

Human Neural Stem Cells Target Experimental Intracranial Medulloblastoma and Deliver a Therapeutic Gene Leading to Tumor Regression

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Abstract Purpose: Medulloblastoma, a malignant pediatric brain tumor, is incurable in about one third of patients despite multimodal treatments. In addition, current therapies can lead to long-term disabilities. Based on studies of the extensive tropism of neural stem cells (NSC) toward malignant gliomas and the secretion of growth factors common to glioma and medulloblastoma, we hypothesized that NSCs could target medulloblastoma and be used as a cellular therapeutic delivery system.

Experimental Design: The migratory ability of HB1.F3 cells (an immortalized, clonal human NSC line) to medulloblastoma was studied both *in vitro* and *in vivo*. As proof-of-concept, we used HB1.F3 cells engineered to secrete the prodrug activating enzyme cytosine deaminase. We investigated the potential of human NSCs to deliver a therapeutic gene and reduce tumor growth.

Results: The migratory capacity of HB1.F3 cells was confirmed by an *in vitro* migration assay, and corroborated *in vivo* by injecting chloromethylbenzamido-Dil – labeled HB1.F3 cells into the hemisphere contralateral to established medulloblastoma in nude mice. *In vitro* studies showed the therapeutic efficacy of HB1.F3-CD on Daoy cells in coculture experiments. *In vitro* therapeutic studies were conducted in which animals bearing intracranial medulloblastoma were injected ipsilaterally with HB1.F3-CD cells followed by systemic 5-fluorocytosine treatment. Histologic analyses showed that human NSCs migrate to the tumor bed and its boundary, resulting in a 76% reduction of tumor volume in the treatment group ($P < 0.01$).

Conclusion: These studies show for the first time the potential of human NSCs as an effective delivery system to target and disseminate therapeutic agents to medulloblastoma.

Medulloblastoma is the most common childhood malignant brain tumor. Although multimodal treatments, including radical surgical resection followed by radiation and chemotherapy, have substantially improved the survival rate for this disease, it remains incurable in about one third of patients.

These treatments are also toxic and can lead to long-term disabilities (1, 2). The main cause of death is recurrence associated with tumor dissemination, at which point current therapeutic options have little efficacy (3, 4). Consequently, there is substantial need for novel, effective, low-toxicity therapies for children with medulloblastoma.

The discovery of the inherent tumor-tropic properties of neural stem cells (NSC) could serve as a novel adjuvant strategy to current medulloblastoma treatments. Recent studies have shown that NSCs have the capacity to target therapeutic genes to brain tumors, such as malignant glioma (5–13) and melanoma brain metastasis (14). We have expanded these investigations to determine whether NSCs are capable of targeting medulloblastoma in an orthotopic xenograft animal model. Therapeutic proof-of-concept studies were done using cytosine deaminase (CD)–producing NSCs and systemic 5-fluorocytosine (5-FC) prodrug administration. Our results show for the first time the potential of NSCs as an effective delivery system to target and disseminate therapeutic agents to medulloblastoma. This provides a rationale for further evaluation of the NSC-based cellular delivery system for human medulloblastoma.

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Materials and Methods

Cell culture. HB1.F3 is an immortalized human NSC (hNSC) line derived from the human fetal brain (ventricular zone) at 15 weeks of

gestation by an amphotropic, replication-incompetent retroviral vector containing ψ -myc (15–18). This is a well-established and well-characterized NSC line that is multipotent, migratory, and non-tumorigenic *in vivo* (12, 15–22). HB1.F3 cells were maintained as adherent cultures in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100 units/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B (Invitrogen, Grand Island, NY).

The human medulloblastoma cell line Daoy was obtained from S.L. Pomeroy (Children's Hospital, Boston, MA). The human glioblastoma cell lines U87MG and U251MG and the fibroblast line NIH 3T3 were obtained from the American Type Culture Collection (Manassas, VA). Daoy, U251MG, and NIH 3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 2 mmol/L sodium pyruvate, and 100 units/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B. U87MG cells were cultured in MEM α (Invitrogen) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 2 mmol/L nonessential amino acids, 2 mmol/L sodium pyruvate, and 100 units/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B. All cells were maintained in humidified atmosphere containing 5% CO₂ at 37°C.

In vitro migration assay. The directed migration ability of HB1.F3 to Daoy cells was determined using a modified transwell migration assay. Daoy cells were incubated in serum-free medium for 24 hours, and the resulting conditioned medium was collected and placed in the lower well of fibronectin-coated (Chemicon, Temecula, CA) 10-mm tissue culture Transwell plates (8 μ m; Nunc International, Rochester, NY). HB1.F3 cells in serum-free medium (50,000/0.5 mL) were seeded in the upper well of the transwell plates. NIH 3T3 cells were plated as a negative control. Following incubation for 6 hours at 37°C, non-migrated cells were scraped off the upper side of the filter and the filters were then stained with Three-Step Stain Set (Richard-Allan Scientific, Kalamazoo, MI). All experiments were conducted in quadruplicate. Nuclei of migrated cells were counted in five high-power fields ($\times 200$) and the values expressed as the mean \pm SE.

