Targeting CD146 using folic acid–conjugated nanoparticles and suppression of tumor growth in a mouse glioma model

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OBJECTIVE Glioma stem cells (GSCs) are responsible for tumor initiation, therapeutic resistance, and recurrence. *CD146* is mainly expressed in dividing GSCs and regulates cell cycle progression. However, the evaluation of the efficacy of targeted therapy against *CD146* in vivo remains to be investigated. In this study, the authors aimed to develop gene therapy targeting GSCs using chitosan oligosaccharide lactate (COL) nanoparticles (NPs) conjugated with folic acid–polyethylene glycol (FA-PEG-COL NPs) for in vitro and in vivo delivery of *CD146* small-interfering RNA (siCD146) and to determine the effect of *CD146* knockdown on tumor growth.

METHODS To examine the uptake of NPs by tumor cells, immunofluorescence staining, flow cytometry, and in vivo imaging were performed. The knockdown effect of siCD146 was measured by western blot and water-soluble tetrazolium salt–8 assay in mouse glioma cells. The efficacy of siRNA therapy–targeted GSCs was evaluated by monitoring tumor growth through in vivo imaging and histological analysis.

RESULTS In vivo accumulation of the FA-PEG-COL NPs in subcutaneous and intracranial gliomas following NP administration via a mouse tail vein was observed. Additionally, in vitro delivery of siCD146 ionically cross-linked NPs, reduced *CD146* levels, and suppressed growth in the glioma tumor sphere. Evaluation of the in vivo therapeutic effects of siCD146–cross-linked NPs in a mouse glioma model revealed significant suppression of intracranial tumor growth, with complete removal of the tumor observed in some mice on histological examination. Furthermore, delivery of siCD146 significantly reduced the Ki-67 index in residual tumor tissues relative to that in control mice.

CONCLUSIONS *CD146* is a potential therapeutic target, and folic acid–conjugated NPs delivering siRNA may facilitate gene therapy in malignant gliomas.

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KEYWORDS glioblastoma; glioma stem cell; *CD146*; gene therapy; siRNA; nanoparticle; oncology

GLIOMASTOMA (GBM) is the most common type of primary brain tumor, with GBM prognosis continuing to be poor, regardless of multimodal therapy. The failure to cure this disease is mainly due to therapeutic resistance and tumor recurrence after surgical removal. One explanation for its resistance to various therapies is that GBM contains tumor stem cells or tumor-initiating cells possessing high tumorigenicity and resis-

tance to chemotherapy and radiotherapy.¹ GBM exhibits a high degree of histological variability and morphological diversity in the cell population that is possibly generated by stem and differentiated cells. The cellular heterogeneity in gliomas and adjacent tissue contributes to therapeutic resistance and elevates immune escape in the tumor microenvironment.² Therefore, because of their heterogeneity, potential to contribute to tumor development, and dem-

ABBREVIATIONS BBB = blood-brain barrier; COL = chitosan oligosaccharide lactate; EPR = enhanced permeability and retention; FA = folic acid; FA-PEG-COL = FA–polyethylene glycol COL; FITC = fluorescein isothiocyanate; FOLR1 = folate receptor 1; GBM = glioblastoma; GSC = glioma stem cell; NP = nanoparticle; PBS = phosphate-buffered saline; RT-PCR = reverse transcription polymerase chain reaction; siCD146 = *CD146* siRNA; siRNA = small-interfering RNA; TS = tumor sphere; WST-8 = water-soluble tetrazolium salt–8.

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onstrated promise as attractive target cells for improving therapeutic efficacy, glioma stem cells (GSCs) should be further investigated.

Several molecules have been proposed as GSC targets. Notch, WNT, Sonic hedgehog, STAT3 (signal transducer and activator of transcription 3), aldehyde dehydrogenase 1 family member A3, and microRNA-145 are involved in GSC self-renewal, differentiation, drug resistance, tumor-specific metabolism, and survival,^{3–8} and clinical trials of inhibitors of these molecules are currently underway. Spheroid culture is a method used to increase the stem cell population and characterize GSCs. We previously reported that *CD146* expression, as well as expression of other metastasis-related genes, was elevated by spheroid culture of mouse RSV-M glioma cells,⁹ and revealed a role for CD146 in cell cycle regulation in GSCs.¹⁰ CD146 (also known as melanoma-adhesion molecule; MCAM) belongs to the immunoglobulin superfamily and was initially identified as a marker of melanoma progression and metastasis.¹¹ *CD146* is highly expressed in other tumors and correlates with tumor invasion, metastasis, and drug resistance. In our previous report, *CD146* silencing by small-interfering RNA (siRNA) suppressed GSC growth via cell cycle regulation.¹⁰ Moreover, immunohistochemical analysis of various glioma tissues showed that *CD146* expression correlated with tumor grade and was particularly elevated in WHO grade III and VI tumors; however, gene therapy strategies targeting CD146 have yet to be established.

