generally responds well to multidrug chemotherapy regimens. However, for the 5% of women with chemotherapy-unresponsive CCA the disease is usually fatal. GTN also includes the rare disorders of placental site trophoblastic tumor (PSTT) and epithelioid trophoblastic tumor (ETT). PSTT and ETT are metastatic in 10–20% of cases (Shih Ie 2009) and respond poorly to conventional chemotherapies (Shih Ie & Kurman 2002). The molecular origins of GTD are not well understood.

There are several challenges in diagnosing hydatidiform moles. Distinguishing PM from CM relies on histologic assessment of the degree of trophoblast proliferation and hydropic changes of the villi. Additionally, hydatidiform moles can be difficult to distinguish from hydropic abortuses (HA; Fox 2000, Baergen 2005). HA are miscarriages with edematous changes in the placental villi without excess trophoblast proliferation. Because of the worse prognosis and follow up required for CM compared with PM and both types of moles compared with HA, it is important to accurately distinguish these three entities. Currently, FISH, flow cytometry and p57 staining can assist in distinguishing HA, PM and CM. However, none of these techniques alone can distinguish all three entities. Furthermore, results are not always conclusive and can be misleading (Sebire & Lindsay 2006, Paul *et al*. 2010). Another clinical issue is that there is currently no way to predict which hydatidiform moles will persist after conventional treatment or which will develop into CCA. A better understanding of the molecular biology of hydatidiform moles and GTN may provide useful diagnostic and predictive markers. Further, understanding the molecular mechanisms leading to GTN will be necessary for developing targeted therapies for chemoresistant GTN.

In order to address the diagnostic challenges and further the molecular understanding of GTD, we evaluated sialic acid immunoglobulin-like lectin (Siglec)-6 expression and function in GTD. Siglec-6 is a transmembrane receptor in the CD33-like family of rapidly evolving Siglecs. As a result of rapid evolution, the *SIGLEC6* gene is present only in primates. Among primates, only humans express Siglec-6 in their placenta whereas all primates evaluated to date express Siglec-6 in their B cells. The unique placental expression of Siglec-6 by humans may be due to promoter elements for human *SIGLEC6* gene that are absent in other primates (Brinkman-Van der Linden *et al*. 2007). Siglec-6 overexpression has been found in two disorders of placentation – CCA (Skotheim *et al*. 2006) and preeclampsia (PE; Winn *et al*. 2009). The overexpression of Siglec-6 mRNA in CCA was reported as part of a publically available microarray data set, but has not been verified by other techniques. Interestingly, both PE and GTD are placental disorders almost exclusively limited to human pregnancy (Benirschke & Kaufmann 2000). Also of note, the *SIGLEC6* gene is located on 19q13.4, in close approximation to a region found relevant to familial hydatidiform moles (Moglabey *et al*. 1999, Sensi *et al*. 2000).

Siglec-6 binds sialoglycans, such as sialyl-TN, and leptin (Patel *et al*. 1999) despite the absence of a sialyl-TN modification on leptin (Cohen *et al*. 1996). The consequences of leptin–Siglec-6 interactions are unknown. Interestingly, patients with hydatidiform moles have approximately twofold higher serum leptin levels compared with normal pregnancies of the same gestation. Indeed, leptin has been localized to trophoblasts in both normal and hydatidiform molar placentas (Sagawa *et al*. 1997, Li *et al*. 2004) raising the question of leptin being involved in GTD pathogenesis. Siglec-6 has no similarity with the leptin receptor LepR, which is expressed in most tissues including placenta (Patel *et al*. 1999). LepR is overexpressed in GTD tissues, but does not predict disease severity (Li *et al*. 2004). Siglec-6 expression in GTD has not been evaluated. Nor has placental Siglec-6 expression been characterized in normal placentas prior to 24 weeks gestation, the time period during which hydatidiform moles (HM) form.

Our goal was to investigate the roles of Siglec-6 and leptin in GTD. We hypothesized that Siglec-6 would be overexpressed in GTD and expression would increase with disease severity. That is, Siglec-6 was predicted to be highest in GTN followed by CM and then PM. We anticipated that expression would be low in both hydropic abortus and normal placentas. To test this hypothesis, we evaluated the expression of Siglec-6 protein in normal, HA, PM, CM and GTN biopsies by immunohistochemistry and immunoblotting. Whether Siglec-6 expression would distinguish persistent vs non-persistent CM was also evaluated. We further hypothesized that in addition to being a marker of disease, Siglec-6 and leptin would have functional consequences in GTD with regard to promoting proliferation, inhibiting apoptosis and/or promoting invasion. Toward addressing the latter hypothesis, we modified BeWO choriocarcinoma cells to overexpress Siglec-6. The effects of Siglec-6 expression and leptin treatment on the proliferation, apoptosis and invasion of BeWO cells were determined.

Materials and methods

Tissue collection

The University of California San Francisco (UCSF) Committee on Human Research and University of Colorado (CU) Institutional Review Board approved these studies. GTD cases were identified at UCSF and CU from their respective pathology databases (through 2005 for UCSF and through 2010 for CU). Cases with missing paraffin blocks or treated outside of the respective institutions were excluded. All pathology was reviewed by two pathologists. Samples were further limited to those that both pathologists agreed on the histologic classification. Normal control placentas were collected from elective terminations between 5 and 24 weeks gestation. Tissue was washed extensively in cold PBS (RICCA Chemical Company, Arlington, TX, USA) and processed within 1 h of the procedure. Tissue was snap frozen and stored at −80 °C for later protein isolation or formalin fixed for histology. Exclusion criteria for normal controls included any evidence of maternal infection and known genetic or fetal anomalies. Placentas with hydropic changes were used for a second comparison group (HA). In total, samples were collected from normal ($n=32$), HA ($n=7$), PM ($n=11$), CM ($n=24$), CCA ($n=3$), metastasis of CCA to lung ($n=1$), metastasis of CCA to vagina ($n=1$) and PSTT ($n=1$). No ETT were available from either institution. A non-gestational choriocarcinoma from a male patient and an endometrial tumor were also evaluated. With the exception of GTN, gestational age of specimens was determined by dating from last menstrual period, standard ultrasound or foot length measurements (Drey *et al*. 2005). CM samples were further divided into persistent ($n=15$) vs non-persistent ($n=9$) disease. Samples for which dilation and curettage (D&C) were curative (human chorionic gonadotropin; hCG returned to baseline without further intervention) were classified as non-persistent. Samples that required chemotherapy to achieve return of hCG to baseline or total abdominal hysterectomy and bilateral salpingo-oophorectomy to treat locally invasive disease were classified as persistent. Subject characteristics are presented in Table 1.