In vivo migration assay. To assess the migratory ability of HB1.F3 cells *in vivo*, the cells were injected intracranially, contralateral to the experimental tumor. Briefly, Swiss nude male mice ($n = 8$; 6–8 weeks old; Charles River, Wilmington, MA) were anesthetized (100 mg/kg ketamine and 5 mg/kg xylazine) and stereotactically implanted with 120,000 Daoy cells in 3 μ L of PBS via a 30-gauge Hamilton syringe into the left forebrain (2.5 mm lateral and 1 mm anterior to bregma, at a 2.5 mm depth from the skull surface). Four weeks after tumor cell implantation, HB1.F3 cells (240,000 in 6 μ L PBS) were injected into the right forebrain (2.5 mm lateral and 1 mm anterior to bregma, at a 2.5 mm depth from the skull surface; $n = 4$). As a control, a group of medulloblastoma-bearing animals ($n = 4$) were injected with NIH 3T3 cells in the right forebrain using the same stereotactic coordinates. For this study, the ratio of cells tested for their migratory ability (HB1.F3 or NIH 3T3) to tumor cells was 2:1. HB1.F3 or NIH 3T3 cells were labeled with the chloromethylbenzamido-Dil (CM-Dil, Molecular Probes, Eugene, OR) before injection for 30 minutes according to the protocol of the manufacturer. CM-Dil is nondiffusible by virtue of its covalent binding to cellular thiols and has been shown to be particularly suitable for labeling and *in vivo* tracking of cells for a minimum of 10 weeks (23, 24). Fourteen days after intracranial, contralateral injection of HB1.F3 or NIH 3T3 cells, animals were perfused with 4% paraformaldehyde under deep anesthesia. The brains were harvested, placed in sucrose gradient solution, embedded in optimum cutting temperature compound (Sakura Finetek USA, Torrance, CA), and stored at -80°C . Brains were cryosectioned coronally (10 μ m), mounted on slides, and stained with 4',6-diamidino-2-phenylindole per standard protocol. Intracranial distribution of CM-Dil-labeled NSCs was assessed with fluorescent microscopy. All animal studies were carried out in the animal facility at Brigham and Women's Hospital in accordance with federal, local, and institutional guidelines.

Confirmation of chemoattractant ligands and receptors. Recent studies suggest that the brain tumor-targeting behavior of NSCs is mediated by chemoattractant molecules and their respective receptors including stem cell factor (SCF)/c-Kit (25), stromal cell-derived factor 1 (SDF-1)/CXCR4 chemokine receptor 4 (CXCR4; ref. 26) and vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR)-1 and VEGFR2 (22). Therefore, the expression of these chemoattractant ligands and their associated receptors was examined in Daoy and HB1.F3 cells by reverse transcription-PCR.

Total cellular RNA was prepared using the RNeasy Mini Kit protocol (Qiagen, Valencia, CA). Single-stranded cDNA was prepared from 1 μ g of total RNA using oligo-p(dT)₁₅ primer following the First Strand cDNA Synthesis Kit for reverse transcription-PCR (avian myeloblastosis virus) protocol (Roche, Indianapolis, IN). cDNA from the reverse transcription reaction was used in the subsequent PCR reactions in the presence of 0.2 μ mol/L each of 5' and 3' primers, 2.5 units of Taq polymerase, 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleotide mix, and 1 \times PCR buffer. Touchdown PCR for these chemoattractant factors (ligands and receptors) and β -actin, a positive control, was carried out for 25 cycles. The reaction products were analyzed on a 2.5% agarose gel stained with ethidium bromide. The sense and antisense primers and the predicted sizes of the reverse transcription-PCR reaction products were as follows: SCF, 5'-ACTTGGATTCTCACTTGCATTT-3' (sense) and 5'-CTTTCTCAGGACITAAATGTTGAAG-3' (antisense; 505 bp); c-Kit, 5'-GCCACAAATAGATTGGTATTT-3' (sense) and 5'-AGCAT-CTTTACAGCGACAGTC-3' (antisense; 570 bp); SDF-1, 5'-ATGAACGC-CAAGTCCGTGGTC-3' (sense) and 5'-GGCTGTTGTGCTTACTTGTTT-3' (antisense; 281 bp); CXCR4, 5'-CTCTCCAAAGGAAAGCGAGGTGGACAT-3' (sense) and 5'-AGACTGTACTGTAGGTGCTGAAATCA-3' (antisense; 558 bp); VEGF, 5'-AAGCCATCTGTGTGCCCTGATG-3' (sense) and 5'-GCTCCTTCCTCTGCCCGGCTCAC-3' (antisense; 377 bp); VEGFR1, 5'-GCAAGGTGTGACTTTTGTTC-3' (sense) and 5'-AGGATTTCTTCCCCTGTGTA-3' (antisense; 512 bp); VEGFR2, 5'-ACGTGACATGTACCGTCTAT-3' (sense) and 5'-GCCAAGCTGTACCATGTGAG-3' (antisense; 438 bp); and β -actin, 5'-GCCAGAGCAAGAGAGGCAT-3' (sense) and 5'-GGCCATCTCTTGCTCGAAGT-3' (antisense; 513 bp).

Engineering of HB1.F3-CD. The clonal HB1.F3-CD line was derived from parental HB1.F3 cells. An expression plasmid was constructed with the retroviral pBabePuro backbone to include the *E. coli* CD cDNA (1.5-kb fragment) transcribed from the long terminal repeat. Vectors were packaged by cotransduction of the CD puro plasmid with the MV12 envelope coding plasmid cDNA into pA317 cells. CD puro retroviral supernatant was used for multiple infections of the HB1.F3 cells. Transduced HB1.F3 cells (HB1.F3-CD) were selected with 3 μ g/mL puromycin for 4 weeks.

