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PCDH10 is required for the tumorigenicity of glioblastoma cells



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ABSTRACT

Protocadherin10 (PCDH10)/OL-protocadherin is a cadherin-related transmembrane protein that has multiple roles in the brain, including facilitating specific cell–cell connections, cell migration and axon guidance. It has recently been reported that PCDH10 functions as a tumor suppressor and that its overexpression inhibits proliferation or invasion of multiple tumor cells. However, the function of PCDH10 in glioblastoma cells has not been elucidated. In contrast to previous reports on other tumors, we show here that suppression of the expression of PCDH10 by RNA interference (RNAi) induces the growth arrest and apoptosis of glioblastoma cells *in vitro*. Furthermore, we demonstrate that knockdown of PCDH10 inhibits the growth of glioblastoma cells xenografted into immunocompromised mice. These results suggest that PCDH10 is required for the proliferation and tumorigenicity of glioblastoma cells. We speculate that PCDH10 may be a promising target for the therapy of glioblastoma.

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1. Introduction

Protocadherin10 (PCDH10)/OL-protocadherin is a transmembrane protein that has 6 cadherin domains in the extra cellular region and belongs to the $\delta 2$ -protocadherin family of proteins [1–3]. PCDH10 is concentrated at cell–cell junctions and promotes aggregation, although its aggregative strength is weaker than that of classical cadherins [4]. Furthermore, PCDH10 recruits the Nap1-WAVE complex at the sites of cell–cell contact and promotes reorganization of the actin cytoskeleton, which results in accelerated cell migration on confluent cell monolayers [5]. In neuronal cells, PCDH10 is localized along the axons, is concentrated in the growth cones and is involved in axon guidance [5,6].

It has recently been reported that CpG islands within PCDH10 are highly methylated and epigenetically silenced in many tumors, including breast cancer, nasopharyngeal, esophageal carcinoma and haematological malignancies and this is associated with poor prognosis [7–12]. It has also been reported that overexpression

of PCDH10 significantly inhibits proliferation or invasion of multiple tumor cells *in vitro* [9,11].

Glioblastoma is a highly invasive and progressive tumor type, with an overall 5-year survival rate of less than 5% [13]. In the present study, we examined whether PCDH10 also exerts a tumor suppressive function in glioblastoma cells. We unexpectedly found that PCDH10 is required for the proliferation and tumorigenicity of glioblastoma cells.

2. Materials and methods

2.1. Cell lines and tumor specimens

Following informed consent, tumor samples classified as primary glioblastoma were obtained from patients undergoing surgical treatment at Kanazawa University Hospital and the University of Tokyo Hospital as approved by the Institutional Review Board. The human astrocytoma cell lines U87, U251, T98G and LN229 (ATCC) were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum. Cell lines GB1–16 were cultured in DMEM/F-12 containing B27 supplement (GIBCO), EGF and FGF2 (20 ng/ml each, Wako) on laminin-coated culture dishes [14–16].

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2.2. Antibodies

Mouse monoclonal antibody (mAb) to PCDH10 (5G10) was prepared as described previously [6]. mAbs to α -tubulin, Flag M2 and Ki67 were purchased from CALBIOCHEM, Sigma–Aldrich and Leica Biosystems, respectively. Rabbit polyclonal antibody to GFP was from Santa Cruz.

2.3. Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue blocks were sectioned (6 μ m thick) onto slides and then deparaffinized. Slides were microwaved for 15 min in target retrieval solution (pH 6.0; Dako). Internal peroxidases were blocked by incubation in 0.3% H₂O₂ solution in methanol for 20 min. Non-specific staining was blocked by a 30 min incubation with blocking solution (5% skim milk, 0.1% Tween20 in TBS). Sections were immunostained with the ABC kit (VECTOR). Anti-PCDH10 (5G10), anti-Ki67 and anti-GFP antibodies were used at a dilution of 1:200. Sections were exposed to diaminobenzidine peroxidase substrate (Funakoshi) for 5–10 min and counterstained with Mayer's hematoxylin. Non-immune rat IgG was used as a negative control. All images were taken by bright field microscopy AX80 (Olympus). Ki67 index was calculated as the number of Ki67-positive cells per 1.0×10^3 cells in each of 6 fields per sample.