RNA interference using siRNA is a powerful tool for reducing target-molecule expression, and thereby represents a potential technique for selectively targeting RNA during gene therapy for many diseases. One disadvantage of siRNA-based gene therapy is the unstable nature of RNA in vivo. To address this issue, nanoparticles (NPs) have been developed as both carriers and stabilizers of nucleic acids. Additionally, the small size of NPs allows enhanced permeability and retention (EPR) effects, which enable the selective delivery of molecules via immature vasculature in tumor tissues. Our previous studies reported the efficient delivery of siRNAs targeting ovary and pancreatic cancer using NPs formulated with chitosan, a cationic polysaccharide derived from a natural substance, followed by conjugation with folic acid (FA) to promote cell-specific targeting.^{12,13} FA plays a central role in DNA synthesis and methylation, and folate receptor 1 (FOLR1) is highly expressed in several cancers and is a promising target for cancer treatment and detection.¹⁴ Furthermore, *FOLR1* is extensively detected in GBMs, with levels positively correlated with tumor grade,¹⁵ and a previous study showed that targeted delivery of etoposide-loaded NPs cross-linked with FA inhibited glioma cell progression.¹⁶ These findings suggest that a strategy involving targeting of the folate receptor might be useful for gene therapy of glioma.

In this study, we employed chitosan oligosaccharide lactate (COL) NPs conjugated with FA–polyethylene glycol (FA-PEG-COL NPs) to deliver *CD146* siRNA (siCD146) to brain tumors in a mouse glioma model in order to demonstrate the efficacy of *CD146*-targeted gene therapy for malignant gliomas.

Methods

NPs and siRNA

FA-PEG-COL was synthesized as previously reported.¹² A detailed protocol for synthesis of FA-PEG-COL NPs and sequences of siRNA used in this study are described in Supplemental Materials and Methods.

Animals

C3H/HeN and Balbc nu/nu mice were obtained from Japan SLC Inc. All animal experiments were approved by the Animal Care and Use Committee of Kochi University.

Tissues

All brain tumor tissues used for expression analysis were obtained from Kochi Medical School (Nankoku, Japan). Written informed consent was obtained from patients prior to tumor specimen collection and from healthy donors before the use of blood samples. The ethics committee of Kochi Medical School approved this study.

Cell Culture

We used RSV-M mouse glioma cells, a cell line established from brain tumors induced in C3H/HeN mice by intracerebral implantation of chicken sarcoma cells producing the Schmidt-Ruppin strain of Rous sarcoma virus, which were kindly gifted by Dr. Kumanishi.¹⁷ Human glioma U87MG, T98G, and U251MG cells were purchased from ATCC, and GBM-PCC1/2/3 cells were primary cultured cells isolated from GBM patients. All cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 50 µg/ml streptomycin in a humidified incubator at 37°C and 5% CO₂. RSV-M tumor sphere (TS) cell culture was established from RSV-M cells using neurosphere-culture methods in DMEM/F12-containing N2 supplement, 20 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 1 mM L-glutamine, and antibiotics. RNAiMAX reagent was purchased from Thermo Fisher Scientific. RSV-M cells stably expressing luciferase were established by transfection of pGL4.50 cells (Promega) and cultured in the presence of hygromycin (InvivoGen).

Water-Soluble Tetrazolium Salt–8 Assay

Water-soluble tetrazolium salt–8 (WST-8) cell-proliferation assays were performed using a Cell Counting Kit-8 (Dojindo) according to the manufacturer's instructions. Cells were suspended in medium at 3×10^4 cells/ml and then plated at 100 µl/well in 96-well plates. Following the addition of siRNA (8.37 µg/1 mg NPs; 30–100 µg/well), cells were incubated in a humidified CO₂ incubator at 37°C for 3 days, and cytotoxicity was determined by WST-8 assay.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA from a normal brain was purchased from TaKaRa. Other RNA was isolated from cultured cells and brain tumor tissues using an RNA isolation kit (QIAGEN).