Siglec-6 immunohistochemistry

Formalin-fixed, paraffin-embedded biopsy tissues were serially sectioned (5 μm thickness). The murine Catalyzed Signal Amplification staining kit (Dako, Carpinteria, CA, USA) was used with a mouse monoclonal anti-human Siglec-6 antibody (1:25) (Brinkman-Van der Linden *et al*. 2007). Visualization was with diaminobenzidine (DAB, Dako). Hematoxylin QS (Vector Laboratories, Burlingame, CA, USA) was used for the nuclear counterstain. Negative controls were performed in the absence of the primary antibodies. A Nikon Eclipse 80i microscope equipped

Table 1 Subject characteristics for Siglec-6 expression studies

	n	**Maternal age** (years ± S.D.)	**Gestational age** (weeks ± S.D.)	**Gravidity** (± S.D.)	**Parity** (± S.D.)
Figure 1A, B, C, D and E					
<8 Weeks	11	24.8±5.3	6.5±0.8	2.2±1.3	0.8±1.4
≥8 Weeks	21	24.1±6.2	11.4±3.4	2.9±2.4	1.1±1.3
		NS	$P<0.001$	NS	NS
Figure 1F[a]					
<8 Weeks	5	25.3±2.3	7.0±0.3	2.8±0.7	1.3±0.8
≥8 Weeks	23	23.8±1.4	17.1±0.9	3.5±0.4	1.0±0.3
		NS	$P<0.001$	NS	NS
Figure 2					
Normal	21	24.1±6.2	11.4±3.4	2.9±2.4	1.1±1.3
Partial moles	11	28.91±5.7	14.2±5.1	3.4±2.7	0.7±1.1
Complete moles	24	26.83±9.6	11.9±2.9	2.7±2.5	1.1±2.2
		NS	NS	NS	NS
Complete moles (non-persistent)	15	23.3±7.9	12.3±3.0	2.0±1.1	0.6±0.7
Complete moles (persistent)	9	32.8±8.9	10.9±2.7	3.5±3.5	1.9±3.3
		$P=0.01$	NS	NS	NS

NS, nonsignificant difference.
[a]Nine samples are in common with the IHC samples, three <8 weeks, six ≥8 weeks. Data not shown for HA because complete clinical data were available for only one sample.

with a Q-imaging Retiga 100R digital camera and NIS-Elements Advanced Research version 2.30 software was used for visualization and photography. Immunoreactivity was evaluated by semiquantitative scoring in all samples except GTN. Intensity was characterized between 0 and 3 where 0 is the absence of staining and 3 is the most intense staining. Percent positive trophoblasts were also scored (1 = <10%, 2 = 10–50%, 3 = 50–90%, 4 = >90%). The intensity and percent positive trophoblast values were multiplied to give the immunoreactivity score (IRS). Scoring was limited to chorionic villi because normal gestation samples <14 weeks gestation generally do not contain basal plate and the pathologic samples did not always contain basal plate. However, all samples (excluding GTN) contained chorionic villi. Siglec-6 immunoreactivity in each sample was independently scored by two people who were blinded to sample identity. Spearman's correlation between the two scores yielded $R = 0.71$ ($P < 0.0001$). The average score for each sample was used.

Siglec-6 immunoblotting

Snap-frozen placental biopsy tissue from normal placentas (gestational age 5–24 weeks, $n = 28$) was homogenized in 1% SDS, sonicated for 5 s and samples centrifuged for 10 min at 13 000 ***g*** to remove insoluble material. Protein concentrations were determined with Bio-Rad DC protein concentration assay as per manufacturer's protocol. Protein lysates (60 μg) were separated on a 7.5% polyacrylamide gel under reducing conditions and transferred onto PVDF membrane. The membrane was incubated in 5% (w/v) nonfat dry milk (NFDM) in TBS–T (10 mM Tris Base, 150 mM NaCl and 0.05% Tween 20 (v/v), pH 8.4) for an hour to block nonspecific binding. The membrane was incubated overnight at 4 °C in the presence of anti-Siglec-6 antibody (1:1000, R&D Bioscience, Minneapolis, MN, USA). After washing, the membrane was incubated with the HRP-conjugated anti-sheep Trueblot antibody (1:1000, eBioscience, San Diego, CA, USA) in 5% NFDM for an hour at room temperature. Visualization of the immunoreactive bands was achieved by using chemiluminescence (Western Lightening Plus-ECL; Perkin Elmer, Waltham, MA, USA) and exposure to XR film (Kodak). To evaluate for protein loading, the PVDF membrane was stripped (Reblot Plus Mild; Millipore, Billerica, MA, USA) and incubated with anti-actin antibody (1:10 000; Sigma) in 5% (w/v) BSA for an hour at room temperature and then processed as described above (secondary antibody: HRP conjugated anti-chicken immunoglobulin 1:100 000 in 5% BSA; Sigma). Band densitometry with local background correction was performed using a Bio-Rad (Hercules, CA, USA) Chemi Doc XRS and Quantity One software. Siglec-6 signal was normalized to actin signal. Samples run on three different blots were normalized to a 7-week sample run on each blot.

Cell culture and transfections

BeWO human choriocarcinoma trophoblast cells (American Type Culture Collection, Manassas, VA, USA) were cultured in growth medium (F12K medium (Cellgro, Manassas, VA, USA) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen)). Cells were maintained in monolayer cultures at 37 °C in 5% CO_2, 95% air and 100% humidity. To generate Siglec-6 overexpressing cells, BeWO cells were transfected with empty PCDNA3.1+ vector or PCDNA3.1+ vector containing full-length Siglec-6. Full-length Siglec-6 cDNA was transferred from a p-Babe plasmid (Patel *et al*. 1999) into the PCDNA3.1+ vector using EcoRI and NotI restriction sites. Transfections were performed according to the manufacturer's protocols with Lipofectamine (Invitrogen) and 2 μg of DNA. Briefly, cells were grown to 80% confluence and then cultured in OptiMEM containing Lipofectamine and DNA plus serum-free, antibiotic-free F12K medium for 12 h. Cells were allowed to recover for 72 h before passaging into growth medium with 200 μg/ml G418 (Invitrogen). This concentration of G418 was sufficient to kill un-transfected BeWO cells within 72 h as determined in a kill curve assay. Empty vector and Siglec-6 clones were generated via dilution cloning. Siglec-6 and LepR expression levels were determined using quantitative RT-PCR. Total RNA was isolated using Trizol (Invitrogen) and then cDNA generated with ImProm-II reagents (Promega) according to the manufacturer's protocols. Templates were amplified with Assay on Demand Kits (Applied Biosystems) as previously described (Winn *et al*. 2007), samples were run in triplicate. Relative quantification was determined with the standard curve method (Applied Biosystems User Bulletin #2). 18S rRNA was used for amplification normalization.