2.4. Lentivirus production

The entry vector pENTRH1 (obtained from K. Ui-Tei), the lentiviral vector CS-RfA-CG and the packaging vectors pCAG-HIV-gp and pCMV-VSV-G-RSV-Rev (provided by H. Miyoshi) were used for lentivirus production. The target sequences for shRNA are as follows: luciferase, 5'-GATTCGAGTCGCTTAATGT-3'; DsRed, 5'-GCCCGT AATGCAGAAGAAGA-3'; human PCDH10#2, 5'-GTGCGTGGCAAC-GAAATGAAC-3'; human PCDH10#4, 5'-GAGAAGAAGCTCAACATC-TAT-3'. Infection efficiency was monitored by GFP expression driven by the CMV promoter.

2.5. Single-cell migration assay

Time-lapse images were obtained at 5-min intervals for 4 h using a microscope (IX81; Olympus) equipped with a cooled CCD camera CoolSNAP K4 (Photometrics). GB2 cells (1×10^3 cells) infected with shRNA-expressing lentiviruses for 3 days (MOI = 6) were plated on laminin-coated, glass-bottom, 24-well plates (Iwaki) and GFP signals were traced. Trace drawing and statistical analyses were performed with IMARIS Track (BitPlane) and Excel (Microsoft), respectively. The box plots were presented as described previously [5].

2.6. In vitro invasion assay

In vitro invasion assays were performed using 24-well Biocoat Matrigel invasion chambers (BD Biosciences). GB2 cells (1.0×10^5 cells per well) infected with shRNA-expressing viruses for 4 days (MOI = 3) were starved overnight with DMEM/F-12 medium and allowed to migrate toward the underside of the top chamber for 24 h. The lower chamber was filled with DMEM/F12 containing 10% FBS. Cells in the top chamber were removed and the number of infiltrated cells was counted by Cell Titer-Glo assays. Infiltrated cells were fixed with methanol and subjected to HE staining (Merck).

2.7. Cell proliferation assay

Cell viability was determined using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega). Luminescence was measured on a Mithras LB 940 (Berthold).

2.8. Pcdh10

The full-length PCDH10 variant 1 cDNA (obtained from Q. Tao) was subcloned into the pIRES hrGFP 3 \times Flag vector (Adigent). 3 \times Flag tagged PCDH10 was subcloned into the pENTR4 dual selection vector (Invitrogen).

2.9. Apoptosis assay

Apoptotic cells were detected using the Annexin V-Biotin Apoptosis Detection Kit (MBL) and labeled with Streptavidin-APC conjugates (S888, Invitrogen). A minimum of 5×10^3 cells were analyzed with flow cytometer Canto II (BD Bioscience) and FlowJo 8.8.7 software (TreeStar, Ashland, OR).

2.10. Sphere formation assay

GB2 cells infected with shRNA-expressing lentiviruses (MOI = 3) for 3 days were plated on 96-well tissue culture plates (1000, 500 or 250 cells per well) and cultured for 2 weeks. Spheres were photographed by In Cell Analyzer 2000 (GE Healthcare) and analyzed by Developer 1.9.1 software (GE Healthcare).

2.11. Real time PCR

Real time PCR was performed as described previously [14]. The results were normalized to the detected values for GAPDH. Primer sequences are as follows: PCDH10 sense, 5'-AGGCCCTTCACAGCACTCT-3'; antisense, 5'-GACTAGCATATCCTTTCCGTGTC-3'; GAPDH sense, 5'-TGGTGAAGACGCCAGTGGA-3'; antisense, 5'-GCACCGTCAAGGCTGAGAAC-3'.

