Original Research

Migration Capacity of Stem Cells from Human Exfoliated Deciduous Teeth Towards Glioma

Taisuke Yamamoto[1], Shinichiro Koizumi[1], Tomoya Oishi[1], Makoto Horikawa[1], Tetsuya Asakawa[1], Tomohiro Yamasaki[1], Tetsuro Sameshima[1], Yasuyuki Mitani[2], Hiroki Namba[3], Kazuhiko Kurozumi[1],*

[1]Department of Neurosurgery, Hamamatsu University School of Medicine, 431-3192 Hamamatsu, Japan
[2]Regenerative Medicine Unit, Kidswell Bio Corporation, 104-0033 Tokyo, Japan
[3]Department of Neurosurgery, Enshu Hospital, 430-0929 Hamamatsu, Japan
*Correspondence: kurozu20@hama-med.ac.jp (Kazuhiko Kurozumi)

Abstract

Background: Stem cells from human exfoliated deciduous teeth (SHED) are a mesenchymal stem cell type and have recently attracted attention for their high proliferative rate, multipotency, and immunosuppressive properties. However, SHED have not yet been investigated for anticancer properties. We therefore investigated whether SHED can be used as a treatment modality, particularly for anti-glioma therapy. **Methods**: *In vitro*, we examined the mobility of SHED and their ability to migrate towards glioma-conditioned medium and specific growth factors secreted by malignant gliomas. *In vivo*, we transplanted SHED into the left hemisphere of nude mice that had been previously implanted with human malignant glioma U87 cells into the right hemisphere. We assessed whether SHED had tumorigenic potential. **Results**: SHED exhibited strong migration ability towards malignant glioma in both *in vitro* and *in vivo* assays. *In vitro*, SHED migrated towards glioma-conditioned medium and specific growth factors such as stem cell factor, platelet-derived growth factor BB, C-X-C motif chemokine ligand 12, and vascular endothelial growth factor. SHED were accumulated around tumor cells in the contralateral hemisphere 1 week after transplantation. Moreover, SHED remained in the brains of nude mice 150 days after transplantation. Finally, we verified that SHED had no malignant transformation or engraftment of SHED in the mouse brain. **Conclusions**: Our findings indicate that SHED can potentially be applied to track malignant glioma.

Keywords: stem cells; human exfoliated deciduous teeth; glioma; cell migration

1. Introduction

Glioma accounts for approximately 20% of primary brain tumors. Recent advances in genomic analysis have made it possible to more accurately treat patients with effective chemotherapies. Additionally, the evolution of awake surgery, devices that improve tumor removal rates, and advances in radiation technology have remarkably improved clinical outcomes. Nevertheless, the 5-year survival rate for glioblastoma remains only 6.8% [1], and recurrences from any remnant tumor after treatment will affect prognosis [2]. Therefore, localized treatment is required to completely destroy all residual tumor cells, particularly any that have invaded the surrounding normal brain tissue. One promising approach to this is the development of delivery modalities that can bring antitumor agents to remnant tumor cells.

There are various strategies for localized glioma therapy such as photodynamic therapy, gene therapy, and oncolytic viral therapy. Of those, gene therapy is a good candidate to control localized tumor cells [3]. One of the gene therapy techniques that has recently been introduced is suicide gene therapy using the *herpes simplex virus–thymidine kinase (HSV-TK)*/ganciclovir (GCV) combination [4], or

the *cytosine deaminase*/5-FC system [5,6]. The key mechanism for these treatments is the so-called "bystander effect", in which cancer cells are driven into apoptosis by genetically modified cells [4]. Several studies have investigated suicide gene therapy in recent years [7–10]; however, a transporter that can carry the suicide gene to tumor cells and track malignant cells throughout the body has not been identified.

Stem cells are recognized to have the ability to migrate to tumors, and previous reports have introduced exogenous suicide genes into stem cells, and then allowed them to migrate to residual tumor cells for treatment [10–12]. This approach has also been attempted for malignant gliomas [13]. Neural stem cells (NSC), induced pluripotent stem (iPS) cells, multilineage-differentiating stress-enduring (Muse) cells, and mesenchymal stem cells (MSC) have all been shown to migrate to malignant gliomas [8,9,14], and the principle of tumor tropism has also been investigated in these contexts. Subsequent studies have confirmed that the growth factors, cytokines, and chemokines secreted by tumors attract stem cells [15–17].

Dental pulp stem cells (DPSC) are a type of MSC that exhibit several advantages over other types of stem cells. *In*

vitro DPSC are easier than for other MSC because they have better growth properties [18–21]. DPSC have been found to have the ability to differentiate into both mesoderm and ectoderm tissues [22]. Stem cells from human exfoliated deciduous teeth (SHED) are a type of DPSC and have similar proliferative potential as DPSC. SHED are more easily and noninvasively collected when deciduous teeth start falling out during childhood compared with DPSC [23]. SHED have been used for various clinical trials in the field of the regenerative therapy because of their increased proliferative capacity [24]. However, no report has verified whether SHED can be used as a vector for suicide gene therapy in an anticancer regimen for malignant glioma.

In this study, to investigate whether SHED could be used as a treatment modality for anti-glioma therapy, we examined the tropism of SHED for malignant gliomas *in vitro* and *in vivo*. Our findings confirmed the tumor tropism and safety of SHED, which may contribute to verifying whether modified SHED can be developed into a novel anti-glioma therapy.

2. Materials and Methods

2.1 SHED Preparation

SHED isolated from a 9-year-old girl's mobile deciduous tooth were provided by Kidswell Bio Corporation (formerly Gene Techno Science Co., Ltd., Tokyo, Japan).

The specifications of SHED such as surface protein expression and multilineage potential are available at https://www.summitpharma.co.jp/japanese/service/s_ATCC_msc.html. Regarding surface protein expression, SHED were positive for CD73, CD90, and CD105 and negative for CD14, CD19, CD34, and HLA-DR. SHED were cultured in expansion medium consisting of Minimum Essential Medium-alpha (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS; Nichirei, Tokyo, Japan), 1% Penicillin–Streptomycin (10,000 U/mL; Thermo Fisher Scientific), and 100 μM ascorbic acid (Wako, Osaka, Japan) at 37 °C under 5% CO_2. SHED were dissociated using TrypLE™ Select (Thermo Fisher Scientific). Culture medium was changed every 3 d, and the cells were passaged when they reached 70% to 80% confluence. SHED were used within 10 passages [25].

2.2 Glioma Cell Lines, MSC, hDF, and Astrocytes

The human glioma cell lines U87 and U251 were purchased from the American Type Culture Collection (Manassas, VA, USA) and from the Japanese Collection of Research Bioresources (Ibaraki, Osaka, Japan), respectively. The human glioma cell line U87-luc (Bioware Ultra Cell Line U-87 MG-luciferase2) was purchased from Caliper Life Sciences (Waltham, MA, USA). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. Glioma cell lines were dissociated using Accutase (Sigma-

Aldrich). Half of the medium as changed every 3 or 4 d. When a confluent cell monolayer was observed, the cells were passaged at a ratio of 1:3. Glioma cell lines were used within 15 passages [26].

Human fibroblasts derived from fetal skin (human dermal fibroblast: hDF; RCB1139) were obtained from Riken BioResource Center (Tsukuba, Japan) and cultured in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. Human MSC from bone marrow (hMSC-BM; RCB2163) were obtained from Riken BioResource Center (Tsukuba, Japan), and human MSC from adipose tissue (hMSC-AT; #C-12977, Promo-Cell, Heidelberg, Germany) were provided by Kidswell Bio Corporation (formerly Gene Techno Science Co., Ltd., Tokyo, Japan). Cells were cultured in Mesenchymal Stem Cell Growth Medium II (Ready-to-use) (Takara Bio, Shiga, Japan) at 37 °C in a humidified atmosphere of 5% CO_2. All cells were used within 10 passages [11,27].

