Inhibition of motility and invasiveness of renal cell carcinoma induced by short interfering RNA transfection of β1,4GalNAc transferase

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Abstract Human renal cell carcinoma (RCC) has been characterized by remarkable changes in ganglioside composition. TOS1 cells, typical of metastatic RCC, are characterized by predominance of GM2 as monosialoganglioside, and β1,4GalNAc disialyl-Lc_4 (RM2 antigen) as disialoganglioside [J. Biol. Chem. 276 (2001) 16695]. In order to observe the functional role of gangliosides in RCC malignancy, TOS1 cells were transfected with short interfering RNA (siRNA) based on open reading frame sequence of β1,4GalNAc transferase (β1,4GalNAc-T), and its disordered sequence of siRNA (dsiRNA) as control. In siRNA transfectant, β1,4GalNAc-T mRNA level and GM2 expression were greatly reduced, whereby GM3 expression appeared. In contrast, RM2 antigen level was unchanged, even though it has the same β1,4GalNAc epitope at the terminus. dsiRNA transfectant showed no change of β1,4GalNAc-T mRNA and did not express GM3. Concomitant with reduction of GM2 and appearance of GM3, siRNA transfectant showed greatly reduced motility and invasiveness, although growth rate was unaltered. Both transfectants with siRNA and dsiRNA expressed the same level of tetraspanin CD9. Since CD9/GM3 complex is known to reduce integrin-dependent motility and invasiveness [Biochemistry 40 (2001) 6414], it is plausible that motility and invasiveness of siRNA transfectant of TOS1 cells may be reduced by enhanced formation of such complex.

Keywords: Short interfering RNA; β1,4 *N*-Acetylgalactosaminyl-transferase; GM3; Renal cell carcinoma; Invasion inhibition

1. Introduction

Metastatic/invasive properties of renal cell carcinoma (RCC) have been suggested to correlate with ganglioside profile [1,2]. RCC TOS1 cells [3] are highly metastatic and are characterized by predominance of GM2 (β1,4GalNAc-GM3) as monosialoganglioside, and by the absence of GM3. TOS1 cells also contain a novel disialoganglioside β1,4GalNAc disialyl Lc_4 [4] defined by monoclonal antibody (mAb) RM2 [5]. GM2 and RM2 antigens have common terminal β1,4GalNAc structure. RM2 antigen was previously claimed to be an adhesion receptor [3], or to be recognized by siglec-7, promoting metastasis to lung [6]. On the other hand, GM2 in other types of tumors has been implicated as a promoter of tumor cell invasiveness (see Section 4). In order to clarify the role of GM2 and β1,4GalNAc disialyl Lc_4 in defining TOS1 cell phenotype, we tried to "knock down" these gangliosides by sequence-specific inhibition of β1,4GalNAc transferase (β1,4GalNAc-T) gene [7], by transfecting short interfering RNA (siRNA) [8]. GM2 synthesis was clearly knocked down, with concomitant appearance or enhancement of GM3, leading to reduction of cell motility and invasiveness, presumably due to formation of a complex with tetraspanin CD9, which inhibits integrin-dependent cell motility and invasiveness [9–11]. However, the level of RM2 antigen was unchanged by siRNA of β1,4GalNAc-T, even though it has the same terminal structure (see Section 4).

2. Materials and methods

2.1. Synthesis of siRNA

The sequence of siRNA for human β1,4GalNAc-T cDNA (GenBank Accession No. M83651) according to the AA-N19 [N, any nucleotide (nt)] rule was 5′-GGAGCAAGUAGUGGGGCUGdTdT-3′ (sense), 3′-dTdTCCUCGUUCAUCACCCCGAC-5′ (antisense).

Disordered siRNA (dsiRNA) of scrambled nucleotide sequence, used as negative control, was 5′-GCGCGCUUUGUAGGAUUCGdTdT-3′ (sense), 3′-dTdTCGCGCGAAACAUCCUAAGC-5′ (antisense).

Specificity of the 21-nt sequences was confirmed by BLAST search against the human genome sequence. The RNA duplexes were purchased from B-Bridge Japan (Tokyo, Japan).