Immunoblotting analysis and tumor formation assays were performed as described previously [14].

3. Results and discussion

3.1. PCDH10 is expressed in glioblastoma cells

We established eight glioblastoma cell lines under serum-free conditions [14–16]. These cell lines retained clonogenic potential and were highly tumorigenic when transplanted into immunocompromised mice. Immunoblotting analysis with anti-PCDH10 antibody revealed that most of these cell lines expressed substantial levels of PCDH10 protein (Fig. 1A), in contrast to previous reports showing weak or no expression in many other tumor types. In particular, PCDH10 was abundantly expressed in GB2 and GB3 cells. By contrast, PCDH10 was not expressed at detectable levels in four commonly used glioblastoma cell lines, LN229, T98G, U251 and U87MG, which are cultured in serum-containing medium. Furthermore, immunohistochemical analysis showed that 11 out of 19 patient specimens (57.9%) were positive for PCDH10 (Fig. 1B and Supplementary Table 1). In addition, we found that PCDH10 was expressed in neurons but not in astrocytes or oligodendrocytes from normal human brain (data not shown).

3.2. PCDH10 is required for glioblastoma cell migration and invasion

It has been reported that PCDH10 binds to the Nap1-Wave complex and controls the migration of U251 cells [5]. We therefore

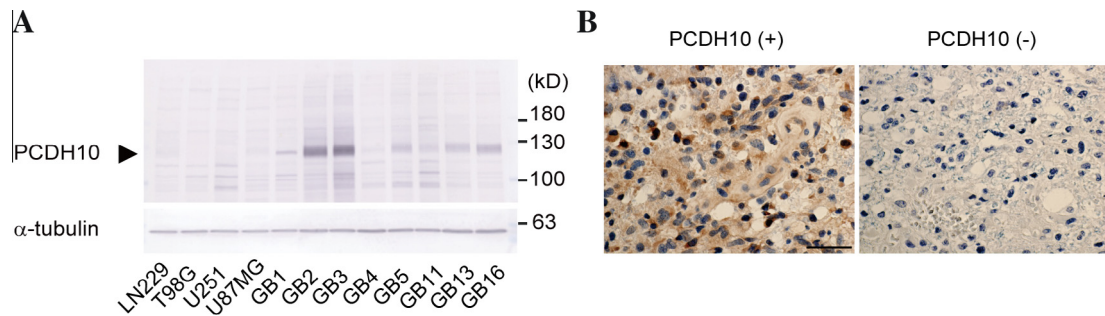


Fig. 1. Expression of PCDH10 in glioblastoma cells. (A) Lysates from glioblastoma cells were subjected to immunoblotting analysis with anti-PCDH10 antibody. LN229, T98G, U251 and U87MG were cultured in serum-containing medium, and GB1~16 cells were cultured in serum-free medium. The arrowhead indicates PCDH10. α -Tubulin was used as a control. (B) Histological examination of patients' samples. Tissue sections were stained with anti-PCDH10 antibody. Representative sections from PCDH10-positive (Left) and -negative (Right) tumors are shown. Scale bars, 20 μ m.

used time-lapse microscopy to examine whether PCDH10 plays a role in migration of glioblastoma cells cultured on laminin-coated dishes. We found that infection of GB2 cells with a lentivirus expressing an shRNA targeting PCDH10 resulted in a significant decrease in cell migratory activity (sh-luciferase vs sh-PCDH10#2, $p = 0.021$; sh-luciferase vs sh-PCDH10#4, $p = 0.00059$) (Fig. 2A and B), as well as a decrease in PCDH10 protein levels (Supplementary Fig. 1), but did not affect adhesion to laminin-coated dishes (Fig. 2C). Since glioblastoma is a highly invasive tumor [13,17], we also performed Matrigel invasion assays. We found that knock-down of PCDH10 resulted in a decrease in the invasive activity of GB2 cells (sh-luciferase vs sh-PCDH10#2, $p = 0.034$; sh-luciferase vs sh-PCDH10#4, $p = 0.042$) (Fig. 2D and E). These results suggest

that PCDH10 has the potential to promote migration and invasion of glioblastoma cells.