Reverse transcription reactions using a random hexamer primer were performed using a PrimeScript first-strand cDNA synthesis kit (TaKaRa). Quantitative reverse transcription polymerase chain reaction (RT-PCR) was conducted on an ABI StepOnePlus Real-Time PCR System using a TaqMan probe (Hs01124179) and Universal PCR master mix (Applied Biosystems). β -actin was used as a reference mRNA control.

Flow Cytometry

For flow cytometry, cultured cells were collected, washed, and suspended in phosphate-buffered saline (PBS) containing 7-aminoactinomycin D (BD Biosciences) for labeling of dead cells. Data from all samples were obtained using a Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar Inc.).

Western Blot

Cell lysates were prepared using radioimmunoprecipitation assay buffer containing 1× Halt protease inhibitor cocktail (Thermo Fisher Scientific). Protein (20 μ g) from each sample was subjected to 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane, which was subsequently probed with rabbit anti-CD146 monoclonal antibody (Abcam) or mouse anti- β -actin monoclonal antibody (Santa Cruz Biotechnology). Blots were visualized using an enhanced chemiluminescence-plus kit (GE Healthcare) according to the manufacturer's instructions.

In Vivo Imaging

Eight-week-old female C3H/HeN and BalbC nu/nu mice were implanted with RSV-M cells and U87MG cells, respectively. In the intracranial model, 2×10^5 cells suspended in 1 μ l PBS were stereotactically inoculated at a 3-mm depth from the skull surface through a burr hole located 3 mm to the right and 1 mm anterior to the bregma. In the flank model, 10^7 cells suspended in 100 μ l PBS were implanted in subcutaneous tissues. At 3 weeks after implantation, 1 mg AlexaFluor 647–labeled NPs suspended in 100 μ l PBS was administered via the tail vein, and in vivo fluorescence imaging was performed using an IVIS Spectrum system (PerkinElmer). To monitor tumor growth, glioma cells in D-luciferin solution (3.75 mg/200 μ l PBS) were injected intraperitoneally, and bioluminescent signals were measured 15 minutes after injection.

Gene Therapy With NP Administration

The RSV-M mouse glioma model was created according to the procedures described in this section. RSV-M cells expressing luciferase rather than regular RSV-M cells were used to monitor tumor growth. On day 0, RSV-M cells expressing luciferase were intracranially implanted, and tumor growth was measured as bioluminescence using the IVIS Spectrum system on days 3, 10, 17, 24, and 31 after implantation. Tumor-transplanted mice were divided into 3 groups of 6, with equal mean luciferase activity on day 3. One mouse was excluded due to low luciferase activity. The mice were treated with 100 μ l PBS or FA-PEG-COL NPs with control siRNA or siCD146 by intravenous

injection via the tail vein at 1 mg NPs/mouse on days 4, 11, 18, and 25.

Immunohistochemistry

At the endpoint of the study (day 6, 24, or 31) or upon the development of abnormal phenotypes, mice implanted with RSV-M or U87MG cells were euthanized by terminal anesthesia, and perfusion fixation was performed with 4% paraformaldehyde. Perfusion-fixed brain tissue was processed for paraffin embedding or frozen, sectioned, and stained with H & E. For immunohistochemistry, 5- μ m sections were prepared and examined for CD146, vimentin, and Ki-67 expression. Proteins were stained with rabbit monoclonal anti-CD146 antibody (1:200; Abcam), rabbit monoclonal anti-vimentin antibody (1:100; Cell Signaling Technology Inc.), and rabbit monoclonal anti-Ki-67 antibody (1:200; Novus Bio). Slides were scanned microscopically using a Biorevo BZ-9000 and BZ-X800 (Keyence), and three representative fields were photographed (magnification $\times 400$). Ki-67–positive cells were counted using software (BZ-II dynamic cell count) embedded within the BZ-9000 system.

Statistical Analysis

In all in vitro experiments, experimental conditions were run as biological triplicates. Investigators were not blinded to the group allocation. For gene therapy experiments, results are presented as the mean $\pm$ SD. ANOVA was used to determine differences between control and treated groups, and $p < 0.05$ was considered significant. In other experiments, the Student t-test or chi-square test was used. Analyses were performed using IBM SPSS software (version 24.0, IBM Corp.).

