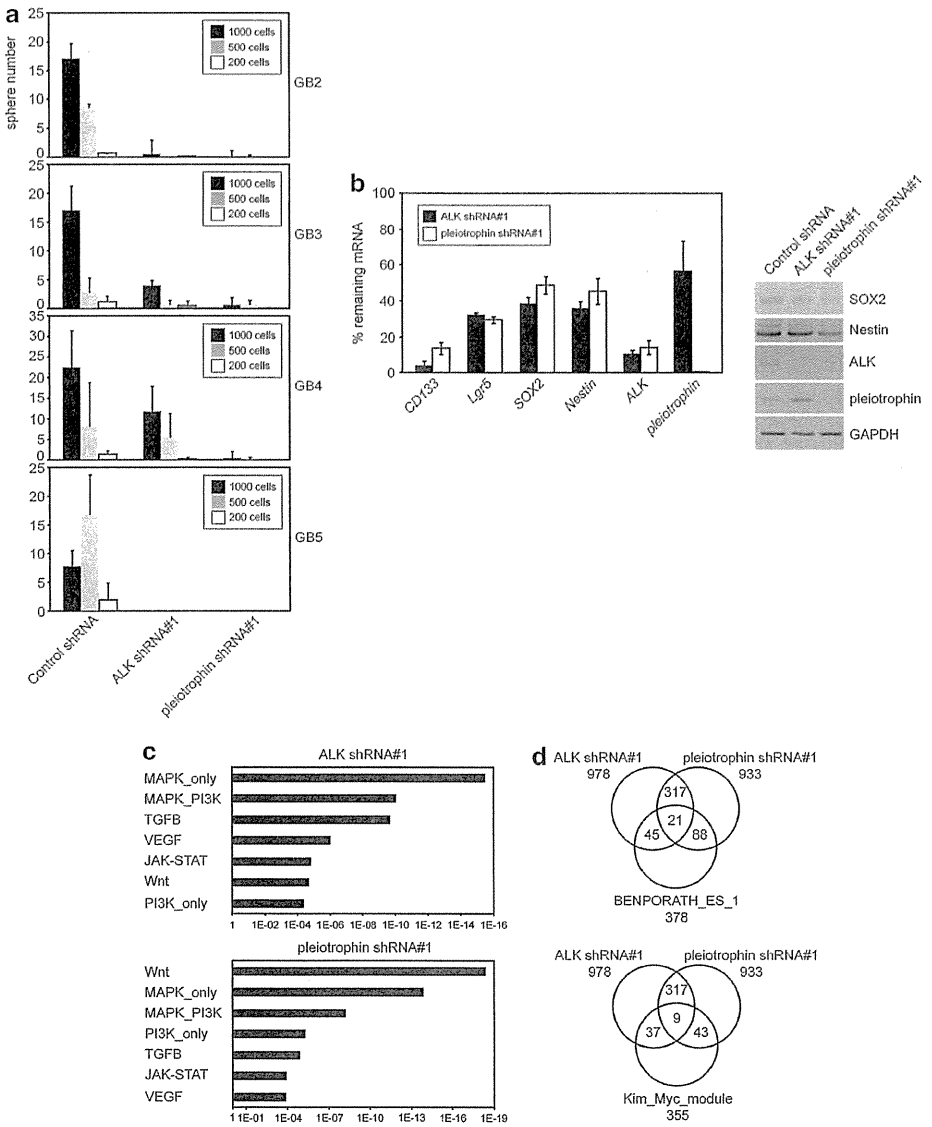

Figure 2. ALK and pleiotrophin are required for the self-renewal and stem-like properties of GSCs. (**a**) GB2–5 cells maintained in serum-free medium were infected with a lentivirus expressing an shRNA-targeting ALK or pleiotrophin. Three days after lentivirus infection, cells were trypsinized and plated at the indicated cell numbers into 96-well tissue culture plates. Two weeks after plating, the number of spheres was counted. Error bars represent the s.d. ($n = 7$–8). (**b**) GB2 cells maintained in serum-free medium were infected with a lentivirus expressing an shRNA-targeting ALK or pleiotrophin. One week after infection, the mRNA levels of the indicated genes were evaluated by quantitative RT–PCR and shown as the percentage of the remaining mRNA compared with cells expressing control shRNA (left). Error bars represent the s.d. ($n = 3$). Cell lysates were subjected to immunoblotting with antibodies to the indicated proteins (right). CD133 and Lgr5 could not be detected by immunoblotting because of their low expression levels. (**c**) GB2 cells maintained in serum-free medium were treated as described in panel (**b**). One week after infection, expression data were generated using HG-U133 plus 2.0 GeneChips. Bar graph represents signaling pathways downregulated by knockdown of ALK (upper) and pleiotrophin (lower). (**d**) Venn diagram showing the overlap among genes downregulated by knockdown of ALK (ALK_signature: ALK shRNA#1), those downregulated by knockdown of pleiotrophin (PTN_signature: pleiotrophin shRNA#1) and those enriched in ESCs (ES signature: BENPORATH_ES_1) (upper) or those targeted by Myc and its related proteins (Myc human: Kim_Myc_module) (lower). The significance of the overlap between each pair of signature is shown in Supplementary Table S4 (hypergeometric *P*-value).

lentivirus-delivered shRNA that stably suppresses ALK or pleiotrophin expression and intracranially transplanted these into immunodeficient mice. Mice receiving the ALK- or pleiotrophin-suppressed cells were found to survive longer than those transplanted with GB2 cells infected with a control lentivirus (Figure 3). Histopathological analysis of the tumor xenografts demonstrated that silencing of either ALK or pleiotrophin inhibited glioblastoma progression, whereas control GB2 cells formed invasive glioblastoma (Supplementary Figure S2). Taken together, these results suggest that the pleiotrophin-ALK axis maintains the tumorigenicity of GSCs.

