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Neural Stem Cell-Mediated Enzyme-Prodrug Therapy for Glioma: Preclinical Studies

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Abstract

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Author contributions: K.S.A, J.N., M.Z.M., and J.P. planned and designed the experiments. K.S.A, J.N., M.Z.M., M.D.A, C.E.B., T.W.S., E.G., S.A., V.V.V., and R.T.F. contributed to data acquisition. L.A.C. supervised the GMP procedures and tests. S.B. assisted with experimental design and carried out statistical analysis of data. S.U.K. generated the HB1.F3 human NSC line and assisted with characterization studies. K.S.A, J.N., M.Z.M., M.G., and J.P. drafted the manuscript and figures. K.S.A. oversaw all aspects of the studies and analysis. All authors contributed to data analysis and critical review of the manuscript.

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High-grade gliomas are extremely difficult to treat because they are invasive and therefore are not curable by surgical resection; the toxicity of currently chemo- and radiation therapies limits the doses that can be used. Neural stem cells (NSCs) have inherent tumor-tropic properties that enable their use as delivery vehicles that can target enzyme/prodrug therapy selectively to tumors. We have used a cytosine deaminase (CD)-expressing clonal human NSC line, HB1.F3.CD, to home to gliomas in mice and locally convert the tumor-localized prodrug 5-fluorocytosine to the active chemotherapeutic 5-fluorouracil. In vitro studies confirmed that the NSCs have normal karyotype, tumor tropism, and CD expression, indicating that these cells are genetically and functionally stable. In vivo biodistribution studies demonstrated that these NSCs retained tumor tropism, even in mice pre-treated with radiation or dexamethasone to mimic clinically relevant adjuvant therapies. We evaluated safety and toxicity after intracerebral administration of the NSCs in non-tumor bearing, and in orthotopic glioma-bearing, immunocompetent and immunodeficient mice. We detected no difference in toxicity associated with conversion of 5-fluorocytosine to 5-fluorouracil, no NSCs outside the brain, and no histological evidence of pathology or tumorigenesis attributable to the NSCs. The average tumor volume in mice that received HB1.F3.CD NSCs and 5-fluorocytosine was approximately one-third that of the average volume in control mice. On the basis of these results, we conclude that combination therapy with HB1.F3.CD NSCs and 5-fluorocytosine is safe, non-toxic and effective in mice. These data have led to approval of a first-inhuman study of an allogeneic NSC-mediated enzyme/prodrug targeted cancer therapy in patients with recurrent high-grade glioma.

INTRODUCTION

Approximately 22,500 people a year in the United States are diagnosed with malignant primary brain tumors, which are highly invasive, neurologically destructive, and considered among the deadliest human cancers (1–3). Despite aggressive multimodal therapy and advances in chemotherapy, imaging, surgical and radiation techniques, these tumors remain virtually incurable. Survival is typically measured in months for patients with recurrent glioblastoma, the most common and aggressive form of high-grade glioma in adults (4, 5). Treatment failure is primarily attributable to the diffuse and invasive nature of glioma cells, ineffective delivery of chemotherapeutic agents across the blood-brain barrier (BBB), and associated dose-limiting systemic toxicities (6–9). Therefore, new tumor-selective therapies are critically needed to improve clinical outcome.

Neural stem cells (NSCs) have inherent tumor-tropic properties that can be exploited for targeted delivery of anti-cancer agents to invasive and metastatic tumors. NSCs can overcome the major obstacles limiting the efficacy of current treatments through their abilities to cross the BBB, to target therapeutic agents to primary and invasive tumor foci throughout the brain, and to minimize toxicity to normal tissues by acting as a local platform for tumor treatment. For use as delivery vehicles, NSCs have been engineered to express a variety of anti-cancer agents, including prodrug-activating enzymes, apoptosis-inducing agents, antibodies, and oncolytic viruses (9–12). NSCs engineered to express various anti-cancer agents and injected intracerebrally exert significant therapeutic efficacy in preclinical brain tumor models of orthotopic glioma (11, 13–16), medulloblastoma (17–19), melanoma brain metastases (20), and breast cancer brain metastases (21). In addition, intravenously administered, genetically engineered NSCs target tumors and are therapeutically effective in mouse models of disseminated neuroblastoma (22, 23) and primary and metastatic breast cancer (21, 24) These observations suggest that the potential clinical applications of NSC-mediated cancer treatment may be quite extensive.