Results

FOLR1 Levels in Glioma Cells and Tissues

FOLR1 is highly expressed in high-grade gliomas, and expression levels correlate with tumor grade.¹⁵ To assess folate receptor–mediated uptake of NPs, we first examined *FOLR1* expression by quantitative RT-PCR in glioma tissues and cell lines (Fig. 1). We detected *FOLR1* expression in most glioma tissues and cells, including primary cultured cells, except for cell line U251MG. This result indicated the possibility for broad-spectrum FOLR1-targeted therapy of gliomas.

FA Conjugation Improves NP Uptake by Glioma Cells

Following FA-PEG-COL NP synthesis, we evaluated NP morphology and size using scanning electron microscopy. The NPs displayed a globular shape with a mean diameter of 104.6 ± 18.05 nm (Fig. 2A). We then investigated their blood-biological effects, including hemolysis, lymphocyte proliferation, and coagulation, because blood compatibility is a mandatory requirement for safe intravenous administration of NPs. Compared with COL NPs, FA-PEG-COL NPs showed reduced hemolysis, lymphocyte cytotoxicity (Supplemental Fig. 1), and aggregation of red blood cells (data not shown). These findings are similar to those from our previous report.^{12,13} We then ex-

FIG. 1. *FOLR1* expression in glioma tissues and cell lines. Relative *FOLR1* mRNA levels were quantified by RT-PCR and normalized against β -actin as a control. Results are from GBM cell lines, gliomatosis cerebri, and anaplastic oligodendroglioma tissues, as well as other glioma cell lines and primary cultured cells (GBM-PCC1–3) derived from GBM patients. Bars represent the mean and error bars, SD.

amined uptake efficiency of FA-PEG-COL NPs in mouse and human glioma cell lines. Two days after the addition of fluorescein isothiocyanate (FITC)–FA-PEG-COL NPs to human glioma U87MG cells, we detected NP-based signals in most cells (Fig. 2A). To evaluate the efficiency of FA conjugation with COL NPs, we analyzed uptake frequency by flow cytometry in mouse glioma RSV-M and U87MG cells. We observed that FA-PEG-COL NP uptake was increased relative to COL NPs by RSV-M cells (Fig. 2B), although there was no clear difference in the percentage of FA-PEG-COL NPs and COL NPs taken up by U87MG cells. However, the mean fluorescence intensity associated with FA-PEG-COL NPs (5346 ± 170 ; $n = 3$) was higher than that of the COL NPs (2658 ± 98 ; $n = 3$) in U87MG cells, suggesting that FA-PEG-COL NP uptake was approximately 2-fold higher than that of COL NPs. Thus, FA conjugation to COL improved NP uptake efficiency in U87MG cells. These results further demonstrated the efficiency of folate receptor–mediated uptake of FA-conjugated NPs by mouse and human glioma cells.

In Vivo NP Accumulation in Tumor Tissues

We then examined the targeting efficiency of FA-PEG-COL NPs to tumor tissue in a syngeneic mouse glioma model (C3H/HeN mice implanted with RSV-M cells) and a human xenograft model (Balb/c nu/nu mice implanted with U87MG cells). At 3 days postadministration of fluorescence-labeled FA-PEG-COL NPs, we observed a clear indication of NP accumulation in tumor tissues according to in vivo imaging of mice administered NPs via the tail vein (Fig. 2C) and intracranial implantation (Fig. 2D). Additionally, strong signals were observed in tumor tissue removed subcutaneously (Fig. 2E), indicating accumulation of large amounts of NPs. Histological examination subsequently confirmed NP accumulation in the mouse brain tumor tissue (Fig. 2E). Furthermore, we verified that FA conjugation to COL improved NP-targeting efficiency to tumor cells in the human xenograft model (Fig. 2F). Based on this observation, COL NPs without FA were eliminated from further experiments. In addition, accumulation of fluorescence-labeled FA-PEG-COL NPs was greater in

tumor tissues from both models at early (3 days) or late (3 weeks) time points (Fig. 2G). These results indicated that the FA-PEG-COL NPs efficiently targeted tumor tissues in glioma mouse models.