High expression of pleiotrophin in GSCs

Aberrant activation of ALK by oncogenic fusion or mutation can drive tumorigenesis.[7] However, we could not identify any

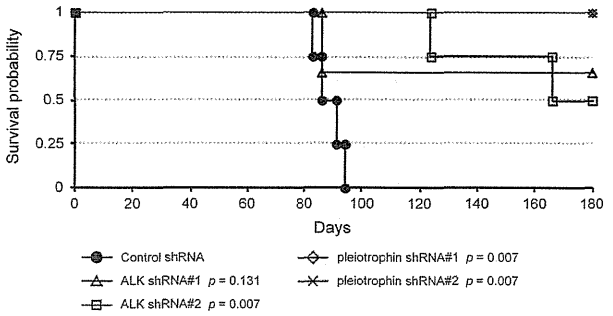

Figure 3. ALK and pleiotrophin are critical for the tumorigenicity of GSCs. GB2 cells maintained in serum-free medium were infected with a lentivirus expressing an shRNA-targeting ALK or pleiotrophin. One week after infection, cells were transplanted into the frontal lobe of immunodeficient mice. *P*-values with comparison to control shRNA by log rank test are shown.

mutation in the *ALK* gene in GB2 cells, consistent with the results of a comprehensive genome analysis reported previously.[29,30] It is known that pleiotrophin is highly expressed in GSCs (Figure 1). We therefore examined the transcriptional control of *pleiotrophin* in GSCs. We found that pleiotrophin protein and mRNA expression levels decreased during serum-induced differentiation, similar to what was observed for the stem cell markers (Figure 4a). Moreover, retinoic acid-induced differentiation also resulted in a decrease in the levels of pleiotrophin[31] (Figure 4b). We also investigated the expression profiles of *pleiotrophin* obtained from a public microarray database.[3] Almost all patient glioblastomas and two GSC lines as well as NSCs expressed substantial levels of *pleiotrophin* (Figure 4c). By contrast, glioma cell lines and two GSC lines cultured in serum-containing medium expressed relatively low levels of *pleiotrophin*, presumably because they had undergone 'differentiation'.[3] These results suggest that high expression of pleiotrophin is a common feature of GSCs.

SOX2 directly transactivates the expression of pleiotrophin in GSCs

To further investigate the mechanisms underlying the expression of *pleiotrophin*, we performed reporter assays using a *luciferase* reporter under the control of the full-length *pleiotrophin* 5′ region (−1401 to +309) and several deletion variants. When transfected into GB2 cells, a reporter containing the region between −251 and +309 showed a substantial level of activity (Figure 5a, left panel). Furthermore, this activity was repressed upon serum-induced differentiation. We therefore attempted to identify the transcription factor(s) involved in modulating this promoter activity. We first searched the region −251 and +309 for transcription factor consensus binding sites and then compared the expression patterns of the selected transcription factors and pleiotrophin using a microarray database.[3] We found that the expression pattern of *pleiotrophin* is similar to that of *SOX2* (Figure 4c). Furthermore, a mutant reporter lacking the SOX2-binding site (−40 to −21) showed reduced promoter activity (Figure 5a, right panel). Chromatin immunoprecipitation analysis demonstrated that endogenous SOX2 was present at the *pleiotrophin* promoter in GSCs (Figure 5b and Supplementary Figure S3A). Consistent with these results, silencing of SOX2 by shRNA resulted in a decrease in the levels of pleiotrophin protein and mRNA (Figure 5c and Supplementary Figure S3B). Similar results were obtained by transfecting two different siRNAs targeting SOX2 (Supplementary Figure S3C). These results suggest that SOX2 stimulates transcription of the *pleiotrophin* gene, thereby maintaining high expression levels of pleiotrophin in GSCs.

DISCUSSION

It has been reported that ALK acquires oncogenic potential when truncated and fused to a partner protein, such as NPM, as can occur via chromosomal rearrangement.[7,8] It has also been reported that ALK is activated by point mutations in its kinase domain in some neuroblastoma.[7,32] Although we could not identify any chromosomal rearrangement or point mutation in ALK in GB2 cells, we found that ALK is highly expressed in GSC lines cultured in serum-free medium, consistent with previous reports.[33] Furthermore, we showed that knockdown of ALK results in a decrease in the self-renewing capacity and tumorigenicity of GB2 cells. We also found that pleiotrophin is overexpressed both in patient glioblastomas and in GSC lines cultured in serum-free medium and that knockdown of pleiotrophin leads to a reduction in the self-renewing capacity and tumorigenicity of GB2 cells. Thus, our results suggest that the pleiotrophin-ALK axis is required for the self-renewal and tumorigenicity of GSCs.

