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Core 2 β1,6-N-acetylglucosaminyltransferases accelerate the escape of choriocarcinoma from natural killer cell immunity

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ABSTRACT

Hyperglycosylated human chorionic gonadotropin (H-hCG) is secreted from choriocarcinoma and contains a core2 *O*-glycan formed by core2 β1,6-N-acetylglucosaminyl transferase (C2GnT). Choriocarcinoma is considered immunogenic as it is gestational and contains paternal chromosomal components. Here we examined the function of C2GnT in the evasion of choriocarcinoma cells from natural killer (NK) cell-mediating killing. We determined that C2GnT is highly expressed in malignant gestational trophoblastic neoplasms. C2GnT KO downregulates core2 *O*-glycan expression in choriocarcinoma cells, which are more efficiently killed by NK cells than control cells. C2GnT KO cell containing tumor necrosis factor-related apoptosis inducing ligand have lower viability than control cells. Additionally, poly-*N*-acetyllactosamine in core2 branched oligosaccharides on MHC class I-related chain A (MICA) and mucin1 (MUC1) is significantly reduced in C2GnT KO cells. Meanwhile, the cumulative survival rate of nude mice inoculated with C2GnT KO tumors was higher than that of the control group. These findings suggest that choriocarcinoma cells may escape NK cell-mediated killing via glycosylation of MICA and MUC1.

1. Introduction

Gestational trophoblastic diseases (GTDs) comprise a generic disease group characterized by aberrant proliferation of atypical placental trophoblasts. Choriocarcinoma accounts for the most aggressive and malignant GTD and can develop after pregnancy. The primary treatment for choriocarcinoma is chemotherapy, including methotrexate, etoposide, and actinomycin-D [1]. The remission rate of choriocarcinoma is ~80% with primary treatment, and the survival rate is ~90% [2,3]. However, the prognosis is poor for the remaining 10% who develop metastasis, except that of the lung, and for those resistant to chemotherapy as the primary treatment [2], and thus, it is important to establish effective treatments for such patients.

Human chorionic gonadotropin (hCG) is produced by trophoblastic cells in pregnant women as well as by GTD patients. Hyperglycosylated human chorionic gonadotropin (H-hCG) is an hCG variant detected in choriocarcinoma patients, but not in molar patients or pregnant women [4]. H-hCG contains *N*- and *O*-glycans that differ from those of hCG and has important functions in the invasion and proliferation of choriocarcinoma cells [4–6]. Core2 β-1,6-N-acetylglucosaminyltransferase (C2GnT) is a key enzyme responsible for the core2 branch of H-hCG *O*-glycans; however, its role in choriocarcinoma remains unclear. Meanwhile, C2GnT is reportedly positively correlated with the progression of several tumor types [7–9] and participates in bladder cancer immunogenicity. Moreover, tumors with high C2GnT expression are resistant to natural killer (NK) cell immunity [9,10]. Since primary trophoblasts from normal pregnant women and choriocarcinoma cell lines rarely express human leukocyte antigen (HLA) class I [11], their primary immune response against choriocarcinoma is believed to involve NK cells. Hence, C2GnT may be associated with NK cell immunity in choriocarcinoma. This study aimed to examine the role of C2GnT in choriocarcinoma cells, particularly in relation to their evasion of NK

Abbreviations: C2GnT, core2 beta 1, 6-N acetylglucosaminyl transferase; DR4, death receptor 4; EVT, extravillous trophoblast; GTD, gestational trophoblastic disease; GTN, gestational trophoblastic neoplasm; hCG, human chorionic gonadotropin; H-hCG, hyperglycosylated human chorionic gonadotropin; HLA, human leukocyte antigen; LEL, *Lycopersicon esculentum* lectin; MICA, MHC class I-related chain A; MUC1, mucin1; NKG2D, natural killer group 2 member D; PSTT, placental site trophoblastic tumor; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

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cell immunity.

2. Materials and methods

2.1. Cells and culture conditions

Human choriocarcinoma cell lines, Jar, BeWo, and JEG3, were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were mycoplasma-free. Human extravillous trophoblast (EVT) cell line HTR-8/SVneo was provided by Dr. Charles H. Graham (Queen's University, Ontario, Canada) [12]. Cell lines were maintained in RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), at 37 °C and 5% CO_2.

2.2. Sample collection

Nine-week placenta samples were obtained from women undergoing elective pregnancy termination; 26- and 39-week samples were collected during elective cesarean section before onset of labor pain. GTD tissues, including hydatidiform mole ($n = 9$), placental site trophoblastic tumor (PSTT; $n = 4$), and choriocarcinoma ($n = 15$), were obtained from patients undergoing surgical treatment. Informed consent was obtained from patients for collection of all samples. All samples were washed with phosphate-buffered saline (PBS), immediately frozen in liquid nitrogen, and stored at −80 °C until protein extraction. This study was conducted in accordance with the ethics committee of Nagoya University Graduate School of Medicine (approval number 2017-0053).