Human astrocytes were obtained from Lonza (Basel, Switzerland). Cells were cultured in Astrocyte Growth Medium SingleQuots™ Supplements and Growth Factors (Ready-to-use) (Lonza, Basel, Switzerland) at 37 °C in a humidified atmosphere of 5% CO_2. All cells were used within 10 passages.

2.3 In Vitro Scratch Assay

To evaluate the migration, hMSC-AT, hMSC-BM, SHED, and hDF alone cells were incubated in 60-mm dishes at 1×10^5 cells per dish, such that the cellular confluence reached approximately 80% after 24-h culture. Then a wound was created with a 1000-μL pipette, and the cellular debris was washed away with phosphate-buffered saline (PBS). Fresh serum-free DMEM was then added to each dish for subsequent culture. The wound healing progress was observed and photographed under an inverted microscope (IX83, Cell Sens, Olympus, Tokyo, Japan) at 12 h, and the rate of wound healing was calculated, which indicated migration ability. The cells were stained with the Diff-Quick kit (International Reagents, Hyogo, Japan). The number of cells that migrated towards the scratch were counted and evaluated as a percentage of the total number of cells. All experiments were repeated three times [28,29].

2.4 In Vitro Transwell Migration Assay

In vitro migration was examined using 24-well Matrigel Invasion Chambers (Corning Discovery Labware, Bedford, MA, USA), which contained 8-μm-pore-sized polyethylene terephthalate membranes. First, DMEM was added to rehydrate the membrane for 2 h at 37 °C in a 5% CO_2 humidified atmosphere. The DMEM was then carefully removed without disturbing the Matrigel on the membrane. SHED, MSC and hDF (control) were washed twice in PBS, and then resuspended at 1×10^5 cells/mL. Then 0.5 mL of the cell suspension (5×10^4 cells) was added to the upper insert. To examine the ability of SHED or other MSC

to migrate towards glioma-conditioned medium (CM), the lower chamber was filled with 0.75 mL of CM from the U87 and U251 glioma cell lines. CM were obtained by collecting, centrifuging, and filtering media from U87 or U251 cells (1×10^6), which were cultured in 10 mL serum-free DMEM for 48 h [15].

To determine the ability of SHED to migrate towards specific growth factors, the lower chamber was filled with 0.75 mL of DMEM and specific growth factors, including stem cell factor (SCF), platelet-derived growth factor BB (PDGF-BB), C-X-C Motif Chemokine Ligand 12 (CXCL12), and vascular endothelial growth factor (VEGF), (Abcam PLC, Tokyo, Japan), which were added at concentrations ranging from 0.1 to 100 ng/mL.

For experiments that involved blocking specific growth factors, U87 and U251 CM were treated with anti-SCF, anti-PDGF-BB, anti-CXCL12, or anti-VEGF neutralizing antibodies (Abcam PLC) at concentrations of 1 to 10 μg/mL, which were added into the lower chamber. Following incubation of the Matrigel invasion chambers for 24 h at 37 °C in 5% CO_2, the non-invading cells and Matrigel were removed from the upper surface of the insert membranes with a cotton swab. Meanwhile, cells that had migrated to the lower surface of the membrane were stained with the Diff-Quick kit (International Reagents) by sequentially transferring the inserts to air dry. The number of cells that had migrated was counted in four high-power fields per membrane ($200\times$ magnification) using an inverted microscope (IX83, Cell Sens, Olympus) [15].

2.5 In Vivo Xenograft Glioma Model

The following experiments were performed and supervised as per guidelines approved by the Animal Care Committee of the Hamamatsu University School of Medicine Animal Care Facility (approval number 2020089).

The mouse model of glioma was based on protocols used in previous reports [8,30]. Briefly, female BALB/c slc nu/nu mice (18–22 g, 8–9-weeks-old, Nippon SLC, Shizuoka, Japan) were subcutaneously injected with an anesthetic mixture comprising 0.75 mg/kg medetomidine (Nippon Zenyaku Kogyo, Fukushima, Japan), 4.0 mg/kg midazolam (SANDOZ, Tokyo, Japan), and 5.0 mg/kg butorphanol (Meiji Seika Pharma, Tokyo, Japan). The human glioma cells U87-luc (1×10^5), which emit luminescence when D-luciferin is administered into the abdominal cavity, were transplanted in 5 μL of PBS. Similarly, human astrocytes (1×10^5) in 5 μL of PBS were injected into the right hemisphere of nude mice on day 0 as a control [9]. A burr hole was placed at the point 2.2-mm lateral for anterior-posterior to bregma. Then, a 10-μL micro-syringe (Hamilton Company, Reno, NV, USA) was inserted at a depth of 3.0 mm from the brain surface and was left in position for 1 min, and then withdrawn to a depth of 2.5 mm, after which cells were infused at a rate of 2×10^4 cells/min by a stereotaxic guidance system [9,31].

2.6 In Vivo Confirmation of Tumor Transplantation

To monitor bioluminescence signals of U87-luc cells, XenoLight Rediject D-luciferin (Summit Pharmaceuticals International, Tokyo, Japan) was injected intraperitoneally at 150 mg/kg body weight, followed by subcutaneous injection of the anesthetic mixture. Then the mice were placed on a stage inside the camera box of the IVIS200 imaging system coupled with a cooled CCD camera (Caliper Life Sciences, Waltham, MA, USA) 20 min after D-luciferin injection. The detected light emitted from U87-luc cells was digitized and electronically displayed as a pseudo color overlay onto a grayscale image of the animal. Images and measurements of luminescence signals were analyzed with Living Image software version 3.0 (Caliper Life Sciences, Waltham, MA, USA) and quantified as photons per second [9]. Images and measurements of luminescence signals were acquired on day 5.

2.7 In Vivo Migration of Superparamagnetic Iron Oxide (SPIO) Nanoparticle-Labelled SHED towards Glioma

To evaluate the ability of SHED to target glioma cells in the brain and to understand the relationship between the number of SHED that accumulated around a glioma lesion and tumor size, we labeled SHED with ferucarbotran (Resovist® Inc., Fujifilm, Tokyo, Japan), in which SPIO was used to label the cell membrane with iron, and the number of SHED surrounding the lesion at the maximum cross-sectional area were counted. SHED were counted within the tumor and within 100 μm of the surrounding area. Counts were performed under a microscope (IX83, Cell Sens, Olympus). These data were then used to evaluate the relationship between tumor size and SHED migration. U87-luc cells (1×10^5) in 5 μL of PBS or Human astrocytes (1×10^5) in 5 μL of PBS (as control) were implanted in the right hemisphere on day 0 (n = 8). On the 5th day after transplantation, bioluminescence signal from the IVIS200 Imaging System confirmed tumor implantation (**Supplementary Data 1**). SPIO-labeled SHED (5×10^5) in 5 μL of PBS or SPIO-labeled hDF (5×10^5) in 5 μL of PBS (as control) were implanted into the left hemisphere of mice on day 7 (n = 4) or 14 (n = 4). On day 14 or 21 (after U87-luc implantation; 7 d after SHED implantation) the mice were sacrificed after the previously mentioned anesthesia was fully in effect by cardiac perfusion with PBS, and their brains were obtained, fixed in 4% paraformaldehyde in 0.1 M PBS, and stained for histologic examination with hematoxylin and eosin (H&E) or Prussian Blue [32].