3.3. Knockdown of PCDH10 induces the growth arrest and apoptosis of glioblastoma cells

It has recently been shown that overexpression of PCDH10 inhibits the proliferation of multiple tumor cells *in vitro* [9,11]. We therefore investigated the significance of PCDH10 in the proliferation of three glioblastoma cell lines, GB2, GB3 and GB16, in which PCDH10 is abundantly expressed (Fig. 1A). Cell Titer-Glo assays revealed that infection of these cells with a lentivirus expressing an shRNA targeting PCDH10 resulted in a decrease in

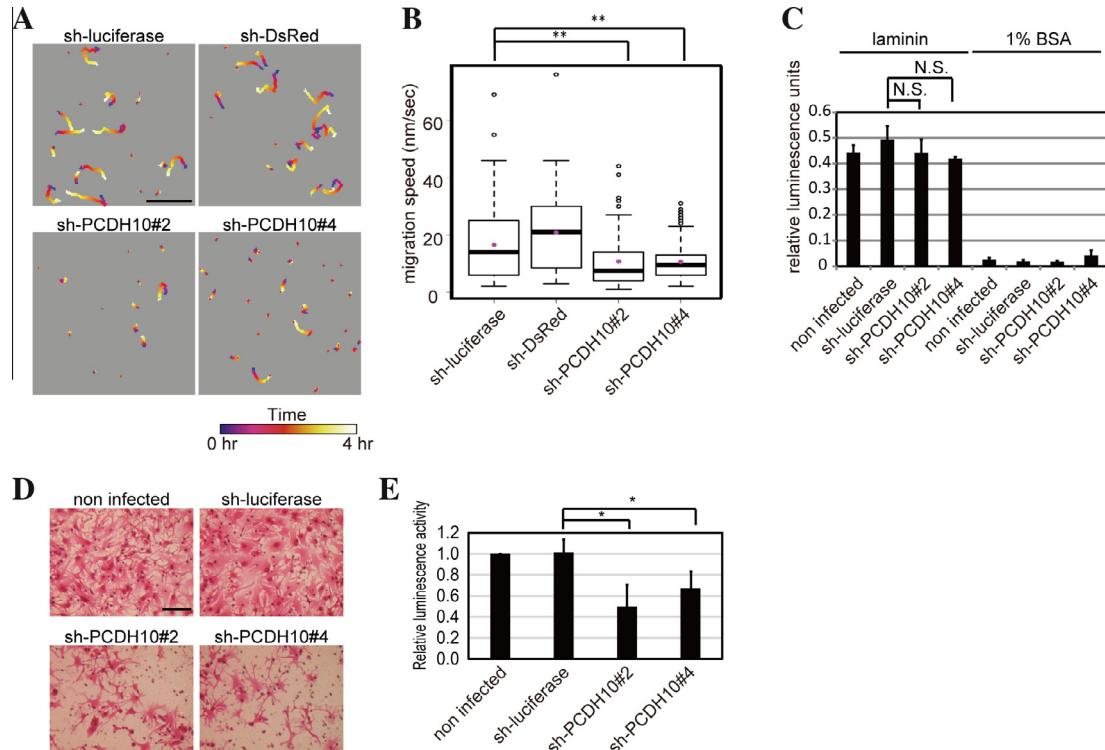


Fig. 2. PCDH10 is required for the migration and invasion of glioblastoma cells. (A and B) GB2 cells were infected with the indicated shRNA-expressing lentivirus at MOI = 6. Time-lapse images were taken at 5-min intervals for 4 h. (A) One representative field of each sample. Tracks of individual cells that neither divided nor contacted other cells were analyzed. Scale bar, 400 μ m. (B) Images were taken from 9 fields per sample. The number of cells analyzed was: sh-luciferase, $n = 140$; sh-DsRed, $n = 131$; sh-PCDH10#2, $n = 138$; sh-PCDH10#4, $n = 158$. Bars indicate medians and magenta dots indicate means ($n = 4$). $**p < 0.01$. (C) GB2 cells were infected with the indicated shRNA expressing lentivirus at MOI = 6. Cells were re-plated 5 days after the infection. Adherent cell numbers were determined by Cell Titer-Glo assays. Dishes coated with 1% BSA were used as controls. Results are shown as average of 4 wells \pm SD. $*p < 0.05$. (D and E) GB2 cells were infected with the indicated shRNA-expressing lentivirus at MOI = 3. 