Proliferation and apoptosis assays

BeWO empty vector and Siglec-6 expressing cell lines were plated on eight-well chamber slides (Lab-TekII; Nunc Brand, Rochester, NY, USA) coated with 10 μl of Matrigel (BD Biosciences, San Jose, CA, USA) (250 000 cells/well) which are the conditions used for the invasion assays (see below). Cells were incubated

in serum-free F12K medium containing human recombinant leptin (Sigma) at 0, 80 or 160 ng/ml. Two wells per concentration were evaluated in each experiment and the experiments were repeated three times. The medium was replaced with fresh leptin-containing serum-free medium at 24 h. Bromodeoxyuridine (BrdU, 10 μM; Roche) was also added to the culture medium after 24 h of culture to assess proliferation. After an additional 24 h culture (48 h total culture time), the slides were washed three times in PBS, fixed in 3% paraformaldehyde for 20 min at 4 °C and permeabilized with ice-cold methanol for 5 min. Slides were stored at −20 °C until subsequent staining. Slides were rehydrated in PBS and incubated for 30 min in 2 M HCl and again washed in PBS. Slides were pre-blocked overnight at 4 °C (10% (w/v) BSA, 10% (v/v) normal goat serum in PBS). Slides were then blocked in 2% (w/v) BSA for 20 min at room temperature and incubated in the presence of anti-BrdU antibody (1:100, Abcam, Cambridge, MA, USA) overnight at 4 °C. After washing in PBS, slides were incubated with anti-cytokeratin-18 caspase-3-specific cleavage peptide antibody (M30, 1:1000; Roche) for 1 h at room temperature to assess apoptosis. Slides were washed again and then incubated in a mixture of anti-rat 488 alexa fluor antibody (for BrdU) and anti-mouse 568 alexa fluor antibody (for M30) (1:1000; Invitrogen) for 1 h at room temperature in the dark. Slides were mounted in DAPI (to label the nuclei) containing mounting medium (Vector Laboratories). Negative controls with no BrdU or no primary antibody were also evaluated. BrdU-positive nuclei, M30-positive cytoplasmic staining and total nuclei were quantified from five high-power fields per well. Counts were summed from all fields per well, averaged across like wells and are reported as percent of total cells positive for BrdU or M30.

Invasion assays

BeWO empty vector and Siglec-6 expressing cell lines were plated on Matrigel-coated invasion chambers in a 24-well format plate (Costar, Corning, NY, USA) (250 000 cells/well). The invasion chambers were incubated in serum-free F12K medium containing 0, 80 or 160 ng/ml leptin in both the upper and lower chambers. Leptin-containing medium was replenished at 24 h and the invasion cultures were harvested at 48 h. The invasion chambers were washed three times in PBS, fixed in 3% paraformaldehyde at 4 °C for 20 min. Cells were then permeabilized for 5 min in ice-cold methanol and stored at −20 °C. The chambers were rehydrated in PBS, invasion membranes were cut out of the chambers and mounted in DAPI-containing mounting medium. Invasion was quantified by counting invasive nuclei for the entire membrane. Average invasion per leptin concentration was normalized to the average at 0 ng/ml leptin in the empty vector cells for each experiment. Invasion was corrected for absolute cell number in each experiment to account for differences that may arise from alterations in apoptosis and proliferation. To determine absolute cell number, cells from each cell line were plated on 96-well plates (BD Biosciences) coated with 10 μl of Matrigel (250 000 cells/well). Cells were incubated in serum-free medium containing human recombinant leptin at 0, 80 or 160 ng/ml. The medium was replaced at 24 h and the cells retrieved by trypsinization and mechanical disruption at 48 h. Cell number was quantified using a hemocytometer. Two counts per well from two to three wells were averaged per experiment at each leptin concentration. Absolute cell number at 48 h was normalized to the cell number of cells plated at the beginning of the experiment.

Statistical analysis

Student's *t*-test was used to compare differences between two groups. One-way ANOVA with Bonferroni *post hoc* test for multiple comparisons were performed to determine statistically significant differences in all other comparisons. Differences were considered significant when $P < 0.05$.

Results

Analysis of Siglec-6 expression in early gestation

We first established the normal pattern of Siglec-6 expression in the human placenta prior to 24 weeks gestation. Immunohistochemical analysis showed that, in normal placentas, Siglec-6 immunoreactivity was restricted to trophoblasts and decreased over the span of early gestation. Four types of trophoblasts were visualized in the normal samples. The stromal core of the chorionic villi is lined by an inner layer of cytotrophoblasts (CTB) that is covered by an outer layer of syncytiotrophoblasts (STB). Siglec-6 immunoreactivity was noted in both CTB and STB, but never in villous stromal cells (Fig. 1A, B, C and D). A third type of trophoblasts, cell column (CC) CTBs, are normally proliferative trophoblasts found at the tips of chorionic villi. CC CTBs were variably positive for Siglec-6 (Fig. 1A, B, C and D). The fourth type of trophoblasts, extra-villous trophoblasts (EVT), are found in the decidua basalis, also referred to as the basal plate. EVT are trophoblasts that have left the CCs and invaded