2.8 Tracking the in Vivo Migration of SHED towards Glioma with Qtracker

For tumor sections from the SPIO experiment, while the Prussian Blue might stain macrophages, Qtracker probe (Thermo Fisher Scientific) was also used to evaluate the in vivo migratory ability of SHED. Malignant glioma cells (U87-luc) were transplanted into the right hemisphere of the
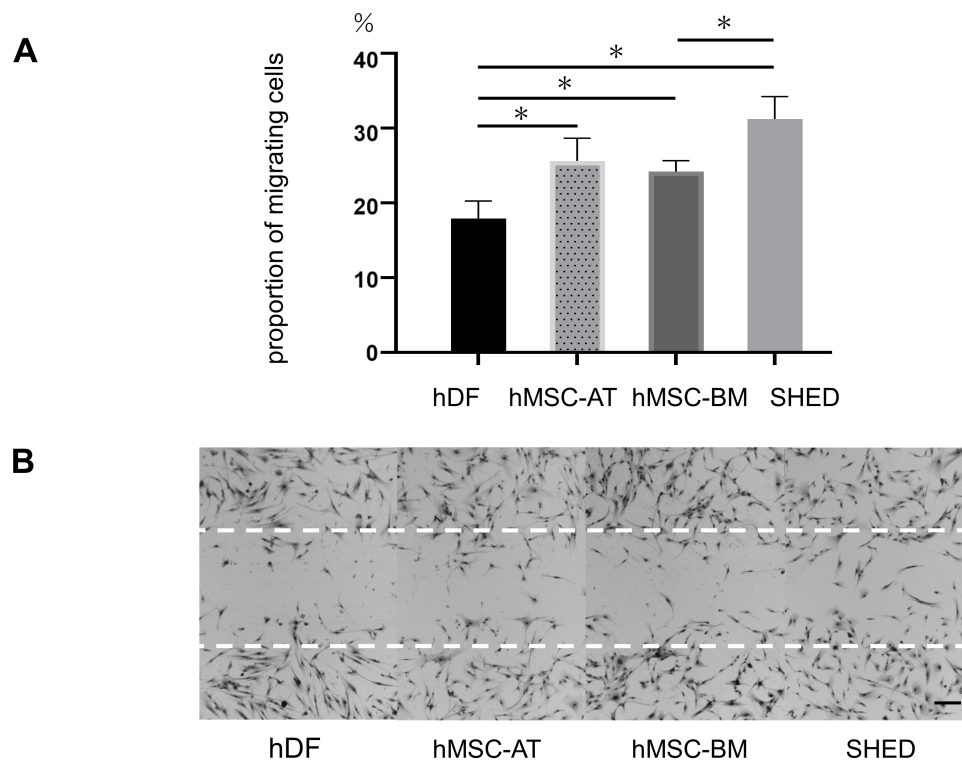
Fig. 1. Verifying the mobility of stem cells from different sources. (A) Quantitative data of cell motility. All stem cells showed some evidence of increase motility, including SHED, hMSC-AT, and hMSC-BM, which were all significantly more mobile than hDF. Data are presented as mean ± SD; *$p < 0.05$. (B) Representative images of scratch assays. The white lines indicate the range of each scratch wound; scale bar: 200 μm.

mice (n = 3), and then SHED were transplanted into the contralateral hemisphere. U87-luc cells (1×10^5) in 5 μL of PBS or PBS only (control) were implanted in the right hemisphere on day 0. On the 5th day after transplantation, bioluminescence signal obtained from the IVIS200 Imaging System confirmed tumor implantation. SHED (5×10^5), labeled with the Qtracker 525 Cell Labeling Kit (Q25049, Thermo Fisher Scientific) following the manufacturer's instructions in 5 μL of PBS, were implanted in the left hemisphere on day 7. On day 14 (after U87-luc implantation; 7 d after SHED implantation), the mice were sacrificed after the previously mentioned anesthesia was fully in effect by cardiac perfusion with PBS, and their brains were obtained for histological examination with H&E staining. The sections were then examined under a fluorescence microscope (IX83, Cell Sens, Olympus) [9].

2.9 In Vivo Evaluation of the Malignant Characteristics of SHED

To use SHED as a transporter for gene therapy in malignant glioma patients, we first assessed whether SHED had tumorigenic potential *in vivo*. SHED (5×10^5 cells) in 5 μL of PBS or 5 μL of PBS alone (control) were implanted in the right hemisphere of female BALB/c slc nu/nu mice to observe alterations in weight and/or behavior. After 7 or 150 d, brain fragments were created, and immunostain-

ing was performed with human anti-mitochondrial antibodies (rabbit monoclonal, Abcam plc, Tokyo, Japan) and secondary antibodies (goat anti-rabbit IgG H&L; Abcam plc, Tokyo, Japan). Tumor formation was evaluated by H&E staining [33].

2.10 Statistical Analysis

The Excel Statistical Program File "ystat 2008.xls" (programmed by Shinya Yamazaki, DDS, Ph.D., Igaku-Tosho-Syuppan, Tokyo, Japan, ISBN4-87151-351-3) was employed for statistical analyses. All data are presented as mean ± standard deviation. $p < 0.05$ was used to indicate significant differences. Enumeration data were analyzed using Friedman's χ^2 test, and quantitative data were analyzed by repeated-measures analysis of variance (ANOVA), followed by Dunnett's test or the Bonferroni method for *post-hoc* corrections.

3. Results

3.1 The Mobility of SHED

The mobility of SHED was compared with those of hMSC-AT and hMSC-BM in scratch assays, with hDF used as a control. The percentages of cells that moved into the scratch range were calculated as follows: SHED, 31.2% ± 2.45%; hMSC-AT, 25.5% ± 2.35%; and hMSC-BM, 24.2%
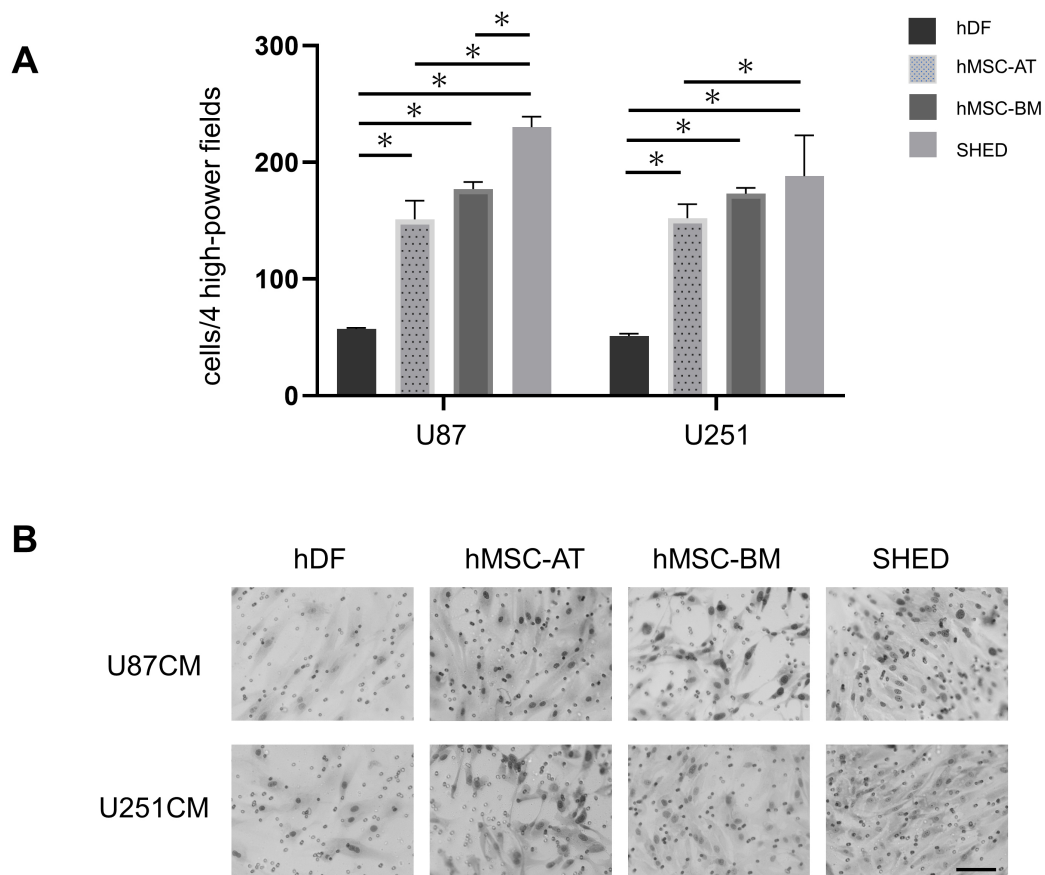
Fig. 2. *In vitro* migration to glioma conditioned medium. Induced migration of mesenchymal stem cells (SHED, hMSC-AT, and hMSC-BM) and hDF (control) in 24-well Matrigel chambers by conditioned medium (CM) from U87 and U251 human glioma cells. After 24 h, the number of cells that had migrated was observed under a microscope. (A) The total number of migrated cells in four high power fields. The number of migrated mesenchymal stem cells was significantly increased compared with that in hDF. Data represent the mean number of migrated cells $\pm$ SD from experiments run in triplicate; $*p < 0.05$. (B) Representative photomicrographs of the micropore membranes of U87CM and U251CM; scale bar: 100 μm.