2.2. Cell line and transfection

Human RCC TOS1 was established from the soft tissue metastatic lesion of RCC (spindle cell type and clear cell mixture type RCC) from a 62-year-old man [3]. This cell line was maintained in MEM (Gibco BRL, Rockville, MD, USA) supplemented with 2 mM L-glutamine, penicillin G (100 units/ml), streptomycin (100 μg/ml), 10 mM HEPES buffer solution (DOJINDO, Kumamoto, Japan) and 10% heat-inactivated fetal bovine serum (ICN Biomedicals, Aurora, OH, USA). TOS1 cells were placed at a density of 5×10^4 per 35-mm diameter dish. One day later, TOS1 cells were transfected with 200 pmol siRNA,

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Abbreviations: C/M, chloroform/methanol; dsiRNA, disordered siRNA; β1,4GalNAc-T, β1,4GalNAc transferase; GAPDH, glyceraldehyde 6-phosphate dehydrogenase; GSL, glycosphingolipid; mAb, monoclonal antibody; PCR, polymerase chain reaction; RCC, renal cell carcinoma; siRNA, short interfering RNA; TLC, thin-layer chromatography

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using Oligofectamine (Gibco–Invitrogen, Rockville, MD, USA) according to the protocol provided by Nihon Bioservice (Saitama, Japan). The medium was replaced with fresh medium 6 h after transfection and gene silencing effect was analyzed 3–4 days after transfection by the following assays. All experiments were performed in triplicate.

2.3. Quantitative real-time polymerase chain reaction

Total RNA was isolated from each transfectant using ISOGEN-LS (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's instructions. cDNA was prepared from 1 μg total RNA using cDNA Synthesis kit for real-time polymerase chain reaction (RT-PCR) (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol, using the principle of FRET-based RT-PCR [12]. Five micrograms of each cDNA was diluted to a volume of 20 μg PCR mix (LightCycler FastStart DNA Master Hybridization Probes, Roche Diagnostics) containing 3 mM $MgCl_2$, primers, and probes. The sequences and final concentrations of primers and probes in the reaction mixtures were: β1,4GalNAc-T forward 5′-CGCTGGGGATTTCT-GACTGT-3′ (0.5 μM); reverse 5′-GAGGACTTGGCACTGGCTGT-3′ (0.5 μM); probe 5′-CCCACCACCCTGTGAGCACTCTACT-fluo-3′ (0.2 μM); probe 5′-LCRed 640-GCTGTCCCTGAGCCTCTAG-TTCCTCA-phos-3′ (0.2 μM); glyceraldehyde 6-phosphate dehydrogenase (GAPDH) forward 5′-TGAACGGGAAGCTCACTGG-3′ (0.5 μM); reverse 5′-TCCACCACCCTGTTGCTGTA-3′ (0.5 μM); probe 5′-TCAACAGCGACACCCACTCCT-fluo-3′ (0.2 μM); probe 5′-LCRed640-CACCTTTGACGCTGGGGCT-phos-3′ (0.2 μM).

Primers and probes were purchased from Nihon Gene Research Labs Inc. (Sendai, Japan). For amplification, initial denaturation at 95 °C for 10 min was followed by β1,4GalNAc-T: 10 s at 95 °C, 15 s at 62 °C, 7 s at 72 °C; GAPDH: 10 s at 95 °C, 15 s at 62 °C, and 9 s at 72 °C, for 40 cycles. The expected size of the PCR products was confirmed by agarose gel electrophoresis (data not shown). The PCR products amplified without the probes from TOS1 transfected with dsiRNA were diluted at various folds and used as references. The threshold cycle C_t was assessed using LightCycler software v. 3.5.