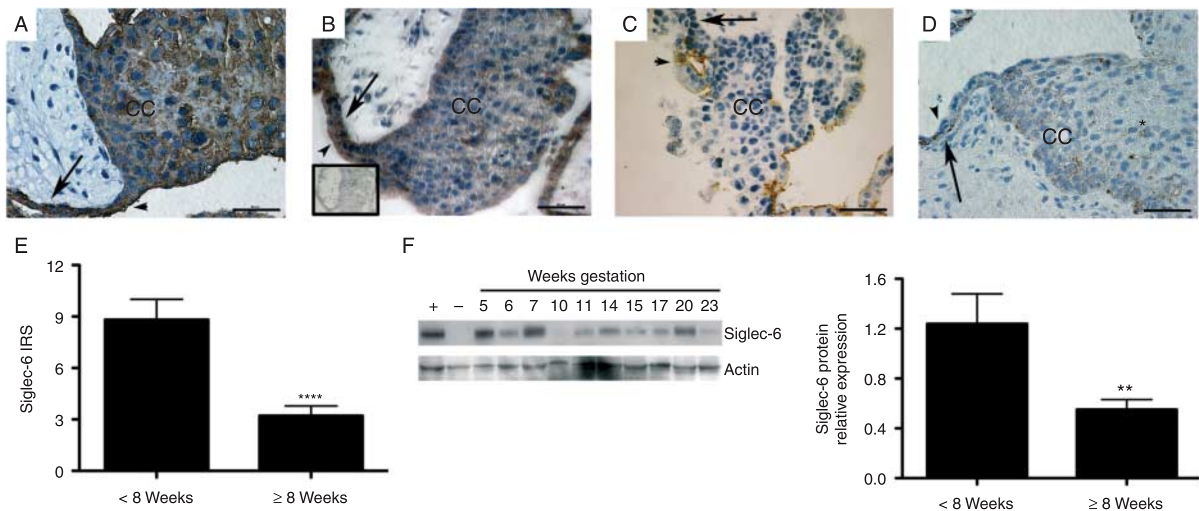

Figure 1 Siglec-6 is expressed in trophoblasts and decreased after 8 weeks gestation in normal placenta. Siglec-6 protein expression was evaluated by immunohistochemistry in formalin-fixed, paraffin-embedded human placental biopsy tissue sections. Representative images across gestation are shown. Blue (hematoxylin) marks nuclei and brown (DAB) indicates Siglec-6. Villous CTB are indicated with arrows, villous STB are indicated with arrowheads, CC denotes the cell column CTBs, EVT are indicated with an asterisk. The scale bar represents 50 μm. (A) 6 Weeks (B) 9 weeks (C) 11 weeks and (D) 16 weeks of gestation. The inset in B shows a representative negative control. (E) Siglec-6 immunoreactivity, as demonstrated by semiquantitative IRS, is higher in normal tissue prior to 8 weeks gestation ($n=11$) and lower thereafter (8–20 weeks gestation $n=21$). (F) Representative immunoblots of Siglec-6 and actin (loading control) protein expression across gestation are shown. Siglec-6 overexpressing BeWO cells are shown as the positive control (+), and BeWO cells transfected with empty vector are shown as the negative control (−). Three immunoblots with different samples showed consistent results. Quantitation of Siglec-6 protein levels was determined from immunoblots (normalized to actin and relative to expression of 7 week gestation sample run on each blot). <8 weeks ($n=5$), 8–24 weeks ($n=23$). ** $P<0.01$, **** $P<0.001$.

into the maternal decidua. Siglec-6 immunoreactivity was largely absent in the basal plate, as maternal decidual cells did not express Siglec-6 and rarely some EVT were positive for Siglec-6 (Fig. 1D). Siglec-6 immunoreactivity in all trophoblast populations was greatest in early gestation and decreased as gestation progressed. Siglec-6 IRS, based on stain intensity and percent positive trophoblasts, was carried out to provide semiquantitative analysis. Based on IRS, the decline in immunoreactivity occurred at 8 weeks gestation (Fig. 1E). Immunoblotting analysis of placental villous lysates for Siglec-6 protein confirmed the observation that Siglec-6 expression was greatest before 8 weeks gestation and lower thereafter (Fig. 1F).

Analysis of Siglec-6 expression in GTD

Having established the normal expression pattern of Siglec-6 in normal human placental tissue we next evaluated Siglec-6 expression in HA. HA, like molar placentas, have villi with edematous changes. However, HA lack abnormal trophoblast proliferation characteristic of molar placentas. Nonetheless, HA are often challenging to distinguish from molar placentas. This is especially true in early gestation when normally proliferative CC can be mistaken for abnormal molar trophoblast proliferation. In HA, immunoreactivity was present on few villi and was discontinuous along the villous trophoblast layer (Fig. 2B). This expression pattern in HA was similar to what was observed in normal samples after ∼8 weeks gestation, suggesting that the edematous changes characteristic of HA do not alter Siglec-6 expression. All but two of the pathologic samples collected were ≥8 weeks gestation (one PM and one CM were <8 weeks gestation); molar pregnancies are rarely diagnosed and treated before 8 weeks gestation. Because of this clinical reality as well as the high levels of Siglec-6 before 8 weeks gestation in normal placentas, data presented in Fig. 2 include only samples ≥8 weeks gestation. As in normal placental samples, Siglec-6 immunoreactivity in pathologic samples was limited to trophoblast populations. In PM samples, Siglec-6 immunoreactivity was present on some but not all villi. In contrast to HA, however, the immunoreactivity was generally more intense and circumferential including all villous trophoblasts (Fig. 2C). In CM, Siglec-6 immunoreactivity was present on the majority of the villi and again generally circumferential (Fig. 2D). Given this differential Siglec-6 expression pattern semiquantitative analysis was performed.

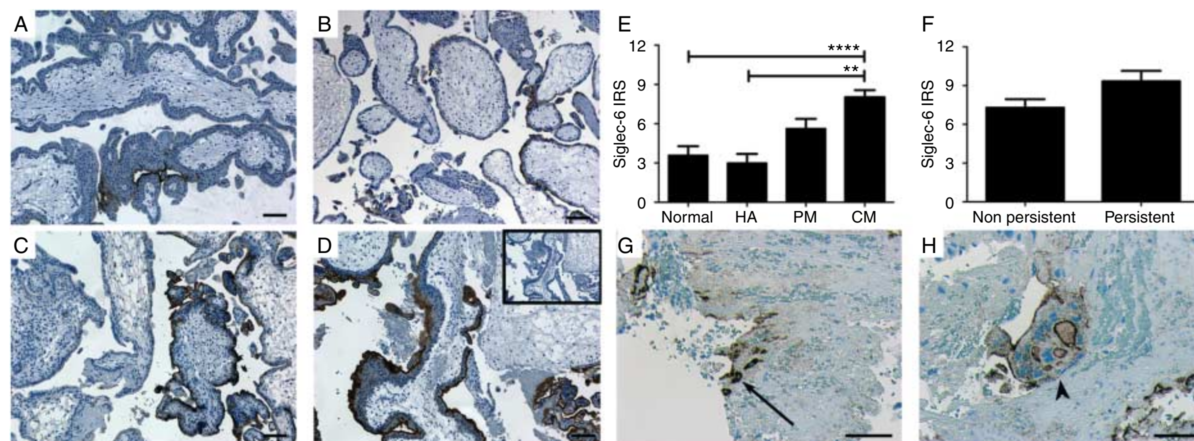

Figure 2 Siglec-6 immunoreactivity is highest in complete molar placentas. Siglec-6 protein expression was evaluated by immunohistochemistry in formalin-fixed, paraffin-embedded human placental biopsy tissue sections. Blue (hematoxylin) marks nuclei and brown (DAB) indicates Siglec-6. Representative images from each group are shown. The scale bar represents 50 μm. (A) Normal placenta, (B) hydropic abortus (HA) placenta, (C) partial molar (PM) placenta and (D) complete molar (CM) placenta. The inset in D shows a representative negative control. (E) Semiquantitation of Siglec-6 expression by immunoreactivity score (IRS) for normal ($n=21$, as for Fig. 1), HA ($n=7$), PM ($n=11$) and CM ($n=24$). (F) Comparison of Siglec-6 levels by IRS for CM samples separated into groups of non-persistent disease ($n=15$) and persistent disease ($n=9$). Choriocarcinoma showing (G) Siglec-6 positive CTBs (indicated by an arrow); and (H) Siglec-6 positive STBs (indicated by an arrow head) ($n=3$). $^{**}P<0.01$, $^{****}P<0.001$.