$\pm$ 1.22%. All cell lines exhibited significantly more movement than hDF (17.7% $\pm$ 1.59%), and SHED were significantly more mobilized than hMSC-BM ($p < 0.05$) (Fig. 1). SHED showed greater mobility than hMSC-AT and hMSC-BM.

3.2 Migration of SHED towards Glioma CM and Growth Factors

The migration ability of SHED towards the CM of malignant glioma cells was compared with those of hMSC-AT and hMSC-BM in transwell assays. hDF were used as a control. The numbers of migrating cells towards the CM of malignant glioma were calculated as follows: SHED, U87: 221.7 $\pm$ 13.1 and U251: 194.7 $\pm$ 14.8; hMSC-AT, U87: 149.3 $\pm$ 10.47 and U251: 153.7 $\pm$ 5.55; hMSC-BM, U87: 177.3$\pm$ 3.18 and U251: 171.3 $\pm$ 4.41; hDF, U87: 55.3 $\pm$ 2.19 and U251: 51.7 $\pm$ 0.67. All SHED, hMSC-AT, hMSC-BM cells migrated significantly more towards the CM of malignant glioma cell lines compared with hDF. Additionally, SHED migrated more significantly towards

glioma CM compared with hMSC-AT (U87 and U251) and hMSC-BM (U87) ($p < 0.05$) (Fig. 2).

We also evaluated the migration of SHED to four specific growth factors with transwell assays. For all four growth factors, the number of migrating SHED cells increased in a concentration-dependent manner (Fig. 3).

3.3 Inhibition of SHED Migration towards Growth Factors with Neutralizing Antibodies

The number of migrating SHED towards CM of both U87 and U251 cells (malignant glioma cell lines) was decreased in a concentration-dependent manner by adding growth factor-neutralizing antibodies. This suggested that SHED might be attracted to these growth factors secreted by malignant gliomas (Fig. 4).

3.4 In Vivo Migration of SHED to Glioma from the Contralateral Hemisphere

We evaluated the migration of SHED from the contralateral hemisphere to malignant glioma in the brains of
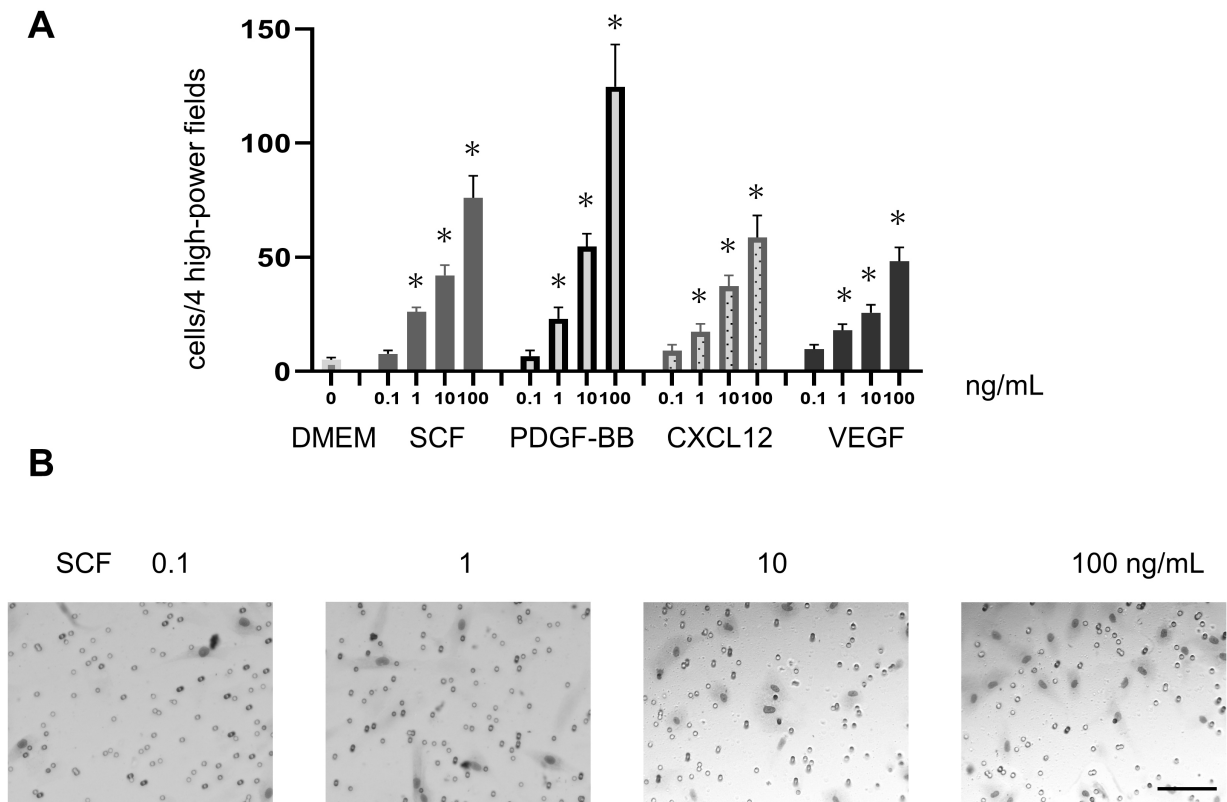
Fig. 3. *In vitro* migration of SHED towards growth factors secreted by gliomas. (A) Migration of SHED towards four specific growth factors (SCF, PDGF-BB, CXCL12, and VEGF) in a 24-well Matrigel chambers. The migration of SHED was significantly increased following stimulation with each growth factor in a concentration-dependent manner (concentrations of 0.1, 1, 10, and 100 ng/mL compared with that with Dulbecco's modified Eagle's medium [DMEM]). Data represent the mean number of migrated cells $\pm$ SD from experiments run in triplicate; *$p < 0.05$. (B) Representative photomicrographs of the micropore membranes of SCF at concentrations from 0.1 to 100 ng/mL. The number of SHED on the membrane was increased depending on the concentration of SCF; scale bar: 100 μm.

nude mice. Brain sections harvested 7 d after SHED implantation showed that SHED accumulated around the tumor in the contralateral hemisphere of the brain (Fig. 5A). In contrast, hDF implanted as a control did not migrate to U87 tumors. Additionally, SHED did not migrate around human astrocytes transplanted as a control for U87 cells (**Supplementary Data 2**).

By labeling SHED with SPIO, we could count the number of cells that had accumulated around the tumor. The number of SHED that accumulated was positively correlated with tumor size (r = 0.74, $p < 0.05$) (Fig. 5B). Moreover, SHED labeled with Qtracker also migrated to the tumor area in the contralateral hemisphere (Fig. 5C,D).

3.5 In Vivo Tumorigenesis of SHED in the Mouse Brain

SHED were implanted into the brains of nude mice, which were evaluated for tumor formation. The mice implanted with SHED showed the same trend of weight gain as the control group, and there were no obvious changes in their health conditions. SHED implanted in the mouse brain were confirmed to be present in the mouse brain after 7 d

(Fig. 6A) and 150 d (Fig. 6B) with human mitochondrial antibodies. After 150 d of SHED transplantation, there was no evidence of SHED forming tumors in brain sections from the mice (Fig. 6B).