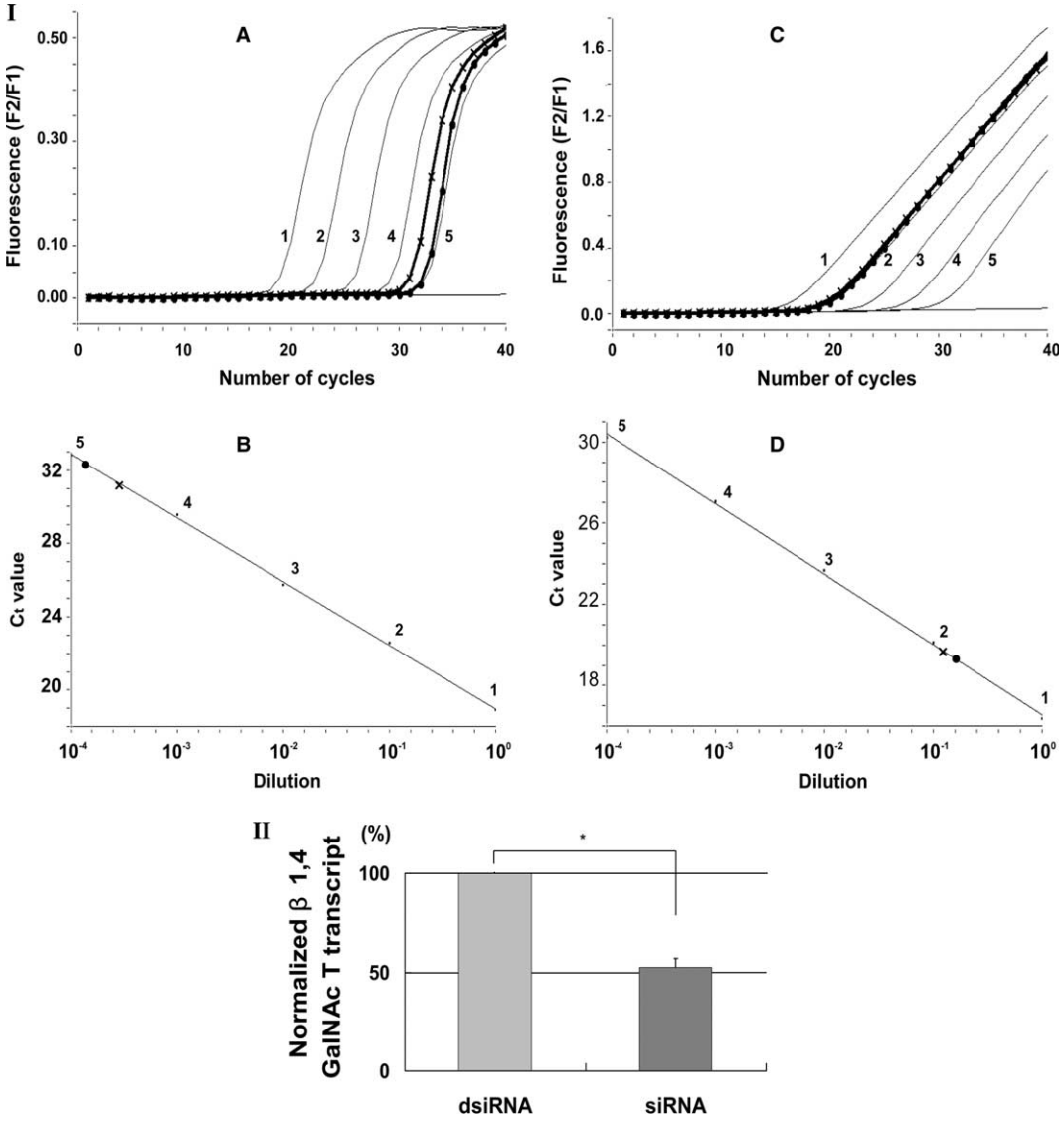

Fig. 1. Reduced β1,4GalNAc-T gene expression in siRNA transfectant. Panel I. Quantitative RT-PCR analysis of β1,4GalNAc-T gene (A) and of control GAPDH gene (C) in siRNA transfectant (●) and dsiRNA transfectant (×). RT-PCR product from the control transfectant was diluted at various concentrations and used as references [1–5]. The acceptor LCRed640 signal yielded by fluorescence resonance energy transfer (FRET) was detected on Channel 2 (F2) and normalized with fluorescein signal detected on Channel 1 (F1). C_t values of the reference were plotted vs. dilutions for β1,4GalNAc-T (B) and for GAPDH (D). C_t values for siRNA transfectant (●) and dsiRNA transfectant (×) were plotted on the standard curves. Panel II. The expression of β1,4GalNAc-T was normalized to GAPDH level. Percent expression was calculated relative to control dsiRNA transfectant, defined as 100%. P values calculated from Welch test were <0.01.