2.3. Immunohistochemistry

Formaldehyde-fixed and paraffin-embedded tissue sections (4-μm-thick) were immunostained [13]. Primary antibodies against C2GnT1 (Wako, Osaka, Japan), human CD57 (BD Biosciences, San Jose, CA, USA), MHC class I-related chain A/B (MICA/B) (Biolegend, San Diego, CA, USA), and mouse NK1.1 (Biolegend) were used. The tissue sections were incubated for 30 min at room temperature (23–25 °C) with HRP-conjugated anti-mouse or rabbit IgG secondary antibodies (EnVision System; Dako), followed by signal detection using 3,3′-diaminobenzidine solution (Dako).

2.4. Western blot

Western blotting was performed as previously described [14] using the anti-C2GnT1 (Wako), anti-MICA/B (Biolegend), anti-death receptor 4 (DR4) (Funakoshi, Tokyo, Japan), and anti-mucin1 (MUC1) (Abcam, Cambridge, UK) antibodies. Immunoreactive proteins were stained using a chemiluminescence detection system (ECL; Amersham, Chicago, IL, USA). An antibody against β-actin (AC-15, Sigma-Aldrich) was used as the loading control.

2.5. Cytotoxicity assay

Human primary NK cells were purified from peripheral blood mononuclear cells using the NK Cell Isolation kit (Miltenyi Biotec, Auburn, CA, USA). Mouse primary NK cells were purified from BALB/c mouse spleens and femoral bones using the Mouse NK Cell Isolation kit (Miltenyi Biotec). Cytotoxicity was assessed using the Cytotox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, MI, USA). NK cells (1×10^6 cells/mL) were cultured for 5 days in NK MACS medium (Miltenyi Biotec) supplemented with 5% FBS and 1000 units/mL human recombinant IL-2 (Wako, Osaka, Japan). Target choriocarcinoma cells were incubated with IL-2-activated NK cells for 4 h at 37 °C, at three effector-to-target ratios (20:1, 10:1, 4:1). Lactate dehydrogenase released from lysed target cells was measured. To investigate the interaction between galectin-3 and NK cells in choriocarcinoma, cells were incubated with endo-β-galactosidase (80 mU/mL) at 37 °C for 8 h before assaying.

2.6. Cell viability assay

Cells (5×10^3 per well) were seeded in 96-well plates and incubated with the indicated concentration of recombinant tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (R&D Systems, Minneapolis, MN, USA) at 37 °C for 24 h. Cell viability was determined using the modified tetrazolium salt (MTS) assay with the CellTiter 96 Aqueous One Solution Proliferation Assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. To investigate the effect of galectin-3 under TRAIL treatment in choriocarcinoma, cells were incubated with endo-β-galactosidase (80 mU/mL) at 37 °C for 8 h and treated with 1 μg/mL TRAIL for 24 h before assaying.

2.7. Knockout of C2GnT expression in Jar and BeWo

Since *GCNT1* regulates C2GnT expression, C2GnT knockout (KO) clones were established by transfection of Jar and BeWo cells with 1 μg of *GCNT1* guide RNA vector (Santa Cruz Biotechnology, Santa Cruz, CA, USA), conferring puromycin resistance, using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Control cells were established by transfection of Jar and BeWo cells with 1 μg of Control CRISPR/Cas9 plasmid (Santa Cruz Biotechnology). After 24 h of incubation, selection with puromycin was performed and single cell clones were established. The stable KO of GCNT1 in each clone was validated by analyzing C2GnT expression by western blot using the C2GnT primary antibody.

2.8. *Lycopersicon esculentum* lectin blot analysis

Lectin blot analysis was performed [15] using biotin-conjugate *Lycopersicon esculentum* lectin (LEL; Vector Laboratories, Burlington, Canada), which recognizes poly-*N*-lactosamine of *O*-glycan.

2.9. CD107a degranulation assay

As CD107a is a marker of NK cell functional activity [16], the CD107a Detection kit (MBL, Nagoya, Japan) was used to quantify NK degranulation. NK cells were treated with 6 μg/mL monensin and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD107a monoclonal antibody for 1 h at 37 °C. Simultaneously, control and C2GnT KO Jar cells were co-incubated with NK cells for degranulation stimulation. CD107a expression on NK cells was analyzed using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (FlowJo, LLC).

2.10. Immunoprecipitation

Protein (1 mg) was extracted from each sample and incubated with 5 μL LEL overnight at 4 °C. Immune complexes were collected with 100 μL protein G-Sepharose 4EF beads (GE Healthcare, Buckinghamshire, UK), released by boiling with sampling buffer without a detergent, and separated by SDS-PAGE on a 10% polyacrylamide gel, transferred onto a nitrocellulose membrane, and immunoblotted with antibodies. Immunoreactive proteins were stained using the ECL.

2.11. Mouse studies

All animal procedures were reviewed and approved by the Nagoya University institutional Animal Experimentation Committee (approval number 31241) and performed in accordance with the institutional guidelines of Nagoya University Division of Experimental Animals. Twenty-eight, 5-week-old female BALB/Slc-*nu/nu* mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and housed in six cages with an inverse 12 h day-night cycle (lights on at 9:00 p.m.) in a temperature

(23.5 ± 2 °C) and humidity (50 ± 10%) controlled room. The mice were randomized into two groups. Under anesthesia with 2–3% isoflurane mixed with 30% oxygen and 70% nitrous oxide inhalation, mice were subcutaneously injected with 5×10^5 control or C2GnT KO Jar cells in 200 μL PBS (n = 5/group) for analysis of tumor volume and immunohistochemistry. Tumor diameter was measured every 1–3 days until the 14th day. Estimated volumes were calculated as follows: tumor volume = length × width × width × 1/2. Mice were sacrificed with CO_2 on day 1 and tumors were resected. Similarly, mice were subcutaneously injected with 2×10^6 control or C2GnT KO Jar cells in 200 μL PBS (n = 11/group) for analysis of overall survival, defined as the time between date of inoculation and date of euthanasia following poor condition.