NPM-ALK, the most thoroughly studied ALK-fusion protein, has been reported to signal via the MAP kinase, phospholipase Cγ, PI3-kinase and JAK/STAT pathways.[7] Consistent with these reports, our DNA array analysis revealed that the MAP kinase, PI3-kinase and JAK/STAT pathways are activated in GSCs. Furthermore, we found that ALK and pleiotrophin activate the Myc and ESC-like transcriptional programs, which are known to be associated with more aggressive phenotypes in human cancers.[25,27] Activation of these pathways by ALK has not been previously reported, presumably because earlier studies utilized cultured cell lines grown in serum-containing media that do not exhibit any stem cell-like properties. We found that there was only about a 30% overlap between the genes dependent on ALK and pleiotrophin expression. Furthermore, only pleiotrophin, but not ALK, activated the Wnt signaling pathway. These results appear to be in line with the fact that pleiotrophin signals not only via ALK but also via other receptors, such as RPTPβ/ζ (receptor protein tyrosine phosphatase β/ζ) and N-syndecan.[34,35] Interestingly, pleiotrophin is known to stimulate tyrosine phosphorylation of β-catenin through inactivation of RPTPβ/ζ.[34] Furthermore, pleiotrophin stimulates tumor angiogenesis and remodeling of the microenvironment.[36] Consistent with these findings, we found that repression of pleiotrophin has a more dramatic effect on the tumorigenicity of GSCs than repression of ALK.

It has been reported that SOX2 is involved in the tumorigenesis of several cancers, including lung cancer and breast cancer.[37–39] We found that SOX2 directly transactivates the expression of pleiotrophin in GSCs. Our results appear to be consistent with a previous report showing that knockdown of SOX2 suppresses the tumorigenicity of GSCs in immunodeficient mice.[17] Thus, SOX2-mediated transactivation of pleiotrophin may be important for the tumorigenicity of GSCs. However, knockdown of SOX2 only partially reduced the expression levels of pleiotrophin (Figure 5c and Supplementary Figures 3B and C), and mutation of the SOX2-binding site in the pleiotrophin promoter only partially reduced activity of a luciferase reporter (Figure 5a, right panel). In addition, we found that the levels of pleiotrophin decreased faster than those of SOX2 during serum-induced differentiation (Figure 4a) and that retinoic acid-induced differentiation resulted in a drastic decrease in the expression levels of pleiotrophin but not of SOX2 (Figure 4b). Thus, the pleiotrophin-ALK axis may be regulated by other transcription factors in addition to SOX2.

SOX2 is well known to have critical roles in the maintenance of neural stem and progenitor cells.[14,15] For example, it has been reported that multipotent neural stem-like cells transfected with an siRNA targeting SOX2 express increased neurofilaments but decreased GFAP and Nestin levels.[40] These results suggest that SOX2 inhibits the differentiation of neural stem-like cells into neurons and maintains their stem-like properties. Intriguingly, our results appear to be consistent with these results. We found that