Suppression of RSV-M TS Cell Growth by *CD146* Knockdown

Based on a recent study reporting growth suppression of human glioma TS cells following *CD146* knockdown, we evaluated the effects of *CD146* knockdown in mouse glioma RSV-M and TS cells. We confirmed reductions in *CD146* protein following si*CD146* transfection in RSV-M cells (Fig. 3A). RSV-M TS cells cultured in the presence of epidermal growth factor and fibroblast growth factor–2 in the absence of serum demonstrated highly invasive activity and tumorigenic potential; however, transfection of si*CD146* by lipofection suppressed growth of RSV-M TS cells but not RSV-M cells (Fig. 3B). Moreover, the addition of FA-PEG-COL NPs cross-linked with si*CD146* resulted in similar suppression of RSV-M TS cell growth. These results imply that siRNA delivery using FA-PEG-COL NPs induces *CD146* knockdown, thereby suggesting its efficacy for use in RNA-based gene therapy.

Correlation of Reduced *CD146* Expression With Uptake of si*CD146*-Loaded NPs by Tumor Cells

To assess knockdown efficiency in tumor tissues, FITC-labeled NPs cross-linked with siRNAs were administered to mice bearing RSV-M or U87MG tumors and distribution of NPs and *CD146* expression were examined in tumor tissues 3 days postadministration (Fig. 4A and B). A majority of endothelial cells with tiny nuclei in the tumor vasculature expressed *CD146* regardless of control siRNA or si*CD146* delivery and showed no fluorescent signals of NPs, revealing the tumor-specific potential of FA-PEG-COL NPs. In *CD146*-positive tumor cells, possibly including GSCs, uptake of FA-PEG-COL NPs cross-linked with control siRNA was observed along with other tumor cells, suggesting the targeting potential of NPs to GSCs (Table 1). *CD146*-positive/NP-positive tumor cells were frequently observed in control siRNA–delivered tumor tissues, but not in si*CD146*-delivered tumors. Most NP-positive tumor cells did not express *CD146* in si*CD146*-delivered tissues. Similar knockdown effects after si*CD146* delivery were observed at 3 days and 3 weeks after implantation of tumor cells in both mouse RSV-M and U87MG tumors. Thus, FA-PEG-COL NPs cross-linked with si*CD146* were taken up by glioma cells and effectively silenced *CD146* mRNA.

Efficacy of si*CD146*-Based Gene Therapy Using FA-PEG-COL NPs in a Mouse Glioma Model

The mice were intracranially implanted with mouse glioma RSV-M cells expressing luciferase, followed by administration of FA-PEG-COL NPs cross-linked with control siRNA or si*CD146* via the tail vein once per week for 4 weeks, starting on day 4 postimplantation (Fig. 5A). Tumor growth was then monitored weekly by in vivo imaging to measure bioluminescence in control and si*CD146*-treated mice ($n = 6$ /group), as well as mice receiving no

FIG. 2. Accumulation of FA-PEG-COL NPs in glioma cells and tissues. **A:** Scanning electron microscopy image of FA-PEG-COL NPs (*left*). Bar = 300 nm. Human glioma U87MG cells were incubated with fluorescence-labeled FA-PEG-COL NPs for 2 days (*right*), and *green signals* for the NPs were detected in the perinuclear region. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (*blue*). Bar = 50 μ m. **B:** Flow cytometric analysis of NP uptake in mouse glioma RSV-M and human glioma U87MG cells. The *blue line* represents uptake of AlexaFluor 647-labeled FA-PEG-COL NPs, and the *red and green lines* represent uptake of AlexaFluor 647-labeled COL NPs and no NPs, respectively. The table shows the percentage of NPs taken up by glioma cells. **FIG. 2. (continued)**→

FIG. 2. C: Accumulation of FA-PEG-COL NPs in subcutaneous and intracranial tumors. Three weeks postimplantation of RSV-M cells into syngeneic C3H/HeN mice, AlexaFluor 647-labeled FA-PEG-COL NPs were administered intravenously via the tail vein, and NP accumulation in tumors was measured. **D:** Increase in NP signals in intracranial tumors. **E:** At 3 days postadministration, frank tumor tissue was removed, and strong fluorescence was observed (*left*). Histological observation of tumor tissue from intracranial tumors revealed NP accumulation (*red signal*; *right*). Nuclei were counterstained with DAPI (*blue*). Bar = 100 μ m. **F:** FA conjugation to COL via a PEG linker improved NP accumulation efficiency in the mouse xenograft model established with human glioma U87MG cells. **G:** NP accumulation in tumor tissues of mouse RSV-M glioma and U87MG xenograft models. At 3 days or 3 weeks postimplantation of tumor cells into mouse brain, FITC-labeled NPs were administered via the tail vein. Three days after the administration, NP accumulation was observed (*green signal*). Nuclei were counterstained with DAPI (*blue*). Bar = 200 μ m.