4. Discussion

In this study, we verified the migration ability of SHED toward glioma cells *in vitro* and *in vivo* and produced safety data for using SHED. *In vitro* transwell assays showed that SHED migrated significantly more to the CM of malignant glioma cells than hMSC-AT or hMSC-BM and were attracted to four specific growth factors secreted by malignant glioma. *In vivo*, SHED migrated from the contralateral hemisphere to the corpus callosum and accumulated around the tumor site in the mouse brain. Finally, after implanting SHED, there was no evidence of tumor formation in the mouse brain, even after 150 d. To the best of our knowledge, this is the first report to verify the tumor-targeting potential of SHED, and these data suggest that SHED could be used as a vehicle for future gene therapy against glioma.
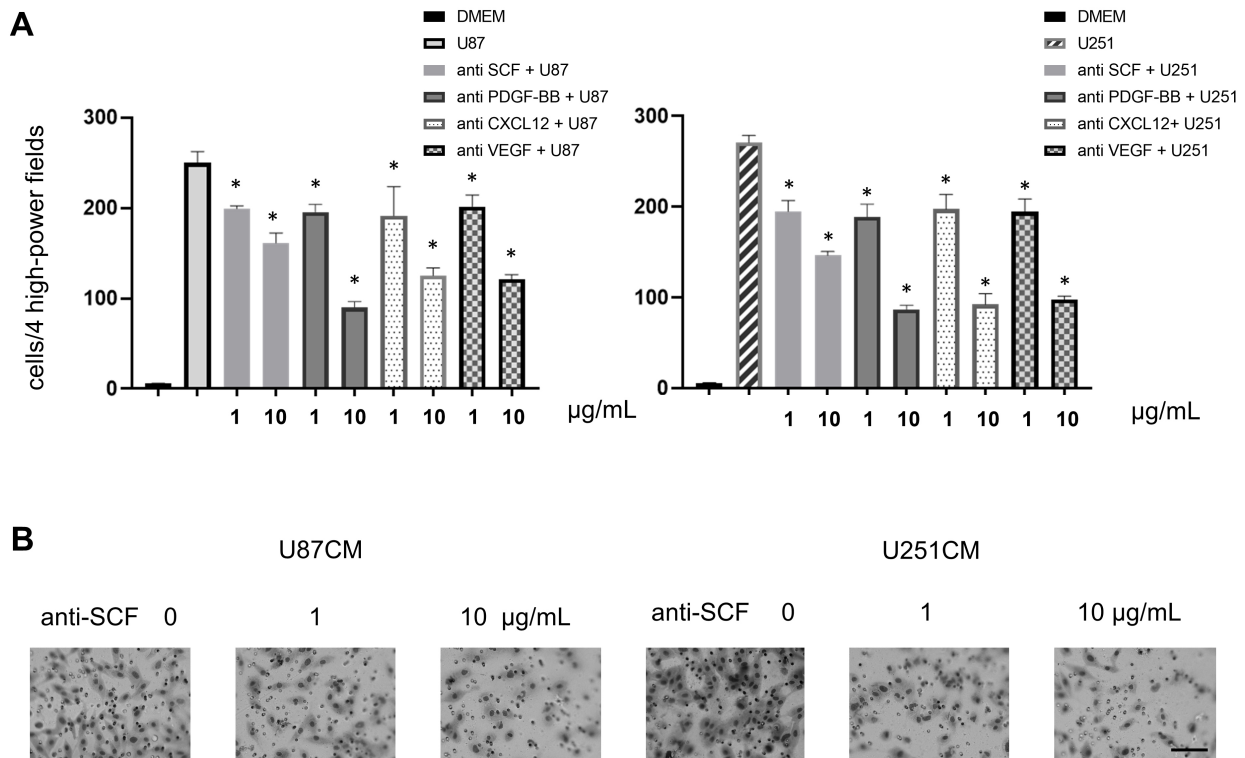
Fig. 4. SHED migration was inhibited by neutralizing antibodies against four growth factors. (A) The migration of SHED towards conditioned media (CM) of U87 and U251 cells was evaluated with inhibitory neutralizing monoclonal antibodies against the indicated growth factors at concentrations of 1 and 10 μg/mL. SHED migration was significantly attenuated by treatment with anti-SCF, anti-PDGF-BB, anti-CXCL12, and anti-VEGF antibodies. Data represent the mean number of migrated cells $\pm$ SD from experiments run in triplicate; *$p < 0.05$. (B) Representative photomicrographs of the micropore membranes of U87CM and U251CM treated with anti-SCF antibody (0, 1, and 10 μg/mL); scale bar: 100 μm.

4.1 SHED

MSC are self-renewing, pluripotent and have been identified in many human tissues, including bone marrow, fat, cord blood, amniotic fluid, and dental pulp. In recent studies, MSC have received particular attention in regenerative medicine and stem cell therapy [27]. Among the MSC, DPSC have recently been used for treatment as multipotent stem cells because they have neuroprotective and anti-inflammatory effects. DPSC are also being applied to treat trauma and stroke [34]. DPSC are also known to have high migratory capacity, and there have been reports on factors affecting DPSC migration [35]. SHED are a type of DPSC that were recently identified as an ideal cell type for regenerative medicine [36]. SHED are known to have no risk of tumorigenesis, high proliferative potential, multipotency, and a low risk of immunorejection [37–39]. SHED have also been investigated for treating neonatal hypoxic brain injury [40], ulcerative colitis, spinal cord injury, and Parkinson's disease [41]. SHED can be managed by cryopreservation [36], and SHED banks have been established for autologous and allogeneic cell therapies [38,39,42].

4.2 Migration Ability of SHED to Specific Growth Factors

Glioma microenvironment plays an essential role in controlling tumor growth and spreading. This microenvironment is made up of different cell types: tissue-resident cells, myeloid cells, bone marrow-derived cells, immune cells, endothelial cells, pericytes, and fibroblasts. In addition, glioma cells produce cytokines, chemokines, and growth factors to form tumor microenvironment [43–45].

There have been many reports on the migration of MSC to gliomas, and it is likely that various factors secreted by gliomas affect the migration of MSC to this disease [17,46]. In this study, SHED showed significant migratory ability to the CM of two human glioma cell lines and to four specific growth factors secreted by glioma. In blocking experiments performed on CM of human glioma cells with inhibitory monoclonal antibodies against the four growth factors, SHED migration to the CM of U87 and U251 cells was significantly inhibited by all four neutralizing antibodies, indicating that these four specific growth factors significantly affect the migration of SHED.