Successful transduction of HB1.F3-CD cells was confirmed by reverse transcription-PCR. The CD transcript (559 bp) was amplified by touchdown PCR with the following primers: sense, 5'-GCGCGAGT-CACCGCCAGCCACACCACGGC-3'; antisense, 5'-GTTTGTAATCGAT-GGCTTCTGGCTGC-3'. β -Actin controls confirmed equal RNA loading.

To confirm the bioactivity of CD produced from HB1.F3-CD, the cytotoxic effect of 5-FC and 5-fluorouracil (5-FU) on HB1.F3-CD was analyzed by cell viability assay. Sensitivity to 5-FC or 5-FU was also assessed for HB1.F3, Daoy, U87MG, and U251MG cells. For all studies, cells (4×10^3 per well) were plated in 96-well plates (Corning, Inc., Acton, MA). After 24 hours, cells were treated with 5-FC (Sigma-Aldrich, St. Louis, MO) or 5-FU (Sigma-Aldrich) with final concentrations in the range of 0 to 100 μ g/mL. Cells were incubated at 37°C for 4 days and quantification of cell viability was done with a colorimetric assay using Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD). All experiments were conducted in quadruplicate. Viability determination was based on the bioconversion of the tetrazolium compound, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, into formazan, as determined by absorbance at 450 nm using a multiwell scanning spectrophotometer. Cell viability was expressed as the mean \pm SE in percentage of the control viability (=100%).

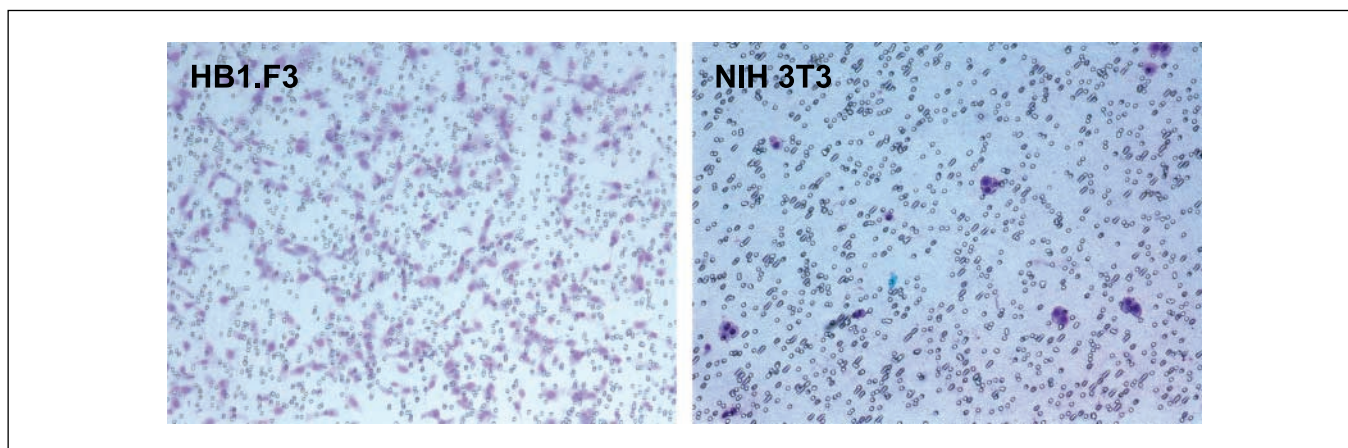


Fig. 1. *In vitro* migration assay. The migratory ability of HB1.F3 or NIH 3T3 cells to Daoy cells was determined using a modified transwell migration assay. Daoy conditioned medium significantly stimulates the directional migration of HB1.F3 cells compared with NIH 3T3 fibroblast cells (36.8 ± 7.93 cells per high-power field for HB1.F3 cells versus 4.2 ± 1.30 cells per high-power field for NIH 3T3 cells; $P < 0.05$, Mann-Whitney U test). Magnification, $\times 100$.

In addition, the migratory capacity of HB1.F3-CD cells was assessed *in vitro* and *in vivo* as described above.

In vitro therapeutic efficacy of HB1.F3-CD. Therapeutic efficacy of HB1.F3-CD cells after 5-FC treatment was analyzed by coculture experiments. Daoy cells (4×10^3 per well) were plated in 96-well plates and experiments were done as follows: on day 1, increasing amounts of HB1.F3 or HB1.F3-CD cells (0 , 2×10^3 , 4×10^3 , 8×10^3 , and 16×10^3 per well) were added to the tumor cell cultures; day 2, 5-FC ($100 \mu\text{g/mL}$) was added to the mixed cell cultures; day 6, quantification of cell viability was done as described above. All experiments were conducted in quadruplicate. Cell viability was expressed as the mean \pm SE in percentage of the control viability (=100%).

In vivo therapeutic efficacy of HB1.F3-CD. Animals received stereotactic implantation of Daoy cells ($120,000$ in $3 \mu\text{L}$ PBS) into the left forebrain as described above. Two weeks after tumor cell implantation, the animals were randomized into five groups: group 1, treated with ipsilateral intratumoral injection of PBS ($8 \mu\text{L}$; $n = 6$); groups 2 and 3, treated with ipsilateral intratumoral injection of $480,000$ HB1.F3 cells in $8 \mu\text{L}$ of PBS ($n = 6$ for each group); groups 4 and 5, treated with ipsilateral intratumoral injection of $480,000$ HB1.F3-CD cells in PBS ($n = 6$ for each group). In this study, the ratio of stem cells/tumor cells was 4:1. HB1.F3 and HB1.F3-CD were labeled with the CM-Dil cell tracker before injection. One week after hNSC injection, groups 3 and 5 received i.p. injections of 5-FC (500 mg/kg) twice a day for 14 days. Three weeks after the last 5-FC

