Expression of the Clustered NeuAcα2–3Galβ O-Glycan Determines the Cell Differentiation State of the Cells

Human embryonic stem cells (hESCs) are pluripotent stem cells from early embryos, and their self-renewal capacity depends on the sustained expression of hESC-specific molecules and the suppressed expression of differentiation-associated genes. To discover novel molecules expressed on hESCs, we generated a panel of monoclonal antibodies against undifferentiated hESCs and evaluated their ability to mark cancer cells, as well as hESCs. MAb7 recognized undifferentiated hESCs and showed a diffuse band with molecular mass of >239 kDa in the lysates of hESCs. Although some amniotic epithelial cells expressed MAb7 antigen, its expression was barely detected in normal human keratinocytes, fibroblasts, or endothelial cells. The expression of MAb7 antigen was observed only in pancreatic and gastric cancer cells, and its levels were elevated in metastatic and poorly differentiated cancer cell lines. Analyses of MAb7 antigen suggested that the clustered NeuAcα2–3Galβ O-linked oligosaccharides on DMBT1 (deleted in malignant brain tumors 1) were critical for MAB7 binding in cancer cells. Although features of MAB7 epitope were similar with those of TRA-1–60, distribution of MAb7 antigen in cancer cells was different from that of TRA-1–60 antigen. Exposure of a histone deacetylase inhibitor to differentiated gastric cancer MKN74 cells evoked the expression of MAB7 antigen, whereas DMBT1 expression remained unchanged. Cell sorting followed by DNA microarray analyses identified the down-regulated genes responsible for the biosynthesis of MAB7 antigen in MKN74 cells. In addition, treatment of metastatic pancreatic cancer cells with MAB7 significantly abrogated the adhesion to endothelial cells. These results raised the possibility that MAB7 epitope is a novel marker for undifferentiated cells such as hESCs and cancer stem-like cells and plays a possible role in the undifferentiated cells.
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hESC markers have been reported to overlap with those of cancer stem cells. TRA-1–60 has originally been raised against human embryonal carcinoma cells, and a previous study showed that the epitope of TRA-1–60 is the sialylated keratan sulfate proteoglycan on podocalyxin (6). SSEA-3 was shown to be expressed in human teratocarcinoma cells, and its epitope has been identified as globo-series ganglioside (7).

To discover novel cell surface molecules expressed on hESCs, we generated a panel of monoclonal antibodies (MAbs) against undifferentiated hESCs using the mouse medial iliac lymph node method and evaluated their ability to mark cancer cells as well as hESCs. In this study, we found that MAb7 strongly recognized not only undifferentiated hESCs but also liver-metastatic pancreatic cancer cells and poorly differentiated gastric cancer cells. Although features of MAb7 epitope were similar with those of TRA-1–60, distribution of MAb7 antigen in cancer cells was different from that of TRA-1–60 antigen. Analyses of MAb7 antigen revealed that the clustered NeuAco2–3Galβ O-linked oligosaccharides on DMBT1 (deleted in malignant brain tumors 1) were critical for mouse IgM was purchased from Invitrogen. Neuraminidases, (Biolegend, San Diego, CA) were obtained. Dynabeads rat anti-

EXPERIMENTAL PROCEDURES

Cell Cultures, Plasmids, and Reagents—hESCs, Khes-1, were used following the hESC research guidelines of the Japanese government and maintained as previously described (8). For neural differentiation of hESCs, the quick serum-free culture of embryoid body-like aggregates (SEEBQ) method was used as previously described (9). The combination of three-dimensional cell aggregation and cAMP signaling induced the hepatic differentiation of hESCs (10). Human cancer cell lines and human umbilical vein endothelial cells (HUVECs) were obtained from Health Science Research Resources Bank (Osaka, Japan) and maintained in accordance with the supplier’s protocol. Primary human brain microvascular endothelial cells were purchased from DS Pharma Biomedical (Osaka, Japan). Human amniotic epithelial cells were purchased from ScienCell Research Laboratories (Carlsbad, CA) and maintained in accordance with the supplier’s protocol. Normal human epidermal keratinocytes (Kurabo, Osaka, Japan) and dermal fibroblasts (Clontech) were obtained. FLAG-tagged UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalac- tosaminyltransferase 1 (GALNT1) and like-glycosyltransferase (LARGE) expression plasmids were obtained by subcloning the entire coding sequence into a pFLAG-CMV-2 expression vec-
tor (Sigma-Aldrich). The antibodies TRA-1–60 (Millipore, Danvers, MA), Pax-6 (Covance, Princeton, NJ), DMBT1 (Santa Cruz Biotechnology, Dallas, TX), DYKDDDDK tag (Cell Signaling Technology, Danvers, MA), and control mouse IgM (Biolegend, San Diego, CA) were obtained. Dynabeads rat anti-

peptide-N-glycanase F (PNGase F), heparinase I, keratanase, chondroitinase ABC, AG-108, and Scriptaid were purchased from Sigma-Aldrich. 3′-O-Acetyl-2′,7′-bis (carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxyethyl ester was purchased from Dojindo (Kumamoto, Japan). Glycan polymers conjugated with polyacrylamide (PA) were purchased from GlycoTech (Gaithersburg, MD).