By IRS, CM had significantly higher Siglec-6 immunoreactivity than either HA or normal placentas. There was no difference in IRS between normal controls and HA samples. IRS for PM was intermediate between controls and CM, but not statistically different from these groups (Fig. 2E). CM samples were further analyzed to determine if Siglec-6 immunoreactivity is predictive of persistent disease. CM were grouped by those cured by D&C alone (non-persistent) or those requiring D&C plus subsequent chemotherapy or surgery for cure (persistent disease). All samples were taken at the time of D&C, before any initiation of chemotherapy. Subject characteristics of the non-persistent and persistent CM samples are presented in Table 1 and differed with respect to maternal age. Although persistent CM had a higher average Siglec-6 IRS, the difference was not significant (Fig. 2F). Therefore, Siglec-6 expression does not show utility in predicting CM persistent disease.

The expression of Siglec-6 in GTN was also evaluated. Siglec-6 expression was absent or only focally present in a minority of cells in GTN samples, unlike in molar placentas where Siglec-6 expression was abundant. In CCA, Siglec-6 expression was detected in both CTB (Fig. 2G) and STB (Fig. 2H). PSTT, which lack STB, had rare, focal immunoreactivity in CTB (data not shown). Metastases of CCA to lung and vagina showed very low, infrequent Siglec-6 immunoreactivity (data not shown). Siglec-6 immunoreactivity was always negative in the normal tissues surrounding the GTN lesions with the exception of infiltrating B-cells, which are known to be Siglec-6 positive (Brinkman-Van der Linden *et al*. 2007). Reproductive tumors of nongestational origin were also evaluated as additional controls. A nongestational choriocarcinoma from a male patient and an endometrial sarcoma both lacked Siglec-6 immunoreactivity (data not shown). Contrary to our hypothesis we did not see an increase in Siglec-6 expression in GTN compared with molar placentas.

Functional consequences of Siglec-6 expression and leptin treatment on BeWO cells

To determine if the increased expression of Siglec-6 in molar placentas might have functional consequences, we investigated the effects of overexpressing Siglec-6 in BeWO cells. BeWO cells are a trophoblast cell line derived from a choriocarcinoma that lacks any endogenous Siglec-6 expression. BeWO cells transfected with empty vector expressed LepR but not Siglec-6 (empty vector controls), as determined by qPCR. Siglec-6 overexpressing cells expressed both LepR and Siglec-6, as determined by qPCR. LepR expression was not affected by expression of Siglec-6 (data not shown). Siglec-6 protein expression of Siglec-6 overexpressing and empty vector BeWO cell lines are shown in Fig. 1F (+ and − respectively). We also evaluated how leptin, a known Siglec-6 ligand, modified Siglec-6 effects on BeWO proliferation, apoptosis and invasion. Outside of pregnancy, serum

leptin levels increase in proportion to a woman's BMI. During pregnancy, serum leptin levels are further augmented by placental production and increase throughout gestation. In both molar pregnancies and GTN, serum leptin levels are higher compared with normal pregnancies (Masuzaki *et al*. 1997, Sagawa *et al*. 1997). To test serum leptin levels similar to those reported for normal pregnancy (20–80 ng/ml range) and GTD pregnancies (25–120 ng/ml range), we evaluated 0, 80 and 160 ng/ml leptin.

Siglec-6 and leptin act together to promote BeWO proliferation

Leptin treatment of Siglec-6 expressing BeWO cells promoted proliferation. Proliferation was evaluated by quantifying cells that incorporated BrdU after culture on Matrigel in the presence or absence of leptin (Fig. 3A). Leptin had no effect on proliferation of empty vector control cells. In cells that express Siglec-6 but were not treated with leptin, proliferation was no different from controls. However, leptin treatment of Siglec-6 expressing cells increased proliferation (Fig. 3B). This finding suggests that leptin acts through Siglec-6, not LepR, to stimulate proliferation, demonstrating that Siglec-6 produces physiologic effects in response to leptin.

Siglec-6 expression decreases BeWO apoptosis

Siglec-6 expression protected BeWO cells from apoptosis, independent of leptin treatment. Apoptotic cells were identified by immunofluorescently labeling cells with the M30 antibody that detects a caspase-3-specific cleavage product of cytokeratin-18 (Fig. 4A). In empty vector control cells, apoptosis decreased with increasing leptin dose but these data did not reach statistical significance. Siglec-6 expression, in the absence of leptin treatment, protected cells from apoptosis. The protection from apoptosis in Siglec-6 expressing cells was not affected by leptin dose (Fig. 4B). In contrast to the cooperative effect of leptin and Siglec-6 on proliferation, only Siglec-6 overexpression protected cells from apoptosis. These data demonstrate that Siglec-6 has actions on apoptotic pathways independent of leptin. This effect could be due solely to Siglec-6 overexpression or the effect could be from Siglec-6 interactions with a ligand present within the culturing system – either a *cis* ligand present on BeWO cells or a *trans* ligand in the Matrigel.