2.4. Glycosphingolipid extraction, thin-layer chromatography, and immunostaining

Glycosphingolipids (GSLs) were extracted [4,11] and separated from phospholipids by alkaline degradation of phospholipids [13,14], and GSLs were analyzed by thin-layer chromatography (TLC) with immunostaining [13–16]. Briefly, $2–5 \times 10^7$ cells (pelleted by centrifugation) were extracted twice with 2 ml isopropanol/hexane/water (55:25:20 v/v/v) [4,11]. Extracts were evaporated to dryness under nitrogen stream, and phospholipids in the residue were dissolved in 2 ml of 0.1 M NaOH in methanol (40 °C, 2 h), neutralized with 200 μl of 1 N HCl, and shaken repeatedly with 2 ml hexane to eliminate fatty acids. GSLs in the lower layer were further purified and desalted by SepPak C18 cartridge (Millipore) [14]. Total GSL fraction thus obtained was further separated by DEAE–Sephadex A25 column into neutral GSLs, monosialosyl, disialosyl, and other fractions by stepwise elution with various molarities of ammonium acetate in chloroform/methanol (C/M)/water. Each fraction was dialyzed, lyophilized, dissolved in C/M 2:1, and subjected to TLC analysis [4,14]. Aliquot quantities of GSL fractions placed on TLC for analysis were based on equal wet cell weight (~10 mg). E.g., wet weight of pelleted 3×10^7 TOS1 cells is usually ~200 mg; GSL fraction derived therefrom is dissolved in 100 μl C/M 2:1; 5 μl aliquot (equivalent to ~10 mg wet cell weight) is placed on each lane of TLC and analyzed. TLC was developed in C/M/0.5% aqueous $CaCl_2$ (50:40:10) and visualized by spraying with 0.5% orcinol in 2 N sulfuric acid, or immunostained [15,16] using mAbs MK1-8 (anti-GM2) [17], M2590 (anti-GM3) [18], and RM2 (anti-GalNAc disialyl-Lc_4) [5]. Vector avidin–biotin solution with Konica HRP-1000 was used, as described previously [4].

Mean density profile of MK1-8 and RM2 of dsiRNA and siRNA were quantified using Scion Image.

2.5. In vitro cell proliferation assay

72 h after transfection, cells were seeded in 96-well plates at a density of 10^4 cells/ml in MEM containing 10% FCS and cultured. Cell number was measured daily using a Cell Counting Kit (Wako Pure Chemical Industries, Tokyo, Japan).

2.6. In vitro motility assay and invasion assay

Motility assay. 72 h after transfection, cells were detached, aliquots of 1×10^5 cells per 100 μl were placed in the inner compartment of Transwell chamber (Costar, Cambridge, MA, USA; polycarbonate filter, pore size 8 μm), and 600 μl MEM containing 30 μg fibronectin was placed in the outer compartment, to initiate motility assay (surface levels of cell suspension and FN solution in inner and outer compartments were the same). Cells migrated to the lower surface of membrane after 3 and 6 h were counted under Giemsa staining, and mean values for 10 microscopic fields were plotted. *P* values were calculated from Student's *t* test.

Invasion assay through Matrigel. For invasion assay, 50 μl of Matrigel stock solution was placed at the upper surface of filter and dried at 37 °C overnight. The Matrigel stock solution was 1 mg/ml (containing laminin-1 and -2, collagen-IV, and heparan sulfate; BD Biosciences, Bedford, MA). Cell suspension was placed in the inner compartment, ad migration was determined as described previously [19].

2.7. Determination of cell surface CD9 expression

Expression of tetraspanin CD9 at the surface of siRNA transfectant and parental TOS1 cells was determined by flow cytometry using anti-human CD9 mouse mAb TP-82 (Nichirei, Tokyo, Japan).

3. Results

3.1. Knock-down of β1,4GalNAc-T mRNA measured by RT-PCR

We examined mRNA level of β1,4GalNAc-T in siRNA transfectant and dsiRNA transfectant by RT-PCR using LightCycler. GAPDH was used as internal control in each sample and normalized by GAPDH expression level (Fig. 1, panel I). Relative expression of β1,4GalNAc-T mRNA in siRNA transfectant as compared with dsiRNA transfectant was ~50%. For details of procedure, see Section 2 and Fig. 1 legend.