treatment ($n = 5$) (Fig. 5B). We observed no suppression of bioluminescence in mice treated with control siRNA, but strong suppression in mice treated with siCD146, suggesting that *CD146* knockdown following administration of siCD146-cross-linked FA-PEG-COL NPs strongly suppressed tumor growth. Interestingly, the therapeutic effect became prominent at 2 weeks after therapy initiation (Fig. 5C). Moreover, histological analysis revealed large tumor masses in control mice, whereas mice receiving 4 weeks of therapy showed small tumors, no tumor, or only a scar at the previous tumor location (Fig. 5D). Furthermore, we observed reduced levels of vimentin, a mesenchymal marker normally elevated in high-grade gliomas, in small residual tumors in treated mice according to immunohistochemical analysis (Fig. 5E). Additionally, we analyzed the Ki-67-labeling index in the tumor-front area and found that this index was higher in tumors of control mice than in residual tumors of treated mice (Fig. 5E and F), suggesting that *CD146* knockdown following siRNA delivery by FA-PEG-COL NPs suppressed tumor-cell proliferation in the mouse glioma model.

Discussion

This study demonstrated that CD146 is a potential target molecule for gene therapy and that FA-PEG-COL NPs are promising delivery devices for gene therapy of glioma. In vitro, CD146 knockdown suppressed cell growth in RSV-M TS cells, but not in RSV-M cells or in GSCs, as previously reported in human cells (Fig. 3).¹⁰ Because of high

expression of CD146 in GSCs, TS cells containing abundant GSCs are more sensitive to CD146 knockdown than parental cells containing fewer CD146-expressing cells. Parental cells grow approximately 2- to 3-fold faster than TS cells and contain a large amount of transient amplifying cells that divide vigorously to sustain the growing cell population. The effects of CD146 knockdown transiently appear in TS cells containing abundant CD146-positive cells, but do not appear in parental cells. Therefore, this strategy for CD146 knockdown specifically targets GSCs.

We selected *CD146* as an siRNA target based on its broad expression in glioma tissues, and because its inhibition suppresses GSC growth, invasion, and metastasis in melanoma,¹⁸ breast cancer,^{19,20} and osteosarcoma.²¹ Importantly, CD146 is an inducer of the epithelial-mesenchymal transition associated with triple-negative breast cancer.²⁰ GBM is classified into 4 groups, including proneural, neural, classical, and mesenchymal, according to transcriptome analysis,²² with the MES phenotype of various tumor cells correlating with carcinogenesis, invasion, metastasis, and poor survival. Moreover, the prognosis of GBM patients assigned to the mesenchymal group is the poorest of any group.^{23,24} A previous study suggested that CD146 might contribute to establishment of the mesenchymal phenotype in GBM, as a subset of proneural GSCs undergo differentiation into a mesenchymal state in some tumor microenvironments.²⁵ Mesenchymal GSCs are aggressive, invasive, angiogenic, and more resistant to radiation relative to other GSCs; therefore, a CD146-targeted therapy

FIG. 3. *CD146* knockdown suppresses RSV-M cell growth in the TS. **A:** Reduced *CD146* expression following siCD146 transfection or FA-PEG-COL NP administration confirmed by western blot analysis. Protein levels were analyzed by densitometry, and the ratio of CD146 to β -actin was calculated. **B:** Effects of *CD146* knockdown following siRNA delivery by FA-PEG-COL NPs on cell growth, as determined by WST-8 cell-proliferation assay. *CD146* knockdown by lipofection suppressed RSV-M TS cell growth but not that of RSV-M parental cells. Bars represent the mean and error bars, SD. All experiments were independently performed in duplicate with similar results. * $p < 0.01$, ** $p < 0.05$ compared with control siRNA (siCont).