2.12. Statistical analysis

We used the statistical program EZR version 1.37 (Saitama Medical Center, Jichi Medical University, Saitama, Japan). Statistically significant differences were determined using the Student's t-test. A value of $p < 0.05$ was considered significant.

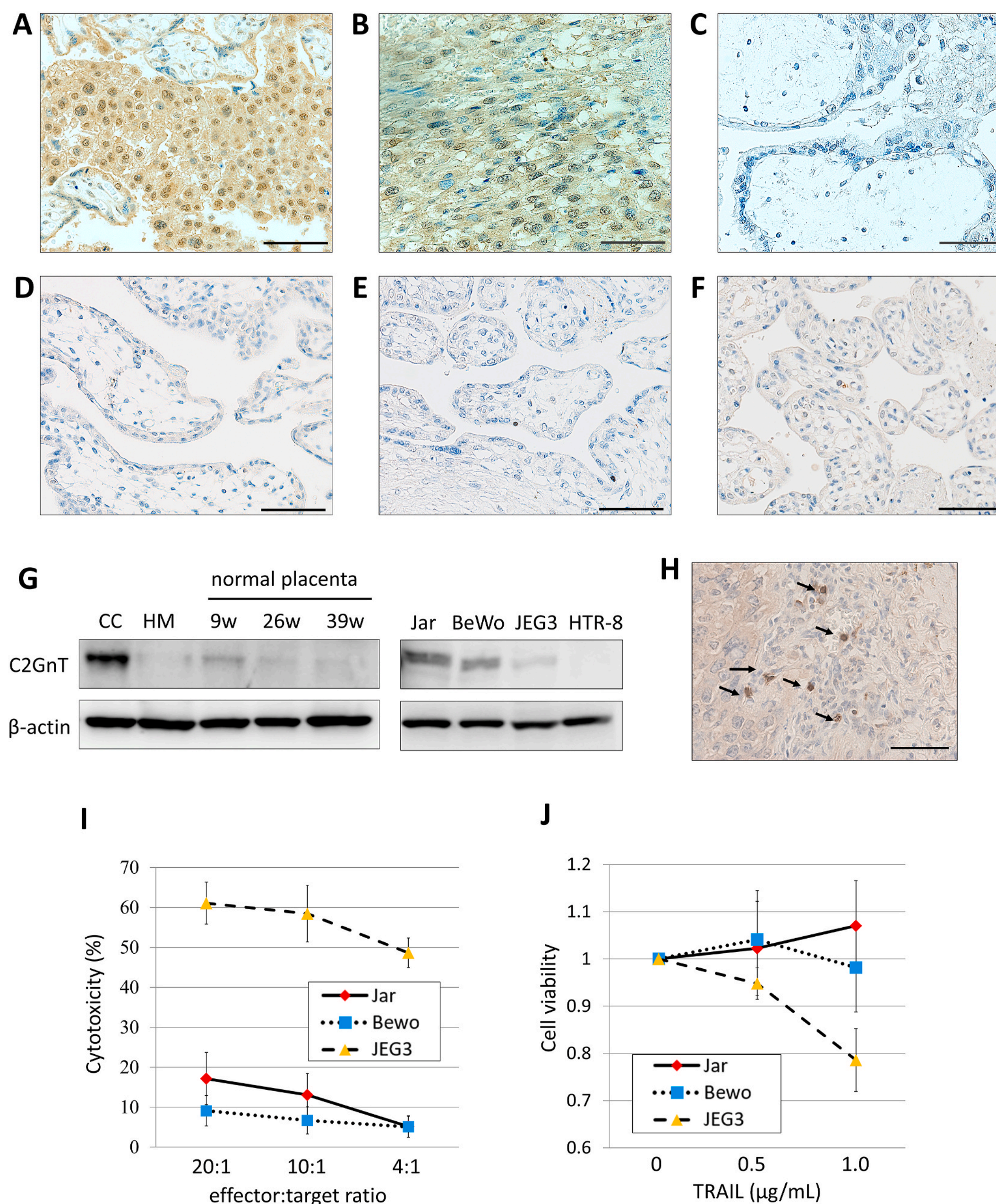

Fig. 1. Expression of C2GnT in various trophoblast cells and tissues and NK cell toxicity against choriocarcinoma cells. (A) Immunohistochemical staining of choriocarcinoma, (B) PSTT, (C) hydatidiform mole; and placenta of various gestational weeks, (D) 9 weeks, (E) 26 weeks, (F) 39 weeks, using the C2GnT antibody. Magnification, 100 ×; Scale bar = 100 μm. (G) Representative western blot demonstrating C2GnT expression in tissues. (H) Choriocarcinoma tissues were immunohistochemically analyzed using the CD57 antibody. Arrows demonstrate CD57-positive cells identified with human NK cells. Magnification, 100 ×; Scale bar = 100 μm. (I) Toxicity of human NK cells against choriocarcinoma cell lines at various effector-to-target ratios. Data were obtained from three individual experiments and each condition was assessed by triplicates. Mean ± SD of cytotoxicity rates are shown. (J) Cells were incubated with TRAIL for 24 h at the indicated concentrations in choriocarcinoma cell lines. Cell viability was determined using a modified tetrazolium salt (MTS) assay. Data were obtained from three individual experiments and each condition was assessed by triplicates. Mean ± SD of cytotoxicity rates are shown.