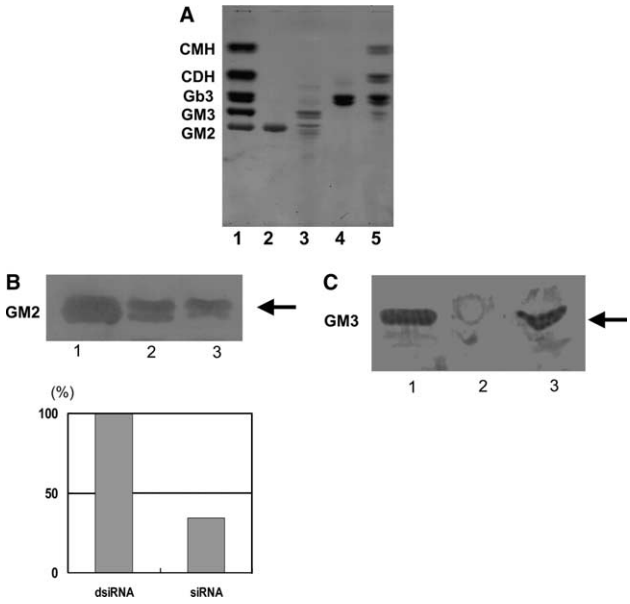

Fig. 2. TLC profile of GSLs from TOS1 cells with or without β1,4GalNAc-T siRNA transfection or dsiRNA transfection Panel A. TLC profile of GSLs shown by orcinol-sulfuric acid staining of TOS1 cells. TOS1 cells transfected with β1,4GalNAc-T siRNA, or transfected with dsiRNA (as control) were extracted, and GSL fractions were separated and analyzed by TLC on "equal cell wet weight" basis, developed in C/M/0.5% aqueous $CaCl_2$ (50:40:10) and stained with orcinol-sulfuric acid. Lane 1: Reference GSLs, from top to bottom: GlcCer (CMH), LacCer (CDH), Gb3, GM3, and GM2. Lane 2: Monosialoganglioside fraction of TOS1 dsiRNA transfectant. Lane 3: Monosialoganglioside fraction of TOS1 β1,4GalNAc-T siRNA transfectant. Lane 4: Neutral GSL fraction of TOS1 dsiRNA transfectant. Lane 5: Neutral GSL fraction of TOS1 β1,4GalNAc-T siRNA transfectant. Note that GM2 is the sole monosialoganglioside in control dsiRNA transfectant (lane 2), but is greatly reduced in siRNA transfectant (lane 3). In contrast, GM3 is nearly absent in lane 2, but appears in lane 3. Panel B. TLC immunostaining with anti-GM2 mAb MK1-8. Total gangliosides of TOS1 cell β1,4GalNAc-T siRNA transfectant and dsiRNA transfectant were stained with mAb MK1-8. Lane 1: Reference GSLs, GM2. Lane 2: Control TOS1 dsiRNA transfectant. Lane 3: TOS1 β1,4GalNAc-T siRNA transfectant. Percent expression was quantified by using Scion Image relative to control dsiRNA transfectant, defined as 100%. Relative mean density of MK1-8 in siRNA transfectant as compared with dsiRNA transfectant was ~30%. Panel C. TLC immunostaining with anti-GM3 mAb M2590. Total gangliosides of TOS1 cell β1,4GalNAc-T siRNA transfectant and dsiRNA transfectant were stained with mAb M2590. Lane 1: Reference GSLs, GM3. Lane 2: Control TOS1 dsiRNA transfectant. Lane 3: TOS1 β1,4GalNAc-T siRNA transfectant.

3.2. Decrease of GM2 expression and concomitant increase of GM3 in siRNA transfectant TOS1 cells

TLC pattern of gangliosides from β1,4GalNAc-T siRNA transfectant showed greatly reduced expression of GM2 and concomitant increase of GM3 (Fig. 2A, lane 3), whereas TLC pattern from dsiRNA transfectant showed GM2 as the sole monosialoganglioside, without GM3 (lane 2). Reduced GM2 and increased GM3 in siRNA transfectant was further confirmed by TLC immunostaining with mAbs MK1-8 (anti-GM2) (Fig. 2B) and M2590 (anti-GM3) (Fig. 2C). Relative mean density of MK1-8 in siRNA transfectant as compared with dsiRNA transfectant was ~30% (Fig. 2B).