Mouse Anti-hESC Monoclonal Antibodies—Mouse anti-

hESC monoclonal antibodies were generated based on the mouse medial iliac lymph node method (11). Briefly, the lysates of hESCs were injected to the rear footpads with Freund’s complete adjuvant. Two weeks later, cells from the lymph nodes of the immunized mice were fused with mouse myeloma Sp2/0-Ag14 cells at a ratio of 5:1 in 50% polyethylene glycol solution. The resulting hybridoma cells were plated onto 96-well plates and cultured in HAT (hypoxanthine aminopterin thymidine) selection medium. Monoclonal antibodies were purified from the desired hybridoma supernatants (11).

Immunofluorescence—The cancer cells were fixed with 4% paraformaldehyde. After blocking, the cells were incubated with primary antibody at 10 μg/ml (12). Localization of anti-
gens was visualized using appropriate secondary antibodies (Alexa Fluor 594 or 488; Molecular Probes, Eugene, OR). Cryo-

sections of SfEBq-cultured hESC aggregates were immuno-
stained with the respective antibodies (9).

Fluorescence-activated Cell Sorting—Cancer cells were disso-
ciated to single cells by trypsin/EDTA, incubated with antibod-
ies at 4 °C, and counted and sorted with FACSAria (Becton Dickinson, Franklin Lakes, NJ).

DNA Microarray Analysis and Real Time RT-PCR Assay—The comprehensive gene expression analyses were performed using the GeneChip system as previously described (13). Briefly, total RNA was extracted using RNeasy Plus Micro kit (Qiagen), and cDNA for in vitro transcription was prepared from 50 ng of total RNA. Fragmented in vitro transcripts were hybridized overnight onto Human Genome U133 plus 2.0 microarrays, stained, washed, and scanned with an Affimetrix Gene Chip Scanner 3000 7G. The obtained image files were analyzed with the Affimetrix data suite system (Expression Console version 1.1).

Expressions of human GALNT1 and LARGE were quantified using MESA Blue qPCR Mastermix (Eurogentec, Seraing, Belgium) as previously described (14). The PCR primers used were as follows: GALNT1 forward, 5′-ATGACCTATG-5′GTTGTTTCAACT-3′ and reverse, 5′-CTGACAGGAAGAGTCCGATCA-3′; LARGE forward, 5′-TCCGAGCAGTGCTAC-3′ and reverse, 5′-TTGCGAAAAACTCCACATG-3′. The relative expression levels were normalized against those of GAPDH gene in the same RNA preparation.

Transient Transfection—Cancer cells were transfected using Lipofectamine 2000 (Invitrogen) in accordance with the manu-

facturer’s protocol. The total amount of DNA in each transfection was kept constant by the addition of appropriate empty vectors.

Cell Proliferation and Migration Assay—Cells were grown in 96-well plates and then treated with different concentrations of antibody for 1–24 h. Cell proliferation was estimated using a CellTiter-Glo luminescent cell viability assay kit (Promega). To
examine the effect of antibody on migration of cancer cells, cells were cultured in 6-well plates and grown until reaching a confluent state. The cell layer with antibody was scratched with a sterile tip, washed with culture medium, and then cultured for 1–24 h. At different time points, the distance between the two edges of the scratch was measured.

Adhesion Assay—The ability of cancer cells labeled with 3'-O-Acetyl-2',7'-bis (carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxyethyl ester to adhere to endothelial cells was determined as previously described (15). Briefly, 1 × 10⁵ labeled cancer cells were incubated with 10 μg/ml of antibody for 30 min at 37 °C. After washing, cells were added to confluent endothelial cells and incubated for 1 h, washed three times with PBS(−) to remove nonadhesive cells, and then incubated with 100 μl of Triton X-100 for 2 h at room temperature. The percentage of adherent cells was calculated from the fluorescence intensity.

Capillary Affinity Electrophoresis—Free O-linked oligosaccharides labeled with Fmoc were prepared from MKN45 cells (16). Capillary affinity electrophoresis was performed on a P/ACE MDQ Glycoprotein System (Beckman Coulter, Fullerton, CA) equipped with a helium-cadmium laser-induced fluorescence detector (excitation, 325 nm; emission, 405 nm) as previously described (17). Briefly, after incubation of labeled O-linked oligosaccharides with antibody for 30 min at room temperature, sample solutions were introduced into the capillary by pressure injection at 1 p.s.i. for 10 s. Separation was performed by applying the potential of 25 kV at 25 °C.