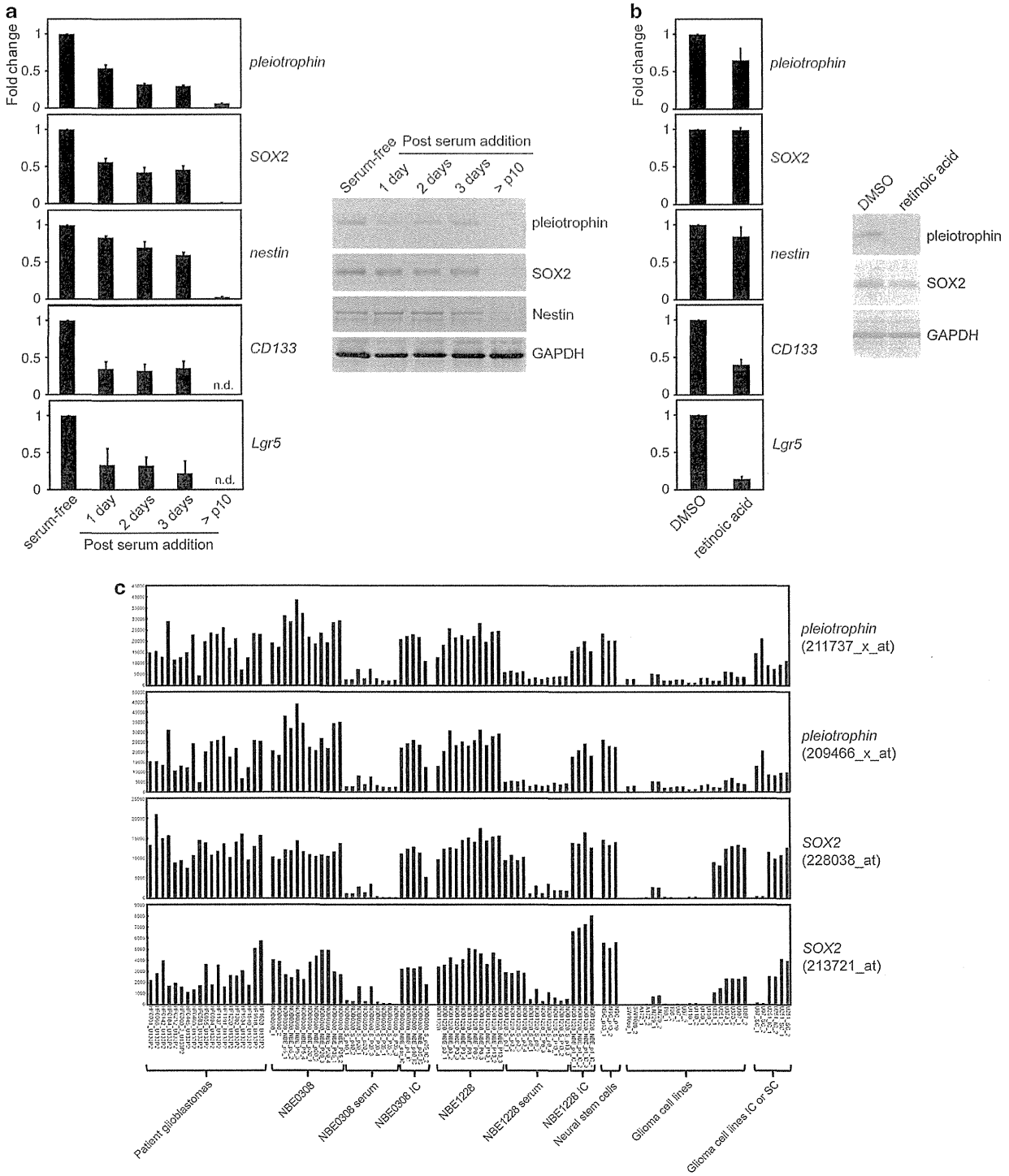

Figure 4. High expression of pleiotrophin in GSCs. (**a**) GB2 cells maintained in serum-free medium were cultured in serum-containing medium for the indicated times. >p10, GB2 cells cultured in serum-containing medium for >10 passages. The mRNA levels of the indicated genes were evaluated by quantitative RT–PCR and shown as the fold change over mRNA levels in GB2 cells maintained in serum-free medium (left). Error bars represent the s.d. ($n = 3$). ND, not detected. Cell lysates were subjected to immunoblotting with antibodies to the indicated proteins (right). CD133 and Lgr5 could not be detected by immunoblotting because of their low expression levels. (**b**) GB2 cells maintained in serum-free medium were cultured in medium containing retinoic acid for 4 days. The mRNA levels of the indicated genes were evaluated by quantitative RT–PCR and shown as the fold change over the vehicle-treated cells (left). Error bars represent the s.d. ($n = 3$). Cell lysates were subjected to immunoblotting with antibodies to the indicated proteins (right). (**c**) Gene expression profiles of *pleiotrophin* and *SOX2* taken from the public microarray database GSE4536 (Lee *et al.*[3]). Data obtained with two independent probes for each gene are shown. NBE0308 and NBE1228 are GSC lines. Serum, GSCs cultured in serum-containing medium; IC, intracranial injection; SC, subcutaneous injection.