3.3. RM2 antigen is unchanged in β1,4GalNAc siRNA transfectant

Relative mean density of RM2 in siRNA transfectant as compared with dsiRNA transfectant was ~60% (Fig. 3A). Since RM2 antigen has β1,4GalNAc at terminal Gal of disialyl-Lc_4, which is the same structure as in GM2 ganglioside, we analyzed the expression of RM2 in β1,4GalNAc siRNA transfectant,and found that RM2 antigen was unaffected (Figs. 3B and C).

3.4. Difference in GM2 and GM3 expression in siRNA transfectant compared to dsiRNA transfectant TOS1 cells

GM2 expression was clear in control dsiRNA transfectant cells, but was much lower in siRNA transfectant (Fig. 4, upper panels). In contrast, immunofluorescence staining for GM3 was much higher in siRNA transfectant than in dsiRNA transfectant (Fig. 4, lower panels). This reciprocal relationship was observed in the majority of cells.

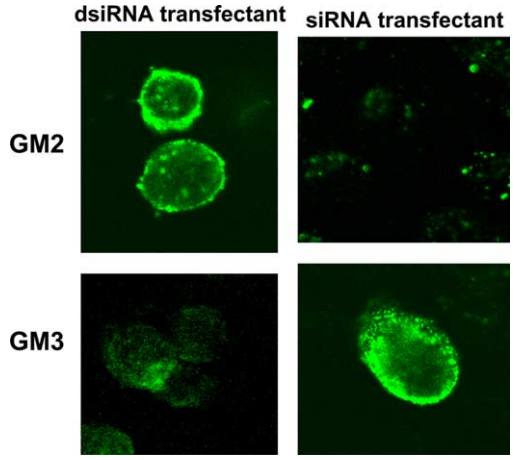

Fig. 4. Confocal laser scanning microscopy of TOS1 cell siRNA transfectant and control dsiRNA transfectant.