1.0×10^5 cells were allowed to migrate toward the underside of the top chamber for 24 h. (D) HE staining of the invaded cells. Scale bar, 100 μ m. (E) Cell Titer-Glo assays of the invaded cells. Results are shown as average of 3 wells \pm SD. $*p < 0.05$.

their proliferation (Fig. 3A). By contrast, overexpression of PCDH10 did not affect proliferation of T98G or U251 cells, which do not express detectable levels of PCDH10 protein (Supplementary Fig. 2). We also performed AnnexinV assays and found that knockdown of PCDH10 induced apoptosis of GB2 cells (Fig. 3B and C). We next performed sphere formation assays to clarify the role of PCDH10 in the self-renewal capacity of glioblastoma cells. We found that knockdown of PCDH10 by shRNA resulted in significant inhibition of sphere formation (Fig. 3D and E). Thus, PCDH10 may be critical for the proliferation, survival and self-renewal of glioblastoma cells.

3.4. Knockdown of PCDH10 suppresses the tumorigenicity of glioblastoma cells

To clarify the role of PCDH10 in the tumorigenicity of glioblastoma cells, we orthotopically transplanted GB16 cells that had been infected with a lentivirus expressing GFP and an shRNA targeting PCDH10 into the frontal lobe of nude mice. We found that the mice transplanted with PCDH10-knockdown GB16 cells survived longer than control mice (sh PCDH10#2, log-rank test $p=0.042$; sh PCDH10#4, log-rank test $p=0.00031$) (Fig. 4A). Histological studies revealed that all mice transplanted with