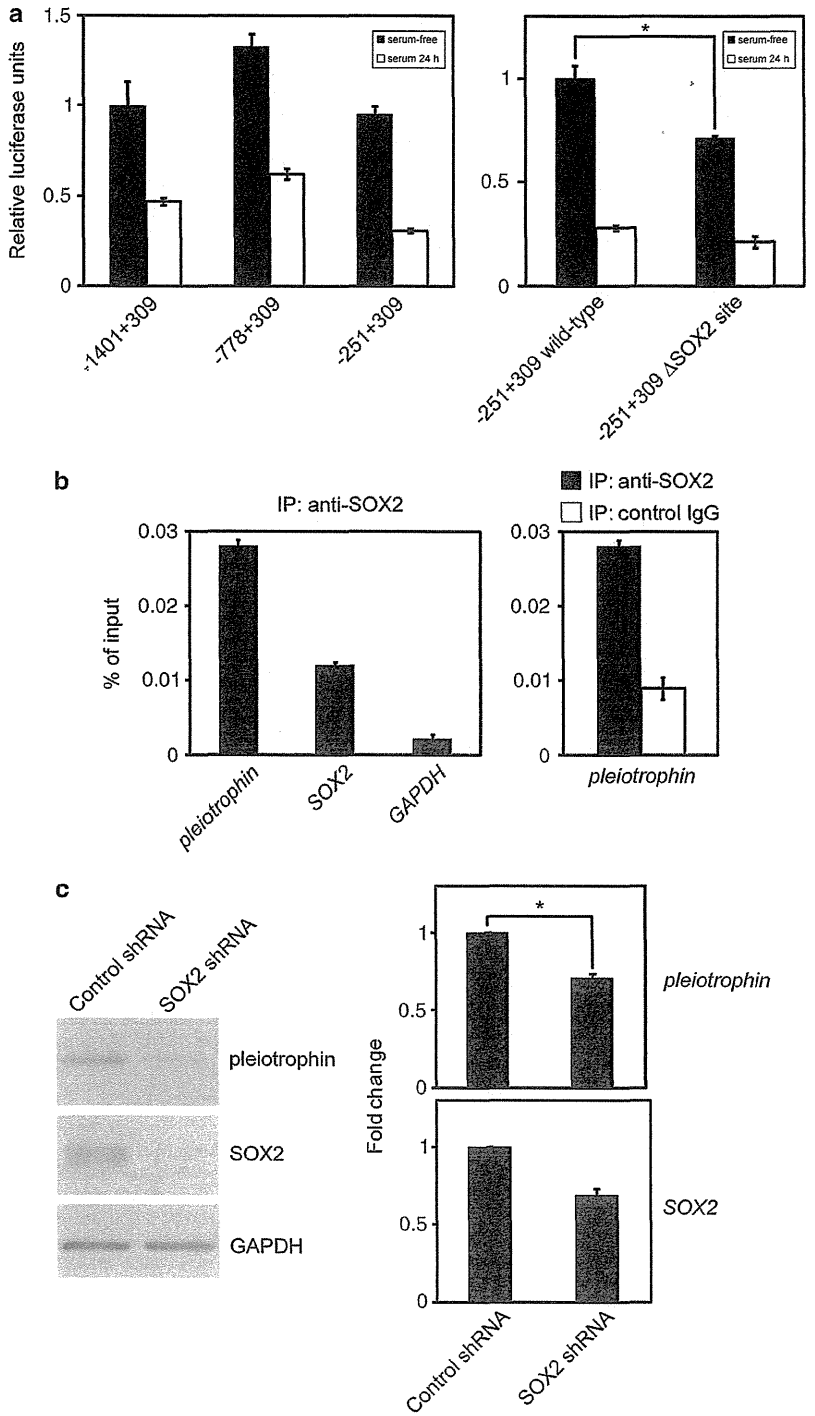

Figure 5. SOX2 directly transactivates the expression of pleiotrophin in GSCs. (**a**) GB2 cells maintained in serum-free medium were transfected with a luciferase reporter driven by the *pleiotrophin* promoter. Luciferase reporter activity is shown. Error bars represent the s.d. ($n = 3$). *$P = 0.0058$ by *t*-test. (**b**) Chromatin immunoprecipitation experiments with anti-SOX2 antibody were performed with GB2 cells maintained in serum-free medium. The precipitated chromatin was amplified by quantitative PCR using primers flanking the indicated gene promoters (*pleiotrophin* and *GAPDH*) or enhancer (*SOX2*) (left). The *SOX2* enhancer was used as a positive control, as SOX2 has been reported to be autoregulated.[50] The *GAPDH* promoter was used as a negative control. Control IgG was used as a negative control (right). Error bars represent the s.d. ($n = 3$). (**c**) GB2 cells maintained in serum-free medium were infected with a lentivirus expressing an shRNA-targeting SOX2. Cell lysates were subjected to immunoblotting with antibodies to the indicated proteins (left). The mRNA levels of the indicated genes were evaluated by quantitative RT–PCR and shown as the fold change over mRNA levels in cells expressing control shRNA (right). Error bars represent the s.d. ($n = 3$). *$P = 0.001$ by *t*-test.

GSCs infected with a lentivirus expressing an shRNA targeting ALK or pleiotrophin have increased MAP2 but decreased GFAP, Olig2 and Nestin levels. These results suggest that the pleiotrophin-ALK axis inhibits neural differentiation of GSCs but maintains their stem-like characteristics. It would be interesting to examine whether the pleiotrophin-ALK axis is also involved in the maintenance of normal neural stem and progenitor cells. SOX2 has also been reported to have essential roles in the neuronal differentiation of subsets of neural stem and progenitor cells.[14,15] For example, it has been reported that SOX2 deficiency causes impaired neurogenesis and neurodegeneration in the adult mouse brain.[41,42] Therefore, it would be interesting to investigate whether the pleiotrophin-ALK axis is also involved in the differentiation of neural stem and progenitor cells.

The ALK inhibitor crizotinib has recently been approved for the treatment of metastatic and late stage ALK-positive NSCLC having translocations of the ALK gene.[9,10] In addition, the ALK inhibitor TAE684 has been reported to inhibit the growth of ALK-positive ALCL, neuroblastoma and NSCLC cell lines.[43] Thus, we speculate that ALK inhibitors may be effective for the treatment of glioblastoma. ALK has been assumed to have a role in the development and function of the central and peripheral nervous system, as ALK is abundantly expressed in the nervous system during mouse embryogenesis,[7] which could raise concerns about the safety of such therapy. However, both ALK- and pleiotrophin-mutant mice survive as long as wild-type mice.[7,44] In addition, although pleiotrophin is highly expressed in NSCs, it seems dispensable for their proliferation *in vivo*.[45] Furthermore, it has been reported that the most common adverse reactions of crizotinib are relatively minor, consisting of vision disorder, nausea, diarrhea, vomiting, edema and constipation.[46] Thus, compounds targeting ALK or pleiotrophin would be expected to have relatively few serious side effects due to their effects on NSCs. In addition, we imagine that antibodies or compounds that specifically target the extracellular domain of ALK or pleiotrophin could also hold promise as novel anti-tumor reagents.