Previously, *in vitro* migration assays have reported that NSC and MSC migrate to specific growth factors, particularly SCF, PDGF-BB [16], CXCL12, and VEGF
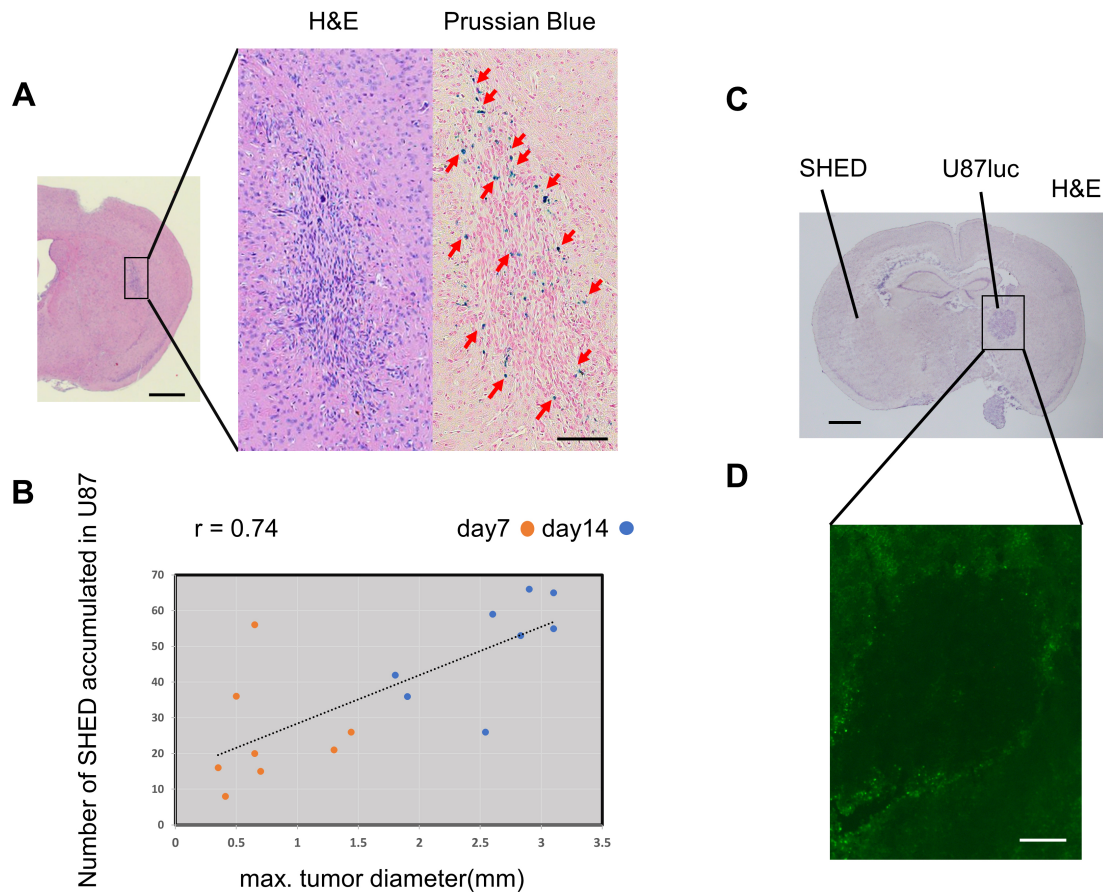
Fig. 5. Tropism of SHED to glioma cells *in vivo*. (A) SPIO-labeled SHED were implanted into the left hemisphere of BALB/c nude mice (n = 8), and the number of SHED that accumulated around along the longest diameter of a section of U87-luc tumors previously implanted in the right hemisphere were counted. Representative Prussian Blue-stained images, in which SPIO-labeled SHED are blue. Hematoxylin and eosin (H&E) staining; scale bar: 1 mm. Prussian Blue-staining; scale bar: 100 μm. (B) The maximum tumor diameter and the number of SHED associated with the tumor were measured. Two slices with the largest tumor diameter were selected from each mouse. Tumor diameter was significantly correlated with the number of SHED (r = 0.74, *p* < 0.05). (C) U87-luc were implanted in the right hemisphere of BALB/c nude mice on day 0, and Qtracker-labeled SHED were implanted in the left hemisphere on day 7; scale bar: 1 mm. (D) SHED were confirmed to have accumulated around the tumor after 7 d; scale bar: 200 μm.

[47,48]. We also found that iPS cells recognized a variety of factors secreted by malignant glioma cells as triggers for migration [15]. Hence, SHED could likely serve as carriers for gene therapy treatments for malignant gliomas if they are genetically modified to express therapeutic transgenes.

Regarding future studies, we are in the process of verifying the receptors for the four specific growth factors (SCF, PDGF-BB, CXCL12, and VEGF), and comparing the levels of their expression between SHED and other MSCs. Additionally, we also need to confirm other factors for migration into glioma.

4.3 Comparison with Other Stem Cells

In vivo, SHED accumulated around tumors implanted in the contralateral hemisphere. Moreover, the number of SHED that migrated to the tumor was increased with tumor size.

NSC and MSC are excellent carriers for gene transfer into gliomas, and thus could be used to transport localized gene therapy to residual tumor cells after surgical removal, and several studies have shown that this is true for NSC, MSC, iPS cells, and Muse cells [7–9,15]. In rodent experiments, intratumoral injection of NSC or Muse cells expressing the *TK* gene followed by systemic GCV administration has shown therapeutic potential. However, ethical and practical issues with transporter collection, as well as the safety and stability of the vectors remain problems that need to be resolved. *TK*-transduced bone marrow cells or MSC-BM also demonstrate the bystander effect [7]; however, they also contain miscellaneous factors and lack stability [49].

Murakami *et al*. [50] reported the self-migrating ability of MSC harvested from the same individual dog and found that DPSC showed higher migratory ability than MSC-AT and MSC-BM. Both SHED and DPSC are de-
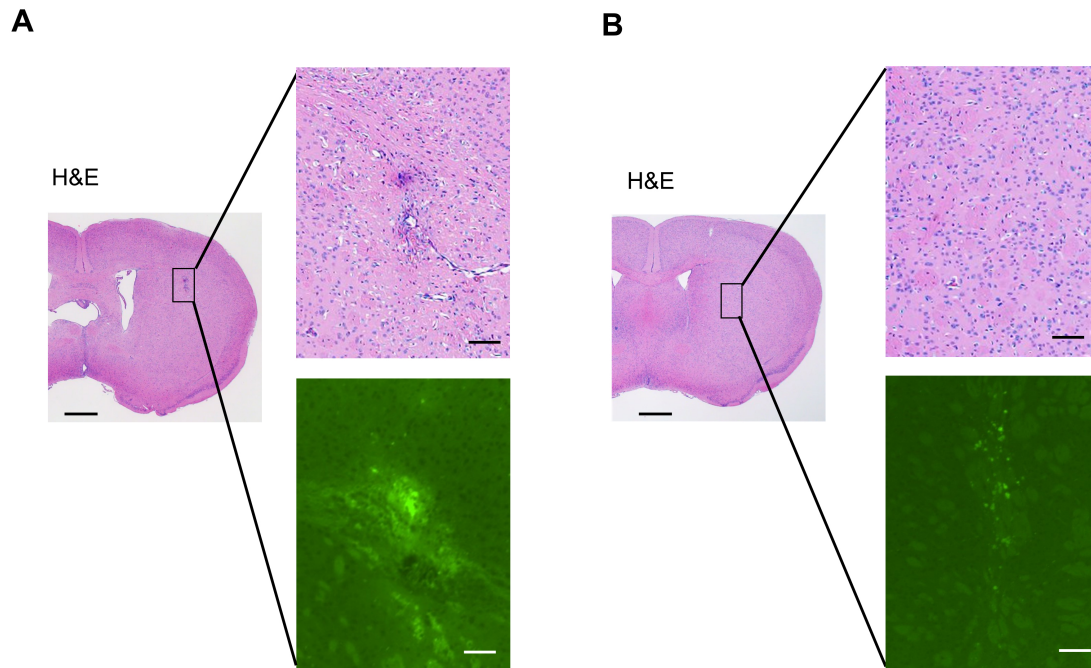
Fig. 6. The situation of tumor formation after SHED implantation. Brain sections of mice at 7 d (A) and 150 d (B) after SHED implantation are presented. Upper column is hematoxylin and eosin (H&E) staining, and lower is immunostaining for anti-human mitochondrial antibodies. As shown in the H&E staining, SHED showed no malignant transformation or engraftment in the mouse brain. As shown by the immunostaining, SHED were still present in the mouse brain 150 d after transplantation. H&E staining; scale bar: 1 mm. Immunostaining for anti-human mitochondrial antibodies; scale bars: 100 μm.

rived from neural crest cells that migrate throughout the body during development to form tissues [51]. Neural crest cells have been found to have excellent migratory ability [52]. Therefore, we consider that SHED and DPSC derived from neural crest cells show higher migratory ability than MSC-AT and MSC-BM derived from mesoderm. In a study comparing SHED and DPSC, Wang *et al.* [53] found no significant difference in the migratory capacity of SHED and DPSC. Similarly, the age of the cell donor may affect the migration of the harvested stem cells.

SHED can be collected from deciduous teeth; hence, they have no invasiveness to the human body and no ethical issues are involved in their collection. Therefore, SHED are likely to be more ideal in terms of collection. Furthermore, dental pulp containing SHED are protected by hard teeth, and their DNA is not easily damaged; thus it remains stable [54]. SHED are therefore a potential practical candidate for a stem cell-based suicide gene therapy for glioma. With respect to the effects of SHED on the normal brain, histological sections of mouse brains 150 d after SHED implantation showed no tumorigenic tissue or remnant SHED, indicating that the safety of SHED transplantation.