Both Siglec-6 and leptin impact BeWO invasive capacity

Leptin treatment and Siglec-6 expression independently promoted BeWO invasion. However, the combined effect impaired the Siglec-6-induced

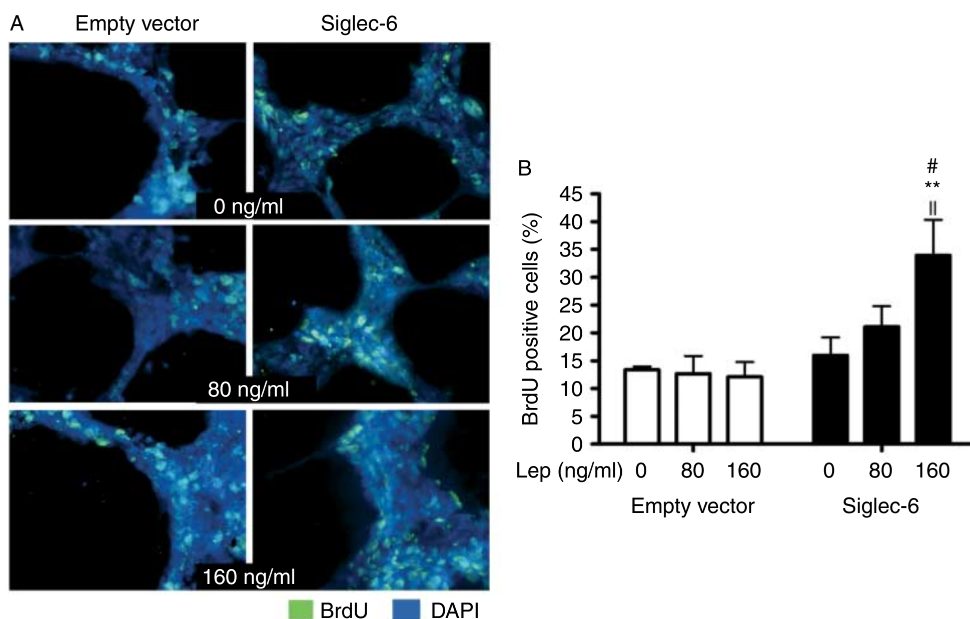

Figure 3 Leptin promotes proliferation of Siglec-6 overexpressing BeWO cells. (A) Representative images of BeWO empty vector and Siglec-6 overexpressing cells cultured on Matrigel and treated with the indicated leptin concentrations. Green fluorescence shows proliferative cells that have incorporated BrdU. DAPI labeling marks nuclei blue. (B) Percent of cells positive for BrdU ($n=6$ per concentration per cell line). $^{\|}P<0.01$ vs empty vector cells treated with 160 ng/ml leptin, $^{**}P<0.01$ vs Siglec-6 cells treated with 0 ng/ml leptin, $^{\#}P<0.05$ vs Siglec-6 cells treated with 80 ng/ml leptin.

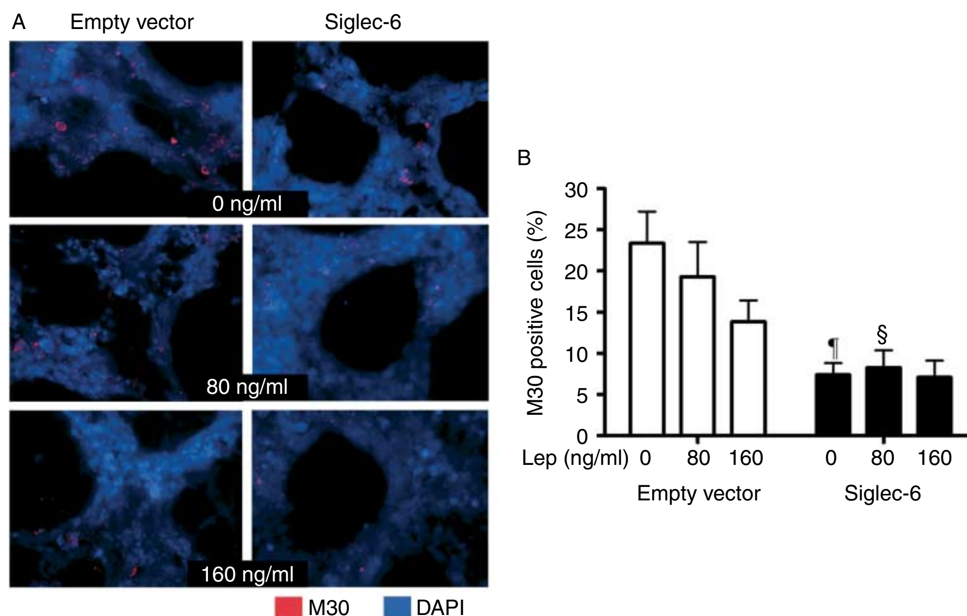

Figure 4 Siglec-6 protects BeWO cells from apoptosis. (A) Representative images of BeWO empty vector and Siglec-6 overexpressing cells cultured on Matrigel and treated with the indicated leptin concentrations. Red fluorescence shows apoptotic cells as assessed by immunoreactivity with M30 antibody, which is specific to a caspase-3-specific cleavage product of cytokeratin-18. DAPI labeling marks nuclei blue. (B) Percent of cells positive for M30 immunoreactivity ($n=6$ per concentration per cell line). [¶]$P<0.01$ vs empty vector cells treated with 0 ng/ml leptin, [§]$P<0.05$ vs empty vector cells treated with 80 ng/ml leptin.

invasive response. BeWO invasive capacity was measured using a Matrigel-coated transwell invasion assay. In empty vector control cells, leptin promoted invasion. Siglec-6 expression, in the absence of leptin treatment, also promoted BeWO invasion. However, in Siglec-6 expressing cells, treatment with leptin resulted in decreased invasion relative to no leptin treatment (Fig. 5). To control for the effects of Siglec-6 and leptin on cell proliferation and apoptosis, the invasion data in Fig. 5 have been normalized to absolute cell number. While both leptin and Siglec-6 independently promote invasion, they interact to decrease invasion. This result suggests that leptin signaling through LepR promotes invasion and Siglec-6 expression also independently promotes invasion. However, the Siglec-6 promotion of invasion is decreased in the presence of leptin, suggesting cross-talk that negatively impacts the independent responses.

Discussion

These studies characterized Siglec-6 expression in normal placentas prior to 24 weeks gestation and in GTD. In normal placentas, Siglec-6 was expressed by trophoblasts and expression was high prior to 8 weeks gestation and low thereafter. An important physiologic change that occurs in human placentas around 8 weeks of gestation is initiation of blood flow to the placenta. Blood flow is thought to initially be intermittent and then increases to continual flow by 12 weeks gestation (Jauniaux *et al*. 2000). The increase in oxygenation as well as exposure of chorionic villi to blood components may play a role in the downregulation of Siglec-6 at this time. Downregulation of placental protein expression in early gestation with increased oxygenation has been shown for other molecules such as sFlt-1 (Nevo *et al*. 2006), Cyclin B (Genbacev *et al*. 1997) and annexin II (Hoang *et al*. 2001).