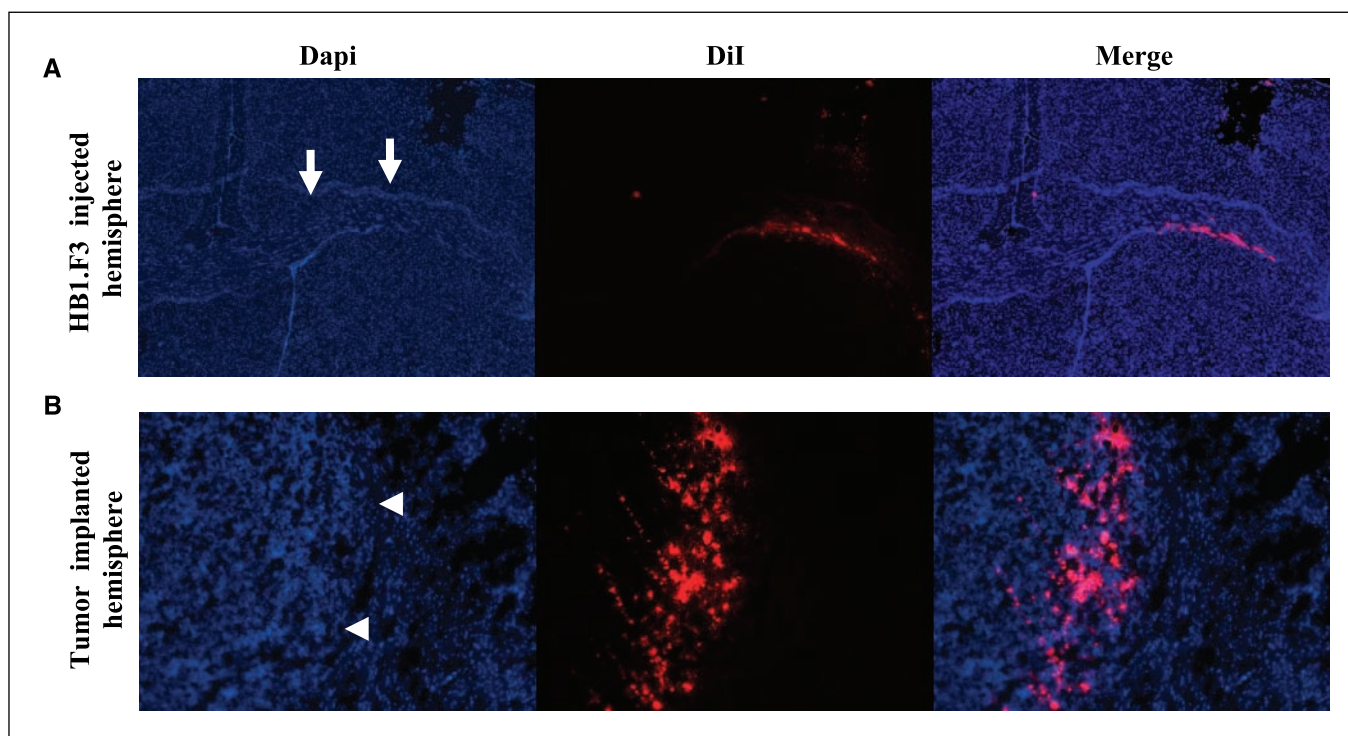


Fig. 2. *In vivo* migration assay. To assess migratory ability of HB1.F3 cells *in vivo*, CM-Dil-labeled HB1.F3 cells were injected into the hemisphere contralateral to the tumor cell implantation (Daoy cells). CM-Dil-labeled HB1.F3 cells (red) are seen migrating across the corpus callosum (arrow; 4',6'-diamidino-2-phenylindole staining; $\times 40$ magnification) in the HB1.F3 injected hemisphere (A) and populating the tumor bed (arrowhead) in the tumor implanted hemisphere (B; $\times 100$ magnification).

treatment, animals were perfused and the brains harvested and processed as described above. Tissue was stained with 4',6-diamidino-2-phenylindole or H&E per standard protocol. Intracranial distribution of CM-Dil-labeled hNSCs was assessed by fluorescent microscopy. Tumor volumes were estimated using the formula for ellipsoid and expressed as mean \pm SE as previously described (27).

Immunohistochemistry was carried out with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Brain sections were fixed in cold acetone. Primary antibody included anti-Ki67 nuclear antigen (1:100; DAKO, Carpinteria, CA) for proliferating cells. Sections were counterstained with hematoxylin, and negative control slides were obtained by omitting the primary antibody. The proliferative index was defined as the percentage of positively stained cells of 100 nuclei from five randomly chosen high-power fields.

Statistics. All of the values were calculated as mean \pm SE or were expressed as percentage of control \pm SE. Significant differences between assessment of *in vitro* migration, cell viability, and tumor volume were determined using the Mann-Whitney *U* test. $P < 0.05$ was considered significant.

Results

Migratory capacity of hNSCs *in vitro*. To determine the migratory capacity of hNSCs to medulloblastoma cells, an *in vitro* transwell migration assay was done. Daoy conditioned medium significantly stimulated the directional migration of HB1.F3 cells (36.8 ± 7.93 cells per high-power field) compared with that of NIH 3T3 cells (4.2 ± 1.30 cells per high-power field; $P < 0.05$; Fig. 1).