FIG. 4. Effect of siCD146 delivery on *CD146* gene silencing in tumor tissues via FA-PEG-COL NPs. **A:** Loss of *CD146* expression by siCD146 delivery in NP-positive RSV-M and U87MG cells. FITC-labeled NPs were cross-linked with control siRNA (siCont) or siCD146 and were intravenously injected at 3 days or 3 weeks postimplantation of tumor cells into brains. Three days postimplantation, TS cells were implanted to increase the population of GSCs. Three weeks postimplantation, parental cells were used. Three days after NP injection, NP accumulation (green signal), *CD146* expression (red signal), and nuclei (blue) were immunohistochemically examined. Bar = 100 μ m. **B:** Magnified images of the experiments shown in U87MG tumors by optical sectioning. Bar = 20 μ m.

might eradicate mesenchymal-specific GBM by inhibiting acquisition of the mesenchymal phenotype. *CD146* also is thought to contribute to neovascularization in tumors and normal tissues.^{26,27} GSCs are capable of transdiffer-

entiation into endothelial cells, which subsequently form blood vessels and then the perivascular niche,^{28,29} a microenvironment that increases tumorigenic potential and therapeutic resistance of tumor cells. Therefore, a *CD146*-

TABLE 1. Presence of FITC-labeled NPs in CD146-positive cells

Time Point	Group	RSV-M			U87MG		
		Positive (%)	Negative (%)	p Value	Positive (%)	Negative (%)	p Value
3 days	siCont	162 (54)	138 (46)	1.46E-16	206 (54)	178 (46)	5.13E-25
	siCD146	144 (92)	12 (8)		390 (86)	63 (14)	
3 wks	siCont	190 (48)	209 (52)	4.29E-21	168 (47)	187 (53)	1.02E-13
	siCD146	155 (90)	18 (10)		214 (76)	66 (24)	

siCont = control siRNA.

targeting therapy might suppress the neovascularization process and decrease therapeutic resistance.

Our results demonstrated the therapeutic potential of FA-PEG-COL NPs ionically cross-linked to siCD146 and used for targeted gene therapy in a mouse glioma model. Although RNA interference is a promising therapeutic approach for gene silencing, in vivo delivery of siRNA in the absence of its subsequent degradation remains challenging. A previous study showed that siRNA ionically cross-linked to chitosan protected siRNA from degradation in serum;¹² however, the stability of siRNA-cross-linked NPs in cerebrospinal fluid should be investigated prior to its application in central nervous system diseases. In the present study, we demonstrated successful NP uptake mediated by a folate receptor, with increased FA-PEG-COL NP uptake by mouse and human glioma cells observed in vitro relative to uptake of NPs not conjugated with FA. Although NP accumulation in tumor tissues was observed in the absence of FA conjugation via the EPR effect in the human glioma xenograft model, levels were enhanced in the presence of FA. These findings suggest that NPs initially accumulate in tumor tissues by passive targeting via the EPR effect and that NP uptake occurs by active targeting of the folate receptor by FA in glioma cells. Our results indicated that siRNA can be effectively delivered by a combination of the EPR effect and targeting of the folate receptor using FA-conjugated chitosan NPs in brain tumor models.

Chitosan is a highly biocompatible molecule as a non-toxic, biodegradable, natural polysaccharide,³⁰ allowing for a wide range of uses in the life sciences in roles associated with wound healing and for the delivery of tumor vaccines. Previous studies demonstrated roles for chitosan oligosaccharide in inflammation, immunomodulation, and antitumor activity, as well as promise for applications related to drug and nucleic acid delivery.^{31–33} Administration of chitosan NPs to mice with hepatocellular carcinoma resulted in suppressed tumor growth both in vitro and in vivo.³⁴ In the present study, we used a low-molecular-weight chitosan oligosaccharide due to its high degree of water solubility, low viscosity, and deacetylation. Based on previous antitumor activity reports, we expected similar results using FA-PEG-COL NPs with control siRNA; however, we observed no tumor suppression following NP administration in our syngeneic glioma models, suggesting that the tumor-suppressive effect of the chitosan oligosaccharide might have been lost during the process of PEG and FA conjugation or fabrication with pentasodium triphosphate. In order to examine the total therapeutic

efficacy of CD146 knockdown as well as immunological antitumor effects, a mouse glioma model was used in this study. Our previous report showed that knockdown of *CD146* gene effectively suppressed the growth of primary cultured human GSCs.¹⁰ The frequency of CD146 expression, except for endothelial cells in tumor tissues, was positively correlated with the expression of CD133, a cancer stem cell marker in glioma. Hence, CD146 seems likely to be a promising therapeutic target in human glioma. Our future work will evaluate siCD146 gene therapy using NPs in a human glioma xenograft model in order to determine its therapeutic efficacy independent of immune activity in immune-deficient mice.