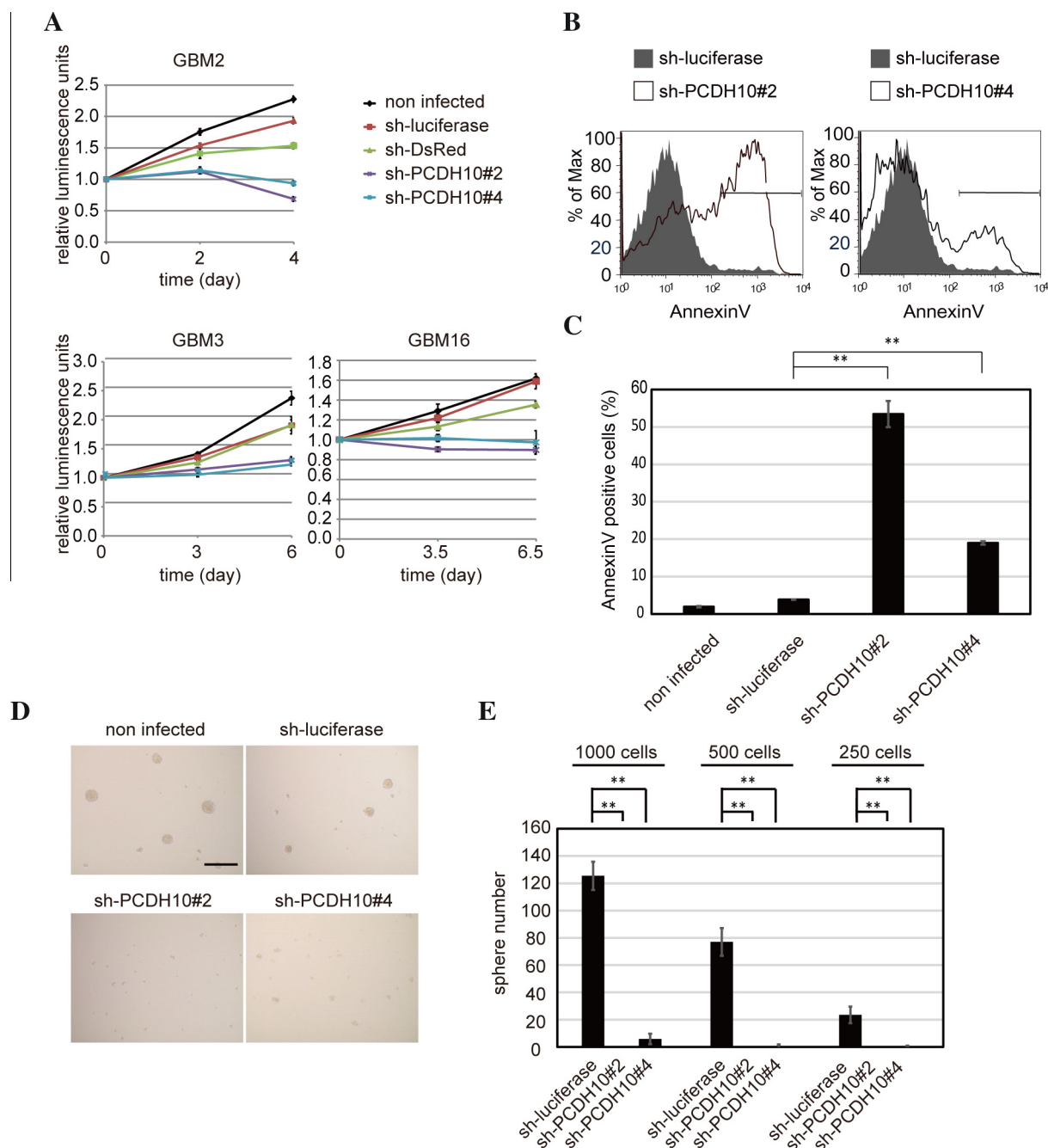


Fig. 3. PCDH10 is required for the proliferation, survival and self-renewal of glioblastoma cells. (A) Cell Titer-Glo assays were performed with GB2, GB3 or GB16 cells infected with a lentivirus expressing an shRNA targeting PCDH10 (MOI = 3). Results are shown as average of 5 wells \pm SD. (B) AnnexinV assays were performed with GB2 cells that had been infected with a lentivirus expressing an shRNA targeting PCDH10 for 8 days. (C) AnnexinV-positive populations in (B) are shown. Results are shown as the mean \pm SD ($n=3$). At least 5000 cells were analyzed per sample. $**p < 0.01$. (D and E) Sphere formation assays were performed with GB2 cells that had been infected with a lentivirus expressing an shRNA targeting PCDH10 for 17 days. (D) Bright field images of spheres. (E) Results are shown as average of 6 wells \pm SD. $**p < 0.01$.