Migratory capacity of hNSCs *in vivo*. To confirm the migratory ability of hNSCs to medulloblastoma *in vivo*, Daoy cells were implanted into the left hemisphere. Four weeks later,

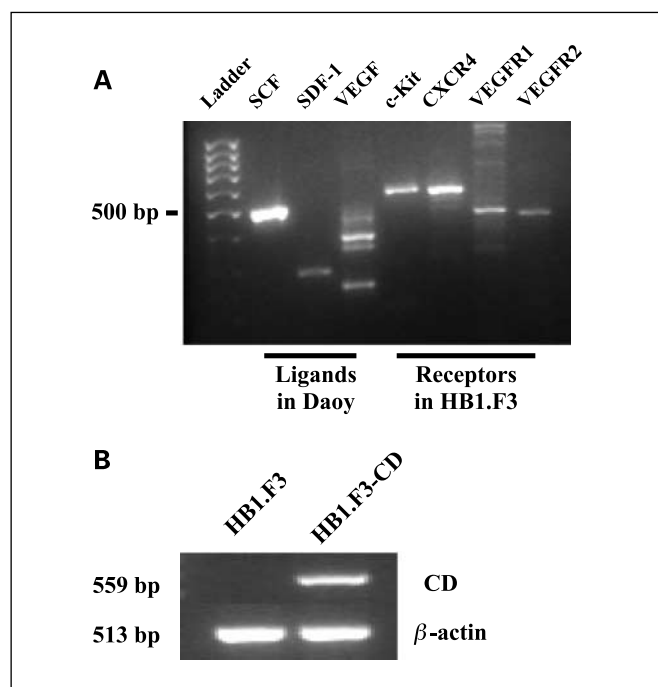


Fig. 3. The analysis of expression of chemoattractant factors (ligands and receptors) and CD using reverse transcription-PCR. *A*, the presence of the ligands SCF, SDF-1, and VEGF in Daoy cells, and of their corresponding receptors c-Kit, CXCR4, VEGFR1, and VEGFR2 in HB1.F3 cells, was confirmed and visualized on 2.5% agarose gel. *B*, the HB1.F3-CD line was derived from HB1.F3 cells by retroviral transduction with *E. coli* CD. The CD transcript was expressed only in HB1.F3-CD cells, but not in HB1.F3 cells.

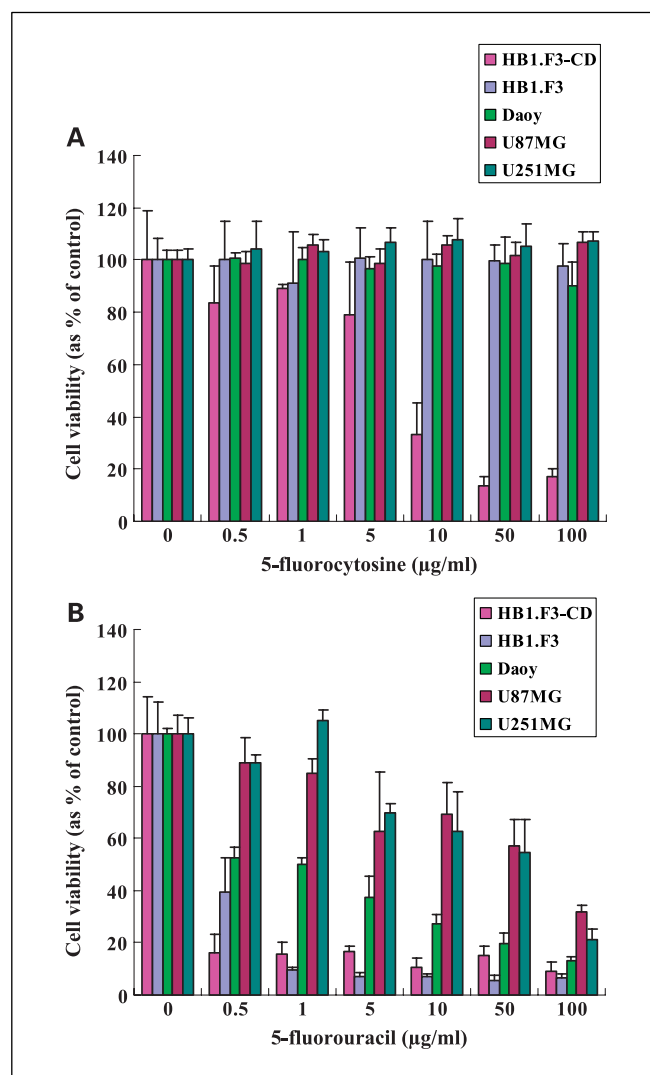


Fig. 4. The bioactivity of 5-FC and 5-FU was determined by cell viability assay. HB1.F3-CD, HB1.F3, Daoy, U87MG, and U251MG cells (4×10^3 per well each) were plated in 96-well cell plates and sensitivity to 5-FC/5-FU was measured by colorimetric assays with Cell Counting Kit-8. Columns, mean cell viability (percentage of the control viability); bars, SE. *A*, only HB1.F3-CD cells are sensitive to 5-FC treatment at a concentration of 10 $\mu\text{g}/\text{mL}$ 5-FC ($P < 0.05$, Mann-Whitney *U* test), confirming the enzymatic conversion of prodrug 5-FC to the toxic 5-FU. The growth of other cells is not affected by 5-FC up to a concentration of 100 $\mu\text{g}/\text{mL}$, indicating the lack of expression of cytosine deaminase in these cells. *B*, after 5-FU treatment, sensitivity is the highest in human neural stem cells, followed by medulloblastoma cells and glioblastoma cells in decreasing order.