Glycan Array—Glycan polymers conjugated with PAA were fixed on glass slides in accordance with a method previously described with minor modifications (16). Briefly, 20 μg of glycan polymers was dissolved in 20 μl of spotting solution for DNA micro arrays (Matsunami Glass Ind., Ltd., Osaka, Japan), and aliquot was spotted on an epoxy-coated glass slide (Schott AG, Mainz, Germany) with a diameter of ~1 mm kept for 16 h at room temperature. After incubation, the unreacted epoxy groups were blocked with Tris-HCl buffer (25 mM Tris-HCl, pH 7.4, containing 0.8% NaCl, 1% Triton X-100, and 4% BSA) at room temperature for 1 h. An 80-μl portion of the probing buffer (25 mM Tris-HCl, pH 7.4, containing 0.8% NaCl, 1% Triton X-100, 1 mM MnCl₂, and 1 mM CaCl₂) containing 0.8 μg of antibody was applied to the array and was kept at room temperature for 3 h. After removing the antibody solution, the probing buffer containing 10 μg/ml of Cy3-conjugated anti-mouse IgM antibody (80 μl) was added, and then fluorescent images were acquired using an Ettan DIGE imager (GE Healthcare, Tokyo, Japan). For inhibition assays, 10 μg/ml of antibody was pretreated with monomer or polymer of oligosaccharide as a competitor for 1 h at room temperature, and mixtures were applied to the glycan array.

Immunoprecipitation and Microsequencing—Five milligrams of KP-3L cell lysates were prepared, and immunoprecipitation was performed using MAb7 followed by Dynabeads rat anti-mouse IgM as previously described (18). Proteins bound to beads were resolved by 7.5% SDS-PAGE and then visualized by silver staining. Desired bands were excised, in-gel-digested with trypsin, and solvent-extracted, and the resulting MS/MS spectra were searched using the MASCOT search engine (18).

Western Blot—Whole cell extracts were resolved by 7.5% SDS-PAGE, transferred to a PVDF membrane, and blocked with 5% skim milk for 30 min at room temperature (19). After incubation with primary antibodies, the proteins of interest on immunoblots were detected using an enhanced chemiluminescence detection system.

Statistical Analysis—Values are expressed as means ± S.D. Student’s t test was used to evaluate the statistical significance of differences between groups. A p value of less than 0.05 was considered significant.

RESULTS

MAb7 Antigen Is a Surface Marker for Undifferentiated hESCs—Because cell surface molecules have been used as markers for isolating, purifying, and targeting cancer cells, we first screened numerous hybridoma supernatants by immunofluorescence (data not shown). The monoclonal antibody, referred to herein as MAb7 (mouse IgM), recognized cell surface antigens of the undifferentiated hESCs (Fig. 1A, Undifferentiated). However, MAb7 hardly reacted with mouse embryonic stem cells (data not shown). hESCs are pluripotent and have the capability to generate all the differentiating cells present in the embryo. A previous study demonstrated an efficient culture method for the selective neural differentiation of hESCs using the SFEBq culture (9). Cryosections of SFEBq-cultured hESC aggregates were immunostained with MAb7. As shown in Fig. 1A, an intense signal of MAb7 was observed on the periphery of the embryoid body-like aggregates of the undifferentiated hESCs (day 0). The expression level of MAb7 antigen was gradually reduced and had almost disappeared by 7 days after the neural differentiation. In contrast, expression of the neural marker Pax-6 was significantly elevated 7 days after neural differentiation of hESCs (Fig. 1A, Day 7). Expression of TRA-1–60 antigen, one of the most specific markers of undifferentiated hESCs, showed similar localization and a time-dependent alteration with that of MAb7 antigen (Fig. 1B). We next examined the expression levels of MAb7 antigen during hESC differentiation into neural cells by Western blotting. As shown in Fig. 1C, a diffuse band with molecular mass of >239 kDa was detected in the undifferentiated hESCs, and the amount of MAb7 antigen was decreased in a time-dependent manner. In addition, a time-dependent decrease in MAb7 antigen expression was observed during hESC differentiation into hepatic cells (data not shown). TRA-1–60 antigen, which has higher molecular weight than MAb7 antigen, was also diminished during hESC differentiation into neural cells (Fig. 1C). Conversely, the amount of Pax-6 was obviously increased 7 days after neural differentiation of hESCs. These results suggested that the >239 kDa molecules strongly expressed in the undifferentiated hESCs were MAb7 antigen. We further investigated the expression levels of MAb7 antigen in normal cultured cells. Human amniotic epithelial cells have been reported to retain stem cell characteristics and express stem cell-specific markers (19). As shown in Fig. 1D, 5–10% of amniotic epithelial cells expressed MAb7 and TRA-1–60 antigens. In contrast, the expressions MAb7 and TRA-1–60 antigens were not observed in normal human keratinocytes, fibroblasts, or endothelial cells (data not shown).
shown). These results suggested that MAb7 marks nontumor undifferentiated cells.