3. Results

3.1. C2GnT expression in trophoblast tissues

Immunohistochemistry showed that trophoblastic cells of choriocarcinoma and PSTT expressed high levels of C2GnT. Alternatively, hydatidiform mole and normal placenta of various gestational weeks expressed low levels of C2GnT (Fig. 1A–F). Western blotting demonstrated that the expression of C2GnT was strong in choriocarcinoma, whereas molar tissue and normal placenta showed weak expression of C2GnT (Fig. 1G). Additionally, all choriocarcinoma cell lines were C2GnT-positive, while HTR-8/SVneo cells were C2GnT-negative. Meanwhile, Jar and BeWo cells showed strong C2GnT expression, and JEG3 cells showed very weak expression of C2GnT (Fig. 1G).

3.2. NK cell toxicity against choriocarcinoma cells

Immunohistochemistry using a CD57 antibody showed NK cells infiltrating choriocarcinoma tumors (Fig. 1H). Moreover, Jar and BeWo cells were less efficiently killed by human NK cells than JEG3 cells at 20:1, 10:1, and 4:1 effector-to-target ratios (Fig. 1I). TRAIL expressed in NK cells induces apoptosis of target cancer cells by stimulating cancer cell-expressing death receptors, such as DR4 [17]. To evaluate the effect of C2GnT expression on TRAIL sensitivity in choriocarcinoma, we measured TRAIL-induced cell death using choriocarcinoma cell lines. In the presence of TRAIL, the viability of Jar and BeWo cells was higher than that of JEG3 (Fig. 1J).

3.3. C2GnT KO and endo-β-galactosidase increase NK cell cytotoxicity in C2GnT-expressing choriocarcinoma cells

C2GnT KO Jar and BeWo cells were established using the CRISPR/Cas9 system (Supplemental Fig. 1A). Lectin blot analysis was performed on total cellular proteins using LEL to determine the level of poly-*N*-lactosamine of *O*-glycan on C2GnT KO cells and control cells. C2GnT KO reduced the level of *O*-glycans to several proteins in whole cell lysate of both Jar and BeWo cells (Supplemental Fig. 1B). C2GnT KO did not affect choriocarcinoma cell proliferation (data not shown). Additionally, C2GnT KO Jar and BeWo cells were more efficiently killed by human NK cells than control Jar and BeWo cells (Fig. 2A). The natural killer group 2 member D (NKG2D)-binding site in MICA is reportedly masked by galectin-3 and MICA modification with poly-*N*-acetyllactosamine in bladder cancer cells [10]. To examine whether galectin-3 interacts with NK cells by binding to MICA in choriocarcinoma cells, we used endo-β-galactosidase, which cleaves internal β1-4 galactose linkages from the N-terminal of repeating poly-*N*-acetyllactosamine structures on