CM-Dil-labeled HB1.F3 cells were injected into the contralateral hemisphere. CM-Dil-labeled HB1.F3 cells were seen migrating across the corpus callosum (Fig. 2A) and populating medulloblastoma tumor in the opposite hemisphere (Fig. 2B). It should be noted that at the time of HB1.F3 injection (4 weeks after Daoy implantation), the established Daoy tumors were small, but the NSCs were still able to migrate to the tumor site. Very few hNSCs were seen beyond the tumor edge, showing the remarkable migratory capacity of NSCs to the infiltrating tumor cell in the peritumoral normal brain. In contrast, the NIH 3T3 fibroblast cells did not migrate and remained localized at the area of the injection site.

Confirmation of chemoattractant ligands and receptors. The brain tumor-targeting behavior of hNSCs has been reported to

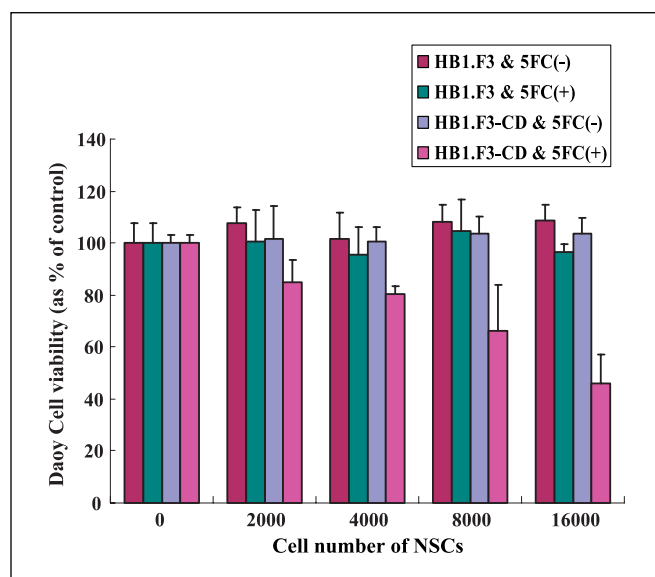


Fig. 5. *In vitro* therapeutic efficacy of HB1.F3-CD. Therapeutic efficacy of HB1.F3-CD cells after 5-FC treatment was analyzed by coculture experiments. Daoy cells (4×10^3 per well) were plated in 96-well plates. After 24 hours, increasing amounts of HB1.F3 or HB1.F3-CD cells ($0, 2 \times 10^3, 4 \times 10^3, 8 \times 10^3$, and 16×10^3 per well) were added to the tumor cell cultures. After 5-FC ($100 \mu\text{g}/\text{mL}$) treatment, cell viability was measured by colorimetric assays with Cell Counting Kit-8. Columns, mean percentage of the control viability; bars, SE. Daoy cells cultured in the presence of HB1.F3-CD cells, but not HB1.F3 cells, show significant growth inhibition after 5-FC treatment, even when the ratio of HB1.F3-CD cells/tumor cells is as low as 1:2 ($P < 0.05$, Mann-Whitney U test). Without 5-FC treatment, Daoy cells cultured with HB1.F3-CD cells show no growth inhibition.

be mediated by a multitude of factors, including the SCF/c-Kit (25), SDF-1/CXCR4 (26), and the VEGF/VEGFR1 and VEGFR2 (22) signaling pathways. Using reverse transcription-PCR, the presence of the ligands SCF, SDF-1, and VEGF in Daoy cells, and of their associated receptors c-Kit, CXCR4, VEGFR1, and VEGFR2 in HB1.F3 cells (Fig. 3A), was confirmed. A β -actin control confirmed RNA quality.

CD-producing HB1.F3 cells. Expression of the CD transcript in HB1.F3-CD was confirmed by reverse transcription-PCR. The CD transcript was found to be only expressed in HB1.F3-CD cells, but not in the parental HB1.F3 cells (Fig. 3B).

Sensitivity of HB1.F3-CD to 5-FC or 5-FU was assessed and compared with that of HB1.F3, Daoy, U87MG, and U251MG cells. Only HB1.F3-CD cells were sensitive to 5-FC treatment at a concentration of $10 \mu\text{g}/\text{mL}$ ($P < 0.05$, Fig. 4A), confirming the enzymatic conversion of prodrug 5-FC to the toxic 5-FU. The growth of the other cell lines was not affected by 5-FC even at a concentration of $100 \mu\text{g}/\text{mL}$, indicating the lack of expression of CD in these cells. In contrast, all cell lines were sensitive to 5-FU. The highest sensitivity was observed in hNSCs with or without CD transduction, followed by medulloblastoma cells, and lastly in glioblastoma cells in decreasing order (Fig. 4B). A notable and highly significant finding is that medulloblastoma cells are more sensitive to 5-FU than glioblastoma cells such as U87MG and U251MG. For example, Daoy cells ($52.7 \pm 4.01\%$) showed a significant decrease in cell viability compared with U87MG ($88.8 \pm 9.54\%$) or U251MG ($89.2 \pm 2.56\%$) cells at the $0.5 \mu\text{g}/\text{mL}$ 5-FU treatment ($P < 0.05$).

The potential for migration of NSCs was not affected by transduction with CD. The migratory pattern of HB1.F3-CD

in vitro and *in vivo* was indistinguishable from that of HB1.F3 cells (Fig. 6A).