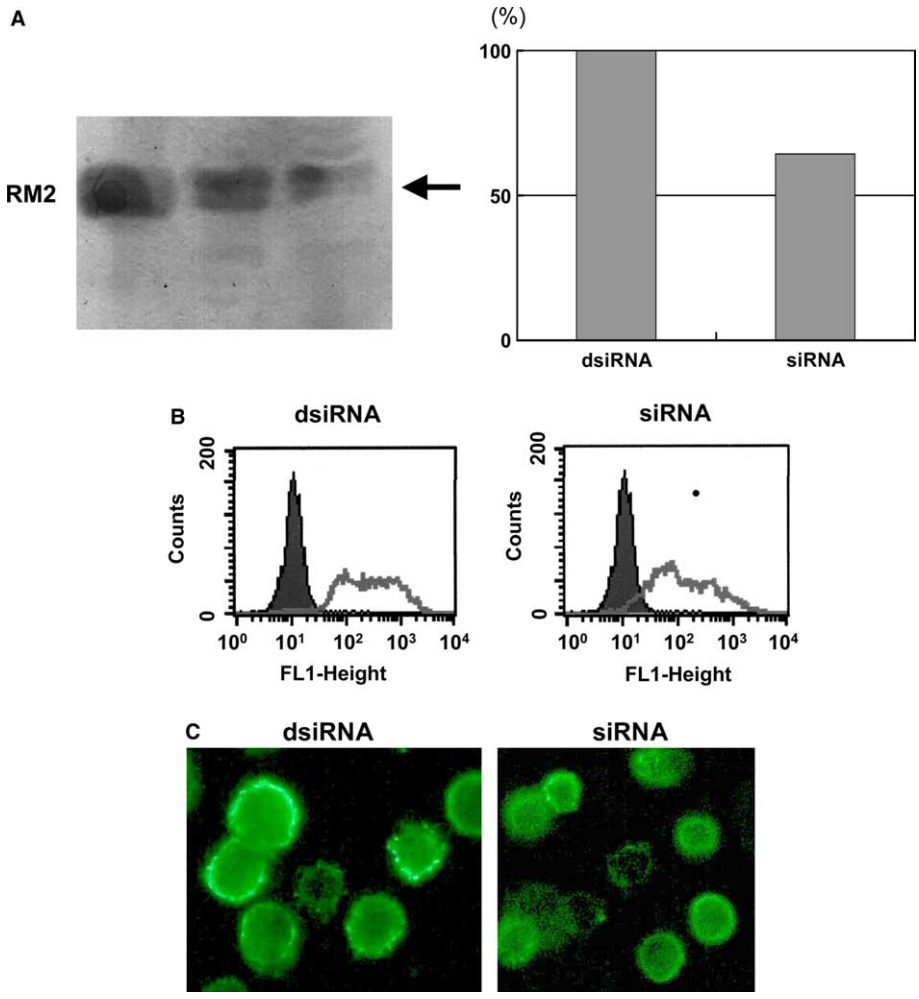

Fig. 3. RM2 antigen expression of TOS1 cell siRNA transfectant and control dsiRNA transfectant. Panel A. TLC immunostaining with mAbs RM2. Total gangliosides of TOS1 cell β1,4GalNAc-T siRNA transfectant and dsiRNA transfectant were stained with mAbs RM2. Lane 1: Reference GSLs, RM2. Lane 2: Control TOS1 dsiRNA transfectant. Lane 3: TOS1 β1,4GalNAc-T siRNA transfectant. Percent expression was quantified by using Scion Image relative to control dsiRNA transfectant, defined as 100%. Relative mean density of RM2 in siRNA transfectant as compared with dsiRNA transfectant was ~60%. Panel B. RM2 expression, determined by flow cytometry, of control TOS1 dsiRNA transfectant (left) and siRNA transfectant (right). Panel C. Confocal laser scanning microscopy of TOS1 cell siRNA transfectant and control dsiRNA transfectant.

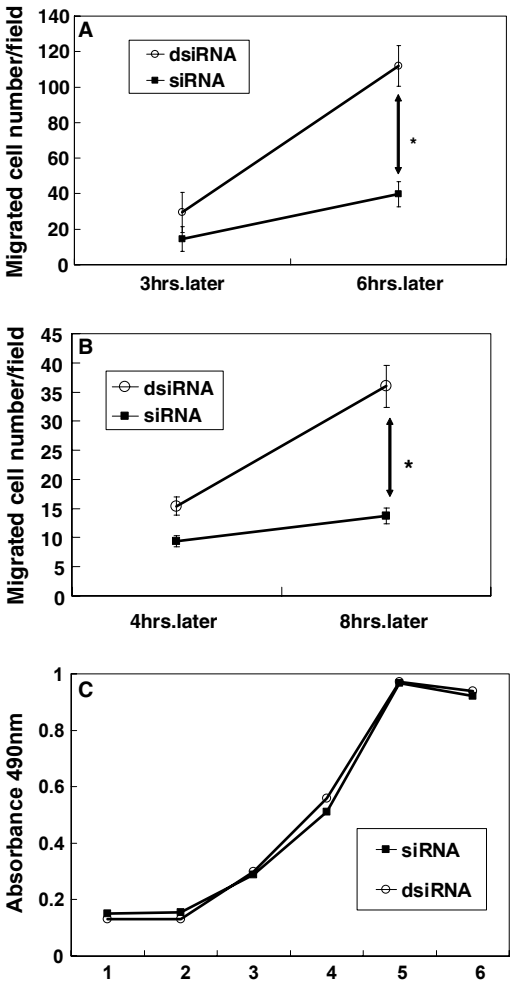

Fig. 5. Motility, invasiveness, and cell growth of TOS1 β1,4GalNAc-T siRNA transfectant compared to control dsiRNA transfectant Panel A. FN-dependent Transwell motility of TOS1 β1,4GalNAc-T siRNA transfectant (black square) compared to that of control dsiRNA transfectant (open circle). Each transfectant was placed on three Transwell plates and motility was determined by counting the number of cells migrated to lower surface of membrane in ten microscopic fields per plate (total 30 fields for each transfectant). Motility values at 3 and 6 h were determined, and Student's t test and S.D. were calculated. In each case, $P < 0.001$. Panel B. Invasiveness through high concentration of Matrigel (containing mainly laminin, collagen-IV, and heparan sulfate) coated on Transwell membrane. Black square, TOS1 β1,4GalNAc-T siRNA transfectant. Open circle, control dsiRNA. Procedures and statistical calculations as in Panel A. In each case, $P < 0.001$. Panel C. Cell proliferation (growth curve) of TOS1 β1,4GalNAc-T siRNA transfectant (black square) compared to that of control dsiRNA transfectant (open circle). Note that the growth rates are essentially the same.