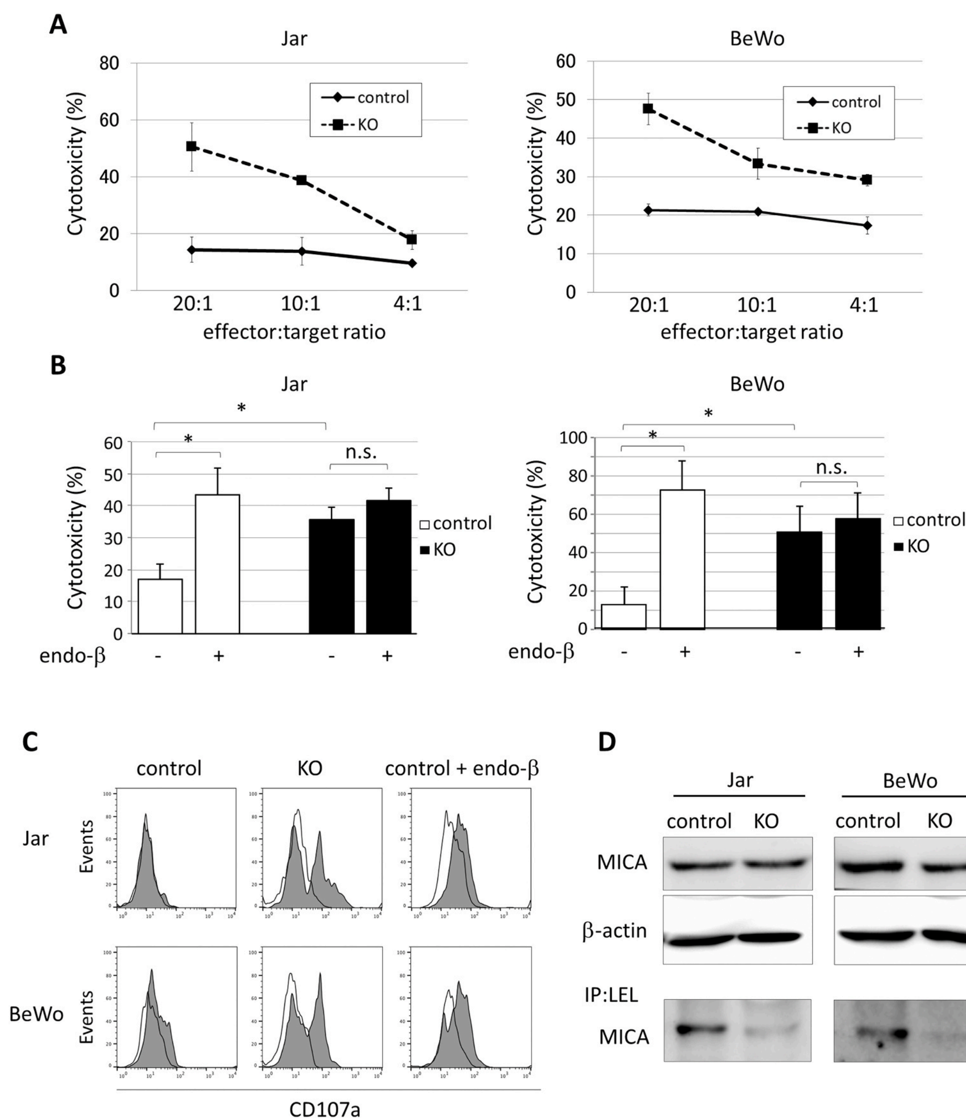

Fig. 2. Association between C2GnT expression and NK cell toxicity via the NKG2D-MICA pathway. (A) Toxicity of human NK cells against control or C2GnT KO Jar and BeWo cells at various effector-to-target ratios. Data were obtained from three individual experiments and each condition was assessed by triplicates. Mean ± SD of cytotoxicity rates are shown. (B) Toxicity of human NK cells against the control and C2GnT KO Jar and BeWo cells at an effector-to-target ratio of 20:1 after endo-β-galactosidase treatment. Data were obtained from three individual experiments and each condition was assessed by triplicates. Mean ± SD of cytotoxicity rates are shown. *$p < 0.05$. n.s., not significant. (C) NK cells were co-incubated with the control or C2GnT KO Jar and BeWo cells, and subjected to the CD107a assay, with and without endo-β-galactosidase treatment. The three independent experiments performed showed similar results. (D) Representative western blot demonstrating MICA protein expression in the control and C2GnT KO Jar and BeWo cells (upper panel). LEL immunoprecipitates were subjected to western blotting with anti-MICA (lower panel).

the cell surface [18]. Jar and BeWo control cells treated with endo-β-galactosidase were more efficiently killed by human NK cells than non-treated choriocarcinoma cells. However, no significant difference in cytotoxicity was observed between C2GnT KO choriocarcinoma cells with and without endo-β-galactosidase treatment (Fig. 2B). Furthermore, the expression level of CD107a in NK cells increased when NK cells were incubated with endo-β-galactosidase or C2GnT KO cells compared to incubation with control Jar or BeWo cells (Fig. 2C).

3.4. O-glycan of MICA on choriocarcinoma cells is reduced in C2GnT KO cells

We examined the *O*-glycosylation of MICA by immunoprecipitation assay using LEL. MICA was barely detected in the LEL immunoprecipitates of C2GnT KO cells although the expression of MICA was the same in C2GnT KO and control Jar and BeWo cells (Fig. 2D), suggesting that galectin-3 binding to MICA through poly-*N*-acetyllactosamine may reduce the affinity of MICA for NKG2D on NK cells and impairs activation of NK cells in Jar and BeWo cells.

3.5. C2GnT KO induces choriocarcinoma cells to react against TRAIL

Control cell viability was higher in the presence of TRAIL than that of C2GnT KO Jar and BeWo cells (Fig. 3A). We studied the function of galectin-3 binding to poly-*N*-acetyllactosamine on MUC1 using endo-β-galactosidase and found that endo-β-galactosidase did not influence Jar or BeWo cell viability in the presence of 1 μg/mL TRAIL (Fig. 3B).

3.6. O-glycan of MUC1 on choriocarcinoma cells is reduced in C2GnT KO cells

Next, we analyzed the expression of MUC1 and DR4, which induce cell apoptosis by interaction with TRAIL [17]. No differences in MUC1 and DR4 expression levels were observed between control and C2GnT KO Jar or BeWo cells. To examine MUC1 *O*-glycosylation, LEL immunoprecipitates were subjected to western blotting with anti-MUC1 antibody. MUC1 was barely detectable in the LEL immunoprecipitates of C2GnT KO Jar and BeWo cells (Fig. 3C).

3.7. In vivo effects of C2GnT KO on choriocarcinoma engraftment

We then analyzed cytotoxicity of mouse NK cells against human choriocarcinoma cells. C2GnT control Jar cells were less efficiently killed by mouse NK cells than C2GnT KO Jar cells at 20:1 and 10:1 effector-to-target ratios (Fig. 4A). Moreover, C2GnT KO tumors grew more slowly than control tumors (Fig. 4B). Overall survival was significantly longer in the C2GnT KO group than in controls ($p = 0.0102$, Fig. 4C). Histopathological examination revealed that tumors contained choriocarcinoma cells in both groups (Fig. 4D, upper panel) and that C2GnT was positive in tumor cells of the control group (Fig. 4D, middle panel). Immunohistochemical staining of tumors with anti-NK1.1 antibody showed that mouse NK cells infiltrated both C2GnT control and KO tumors (Fig. 4D, lower panel).