MATERIALS AND METHODS

Tumor specimens and primary tumor cultures

Tumor samples classified as primary glioblastoma were obtained from patients undergoing surgical treatment at the University of Tokyo Hospital with informed consent and as approved by the Institutional Review Board. Tumors were washed and mechanically and enzymatically dissociated into single cells. Tumor cells were cultured in Neurobasal medium (Life Technologies, Carlsbad, CA, USA) containing B27 supplement minus vitamin A (Life Technologies), EGF and FGF2 (20 ng/ml each; Wako Pure Chemical Industries, Osaka, Japan). For *in vitro* differentiation, tumor cells were cultured in DMEM (Dulbecco's modified Eagle's medium)/F-12 medium (Life Technologies) containing 10% fetal bovine serum or 10 μM all-trans retinoic acid (Sigma, St. Louis, MO, USA). U87 and 293FT cells were cultured in DMEM (Nissui, Tokyo, Japan) containing 10% fetal bovine serum. Transfections were performed using Lipofectamine 2000 Reagent, Lipofectamine LTX Reagent or Lipofectamine RNAiMAX Reagent (Life Technologies).

RNAi

Silencer Select Human Extended Druggable Genome siRNA Library was purchased from Life Technologies (the list is given in Supplementary Table S1). The siRNA oligonucleotide sequences were as follows: SOX2#1 (5′-CAGUAUUUAUCGAGAUAAA-3′), and SOX2#2 (5′-AGUG GAAACUUUUGUCGGA-3′).

The shRNA oligonucleotide sequences were as follows: ALK#1 (5′-GGCCU GUAUACCGGAUAAUGA-3′), ALK#2 (5′-GAAUACAGCACCCAAAUCAAG-3′), pleiotrophin#1 (5′-GGAGCUGAGUGCAAGCAAACC-3′), pleiotrophin#2 (5′-GCAACUGGAAGAAGCAAUUUG-3′), and SOX2 (5′-GUAAGAAACAGCAUG GAGAAA-3′).

Quantitative RT–PCR

Total RNA was extracted using the NucleoSpin RNA Clean-up kit (Takara Bio Inc., Shiga, Japan) and reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara Bio Inc.). Real-time PCR was performed using LightCycler480 SYBR Green I Master and a LightCycler480 Instrument (Roche, Indianapolis, IN, USA). The results were normalized with the detected value for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). Primers used in real-time PCR were as follows: *GAPDH* forward (5′-GCACCGTCAAGGCTGA GAAC-3′), *GAPDH* reverse (5′-TGGTGAAGACGCCAGTGGA-3′); *HPRT1* forward (5′-GGCAGTATAATCCAAAGATGGTCAA-3′), *HPRT1* reverse (5′-GTCA AGGGCATATCCTACAACAAAC-3′); *CD133* forward (5′-AGTGGCATCGTGCA AACCTG-3′), *CD133* reverse (5′-CTCCGAATCCATTCGACGATAGTA-3′); *nestin* forward (5′-GAGGTGGCCACGTACAGG-3′), *nestin* reverse (5′-AAGCTGAGGG AAGTCTTGGA-3′); *Lgr5* forward (5′-GATTTCCTGCTTGACTTTGAGG-3′), *Lgr5* reverse (5′-GCAGGTGTTCACAGGGTTTG-3′); *SOX2* forward (5′-TTGCTGCCTC TTTAAGACTAGGA-3′), *SOX2* reverse (5′-CTGGGGCTCAAACTTCTCTC-3′); *ALK* forward (5′-CACTCCAGGGAAGCATGG-3′), *ALK* reverse (5′-TCGAAATGGGTT GTCTGGA-3′); *pleiotrophin* forward (5′-AACTGACCAAGCCCAAACCT-3′), *pleiotrophin* reverse (5′-GGTGACATCTTTTAATCCAGCA-3′), *MAP2* forward (5′-TCTCCTGTGTTAAGCGGAAAA-3′), *MAP2* reverse (5′-AATACACTGGGAGC CAGAGC-3′), *GFAP* forward (5′-GACCTGGCCACTGTGAGG-3′), *GFAP* reverse (5′-AGGCAGCCAGGTTGTTCTC-3′), *Olig2* forward (5′-AGCTCCTCAAATCGC ATCC-3′), and *Olig2* reverse (5′-ATAGTCGTCGCAGCTTTCG-3′).

Antibodies

Rabbit polyclonal antibody (pAb) to green fluorescence protein (GFP) and goat pAb to SOX2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit pAbs to ALK were from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody (mAb) to pleiotrophin (H-6) was from Santa Cruz Biotechnology. Mouse mAb to Nestin was from R&D systems (Minneapolis, MN, USA). Mouse mAb to GAPDH was from Millipore (Bedford, MA, USA).

Immunoblotting

Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 1 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF and protease inhibitors. Lysates were fractionated by SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to a PVDF (polyvinylidene difluoride) membrane (Immobilon-P, Millipore). The membrane was subjected to immunoblot analysis using alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG (Promega, Madison, WI, USA) as secondary antibodies. Visualization was performed using the NBT/BCIP colorimetric substrate system (Promega).

Lentivirus production

A lentiviral vector (CS-Rfa-CG) expressing an shRNA driven by the H1 promoter was transfected together with the packaging vectors pCAG-HIV-gp and pCMV-VSV-G-RSV-Rev into 293FT cells. All plasmids were kindly provided by H. Miyoshi (RIKEN BioResource Center, Ibaraki, Japan). Virus supernatant was purified by ultracentrifugation at 25 000 r.p.m. for 90 min (SW28 rotor, Beckman Coulter, Brea, CA, USA). Infection efficiency was monitored by GFP expression driven by the CMV promoter.