**MAb7 Marks Undifferentiated and Metastatic Cancer Cells**—We then tested the possibility of using MAb7 antigen as a cancer cell marker. For this purpose, we first screened 20 cancer cell lines by immunofluorescence. As a result, the expression of MAb7 antigen was detected only in pancreatic and gastric cancer cells, whereas its expression was barely observed in cancer cell lines derived from cervix (HeLa), brain (Hs683), breast (MCF-7, MDA-MB-231), lung (HARA, HARA-B, A549), bladder (TCCsup, RT-112), bone (Saos-2), liver (HepG2), prostate (LNCap, PC3), gallbladder (1TKB), colon (HCT-15), connective tissue (HT1080), and skin (A431, G361, A2058) (data not shown). In nonmetastatic pancreatic cancer cell line KP-3, 30% of cells expressed MAb7 antigen in the cytoplasm and on the cell surface (Fig. 2A). Interestingly, MAb7 antigen was strongly expressed on the periphery of colonies in liver-metastatic pancreatic cancer cells, KP-3L. In gastric cancer cells, almost all cells expressed MAb7 antigen on the plasma membrane in the poorly differentiated cancer cell line MKN45, whereas none of cells produced MAb7 antigen in the differentiated cell line MKN74 (Fig. 2A). In contrast, some KP-3 and KP-3L cells expressed TRA-1–60 antigen in the cytoplasm and on the plasma membrane (Fig. 2B). Although TRA-1–60 antigen was not detected in MKN74 cells, 50% of MKN45 cells expressed TRA-1–60 antigen on the plasma membrane (Fig. 2B). Expression of MAb7 antigen on the plasma membrane of KP-3L cells was confirmed by flow cytometry (Fig. 2C). Because MAb7 antigen is distributed on the cell surface of cancer cells, we tried to determine its content in the conditioned medium. The same number of cancer cells was seeded, and equal aliquots of culture medium were supplied for Western blotting. At each time point, the number of cancer cells was almost the same (data not shown). As shown in Fig. 2D, the secreted MAb7 antigen from KP-3L was remarkably elevated in a time-dependent manner. The MAb7 antigen from KP-3 was detectable only 4 days after the seeding. In contrast, bone-metastatic lung cancer cells HARA-B produced no detectable amounts of MAb7 antigen (Fig. 2D), consistent with the results from immunofluorescence (data not shown). These results suggested that MAb7 antigen distinct from TRA-1–60 antigen is a cell type-specific secreted protein.

**MAb7 Recognizes the Clustered NeuAcα2–3Galβ O-Linked Oligosaccharides on DMBTI**—The results described above led us to identify the MAb7 antigen. The features of MAb7 antigen, the diffused pattern by Western blotting, and the distribution on the plasma membrane shown by immunofluorescence raised the possibility of glycoproteins. To determine whether the MAb7 antigen appears to be associated with N- or O-linked oligosaccharides, we first tested the susceptibility to digestion with PNGase F or mild alkaline hydrolysis. Intriguingly, the MAb7 antigen was completely destroyed by mild alkaline diges-
tion (Fig. 3A). In contrast, PNGase F had no effect on its antigenicity. These results suggested that MAb7 recognizes O-linked oligosaccharides. Thus, we further defined the epitope of MAb7 using enzymes that degrade carbohydrates. As shown in Fig. 3B, treatment of KP-3L lysate with keratanase abrogated the antigenic activity of MAb7, whereas the molecular mass of the antigen was not altered by digestion with heparinase I or chondroitinase ABC. Furthermore, neuraminidase from *Arthrobacter ureafaciens* (*H*9251 2–3,6,8,9-specific) efficiently abolished MAb7 binding to the lysate; however, digestion of lysate with other bacterial neuraminidases, *Streptococcus pneumoniae* (*H*9251 2–3-specific) and *Clostridium perfringens* (*H*9251 2–3,6-specific), had no effect on the MAb7 binding (Fig. 3C).