4. Discussion

This study is the first to analyze the expression and effect of C2GnT on NK cell immunity in choriocarcinoma cells. We show that gestational trophoblastic neoplasms (GTNs), including choriocarcinoma and PSTT, strongly express C2GnT, while expression is significantly reduced in normal placenta, EVT, and hydatidiform mole. We also show that higher expression of C2GnT causes choriocarcinoma cells to more effectively evade NK cell injury. Furthermore, mice inoculated with C2GnT KO cells survive longer than those inoculated with control cells *in vivo*. These results suggest that C2GnT may participate in choriocarcinoma cell immune tolerance.

Moreover, NK cell toxicity may decrease in choriocarcinoma with high C2GnT expression through *O*-glycosylation with poly-*N*-acetyllactosamine on MICA by C2GnT. We observed that endo-β-galactosidase treatment did not influence NK cell function in C2GnT KO cells, since MICA on C2GnT KO choriocarcinoma cells had no poly-*N*-acetyllactosamine. One of the major tumor-rejecting mechanisms of NK cells is receptor-tumor ligand interaction-mediated killing. Among several NK-activating receptors, NKG2D is critical for eliminating cancer cells [19]. MICA is expressed in cancer cells and interacts with NKG2D, which stimulates NK cells to secrete cytotoxic granules, such as perforin and granzyme B, thereby killing cancer cells. In this process, glycosylation of the NKG2D-binding site MICA has been proposed to act via interaction between NKG2D and MICA [10,20]. It is assumed, based on the results of this study, and those of others, that galectin-3 binds to poly-*N*-acetyllactosamine of *O*-glycan attached to the NKG2D-binding of MICA by C2GnT and reduces the affinity of MICA for NKG2D thereby impairing NK cell activation in choriocarcinoma [10,21].

In choriocarcinoma, C2GnT may also participate in TRAIL-mediated killing, another tumor-rejecting mechanism of NK cells. Herein, cell surface MUC1 was heavily glycosylated with core 2 *O*-glycans carrying poly-*N*-acetyllactosamine. Moreover, cell viability decreased with TRAIL by C2GnT knockout. TRAIL expressed in NK cells induces apoptosis of cancer cells by stimulating them to express death receptors, such as DR4. MUC1 is also overexpressed in various cancers, which reportedly serves as an independent marker for aggressive tumor behavior and poor survival [22,23]. Clinical trial results suggest that glyco-immune checkpoint inhibitors of glycans on MUC1 enhance NK cell killing [24]. Specifically, poly-*N*-acetyllactosamine on MUC1 interferes with the access of TRAIL on NK cells to DR4 on tumor cells. This molecular shield function was only previously characterized in bladder and prostate cancer cells [9,25]. Herein, the association between galectin-3 and MUC1 did not influence choriocarcinoma cell viability in the presence of TRAIL, suggesting that poly-*N*-acetyllactosamine may function as a barrier for TRAIL-DR4 binding on choriocarcinoma cells.

We also confirmed that the level of NK cell killing is related to the expression level of C2GnT in choriocarcinoma cells. GTDs develop from the placental tissues and involve a paternal allele [26]. They are, therefore, recognized as "non-self" tumors and may induce an immune reaction in patients. However, previous studies show that HLA class I expression in most trophoblasts and choriocarcinoma cell lines is low or undetectable [11]. NK cells can recognize and kill cells with down-regulated HLA class I molecules on their surface [27], including most GTN cells.

Certain limitations were noted in this study. First, we investigated only MICA and MUC1 as substrate proteins influenced by C2GnT, while lectin blot analysis with LEL demonstrated that numerous proteins in choriocarcinoma express poly-*N*-lactosamine. Thereby, other proteins hyperglycosylated by C2GnT could be causing the NK cytotoxicity escape observed in choriocarcinoma. Core2 *O*-glycans on CD43, attached by C2GnT, reduce primary T-cell responses, resulting in immune defects [28]. Similarly, C2GnT expression promotes evasion of CTL immunity via glycosylation of HLA class I with *O*-glycan [29]. Second, the inoculated mouse model might reflect not only the immune response, but also other choriocarcinoma biological processes, including carcinogenesis, progression, and microenvironment. C2GnT KO significantly reduced the potential of tumorigenesis and improved the overall survival of nude mice. Mouse NK cells infiltrated both C2GnT KO tumors and control tumors. Therefore, it is assumed that only choriocarcinoma cells expressing C2GnT can evade NK cells and shorten the overall survival period. However, the mechanism of escaping NK cell attack may be only one of C2GnT functions, it may also influence tumorigenesis or survival. Therefore, further investigation is needed to assess other target proteins glycosylated by C2GnT and their roles in choriocarcinoma. Third, we have not proved the direct association of NKG2D-MICA, TRAIL-DR4, and galectin-3 with the NK killing activity observed in choriocarcinoma cells. In this sense, Sivori et al. have found that the