Oligonucleotide therapeutics are innovative, although the cost is high for clinical application due to the expense of oligonucleotides. Many reports, including the work we present here, require a large amount of nucleic acid. In the future, development of technology that will reduce the amount required for administration or lower costs of mass synthesis of oligonucleotides will be absolutely necessary for implementing gene therapy.

The blood-brain barrier (BBB) hampers the penetration of therapeutic agents into the central nervous system and thus poses an obstacle for brain tumor therapy.³⁵ In the present study, we found that the tumor-suppressive effects of FA-PEG-COL NPs might be dependent on the EPR effect, and that blood flow is important for NP-specific tumor targeting. However, the therapeutic effective dose used in this study did not preclude penetration of the NPs into the BBB. The perivascular niche for cancer stem cells in brain tumors plays an important role in maintaining their self-renewal activity and pluripotency, as well as their involvement in radioresistance.³⁶ Perivascular GSCs might be targeted efficiently by NPs via the EPR effect; however, it is necessary to establish strategies for crossing the BBB in order to target glioma cells invading healthy brain tissue. A recent report suggested that NP conjugation with transferrin or des-octanoyl ghrelin enhances NP transport across the BBB.³⁷ Additionally, convection-enhanced delivery represents a promising strategy to promote the ability of locally administered NPs to overcome BBB restrictions. Further studies are required to determine best strategies for gene delivery to treat brain tumors.

Conclusions

Our results demonstrated that *CD146* silencing could eradicate experimental glioma through the delivery of FA-conjugated NPs targeting the folate receptor on glioma

FIG. 5. siRNA gene therapy targeting *CD146* using FA-PEG-COL NPs in a mouse glioma model. **A:** Gene therapy protocol. **B:** Bioluminescence imaging showing the growth of RSV-M-luc tumors. Mice showing neurological defects were euthanized prior to the end of the study in control groups not administered NPs or those administered NPs with control siRNA. **FIG. 5. (continued)→**

FIG. 5. C: Total radiant efficiency measured following luciferin administration. Bars indicate the mean $\pm$ SD. Statistically significant differences ($p < 0.05$, ANOVA) were observed between treated and untreated groups. **D:** Representative H & E staining of whole-brain slices from treated and untreated mice. Minimal evidence of tumors was observed in some mice treated with siCD146-delivered NPs. The black arrow indicates the needle track. **E:** Immunohistological analysis of glioma tissues in mice treated with siCD146 gene therapy. Representative sections show reduced vimentin and Ki-67 levels in tumors responsive to siCD146 gene therapy using FA-PEG-COL NPs. The brown areas indicate positive staining. Bar = 200 μ m. **F:** Quantification of Ki-67–positive cells/field in untreated ($n = 5$) mice, mice treated with NPs containing control siRNA ($n = 6$), and mice treated with siCD146-delivered NPs ($n = 3$). Experiments were performed independently in duplicate with similar results.

cells, suggesting that NPs are attractive candidates for GBM-targeted therapy. Moreover, this siRNA delivery system might be useful for evaluating various target molecules during the development of glioma therapy. Our work examining *CD146* targeting in multiple tumorigenic processes and therapeutic resistance in various tumors will promote the development of gene therapeutic strategies for other malignant tumors.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Conception and design: Yawata, Fukui, Nakajo, Honke, Ueba. Acquisition of data: Yawata, Fukui, Yamashita. Analysis and interpretation of data: Yawata, Fukui, Aratake. Drafting the article: Yawata. Critically revising the article: Yawata, Nakajo, Higashi, Honke, Ueba. Reviewed submitted version of manuscript: Yawata. Approved the final version of the manuscript on behalf of all authors: Yawata. Statistical analysis: Yawata. Administrative/technical/material support: Yawata, Kawanishi, Honke. Study supervision: Yawata.

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