We investigated an NSC-mediated enzyme/prodrug strategy for the treatment of glioma. We hypothesized that the established cytosine deaminase (CD)-expressing clonal human NSC line HB1.F3.CD, when injected into the brains of glioma-bearing mice, would localize to

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primary and invasive tumor sites where it would locally convert the prodrug 5-fluorocytosine (5-FC) to the active chemotherapeutic 5-flurouracil (5-FU). We characterized the stability, safety and efficacy of HB1.F3.CD NSCs in combination with 5-FC for treatment of glioma. Here, we report these pre-clinical studies, which led to successful filing of an investigational new drug (IND) application with the FDA to initiate a first-in-human clinical trial of this NSC-based cancer treatment strategy.

RESULTS

In vitro characterization and stability of the HB1.F3.CD NSC line

The HB1.F3.CD NSC line used in this study was generated by transducing the well-characterized v-*myc* immortalized HB1.F3 parental line with an amphotropic replication-incompetent retroviral vector encoding *Escherichia coli* CD (25–28). Clonal lines were expanded and frozen. We established a laboratory research cell bank of HB1.F3.CD NSCs at passage 16. This cell bank was used for characterization and biodistribution studies and served as the source for the good manufacturing practice (GMP)-grade master cell bank (MCB, frozen at passage 20) that we used in our IND-enabling studies.

To ensure the genetic stability of both the HB1.F3.CD research cell bank and the MCB, we analyzed the karyotype of these cells through passage 32. All samples had a normal human female karyotype within the limits of banding resolution; no acquired clonal cytogenetic changes were detected (Fig. 1A). We also used linear amplification-mediated (LAM) PCR to identify the genomic DNA insertion sites and copy numbers for the v-*myc* and CD transgenes. The HB1.F3.CD cell line contained a single copy of the v-*myc* gene on chromosome 19 and a single copy of the CD gene on chromosome 9 (Fig. S1A, S1B). Copy numbers were confirmed by Q-PCR. No proto-oncogenes or tumor suppressor genes were found within a 2 Mbp stretch of either transgene. We performed Boyden chamber cell migration assays to assess in vitro tropism of HB1.F3.CD NSCs to conditioned media derived from the established human glioma cell lines U87 and U251. The HB1.F3.CD NSCs retained their tumor tropism at earlier and later passages relative to parental HB1.F3 NSCs (Fig. 1B). Flow cytometry identity tests revealed that >99% of the HB1.F3.CD cells stained positive for human nestin, an NSC marker (Fig. 1C). Furthermore, >89% of HB1.F3.CD cells were positive for CD expression, as assessed by flow cytometry and fluorescence immunocytochemistry (Fig. 1D).

HLA genotyping of HB1.F3.CD NSCs (research cell bank and MCB) to assess potential immunogenicity revealed HLA Class I A*0101, B*0702, C*0701 and HLA Class II DRB1*1001, DQB1*0501, DPB1*0201 genes. However, flow cytometry indicated that only the HLA Class I antigens were expressed on the HB1.F3.CD cells; no Class II antigen expression was detected.

Conversion of 5-FC to 5-FU by HB1.F3.CD NSCs

We next demonstrated in vitro conversion of 5-FC to 5-FU by the HB1.F3.CD cells. We performed high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) analysis on HB1.F3.CD NSCs that had been cultured in the presence or absence of 5-FC. Supernatant and cells were collected 24, 48 and 72 hours after addition of 5-FC, and the concentrations of 5-FC and 5-FU in conditioned media and cell lysates measured. We detected a concentration- and time-dependent increase of 5-FU in the supernatant and cell lysates when 10–50 μg/ml of 5-FC was added to the NSC culture (Table S1).

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Tumor tropism of NSCs with dexamethasone or prior radiation