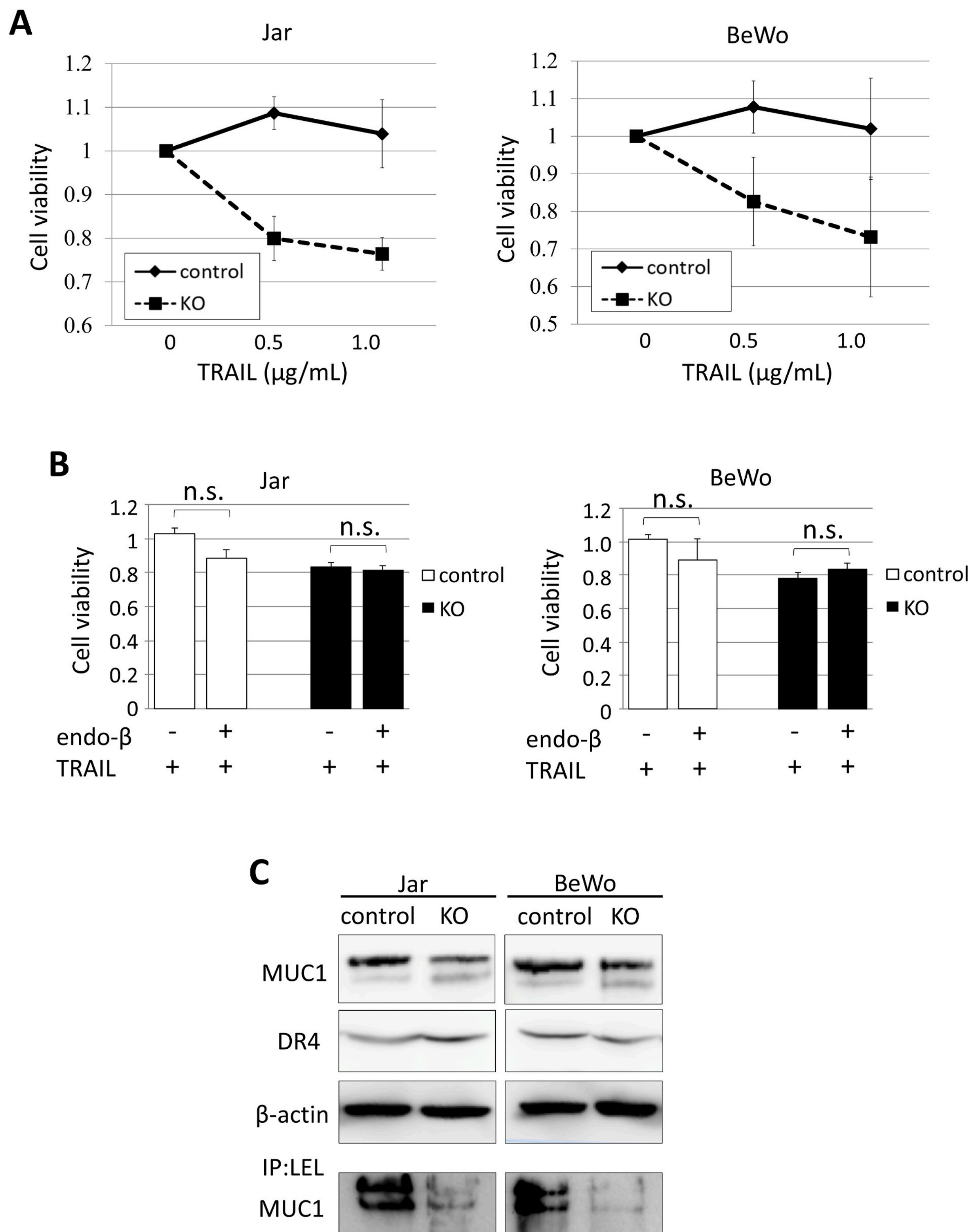

Fig. 3. Association between C2GnT expression and NK cell toxicity via the TRAIL-DR pathways. (A) Viability of control, C2GnT KO Jar and BeWo cells using MTS assay. Data were obtained from three individual experiments and each condition was assessed by triplicates. Mean ± SD of relative cell counts are shown. (B) Cell viability of control, C2GnT KO Jar and BeWo cells treated with endo-β-galactosidase with 1 μg/mL TRAIL for 24 h. Data were obtained from three individual experiments and each condition was assessed by triplicates. Mean ± SD of cytotoxicity rates are shown. n.s., not significant. (C) Representative western blot demonstrating MUC1 and DR4 protein expression in control, C2GnT KO Jar and BeWo cells. LEL immunoprecipitates were subjected to western blotting with anti-MUC1.