We have shown that the heavily glycosylated O-glycans were abundantly present on the cell surface of MKN45 cells (20). To identify the precise structure of MAb7 epitope, we first examined the effects of MAB7 on the binding to the liberated carbohydrates prepared from MKN45 cells by capillary affinity electrophoresis. Asialo, monosialo, disialo, and trisialo glycans were successfully separated as previously reported (16). As shown in Fig. 3D, the electrophoresis profiles of control and MAb7 were almost the same. In contrast, the incubation of MAM (*Maackia amurensis*) lectins recognizing NeuAcα2–3Gal within O-glycans significantly delayed the migration time of sialo glycans under the same conditions (data not shown). Previous studies have shown that interactions of antibodies or lectins with monomeric oligosaccharides are weak (21, 22). A type 1 HIV neutralizing antibody, 2G12, interacts with oligomannose, whereas monomeric oligosaccharides bind to 2G12 weakly (22). To test the possibility that MAb7 interacts with the oligomeric carbohydrates, MAb7 was provided for glycan array spotting with the commercially available glycan polymers. The interactions between MAb7 and glycan polymer were examined using an evanescent-field fluorescence-assisted scanner. Interestingly, MAb7 was bound to a NeuAcα2–3Galβ1–4Glc polymer, whereas no interaction between MAb7 and NeuAcα2–6Galβ1–4Glc, NeuAcα2–8NeuAcα2–8NeuAc, or sialic acid-deficient glycan polymers was observed (Fig. 3E). In addition, MAB7 also recognized NeuAcα2–3Galβ1–4GlcNAc polymer (data not shown). To confirm the specific binding of MAb7 to oligomeric carbohydrate, competition assays were performed. After incubation of MAb7 with several concentrations of NeuAcα2–3Galβ1–4Glc monomer or polymer as competitors, the reaction mixtures were applied to three arrays of either NeuAcα2–3Galβ1–4Glc or NeuAcα2–6Galβ1–4Glc polymer, and the bindings were detected using a fluorescence-assisted scanner (Fig. 3F, upper panel). The interaction between MAb7 and NeuAcα2–3GalβGlc polymer was inhibited by...
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NeuAcα2–3GalβO-linked oligosaccharides in a dose-dependent manner (Fig. 3F, lower panel). In contrast, this interaction was not totally interrupted by a monomer (Fig. 3F, lower panel). These results demonstrated that MAb7 recognizes the clustered NeuAcα2–3Galβ O-linked oligosaccharides.

Next, we searched for the core proteins having MAb7 epitope. After incubation of the KP-3L lysate with MAb7, immunoprecipitation was performed. The results of three independent experiments showed that a band with molecular mass of >239 kDa was consistently detected by Western blotting (Fig. 4A, left panel) and silver staining (Fig. 4, right panel). The relevant band was excised and analyzed by mass spectrometry, and DMBT1 was identified as a candidate protein carrying MAb7 epitope (MASCOT score >70; an observed m/z 730.81 was matched with amino acids 289–302 of DMBT1). Mucin-like protein DMBT1 belongs to a family of proteins that contain scavenger receptor cysteine-rich domains. A previous study showed that DMBT1 carries sialylated, O-linked oligosaccharides (23). To determine whether MAb7 recognizes a carbohydrate on DMBT1, we performed Western blot using anti-DMBT1 antibodies, which are raised against a partial synthetic peptide within DMBT1. As shown in Fig. 4B, a diffuse band with molecular mass of >239 kDa in the KP-3L lysate was detected by MAb7 and anti-DMBT1 antibodies, while appearing as a faint band in the KP-3 lysate observed using both antibodies. In addition, it was evident that anti-DMBT1 antibodies detected a >239-kDa protein band in immunopurified MAb7 precipitates (Fig. 4C). To confirm that DMBT1 is one of the core proteins carrying MAb7 epitope, the immunopurified anti-DMBT1 precipitates from the plasma membrane of KP-3L cells were treated with PNGase F, dilute NaOH, or α2–3,6,8,9-specific neuraminidase. As shown in Fig. 4D, the MAb7 epitope in precipitates was completely destroyed by mild alkaline and neuraminidase digestion. In contrast, treatment of precipitates with PNGase F had no effect on its antigenicity (Fig. 4D).

Furthermore, the transferred anti-DMBT1 precipitates to a PVDF membrane were blotted with MAb7 in the presence of NeuAcα2–3Galβ1–4Glc monomer or polymer as competitors, and the reaction mixtures were applied to three arrays of either NeuAcα2–3Galβ1–4Glc or NeuAcα2–6Galβ1–4Glc polymer. The interactions between MAb7 and glycan polymer were examined using an evanescent-field fluorescence-assisted scanner (upper panel). The relative intensity was calculated from that of noncompetitor as 100% (lower panel). Asterisks signify values that are significantly different compared with control. **, p < 0.01; *, p < 0.05.