Intracranial xenograft

One week after lentivirus infection, 1×10^4 cells were injected stereotactically into the right frontal lobe of 5-week-old nude mice (BALB/cAJcl-*nu/nu*, CLEA Japn Inc., Tokyo, Japan), following administration of general anesthesia ($n = 3$ or 4). The injection coordinates were 2 mm to the right of the midline, 1 mm anterior to the coronal suture and 3 mm deep. Mice were monitored for 6 months. Survival of mice was evaluated by Kaplan–Meier analysis. *P*-values were calculated using log rank test. The distribution of tumor cells was analyzed by GFP immunostaining. Tumors were histologically analyzed after hematoxylin and eosin staining. All animal experimental protocols were performed in accordance with the guidelines of the Animal Ethics Committee of the University of Tokyo.

Immunohistochemistry

Three months after injection of cells, brains were fixed in 3.7% buffered formalin, dehydrated and embedded in paraffin. Sections (6 μm) were rehydrated, and endogenous peroxidases were blocked by incubation in

0.3% H_2O_2 for 5 min. The primary antibody was detected using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA). Slides were lightly counterstained with hematoxylin.

Flow cytometry

Cells were trypsinized, fixed in 70% ethanol and then stained with propidium iodide (Sigma). Cells were passed through a FACSCalibur instrument (BD Biosciences, Billerica, MA, USA).

Microarray analysis

Expression data of cells infected with a lentivirus expressing an shRNA targeting ALK or pleiotrophin were generated using HG-U133 plus 2.0 GeneChips (Affymetrix, Santa Clara, CA, USA). Normalization and analysis of the data were conducted using Gene Spring version 11.5.1 (Agilent Technologies, Santa Clara, CA, USA). Affimetrix CEL files were uploaded to GeneSpring, and RobustMulti-Array normalization was performed. Genes downregulated >1.9-fold by ALK knockdown and those downregulated >2.3-fold by pleiotrophin knockdown were termed 'ALK_signature (ALK-sig)' and 'pleiotrophin (PTN) signature (PTN-sig)' genes, respectively (the gene lists are provided in Supplementary Tables S2 and S3). BENPORATH_ES_1 were taken from the Molecular Signature Database (MSigDB).[25,47] The Kim_Myc_module was taken from Myc human module listed in Supplementary Table S3 of Kim *et al.*[27] The significance of the overlap between sh-ALK, sh-PTN#1, BENPORATH_ES_1 and/or Kim_Myc_module was calculated by the hypergeometric distribution shown in Supplementary Table S4 (Tavazoie *et al.*[48]) Functional characterization of these genes was performed using SPEED.[49] The data derived from microarray analysis has been deposited in the Gene Expression Omnibus database (GSE32482). The expression profiles of *pleiotrophin* and *SOX2* in patient glioblastomas, two GSC lines and glioma cell lines were taken from GSE4536 (Lee *et al.*[5]).

Reporter assay

Cells were transfected with a luciferase-reporter plasmid. For *in vitro* differentiation, fetal bovine serum was added to culture medium at a final concentration of 20% and cultured for an additional 24 h. Cells were lysed and firefly luciferase activity was measured with the Luciferase Reporter Assay System (Promega) and shown as the average of three measurements.

Chromatin immunoprecipitation

Cells were fixed with 1% formalin and then the reaction was stopped by the addition of glycine to a final concentration of 125 mM. Cells were lysed in chromatin immunoprecipitated lysis buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 10 mM EDTA and protease inhibitors). Lysates were sonicated to generate DNA fragments of ~1 kb in length and then diluted 10-fold to reduce the concentration of SDS to 0.1%. Immunoprecipitations were performed with an anti-SOX2 antibody. Samples were washed, reverse-crosslinked and digested by proteinase K. Purified DNA samples were analyzed by real-time PCR, and differences in the DNA content between the bound and input fractions were determined. Primers used for amplification of the gene promoters or enhancer were as follows: *pleiotrophin* forward (5′-CAGCTCTCGAGTGCAAAGC-3′), *pleiotrophin* reverse (5′-AATGGGAGGGATGAGAGGAG-3′); *GAPDH* forward (5′-TGCGTGCCCAGTTGAACCAG-3′), *GAPDH* reverse (5′-AACAGGAGGAGCAGAGAGCGAAGC-3′); *SOX2* forward (5′-TGAAGACAGTCTAGTGGGAGATGT-3′), and *SOX2* reverse (5′-CTCTTTGGCCCAGGAAACT-3′).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Research Program of Innovative Cell Biology by Innovative Technology (Integrated Systems Analysis of Cellular Oncogenic Signaling Networks), Grants-in-Aid for Scientific Research on Innovative Areas (Integrative Research on Cancer Microenvironment Network), Takeda Science Foundation and, in part, by Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms), MEXT, Japan.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

Biochemical and Biophysical Research Communications 444 (2014) 13–18

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

PCDH10 is required for the tumorigenicity of glioblastoma cells

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ARTICLE INFO

Article history:
Received 19 December 2013
Available online 6 January 2014

Keywords:
Protocadherin PCDH10
OL-protocadherin
Glioblastoma
Tumorigenicity
Proliferation

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.