***In vitro* therapeutic efficacy of HB1.F3-CD.** To confirm the "bystander effect" of the CD released from the HB1.F3-CD cells, cell viability studies were done in a coculture system. After 5-FC treatment, Daoy cells cultured in the presence of HB1.F3-CD cells, but not the parental HB1.F3 cells, showed significant growth inhibition even when the ratio of HB1.F3-CD cells/tumor cells was as low as 1:2 ($P < 0.05$; Fig. 5). Without 5-FC treatment, Daoy cells cultured with HB1.F3-CD cells showed no growth inhibition. This indicates that HB1.F3-CD cells can convert sufficient amounts of 5-FC to 5-FU to effectively kill Daoy cells *in vitro*.

***In vivo* therapeutic efficacy of HB1.F3-CD.** To assess the therapeutic efficacy of HB1.F3-CD in medulloblastoma animal models, we injected CM-Dil-labeled HB1.F3-CD cells into tumor-bearing animals. The distribution of HB1.F3-CD cells and tumor volumes were determined on harvested brain tissue 3 weeks after the last 5-FC treatment. *In vivo* studies showed that the majority of intracranially injected CM-Dil-labeled HB1.F3-CD cells localized to the tumor bed and the tumor-normal parenchyma interface (Fig. 6B).

Histologic analysis showed a 76% reduction of tumor volume in the brains of 5-FC-treated HB1.F3-CD animals ($2.6 \pm 1.49 \text{ mm}^3$) compared with PBS-treated control animals ($10.7 \pm 6.80 \text{ mm}^3$; $P < 0.01$; Fig. 6C and D) and all other control groups. We could not detect any abnormalities in the parenchyma surrounding the tumor in treated animals. The proliferative index revealed no significant differences among the groups.

Discussion

The focus of this study is to explore the migratory capacity and therapeutic potential of hNSCs in experimental medulloblastoma. We have previously reported the inherent migratory, tumor-tropic, and therapeutic properties of NSCs (C17.2 and HB1.F3) to intracranial glioblastomas (5, 12). In this study, we have chosen to use human HB1.F3 NSCs as it is a well-established and well-characterized human stem cell line (12, 15–22). No signs of local or systemic toxicity in HB1.F3 and HB1.F3-CD treated groups were observed although the therapeutic effects were assessed 6 weeks after hNSC injection.

The *in vitro* and *in vivo* studies clearly show for the first time the ability of NSCs to target medulloblastoma, indicating that the tumor tropism of NSCs is not limited to gliomas (14). Furthermore, migration of HB1.F3 was not affected by the CD transduction. To better define the crosstalk between medulloblastoma and hNSCs, we investigated the signals that have been shown to modulate the brain tumor tropism of NSCs, such as SCF/c-Kit (25), SDF-1/CXCR4 (26), and VEGF/VEGFR1 and VEGFR2 (22). Our study documented the presence of chemoattractant factors (ligands in medulloblastoma and receptors in hNSCs), which may allow them to communicate with each other and facilitate the migration we observed.

After confirmation of the tropism of hNSCs for medulloblastoma, we tested their therapeutic potential using the CD enzyme/5-FC prodrug system. CD is a bacterial enzyme that converts the nontoxic prodrug 5-FC to the cytotoxic drug 5-FU, a nucleotide analogue that disrupts DNA synthesis in

proliferating cells (28). Because mammalian cells do not express significant amounts of CD, as shown in the present study, 5-FC is nontoxic at concentrations that result in strong antimicrobial activity. Tumor cells transfected with the *E. coli* CD gene become selectively sensitive to the toxic effects of 5-FC; e.g., expression of this enzyme in glioma cells confers their lethal sensitivity to systemically administration of 5-FC (29). In addition, this enzyme/prodrug system has a bystander effect (30, 31), shown by the death of unmodified tumor cells adjacent to genetically modified cells. NSCs engineered to express a suicide gene such as CD are a particularly good choice because they can generate agents that kill tumor cells as well as result in self-elimination if the NSCs start dividing *in vivo*. Although HB1.F3 cells have proved to be a non-tumor-producing line *in vivo* model (12, 15–22), there is possibility of integration of NSCs into the stroma of brain tumors. Recently, more studies of neurogenesis have focused on modulation of endogenous precursors, rather than *in vitro* propagation of cell lines (32).

Our group and others have shown that NSCs transduced with the CD gene retain their tumor tracking properties and are therapeutically effective for malignant gliomas in animal models (5, 6). To our knowledge, these data are the first to show therapeutic efficacy of this hNSC-CD/5-FC system in a medulloblastoma model; the systemic administration of 5-FC in combination with CD-expressing hNSCs resulted in a 76% reduction in intracranial medulloblastoma tumor volume in nude mice.

Although there was a significant tumor volume reduction induced by this therapy, the proliferative index of the remaining tumor was still high. As the therapeutic benefit from one-time drug delivery may be short-lived as the tumor

regrows, additional cycles of HB1.F3-CD and 5-FC therapy can be given to eradicate the cancer cells not removed by the first wave of therapy. Alternatively, NSCs can be engineered with other potentially beneficial therapeutic genes (differentiating agents, immune-enhancing agents, apoptosis-promoting agents, antiangiogenic agents, etc.), which can be given in combination with HB1.F3-CDs or sequentially.