FIGURE 3. MAb7 recognizes the clustered NeuAcα2–3Galβ O-linked oligosaccharides. A, 10 μg of KP-3L lysates were untreated or treated with PNGase at 37 °C or 50 mM NaOH at 25 °C for 24 h. B, 10 μg of KP-3L lysates were untreated or treated with heparinase I, keratanase, or chondroitinase ABC at 37 °C for 24 h. C, 10 μg of KP-3L lysates were untreated or treated with sialidases at 37 °C for 24 h. MAb7 antigen expression levels were determined by Western blotting.

D, antibodies were incubated with Fmoc-labeled O-linked oligosaccharides prepared from MKN45 cells, and then solutions were introduced into a capillary by pressure injection at 1 p.s.i. for 10 s. Capillary affinity electrophoresis was performed on a P/ACE MDQ glycoprotein system equipped with a helium-cadmium laser-induced fluorescence detector. E, glycan polymers conjugated with PAA were fixed on glass slides and incubated with 10 μg/ml of antibody. The array slide was scanned using an Ettan DIGE imager. F, MAb7 recognizes the clustered NeuAcα2–3GalβO-linked oligosaccharides.
ings suggested that MAb7 recognizes the clustered NeuAcα2–3Galβ O-linked oligosaccharides on DMBT1; however, the distribution of MAb7 epitope in cancer cells did not completely overlap with that of DMBT1.

MAb7 Discriminates Epigenetic Status of Cancer Cells—Because our results showed that MAb7 recognizes poorly differentiated and metastatic cancer cells, we next explored the usability and capability of MAb7. Epigenetics plays a major role in cell type specification, because the differentiation process is accompanied by major chromatin remodeling (24). Aberrant methylation of CpG island at a promoter region of tumor suppressor gene is an epigenetic change that induces transcriptional silencing of genes. Inhibition of histone deacetylase activity plays a role in the accumulation of acetylated core histones, leading to a more relaxed chromatin conformation and the transcriptional activation of a limited number of target genes by CpG island hypermethylation (25). To examine the expression level of MAb7 antigen under epigenetic change, MKN74 cells were treated with Scriptaid and RG108, inhibitors of histone deacetylase and DNA methyltransferase, respectively. Interestingly, MAb7 antigen was evoked when the cells were exposed to Scriptaid for 8 days (Fig. 5, A). MAb7 antigen production by Scriptaid was confirmed by Western blotting (Fig. 5, B), whereas DMBT1 expression was almost unchanged (Fig. 5, A and B). On the other hand, treatment of MKN74 cells with RG108 for up to 10 days had no effect on MAb7 antigen expression (data not shown). These results suggested that expression of MAb7 epitope is susceptible to epigenetic modification. To identify the silenced genes that are responsible for MAb7 epitope expression, we first performed comprehensive gene expression analyses using the sorted MAb7-positive and -negative MKN74 cells. As shown in Fig. 5C, only 1% of MKN74 cells expressed MAb7 epitope when cells were exposed to Scriptaid for 8 days (P2 fraction). All cells in P2 fraction were stained with MAb7 (Fig. 5D). Biosynthesis of O-glycan begins with α-GalNAc transfer from UDP-GalNAc to a Ser/Thr residue in an acceptor
polypeptide and proceeds to the elongation of sugars by glycosyltransferases. The results of DNA microarray analysis showed that the expressions of \textit{GALNT1} and \textit{LARGE} were elevated in P2 cells compared with P3 cells (data not shown). GALNTs are glycosyltransferases that catalyze initial reaction of mucin-type linkages (GalNAc\(\beta\)1-\(\text{O}\)-Ser/Thr). LARGE is a putative glycosyltransferase that shows high homology to bacterial GT8 glycosyltransferase and human UDP-GlcNAc:Gal\(\beta\)1,3-N-acetylglucosaminyltransferase. The quantitative analysis showed the higher expression of \textit{GALNT1} and \textit{LARGE} in P2 cells (Fig. 5E). In addition, the expression of \textit{GALNT1} in MKN74 cells was less than one-tenth that in poorly differentiated cancer cell line MKN45 (Fig. 5E). Finally, we examined whether GALNT1 and LARGE are involved in biosynthesis of MAb7 epitope. As shown in Fig. 5F, transfection of GALNT1 or LARGE expression plasmid to MKN74 cells exhibited its distribution in the cytoplasm; however, MAb7 epitope production was not observed. In contrast, supplementation of both genes clearly evoked MAb7 epitope in MKN74 cells (Fig. 5F). These results suggested that elimination of MAb7 epitope in MKN74 cells is in part attributed to the down-regulation of \textit{GALNT1} and \textit{LARGE} genes under epigenetic modification.