Among the pathologic samples evaluated, CM had the highest Siglec-6 expression. Expression was low in HA samples and moderate in PM samples. These results are consistent with the hypothesis that Siglec-6 expression increases with severity among molar placentas. Importantly, Siglec-6 may be useful in distinguishing CM placentas from non-molar placentas (normal and HA) after 8 weeks gestation, a current diagnostic challenge. However, expression was not significantly higher in CM relative to PM. Currently, evaluation of placental histology and occasionally p57 staining is used to confirm clinical suspicion of molar pregnancy and distinguish CM from PM and HA. However, several reports suggest that accurate diagnosis of PM and CM is subject to up to 30% inter-observer differences (Popiolek *et al*. 2006,

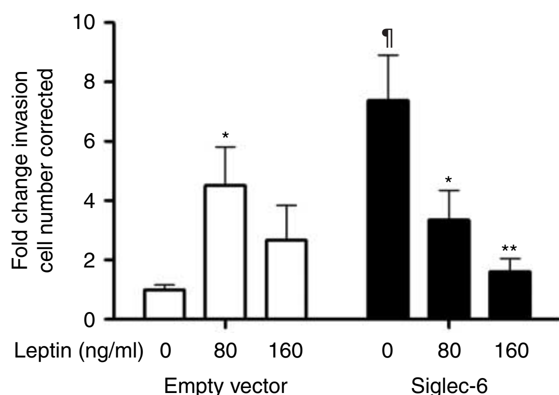

Figure 5 Leptin and Siglec-6 interact to alter BeWO cell invasion. The number of BeWO empty vector and Siglec-6 overexpressing cells that invaded through Matrigel in the presence of the indicated leptin concentrations was quantified and normalized to absolute cell number. Data are reported as fold change from invasion at 0 ng/ml in the empty vector cell line. Leptin treatment promoted invasion of empty vector BeWO cells. Siglec-6 overexpression alone (0 ng/ml) also promoted BeWO cell invasion relative to no Siglec-6 expression. However, in Siglec-6 overexpressing cells, leptin decreased invasion relative to no leptin treatment. Experiments were repeated three times ($n=6$ per concentration and cell line). ¶$P<0.01$ vs empty vector cells treated with 0 ng/ml leptin, *$P<0.05$ vs like cell type treated with 0 ng/ml leptin, **$P<0.01$ vs like cell type treated with 80 ng/ml leptin.

Maggiori & Peres 2007, Hoffner *et al*. 2008, LeGallo *et al*. 2008, McConnell *et al*. 2009, Kipp *et al*. 2010). Although Siglec-6 immunoreactivity does not show utility in distinguishing CM from PM, Siglec-6 may have utility for diagnosis of CM after 8 weeks gestation in conjunction with current methods or as part of a panel of novel markers. Siglec-6 expression did not show utility in identifying CM that will be persistent. Among the CM samples, there was a significant difference in maternal age between the non-persistent and persistent groups. Extremes of age is a known risk factor for molar pregnancy (<20 or >40 years of age). In the CM non-persistent sample group, seven (47%) patients were <20 years of age and none were over 40 years of age. By contrast, among the CM persistent sample group, none were <20 years of age and two (22%) were over 40 years of age. While this result could reflect selection bias, the striking segregation of disease persistence by age group is consistent with previous studies finding age >40 as a risk factor for persistent disease (Parazzini *et al*. 1988, Tangtrakul *et al*. 1990). As with Siglec-6, LepR expression also does not predict persistent disease (Li *et al*. 2004). Distinguishing different molar pregnancies remains an ongoing challenge. Larger prospective studies will be needed to fully evaluate a role for Siglec-6 as a diagnostic marker.

An unexpected finding of the immunohistochemistry studies was that Siglec-6 expression was focal or absent in GTN and metastases. Four of the five GTN samples were from patients with no documented history of prior molar pregnancy. It is possible that the low level of Siglec-6 expression in GTN derived from nonmolar pregnancies reflects the low level of expression in the originating nonmolar pregnancy. For GTN that originate from CM, Siglec-6 may play a role in the early progression of trophoblasts into CCA, consistent with the pro-proliferation, anti-apoptotic, pro-invasion phenotype of Siglec-6 expressing cells. However, Siglec-6 may no longer play an important role at the metastatic site, as suggested by the low Siglec-6 expression in metastasis samples. Such molecular behavior of enhancing tumor formation but not being required for metastasis has been shown for HER2/neu in breast cancer. In a mouse model, induction of HER2/neu led to mammary gland tumor development. Tumors regressed after removal of HER2/neu expression but eventually recurred and metastasized in an HER2/neu-independent manner (Moody *et al*. 2002, 2005). Due to the limited number of samples evaluated, a role for Siglec-6 in GTN remains speculative.

Our studies demonstrate that Siglec-6 and leptin have functional consequences on BeWO cell proliferation, apoptosis and invasion. The *in vitro* studies permitted the evaluation of three different ligand–receptor situations (Fig. 6). In these studies, we cultured the cells on Matrigel, which models conditions of trophoblast invasion into maternal tissues. BeWO cells normally express LepR (our findings and Challier *et al*. (2003) and Wyrwoll *et al*. (2005)) but not Siglec-6 (our findings and Brinkman-Van der Linden *et al*. (2007)). The effects of leptin/LepR interactions on BeWO behavior were evaluated in empty vector control cells (Fig. 6, scenario A). Leptin acting through LepR does not affect proliferation or apoptosis. The inability of leptin at physiologic concentrations to promote BeWO proliferation is consistent with other studies (Magarinos *et al*. 2007). With respect to apoptosis, previous work found that 160 ng/ml leptin can protect cultured choriocarcinoma cells from apoptosis when they are cultured on plastic (Pérez-Pérez *et al*. 2008). While our data at this dose suggested a protection from apoptosis of BeWO cells cultured on Matrigel, the data were not statistically significant. It is possible that culture differences explain the differences between these two studies; trophoblasts cultured on plastic adopt a syncytial phenotype whereas culture on Matrigel induces an invasive phenotype. However, because our studies