4.4 Limitations and Future Directions

In this study, we confirmed that SHED is a potential candidate carrier to deliver genes to glioma cells for gene therapy. However, there are several limitations to our study.

We evaluated only four specific growth factors. The study used a nude mice model, which lacks the effect on the immune system. Furthermore, we did not investigate the migratory behavior of SHED in detail. In the future, the following issues should be further investigated: the relationship between the migratory ability of SHED and other triggering factors and receptors; the immune system and inflammation; migration pathways; and the time required for migration.

5. Conclusions

We confirmed the migratory ability of SHED for malignant glioma cells. Our findings provide experimental evidence that SHED could be a potential therapeutic vehicle to deliver genes or oncolytic viruses, which would be novel treatment approaches for malignant gliomas. These data require further verification in future clinical investigations.

Abbreviations

CM, conditioned media; CXCL12, C-X-C Motif Chemokine Ligand 12; DMEM, Dulbecco's Modified Eagle's medium; DPSC, Dental pulp stem cells; FBS, fetal bovine serum; GCV, ganciclovir; H&E, Hematoxylin and Eosin; hDF, human dermal fibroblast; hMSC-AT, human MSC from adipose tissue; hMSC-BM, human MSC from bone marrow; HSV, herpes simplex virus; iPS, induced pluripotent stem; MSC, mesenchymal stem cell;

Muse, multilineage-differentiating stress-enduring; NSC, neural stem cell; PBS, phosphate-buffered saline; PDGF-BB, platelet-derived growth factor BB; SCF, stem cell factor; SHED, stem cells from human exfoliated deciduous teeth; SPIO, superparamagnetic iron oxide; TK, thymidine kinase; VEGF, vascular endothelial growth factor.

Author Contributions

Conception and design—TYamam, HN, KK. Development of methodology—TYamam, HN, KK. Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.)—SK, TO, MH. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis)—TYamas, TA. Writing, review, and/or revision of the manuscript—TYamam, SK, TO, MH, TA, TYamas, TS, HN, KK. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases)—TYamam, SK, TO, MH, YM. Study supervision—HN, KK.

Ethics Approval and Consent to Participate

This study was performed in accordance with guidelines approved by the Animal Care Committee of the Hamamatsu University School of Medicine Animal Care Facility (approval number 2020089).

Conflict of Interest

Kidswell Bio Corporation (formerly, Gene Techno Science Co., Ltd., Tokyo, Japan) generously provided SHED, and hMSC-AT cell as experimental materials. YM possesses stock options of Kidswell Bio Corporation. TA is serving as one of the Editorial Board members and the guest editor for the special issue titled Exploration of mechanisms in cortical plasticity of this journal. We declare that TA had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to RF. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2201001.

References

[1] Ostrom QT, Cioffi G, Gittleman H, Patil N, Waite K, Kruchko C, et al. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2012-2016. Neuro-oncology. 2019; 21: v1–v100.

[2] Garcia-Ruiz A, Naval-Baudin P, Ligero M, Pons-Escoda A, Bruna J, Plans G, et al. Precise enhancement quantification in post-operative MRI as an indicator of residual tumor impact is associated with survival in patients with glioblastoma. Scientific Reports. 2021; 11: 695.

[3] Mohanam S, Sawaya R, McCutcheon I, Ali-Osman F, Boyd D, Rao JS. Modulation of in vitro invasion of human glioblastoma cells by urokinase-type plasminogen activator receptor antibody. Cancer Research. 1993; 53: 4143–4147.

[4] Namba H, Tagawa M, Miyagawa T, Iwadate Y, Sakiyama S. Treatment of rat experimental brain tumors by herpes simplex virus thymidine kinase gene-transduced allogeneic tumor cells and ganciclovir. Cancer Gene Therapy. 2000; 7: 947–953.

[5] Kurozumi K, Tamiya T, Ono Y, Otsuka S, Kambara H, Adachi Y, et al. Apoptosis induction with 5-fluorocytosine/cytosine deaminase gene therapy for human malignant glioma cells mediated by adenovirus. Journal of Neuro-oncology. 2004; 66: 117–127.

[6] Kosaka H, Ichikawa T, Kurozumi K, Kambara H, Inoue S, Maruo T, et al. Therapeutic effect of suicide gene-transferred mesenchymal stem cells in a rat model of glioma. Cancer Gene Therapy. 2012; 19: 572–578.

[7] Amano S, Li S, Gu C, Gao Y, Koizumi S, Yamamoto S, et al. Use of genetically engineered bone marrow-derived mesenchymal stem cells for glioma gene therapy. International Journal of Oncology. 2009; 35: 1265–1270.

[8] Li S, Tokuyama T, Yamamoto J, Koide M, Yokota N, Namba H. Bystander effect-mediated gene therapy of gliomas using genetically engineered neural stem cells. Cancer Gene Therapy. 2005; 12: 600–607.

[9] Yamasaki T, Wakao S, Kawaji H, Koizumi S, Sameshima T, Dezawa M, et al. Genetically Engineered Multilineage-Differentiating Stress-Enduring Cells as Cellular Vehicles against Malignant Gliomas. Molecular Therapy Oncolytics. 2017; 6: 45–56.

[10] Nakamura K, Ito Y, Kawano Y, Kurozumi K, Kobune M, Tsuda H, et al. Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. Gene Therapy. 2004; 11: 1155–1164.

[11] Menon LG, Kelly K, Yang HW, Kim S, Black PM, Carroll RS. Human bone marrow-derived mesenchymal stromal cells expressing S-TRAIL as a cellular delivery vehicle for human glioma therapy. Stem Cells (Dayton, Ohio). 2009; 27: 2320–2330.

[12] Nakamizo A, Marini F, Amano T, Khan A, Studeny M, Gumin J, et al. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. Cancer Research. 2005; 65: 3307–3318.

[13] Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, Blaese RM. In vivo gene transfer with retroviral vector-producer cells

for treatment of experimental brain tumors. Science. 1992; 256: 1550–1552.

[14] Aboody KS, Brown A, Rainov NG, Bower KA, Liu S, Yang W, *et al*. Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97: 12846–12851.

[15] Koizumi S, Gu C, Amano S, Yamamoto S, Ihara H, Tokuyama T, *et al*. Migration of mouse-induced pluripotent stem cells to glioma-conditioned medium is mediated by tumor-associated specific growth factors. Oncology Letters. 2011; 2: 283–288.

[16] Nistér M, Libermann TA, Betsholtz C, Pettersson M, Claesson-Welsh L, Heldin CH, *et al*. Expression of messenger RNAs for platelet-derived growth factor and transforming growth factor-alpha and their receptors in human malignant glioma cell lines. Cancer Research. 1988; 48: 3910–3918.

[17] Pan J, Sheng S, Ye L, Xu X, Ma Y, Feng X, *et al*. Extracellular vesicles derived from glioblastoma promote proliferation and migration of neural progenitor cells via PI3K-Akt pathway. Cell Communication and Signaling. 2022; 20: 7.

[18] Brar GS, Toor RSS. Dental stem cells: dentinogenic, osteogenic, and neurogenic differentiation and its clinical cell based therapies. Indian Journal of Dental Research: Official Publication of Indian Society for Dental Research. 2012; 23: 393–397.

[19] Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, *et al*. SHED: stem cells from human exfoliated deciduous teeth. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100: 5807–5812.

[20] Kabir R, Gupta M, Aggarwal A, Sharma D, Sarin A, Kola MZ. Imperative role of dental pulp stem cells in regenerative therapies: a systematic review. Nigerian Journal of Surgery: Official Publication of the Nigerian Surgical Research Society. 2014; 20: 1–8.

[21] Kichenbrand C, Velot E, Menu P, Moby V. Dental Pulp Stem Cell-Derived Conditioned Medium: An Attractive Alternative for Regenerative Therapy. Tissue Engineering. Part B, Reviews. 2019; 25: 78–88.

[22] Mattei V, Martellucci S, Pulcini F, Santilli F, Sorice M, Delle Monache S. Regenerative Potential of DPSCs and Revascularization: Direct, Paracrine or Autocrine Effect? Stem Cell Reviews and Reports. 2021; 17: 1635–1646.