\textbf{MAb7 Abrogates the Interaction between Metastatic Cancer Cells and Endothelial Cells}—The selectin family of cell adhesion molecules and their glycoconjugate ligands play an important role in the initial events of inflammation and metastasis. Selectins bind with high affinity to specific macromolecular ligands containing sialyl Lewis x epitope (sLex), but binding to the sLex-containing glycan alone is weak (21). To assess the involvement of MAb7 antigen in the interaction of liver-metastatic pancreatic cancer KP-3L cells with endothelial cells, we employed a cell adhesion assay. Fluorescent labeled HARA-B, KP-3, or KP-3L cells were added to confluent HUVECs. After incubation for 1 h at 37 °C, the percentage of adherent cells was determined. As shown in Fig. 6A, the relative number of adherent KP-3L cells was significantly higher than that of HARA-B.
and KP-3 cells, in parallel with MAb7 epitope expression levels. Next, the fluorescent labeled KP-3L cells were pretreated with MAb7 or anti-DMBT1 antibodies for 30 min, washed with culture medium three times, and then added to confluent HUVEC or human brain microvascular endothelial cell. Treatment of both lines of endothelial cells with IL-1β or TNF-α had negligible impact on the adhesion to KP-3L cells under our experimental conditions (data not shown). As shown in Fig. 6B, preincubation of KP-3L cells with MAb7 significantly abrogated the binding to both lines of endothelial cells. In contrast, pretreatment of KP-3L cells with anti-DMBT1 antibodies had no effect on the adhesion to endothelial cells. Exposure of MAB7 and anti-DMBT1 antibodies to KP-3L cells had no effect on cell proliferation or migration (data not shown). These findings suggest that MAB7 epitope is involved in the adhesion of KP-3L cells to endothelial cells.

DISCUSSION

Many of the stem cell marker antibodies, such as TRA-1–60 and SSEA-3, have originally been raised against human embryonal carcinoma cells, which were used as a model for human pluripotent cells before hESCs were introduced (26). Previous studies have demonstrated that the epitope of TRA-1–60 is the sialylated keratan sulfate proteoglycan on podocalyxin (6). However, recent results of glycan array and mass spectrometry indicated that TRA-1–60 recognizes the minimal epitope Galβ1–3GlcNAcβ1–3Galβ1–4GlcNac, which is also present in hESCs as a part of a mucin-type O-glycan structure (26). In the present study, the analyses of MAb7 epitope using glycan arrays suggested that the clustered NeuAcα2–3Galβ plays a pivotal role in MAB7 binding. However, treatment of cell lysates with α2–3 neuraminidase had little effect on MAB7 recognition by Western blot analyses. Because α2–3 neuraminidase may not release sialic acid from O-linked oligosaccharide on glycoproteins (27), our results led to the conclusion that the epitope of MAB7 is the clustered NeuAcα2–3Galβ O-linked oligosaccharide. As far as we know, antibodies against the clustered NeuAcα2–3Galβ have not been reported. Furthermore, we identified DMBT1 as a carrier protein of MAB7 epitope and showed that expressions of MAB7 and TRA-1–60 antigen differed in frequency and localization in cancer cells.

TRA-1–60 has been widely used in human stem research as a positive indicator of a true pluripotent human stem cell (26). Several lines of evidence have suggested that TRA-1–60 antigen is one of the most specific markers of undifferentiated hESCs, and TRA-1–60 has been used to separate undifferentiated hESCs from differentiated derivatives for hESC-derived cell replacement therapy (28). Our results showed that MAB7 recognized cell surface antigens of undifferentiated hESCs in the same way as TRA-1–60, suggesting that MAB7 antigen could be another pluripotent hESCs marker. Unexpectedly, although anti-DMBT1 antibodies reacted with lysates of KP-3L (Fig. 4E), no apparent bands in the hESC lysates were observed (data not shown). In addition, the results of DNA microarray analysis showed that the expression levels of DMBT1 gene were negligible in hESCs (data not shown). These results implied the presence of other proteins, distinct from DMBT1, carrying MAB7 epitope in hESCs. A recent study suggested the involvement of the terminal sialic acid in the maintenance of embryonic stem cell pluripotency and undifferentiation (29). Future studies to clarify the physiological significances, as well as identification of MAB7 antigen in hESCs, are now under investigation.