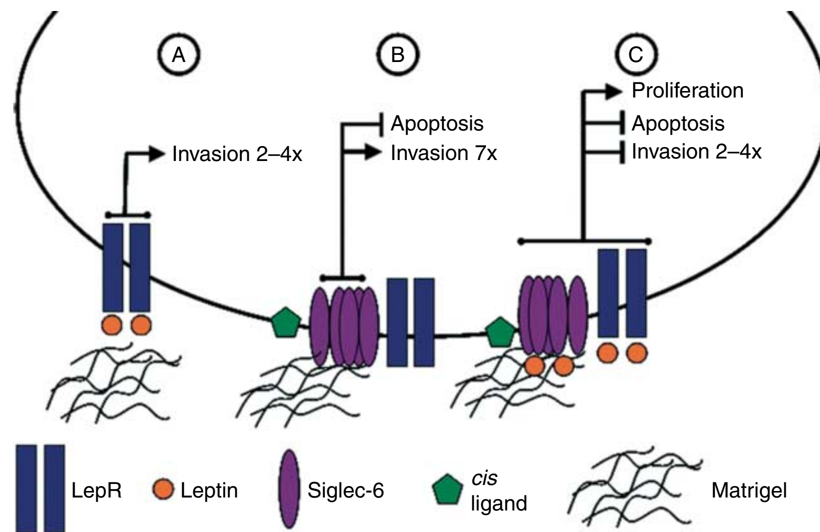

Figure 6 Summary of leptin and Siglec-6 responses in GTD as modeled using the BeWO cell line. In scenario A, empty vector cells are cultured on Matrigel and treated with leptin. Leptin acting through LepR promotes invasion two- to four-fold without affecting proliferation or apoptosis. Scenario B depicts Siglec-6 overexpressing cells cultured on Matrigel in the absence of leptin. Under these conditions Siglec-6 can interact with a ligand in *cis* or with a ligand in Matrigel. Although LepR is present, there is no leptin to engage LepR. Siglec-6 actions result in a seven-fold promotion of invasion and a protection from apoptosis. In scenario C, Siglec-6 overexpressing cells cultured on Matrigel are treated with leptin. Under these conditions, leptin can act through LepR or Siglec-6. Further, Siglec-6 can also engage a *cis* ligand or Matrigel ligand. Invasion is increased two- to four-fold, as in scenario A, suggesting that leptin preferentially engages LepR (as in A) or that leptin acting through Siglec-6 decreases Siglec-6-induced invasion (compared with B). The protection of apoptosis is unchanged from scenario B suggesting that leptin does not alter Siglec-6 protection from apoptosis. Also in scenario C, proliferation is increased. Because proliferation is not changed in scenario A or B, this suggests that leptin acting through Siglec-6 accounts for the promotion of proliferation.

were larger ($n=6$ vs 1), we conclude that leptin does not protect BeWO cells from apoptosis. As shown in scenario A, leptin/LepR interactions promote BeWO invasion. With respect to the expression studies, CCA express LepR but not Siglec-6, and would be exposed to leptin. Thus, leptin action on CCA cells may be part of the highly invasive nature of this type of tumor.

The second ligand/receptor interactions evaluated by our *in vitro* studies were Siglec-6 interactions with non-leptin ligands (Fig. 6, scenario B). BeWO proliferation is unaffected by expression of Siglec-6. However, Siglec-6 expression results in a decrease in BeWO cell apoptosis and sevenfold increase in invasion compared with cells that lack Siglec-6. These data show that Siglec-6 has functions independent of leptin. Siglec-6 could be acting in *cis* with another cell surface ligand or in *trans* with a ligand in the Matrigel. Such ligands, either sialylated or not, are yet to be identified. Evidence for both *cis* and *trans* mechanisms have been demonstrated for other Siglecs including myelin-associated glycoprotein (MAG, Siglec-4) and CD22 (Siglec-2) (reviewed in Crocker (2002)). This study demonstrates that Siglec-6 expression protects trophoblast cells from apoptosis and promotes invasion. These findings expand on the recently published work of Lam *et al*. (2011) showing that in normal trophoblasts, GdA (a maternal protein) interacts with Siglec-6 (a fetal protein) to suppress trophoblast invasion. However, in the context of molar pregnancies, where GdA is low (Makovitzky *et al*. 2009), and Siglec-6 expression is high (our findings), invasion is high. Our data suggest that in molar placentas, excess Siglec-6 would overwhelm the suppressive effects of GdA on trophoblast invasion.

The third ligand/receptor interactions evaluated by our *in vitro* studies were leptin actions through both LepR and Siglec-6 in the context of Matrigel (Fig. 6, scenario C). This scenario would most closely resemble the situation for patients with CM. Because proliferation is not affected by leptin acting through LepR or Siglec-6 expression alone, leptin acting through Siglec-6 promotes cell proliferation. The hyper-proliferation characteristic of molar placentas may well be influenced by leptin and Siglec-6 interactions. In scenario C, apoptosis was unchanged from scenario B, suggesting that Siglec-6 acts independently of leptin to protect cells from apoptosis. In scenario C, there is a decrease in invasion relative to no leptin treatment (scenario B) but equivalent to leptin/LepR promotion of invasion (scenario A). Because leptin has a 10-fold higher affinity for LepR than Siglec-6 (Patel *et al*. 1999), it is possible that

leptin effects on invasion are exclusively through LepR in scenario C. Alternatively, in the presence of leptin there is a crosstalk between these receptors, which results in an intermediate invasive phenotype. Another possibility is that leptin is being sequestered at the cell surface by Siglec-6 and thus altering interactions with LepR.

Overall, these studies characterize the expression pattern of Siglec-6 in first and second trimester human placentas and GTD. Further, they demonstrate that Siglec-6 expression has functional consequences on trophoblast biology both independent of and in response to leptin treatment. Siglec-6 expression changes with gestational age, by disease state, and may be a useful marker for diagnosing CM pregnancies. Because of the high levels of expression in CM placentas and its limited tissue expression in normal tissue (trophoblasts and B-cells), Siglec-6 is an interesting candidate to consider for developing novel treatments for molar pregnancies and may have a reasonable safety profile. Our findings that Siglec-6 protects from apoptosis and promotes invasion, and that the treatment of Siglec-6 expressing cells with leptin results in proliferation suggests that Siglec-6 and leptin play a role in the aberrant proliferation and invasion of trophoblasts in complete molar placentas. Importantly, these *in vitro* studies demonstrate that Siglec-6 expression impacts cellular biology and provides evidence in support that Siglec-6 is indeed a transmembrane leptin receptor.

Declaration of interest

M D Post, R S Larivee, M Zink, J Uyenishi, A Kramer, D Teoh and K Bogart have nothing to declare. K K Rumer and V D Winn have research grants as listed below.

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Author contribution statement

K K Rumer performed the immunostaining, immunoreactivity scoring, Western blotting, invasion assays and wrote the manuscript. M D Post reviewed histopathology, performed immunoreactivity scoring and provided intellectual expertise. R S Larivee performed the proliferation and apoptosis assays. M Zink, J Uyenishi and K Bogart performed immunostaining. A Kramer acquired samples, clinical data and performed immunostaining. D Teoh acquired samples and clinical data. V D Winn supervised all the work, provided material support and intellectual expertise. All authors reviewed and edited the manuscript.

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