[23] Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. Journal of Dental Research. 2009; 88: 792–806.

[24] Shi X, Mao J, Liu Y. Pulp stem cells derived from human permanent and deciduous teeth: Biological characteristics and therapeutic applications. Stem Cells Translational Medicine. 2020; 9: 445–464.

[25] Kenmochi H, Yamasaki T, Koizumi S, Sameshima T, Namba H. Nicotine does not affect stem cell properties requisite for suicide gene therapy against glioma. Neurological Research. 2020; 42: 818–827.

[26] Yu S, Ping Y, Yi L, Zhou Z, Chen J, Yao X, *et al*. Isolation and characterization of cancer stem cells from a human glioblastoma cell line U87. Cancer Letters. 2008; 265: 124–134.

[27] Wong C, LeGrand CF, Kinnear BF, Sobota RM, Ramalingam R, Dye DE, *et al*. In Vitro Expansion of Keratinocytes on Human Dermal Fibroblast-Derived Matrix Retains Their Stem-Like Characteristics. Scientific Reports. 2019; 9: 18561.

[28] Tomita Y, Kurozumi K, Yoo JY, Fujii K, Ichikawa T, Matsumoto Y, *et al*. Oncolytic Herpes Virus Armed with Vasculostatin in Combination with Bevacizumab Abrogates Glioma Invasion via the CCN1 and AKT Signaling Pathways. Molecular Cancer Therapeutics. 2019; 18: 1418–1429.

[29] Meng S, Xu X, Chang W, Lu Z, Huang L, Xu J, *et al*. LincRNA-

p21 promotes mesenchymal stem cell migration capacity and survival through hypoxic preconditioning. Stem Cell Research & Therapy. 2018; 9: 280.

[30] Amano S, Gu C, Koizumi S, Tokuyama T, Namba H. Timing of ganciclovir administration in glioma gene therapy using HSVtk gene-transduced mesenchymal stem cells. Cancer Genomics & Proteomics. 2011; 8: 245–250.

[31] Gao Y, Gu C, Li S, Tokuyama T, Yokota N, Nakayama KI, *et al*. p27 modulates tropism of mesenchymal stem cells toward brain tumors. Experimental and Therapeutic Medicine. 2010; 1: 695–699.

[32] Shahror RA, Wu C, Chiang Y, Chen K. Tracking Superparamagnetic Iron Oxide-labeled Mesenchymal Stem Cells using MRI after Intranasal Delivery in a Traumatic Brain Injury Murine Model. Journal of Visualized Experiments. 2019.

[33] Amano S, Gu C, Koizumi S, Tokuyama T, Namba H. Tumoricidal bystander effect in the suicide gene therapy using mesenchymal stem cells does not injure normal brain tissues. Cancer Letters. 2011; 306: 99–105.

[34] Tsutsui TW. Dental Pulp Stem Cells: Advances to Applications. Stem Cells and Cloning: Advances and Applications. 2020; 13: 33–42.

[35] Rombouts C, Jeanneau C, Bakopoulou A, About I. Dental Pulp Stem Cell Recruitment Signals within Injured Dental Pulp Tissue. Dentistry Journal. 2016; 4: 8.

[36] Ma L, Makino Y, Yamaza H, Akiyama K, Hoshino Y, Song G, *et al*. Cryopreserved dental pulp tissues of exfoliated deciduous teeth is a feasible stem cell resource for regenerative medicine. PLoS ONE. 2012; 7: e51777.

[37] Kunimatsu R, Nakajima K, Awada T, Tsuka Y, Abe T, Ando K, *et al*. Comparative characterization of stem cells from human exfoliated deciduous teeth, dental pulp, and bone marrow-derived mesenchymal stem cells. Biochemical and Biophysical Research Communications. 2018; 501: 193–198.

[38] Taguchi T, Yanagi Y, Yoshimaru K, Zhang X, Matsuura T, Nakayama K, *et al*. Regenerative medicine using stem cells from human exfoliated deciduous teeth (SHED): a promising new treatment in pediatric surgery. Surgery Today. 2019; 49: 316–322.

[39] Arora V, Arora P, Munshi AK. Banking stem cells from human exfoliated deciduous teeth (SHED): saving for the future. The Journal of Clinical Pediatric Dentistry. 2009; 33: 289–294.

[40] Yamagata M, Yamamoto A, Kako E, Kaneko N, Matsubara K, Sakai K, *et al*. Human dental pulp-derived stem cells protect against hypoxic-ischemic brain injury in neonatal mice. Stroke. 2013; 44: 551–554.

[41] Chen Y, Lai P, Chien Y, Lee P, Lai Y, Ma H, *et al*. Improvement of Impaired Motor Functions by Human Dental Exfoliated Deciduous Teeth Stem Cell-Derived Factors in a Rat Model of Parkinson's Disease. International Journal of Molecular Sciences. 2020; 21: 3807.

[42] Suchánek J, Visek B, Soukup T, El-Din Mohamed SK, Ivancaková R, Mokrỳ J, *et al*. Stem cells from human exfoliated deciduous teeth–isolation, long term cultivation and phenotypical analysis. Acta Medica (Hradec Kralove). 2010; 53: 93–99.

[43] Fanelli GN, Grassini D, Ortenzi V, Pasqualetti F, Montemurro N, Perrini P, *et al*. Decipher the Glioblastoma Microenvironment: The First Milestone for New Groundbreaking Therapeutic Strategies. Genes. 2021; 12: 445.

[44] Pasqualetti F, Giampietro C, Montemurro N, Giannini N, Gadducci G, Orlandi P, *et al*. Old and New Systemic Immune-Inflammation Indexes Are Associated with Overall Survival of Glioblastoma Patients Treated with Radio-Chemotherapy. Genes. 2022; 13: 1054.

[45] Piccirillo SGM, Alonso MM, Pasqualetti F. Basic and Translational Advances in Glioblastoma. BioMed Research Interna-

tional. 2018; 2018: 1820345.

[46] Xu F, Zhu J. Stem cells tropism for malignant gliomas. Neuroscience Bulletin. 2007; 23: 363–369.

[47] Schichor C, Birnbaum T, Etminan N, Schnell O, Grau S, Miebach S, *et al*. Vascular endothelial growth factor A contributes to glioma-induced migration of human marrow stromal cells (hMSC). Experimental Neurology. 2006; 199: 301–310.

[48] Schmidt NO, Przylecki W, Yang W, Ziu M, Teng Y, Kim SU, *et al*. Brain tumor tropism of transplanted human neural stem cells is induced by vascular endothelial growth factor. Neoplasia. 2005; 7: 623–629.

[49] Sandhaanam DS, Pathalam G, Dorairaj S, Savariar V. Mesenchymal Stem Cells (MSC): Identification, Proliferation and Differentiation – A Review Article. PeerJ. 2013; 1: e148v141.

[50] Murakami M, Hayashi Y, Iohara K, Osako Y, Hirose Y, Nakashima M. Trophic Effects and Regenerative Potential of Mobilized Mesenchymal Stem Cells From Bone Marrow and Adipose Tissue as Alternative Cell Sources for Pulp/Dentin Regeneration. Cell Transplantation. 2015; 24: 1753–1765.

[51] Abe S, Hamada K, Miura M, Yamaguchi S. Neural crest stem cell property of apical pulp cells derived from human developing tooth. Cell Biology International. 2012; 36: 927–936.

[52] Leonard CE, Taneyhill LA. The road best traveled: Neural crest migration upon the extracellular matrix. Seminars in Cell & Developmental Biology. 2020; 100: 177–185.

[53] Wang H, Zhong Q, Yang T, Qi Y, Fu M, Yang X, *et al*. Comparative characterization of SHED and DPSCs during extended cultivation in vitro. Molecular Medicine Reports. 2018; 17: 6551–6559.

[54] Mai BHA, Drancourt M, Aboudharam G. Ancient dental pulp: Masterpiece tissue for paleomicrobiology. Molecular Genetics & Genomic Medicine. 2020; 8: e1202.