Although systemic 5-FU is not routinely used in the clinical management of brain tumors, as it does not cross the blood-brain barrier, we clearly show that medulloblastoma is sensitive to 5-FU, even more so than glioblastoma. The selective activation of prodrug in tumor tissues by exogenous enzyme for cancer therapy has been accomplished by several ways, including gene-directed enzyme prodrug therapy, virus-directed enzyme prodrug therapy, and antibody-directed enzyme prodrug therapy (33). Our cell-directed enzyme prodrug therapy with NSCs is potentially more flexible than previous methods because of the tumor-tropic capacity of NSCs. Because of an impressive bystander effect, as little as 2% of the tumor mass containing CD-expressing cells may generate significant oncolysis (31). This unique property increases the likelihood of achieving therapeutic efficacy. When cell-directed enzyme prodrug therapy is applied clinically, the interval between enzyme and prodrug administrations should be optimized so that CD/5-FC system could work after NSCs localize to tumor site to avoid systemic toxicity.

In the clinical setting, NSC therapy could be used in conjunction with standard therapies. Especially in the case of recurrence with tumor dissemination in the subarachnoid space, NSCs can be grafted into tumor resection cavity to target residual invading and disseminating tumor cells. Furthermore,

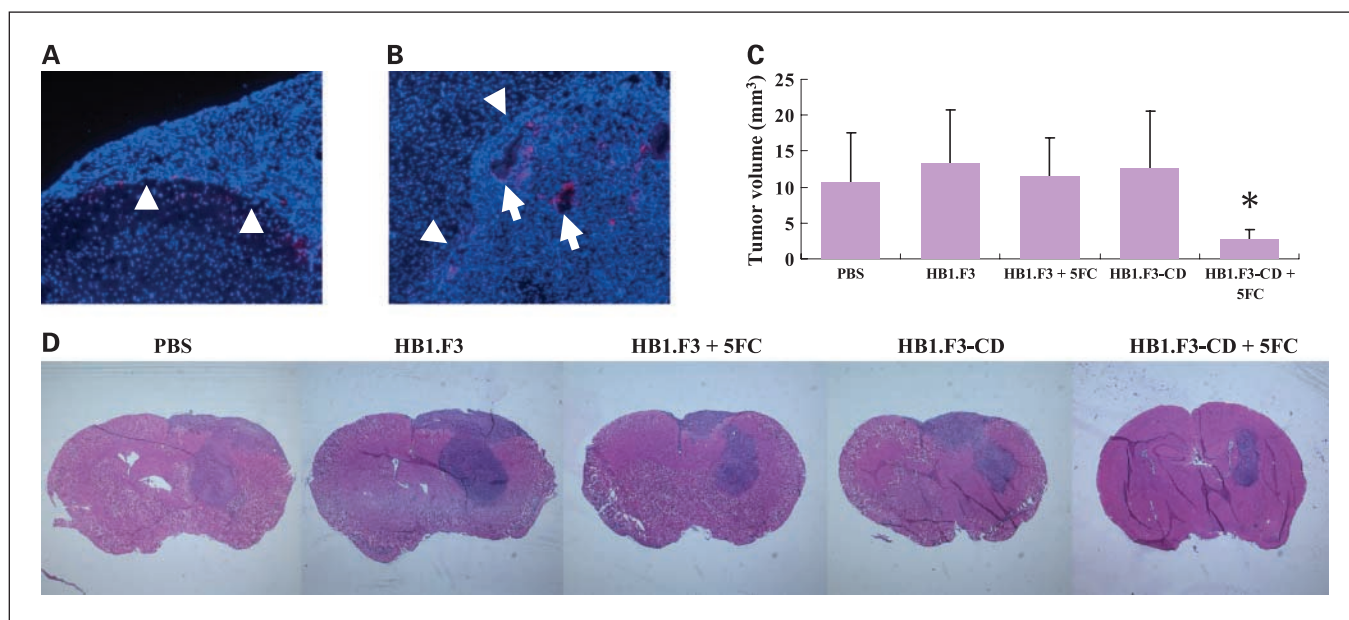


Fig. 6. Intracranial distribution and *in vivo* therapeutic efficacy of HB1.F3-CD. *A*, HB1.F3-CD were labeled with CM-Dil and injected into the hemisphere contralateral to the tumor cell implantation. HB1.F3-CD cells (red) are observed migrating towards intracranial medulloblastoma (arrowhead) through normal brain parenchyma in a similar pattern as parental cells (4',6-diamidino-2-phenylindole staining; merged image; $\times 100$ magnification). *B*, the majority of CM-Dil-labeled HB1.F3-CD cells, injected intracranially, are located at the border between tumor (arrowhead) and normal parenchyma (4',6-diamidino-2-phenylindole staining; merged image; $\times 100$ magnification). Cavity formation (arrow) is more dramatic in areas where the labeled HB1.F3-CD cells are found, suggesting a bystander effect. *C*, tumor volumes were estimated from histologic analysis. Columns, mean; bars, SE. After systemic treatment with 5-FC, 76% reduction of tumor volume is shown in HB1.F3-CD-treated animals ($2.6 \pm 1.49 \text{ mm}^3$) compared with PBS-treated animals ($10.7 \pm 6.80 \text{ mm}^3$; $* P < 0.01$, Mann-Whitney *U* test). *D*, representative histologic photographs (H&E staining; $\times 1$ magnification).

based on our study, the same approach can be exploited using other sources of stem cells and engineered to deliver other antitumor agents for medulloblastoma therapies.

In conclusion, this study indicates that hNSCs have migratory capacity and tropism for medulloblastoma. Fur-

thermore, they can be genetically modified *ex vivo* to express genes that have therapeutic efficacy against human medulloblastoma. These results suggest a potential role of hNSCs as a targeted, therapeutic delivery vehicle for human medulloblastoma.

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