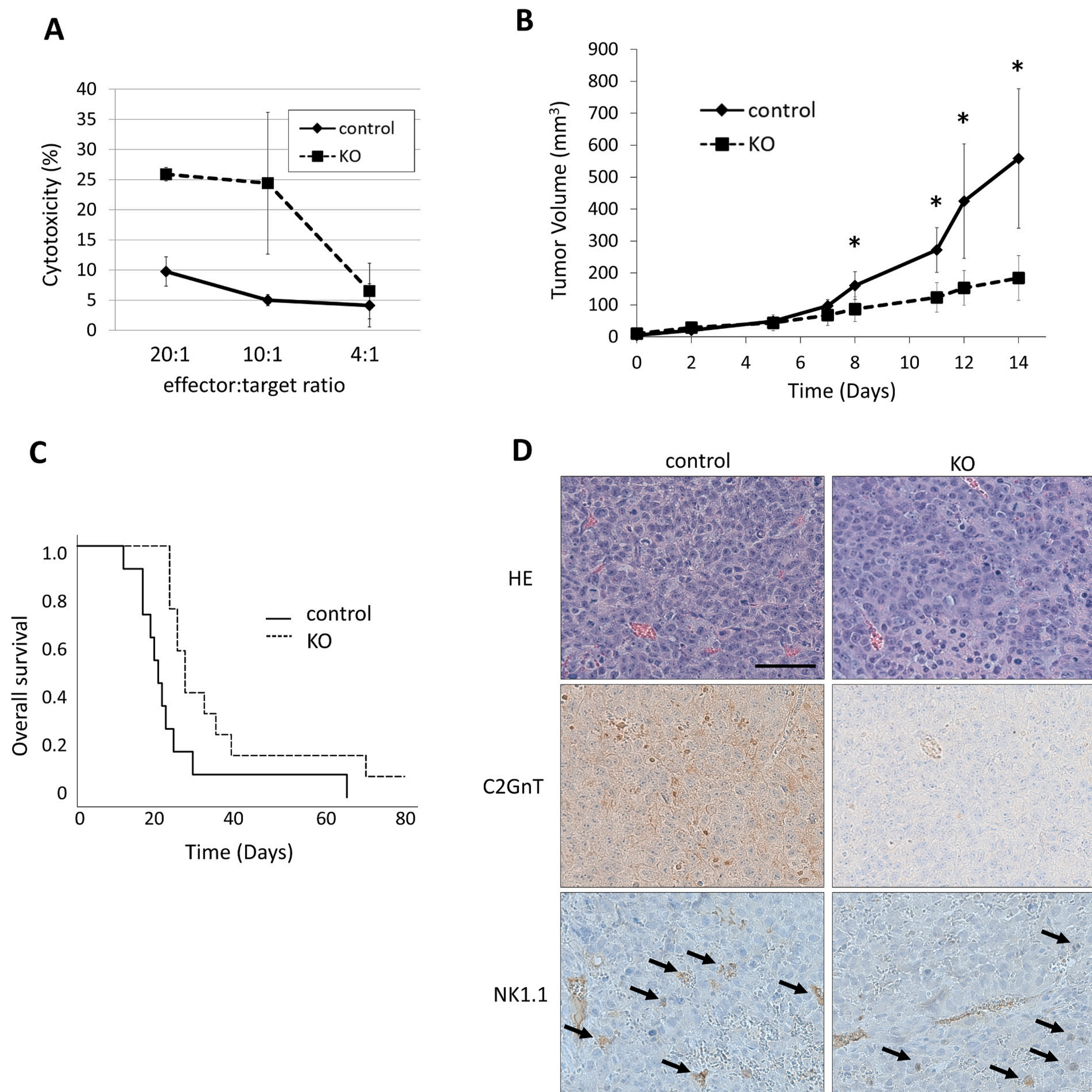

Fig. 4. C2GnT knockout (KO) attenuates tumorigenic activity of Jar cells *in vivo*. C2GnT KO choriocarcinoma cells were susceptible to attack by mouse NK cells. (A) The cytotoxicity of mouse NK cells against control and C2GnT KO Jar cells. Data were obtained from three individual experiments and each condition was assessed by triplicates. Mean ± SD of cytotoxicity rates are shown. (B) Growth of inoculated tumors in nude mice. Control and C2GnT KO Jar cells were used. Mean ± SD of tumor volume are shown. *$p < 0.05$. (C) Cumulative survival rate of mice injected with control and C2GnT KO Jar cells. (D) Hematoxylin and eosin staining of tumors derived from both control and KO groups. Arrows indicate mouse NK cells. Magnification, 100 × ; Scale bar = 100 μm.

anti-NKG2D antibody does not decrease NK cell cytotoxicity to choriocarcinoma cell lines [30]. Therefore, further MICA or MUC1 interference studies and immunoprecipitation experiments with anti-galectin-3 antibody in choriocarcinoma cells are needed to demonstrate the direct binding of these proteins.

In conclusion, this study suggests that in choriocarcinoma cells C2GnT may participate in the NKG2D-MICA and TRAIL-DR4 immunosuppressive pathways through glycosylation of MICA and MUC, respectively. Specific inhibitors that reduce C2GnT activity may restore the susceptibility of choriocarcinoma to NK cells, thereby promoting tumor rejection and suppressing metastasis.

CRediT authorship contribution statement

Kenichi Nakamura: Methodology, Validation, Investigation, Data curation, Writing - original draft, Visualization. **Kaoru Niimi:** Conceptualization, Writing - review & editing, Visualization, Funding acquisition, Project administration. **Eiko Yamamoto:** Resources, Data curation, Project administration. **Yoshiki Ikeda:** Methodology, Investigation, Resources. **Kimihiro Nishino:** Methodology, Validation, Investigation, Data curation. **Shiro Suzuki:** Methodology. **Hiroaki Kajiyama:** Supervision, Project administration. **Fumitaka Kikkawa:** Supervision, Project administration, All authors finally approved the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.100951.

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