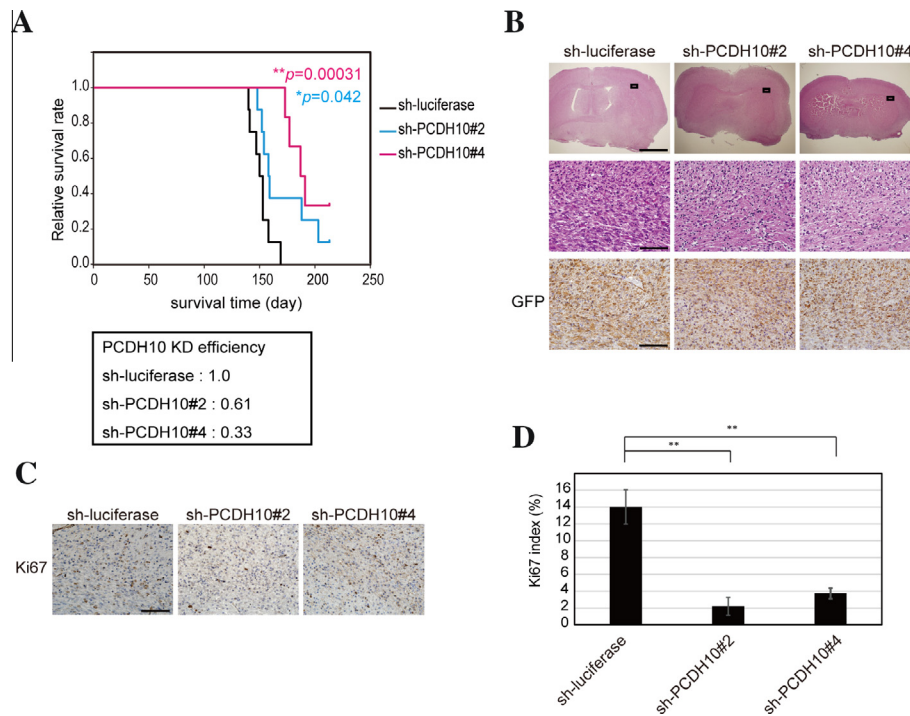


Fig. 4. PCDH10 is required for the tumorigenicity of glioblastoma cells. (A) Kaplan–Meier survival curves of mice transplanted with 1.0×10^4 GB16 cells infected with a lentivirus expressing GFP and shRNA targeting PCDH10 or luciferase (control) (MOI = 1; $n = 8$ for shPCDH10#2 and luciferase; $n = 6$ for shPCDH10#4). (B) Histological analysis of tumors harvested from the mice in (A). Tissue sections were stained with HE or anti-GFP antibody. General views (Upper Panel; Scale bar, 2.0 mm) and magnified views (Middle and Lower panels; Scale bars, 100 μ m) of the region around the corpus callosum in the right hemisphere (near the injection point, black squares in the upper panel). (C and D) Immunohistochemical analysis of tumors harvested from the mice in (A). Tissue sections were stained with anti-GFP and anti-Ki-67 antibodies. Scale bars, 100 μ m. (D) Ki67 indices of the brain sections. 1.0×10^3 cells were counted in each field. Results are shown as average of 6 fields \pm SD. $**p < 0.01$.

GB16 cells had developed tumors with diffuse infiltration into surrounding brain tissues, one of the hallmark features of glioblastoma (Fig. 4B). The density of GFP-expressing cells (injected tumor cells) was slightly lower in the brains of the mice transplanted with PCDH10-knockdown GB16 cells compared to those of control mice (Fig. 4B). Furthermore, we observed that the number of Ki67-positive cells was significantly reduced in the brains of the mice transplanted with PCDH10-knockdown GB16 cells compared to those of control mice (sh-luciferase, $14.0 \pm 2.0\%$; sh-PCDH10#2, $2.2 \pm 1.1\%$; sh-PCDH10#4, $3.7 \pm 0.64\%$) (Fig. 4C and D). These results suggest that knockdown of PCDH10 suppresses the proliferation of glioblastoma cells and extends the survival of tumor-bearing mice.

These results show that PCDH10 is required for the proliferation and tumorigenicity of glioblastoma cells. This suggests that PCDH10 may function differently at the molecular level in glioblastoma cells compared to other tumor cell types. This may mean that PCDH10 associates with different molecules and elicits different downstream signals in glioblastoma cells than it does in other tumor cells. The molecular mechanisms underlying this functional difference remain to be elucidated. It also remains to be investigated whether PCDH10 expression levels are associated with the classification and prognosis of glioblastoma patients. Finally, we speculate that PCDH10 may be a potential therapeutic target for glioblastoma. In particular, monoclonal antibodies that target the extracellular domain of PCDH10 could hold promise as novel anti-tumor reagents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.12.138>.

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