3.5. Changes in cell growth, motility, and invasiveness through Matrigel of β1,4GalNAc siRNA transfectant as compared to dsiRNA transfectant TOS1 cells

Fibronectin-dependent cell motility analyzed with Transwell chamber was reduced in β1,4GalNAc siRNA transfectant as compared to dsiRNA transfectant; the difference was much greater at 6 than at 3 h after transfection (Fig. 5A). Cell migration through a high concentration of Matrigel (regarded as "invasion") was also greatly reduced in β1,4GalNAc siRNA transfectant compared to control (Fig. 5B). Rate of cell proliferation as shown by cell growth curve of siRNA transfectant was essentially the same as that of control dsiRNA transfectant (Fig. 5C).

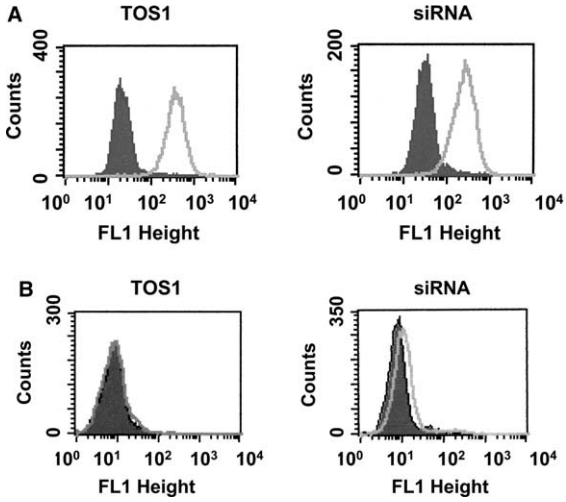

Fig. 6. CD9 expression, and CD9/GM3 expression ratio, in TOS1 β1,4GalNAc-T siRNA transfectant and parental TOS1 cells Panel A. CD9 expression, determined by flow cytometry, of parental TOS1 (left) and siRNA transfectant (right). Panel B. GM3 expression, determined by flow cytometry, of parental TOS1 (left) and siRNA transfectant (right).

3.6. CD9 expression in β1,4GalNAc siRNA transfectant

Tetraspanin CD9, a type of proteolipid protein, was expressed to a similar, high degree in parental TOS1 cells and in β1,4GalNAc siRNA transfectant, as observed by flow cytometry (Fig. 6A). Since GM3 is essentially absent in parental TOS1 cells (Fig. 6B), relative expression of CD9 and GM3 (CD9/GM3 expression ratio) is greatly reduced in siRNA transfectant. Cell motility is controlled by GM3/CD9/integrin complex (see Section 4).

4. Discussion

Tumor malignancy is closely associated with types of gangliosides expressed. Bladder carcinoma with enhanced GM2 and lower GM3 expression showed higher motility and invasiveness [11]. Similarly, malignancy of mouse ependymoblastoma is higher in β1,4GalNAc transferase transfectant expressing GM2 and other a-series gangliosides than in parental cells which express GM3 alone [20]. This relationship between GM3 vs. GM2 expression as a basis of tumor cell invasiveness is clarified by the current studies with RCC TOS1 cells using siRNA approach. Our results show that silencing of β1,4GalNAc-T by transfection of siRNA, but not dsiRNA as control, knocks down GM2 expression and thereby increases level of its precursor GM3. Associated with this change, siRNA transfectant showed significant decrease of FN-dependent cell motility and invasiveness through Matrigel. Since TOS1 cells have high CD9 expression, enhanced level of GM3 in siRNA transfectant may cause formation of GM3/CD9/integrin complex which suppresses integrin-dependent cell motility, as observed originally in ldlD cells with endogenous GM3 expression [9] and subsequently in various types of colorectal

carcinoma showing high CD9 expression, or transfected with CD9 [10]. Extensive studies are necessary to clarify this possibility.

TOS1 cells express novel β1,4GalNAc disialyl Lc_4 (RM2 antigen), having the same terminal β1,4GalNAc as in GM2. However, little change in the level of this ganglioside was caused by siRNA approach with β1,4GalNAc-T, while GM2 was knocked down. This finding suggests that β1,4GalNAc-T for synthesis of GM2 is different from that for synthesis of RM2 antigen in terms of substrate specificity. This point also requires further extensive study.

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