1. Introduction

Protocadherin10 (PCDH10)/OL-protocadherin is a transmembrane protein that has 6 cadherin domains in the extra cellular region and belongs to the δ2-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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http://dx.doi.org/10.1016/j.bbrc.2013.12.138

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 μm 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% H_2O_2 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′-GATTTCGAGTCGTCTTAATGT-3′; DsRed, 5′-GCCCCGTAATGCAGAAGAAGA-3′; human PCDH10#2, 5′-GTGCGTGGCAACGAAATGAAC-3′; human PCDH10#4, 5′-GAGAAGAAGCTCAACATCTAT-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 × Flag vector (Adgilent). 3 × 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′-GACTAGCATATCCTTTTCCGTGTC-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

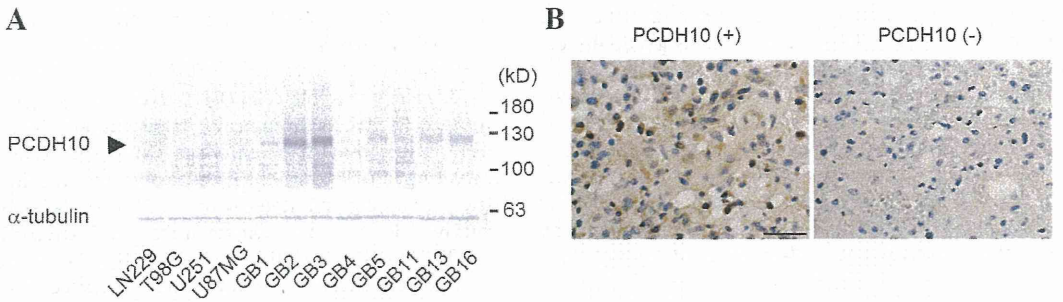

Fig. 1. Expression of PCDH10 in glioblastoma cells. (A) Lysates from glioblastoma cells were subjected to immunoblotting analysis with anti-PCDH10 antibody. LN229, T98G, U251 and U87MG were cultured in serum-containing medium, and GB1~16 cells were cultured in serum-free medium. The arrowhead indicates 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 μm.

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 knockdown 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

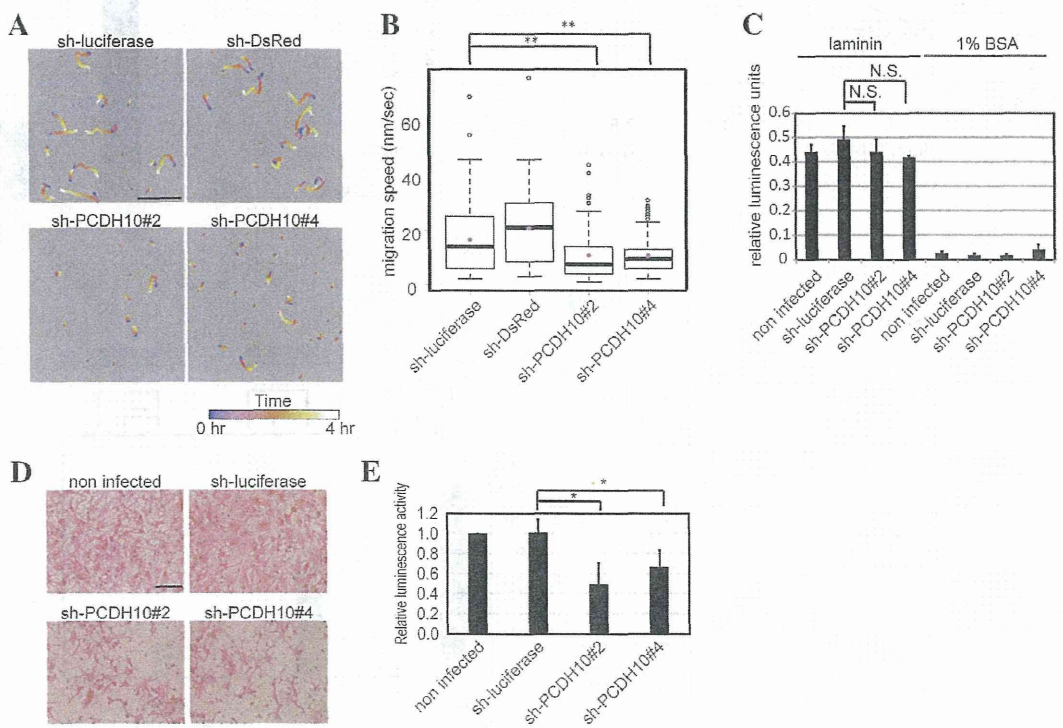

Fig. 2. PCDH10 is required for the migration and invasion of glioblastoma cells. (A and B) GB2 cells were infected with the indicated shRNA-expressing lentivirus at MOI = 6. Time-lapse images were taken at 5-min intervals for 4 h. (A) One representative field of each sample. Tracks of individual cells that neither divided nor contacted other cells were analyzed. Scale bar, 400 μm. (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 ± 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 μm. (E) Cell Titer-Glo assays of the invaded cells. Results are shown as average of 3 wells ± SD. $^{*}p < 0.05$.