To investigate the expression of MAB7 antigen in cancer cells, we first screened 20 cancer cell lines to discover MAB7-positive ones and suggested that the expression of MAB7 antigen is cell type-specific. MKN74 cells derived from the differentiated gastric carcinoma showed morphological characteristics of intestinal differentiation in cell polarity (30). In contrast, MKN45 cells were derived from the poorly differentiated carcinoma and had characteristics of ordinary gastric mucosa. In addition, MKN45 cells seem to have multipotentiality for differentiation and preserved that characteristic well for long periods of culture (30). Consistent with the results of hESCs studies (Fig. 1), we found that MAB7 discriminated poorly differentiated gastric cancer cells from differentiated ones. In addition, we showed that expression of MAB7 epitope is vulnerable to epigenetic modification. Epigenetics plays a major role in cell type specification, because the differentiation process is accompanied by major chromatin remodeling (24). In this study, we identified GALNT1 and LARGE as down-regu-
lated genes that are responsible for biosynthesis of MAb7 epitope in MKN74 cells. GALNT1 catalyzes the initial reaction in O-linked oligosaccharide biosynthesis and has a broad spectrum of substrates for peptides such as mucins (31). LARGE was originally named because it covers ~650 kb of human genomic DNA, despite having a coding region of only 2268 bp (32). On the basis of homologies of the catalytic domains, LARGE might add a repeated glycan structure such as poly-N-acetyllactosamine, which can be attached to O-glycan (32). We previously had shown that the heavily glycosylated O-glycans were abundantly present on the cell surface of MKN45 cells compared with the differentiated gastric cancer cells (20). Considering the results from analyses of MAb7 antigen, a keratanase-sensitive O-glycan, both GALNT1 and LARGE are definitely involved in the biosynthesis of MAb7 antigen.

The mortality rate of pancreatic cancer is so high in part because it usually does not produce symptoms until being metastasized and because there are no sensitive and specific tools to screen for early disease (33). Mucin-like protein DMBT1 binds to a variety of host proteins including IgA, C1q, lactoferrin, mucin 5B, and trefoil factor 2, which are all involved in innate immunity (34). The highly metastatic KP-3L cells were isolated from the liver colonies of pancreatic cancer KP-3 cells after injection into nude mice (35). The present results showed that MAb7 antigen was almost expressed in cytoplasm of some KP-3 cells, whereas all KP-3L cells expressed its antigen on the plasma membrane and in the conditioned medium. These observations implied that the membrane-bound type, as well as the secreted form of the clustered NeuAcc2→3Galβ O-linked oligosaccharide on DMBT1, might play a role in the ability of pancreatic cancer cells to escape from the immune surveillance of the host, leading to metastasis. Further work is necessary to characterize other MAb7-positive pancreatic cancer cell lines. In addition, validation studies to test the possibility of MAb7 antigen as a biomarker for pancreatic cancer are also under investigation.

Cancer stem cells are characterized by undifferentiation, self-renewal, and differentiation potential, which is responsible for tumor occurrence, growth, and metastasis. Previous studies have shown that elevated levels of cell surface sialic acid and α2,3-sialyltransferase activity are associated with liver-metastatic potential of colon cancers (15, 36). In addition, the clustered presentation of sialyl Lewis X on tumor cell mucins is thought to facilitate metastasis through binding to selectin adhesion receptors expressed on platelets, leukocytes, and endothelial cells (37, 38). In this study, we also showed that the clustered sialyl oligosaccharide on the cell surface plays a pivotal role in interaction between cancer cells and endothelial cells. However, adhesions of KP-3L cells to endothelial cells from umbilical vein and brain microvessel were relatively low, and neither IL-1β nor TNF-α stimulated the binding of endothelial cells to KP-3L cells. These results favor the idea of unknown interaction between the clustered sialyl O-linked oligosaccharide on liver-metastatic pancreatic cancer cells and its receptor on endothelial cells in liver.

Aberrant glycosylation occurs in essentially all types of experimental and human cancers, and many oligosaccharide epitopes, particularly sialyl O-glycan, constitute tumor-associated antigens (39). Previous studies indicate that some, if not all, aberrant glycosylation is a result of initial oncogenic transformation, as well as a key event in induction of invasion and metastasis (39). There is emerging evidence that cell surface mucins contribute to the regulation of differentiation and proliferation of cancer cells through processes of ligand-receptor interactions and morphogenetic signal transduction (40). Linkage of GaINAc to the hydroxyl group of serine and threonine in a peptide context initiates the production of O-glycosylation and proceeds to the elongation of sugars step by step. In this study, we showed that DMBT1 protein was uniformly distributed throughout the cancer cells, whereas MAb7 epitope was strongly expressed on the plasma membrane. NetOglyco, a database for predictions of mucin-type O-glycosylation sites in mammalian proteins, shows several clustered O-glycosylation sites within DMBT1. Because MAb7 interacts with the clustered sialyl O-glycan, differences in localization between DMBT1 protein and MAb7 epitope might be explained by the variety of maturation states of DMBT1 after translation. Further studies to identify the maturation level of DMBT1 would determine the differentiation stage of cancer cells.

Altogether, we have suggested that mouse anti-hESCs monoclonal antibody MAB7 marks undifferentiated stem cells such as hESCs and cancer stem-like cells. In addition, our results raised the possibility of MAB7 as a biomarker and an antibody drug for pancreatic cancer. These results provide support for the importance of carbohydrates in stem cells.

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