Brain tumor patients typically receive corticosteroids to control cerebral edema, particularly immediately after surgery, so we determined whether HB1.F3.CD NSCs retained their tumor-tropic properties and remained non-tumorigenic in the presence of dexamethasone. Boyden chamber cell migration assays of NSCs to glioma-conditioned media in the presence or absence of dexamethasone showed that HB1.F3.CD NSCs retained tropism to U251 glioma-conditioned media in vitro (Fig. S2). In vivo studies were carried out in an orthotopic U251 human glioma nude mouse model (n = 8). U251 glioma was selected because it is extensively characterized and its invasive properties more closely resemble those observed in patients' tumors. Tumors were established by stereotactic, intracranial injection of 2×10^5 U251 cells into the frontal lobe (day 0). Dexamethasone treatment (0.2 mg/kg/day, injected intraperitoneally) started on day 7 and continued until mice were euthanized on day 14 or 17. Mice were injected intracranially with 1×10^5 HB1.F3.CD NSCs from the MCB (caudal-lateral to tumor or in the opposite hemisphere at same stereotactic coordinates) on day 10. NSCs were pre-labeled with CM-DiI to enable detection by fluorescence microscopy after tissue sectioning. Mice were perfused transcardially with 4% paraformaldehyde (PFA) in PBS on days 14 or 17 (4 or 7 days after NSC injection), at which point brains were harvested, fixed, cryosectioned and stained with DAPI to assess NSC migration and tumor distribution. NSCs migrated to tumors whether they were injected next to the tumor site (Fig. 2A–C) or into the opposite hemisphere (Fig. 2F–H). NSCs distributed throughout the main tumor bed and at invasive micro-tumor foci. We also assessed HB1.F3.CD fate in the presence of dexamethasone in non-tumor bearing adult nude mice (n = 4). Dexamethasone was administered to mice as described above from day 0 through day 10. HB1.F3.CD NSCs (2.5×10^5) were injected into the frontal lobe on day 3. No viable NSCs were detected (with the exception of a few single cells at the injection site) 30 days after NSC injection. We concluded that HB1.F3.CD NSCs remain tumor-tropic and non-tumorigenic in the presence of dexamethasone *in vivo.*

Because patients enrolled in the planned phase 1 clinical trial would have previously been treated with radiation therapy, we also determined whether HB1.F3.CD cells retained their tumor-tropism in glioma-bearing mice that had received prior radiation to the brain. On day 0, adult nude mice (n = 10) were injected in the frontal lobe with 2×10^5 U251 human glioma cells. On day 10, mice received a single dose of radiation (10 Gy focused to the brain). On day 20 (10 days after radiation), mice were injected intracranially with 1×10^5 HB1.F3.CD NSCs (caudal-lateral to tumor or into the opposite hemisphere). Mice were euthanized on day 24 or 28 (4 or 8 days after NSC administration), and their brain tissue processed for immunohistochemistry with an antibody to CD to assess NSC distribution at the glioma site. HB1.F3.CD NSCs retained their tumor-tropism to glioma and continued to express CD (Fig. 2I, J). We also assessed HB1.F3.CD NSC fate after brain radiation in non-tumor bearing adult nude mice (n = 4). HB1.F3.CD NSCs (2.5×10^5) were injected into the frontal lobe of mice 10 days after radiation. Immunohistochemical staining with an antibody to a 60 kDa non-glycosylated protein component of human mitochondria failed to detect any viable NSCs in brains of these mice 30 days after NSC injection. This antibody does not cross-react with mouse or rat tissues. We concluded that prior radiation therapy is not likely to affect HB1.F3.CD NSC tumor tropism or CD expression.

NSC biodistribution in an immunocompetent, syngeneic glioma model

To evaluate tumor-tropism and how long NSCs remain viable in the absence of immunosuppresion, we determined HB1.F3.CD NSC biodistribution in mice carrying an orthotopic syngeneic murine glioma (GL261). Adult mice (n = 10) received stereotactic injections of 1×10^5 GL261 cells into the frontal lobe (day 0). Seven days later, HB1.F3.CD cells (5×10^4; CM-DiI-labeled) were injected caudal-lateral to the tumor injection site. We

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euthanized mice on days 11 or 22 (4 or 15 days after NSC injection), and assessed NSC distribution by histological examination. We detected CM-DiI-labeled HB1.F3.CD NSCs at the tumor sites, but not in areas of normal brain tissue (Fig. 2D, E). Viable HB1.F3.CD NSCs were detected for at least for 15 days after injection near the tumor (Fig. 2E). These data are consistent with previous publications showing that NSCs are maximally distributed in tumors on day 4 after injection.

Safety of HB1.F3.CD NSCs in non-tumor-bearing mice

To assess the safety and potential tumorigenicity of HB1.F3.CD NSCs in normal brain tissue, we injected up to 10 times the clinically relevant human cell dose into the brains of non-tumor-bearing immunodeficient mice. Sixteen *nu/nu* mice received stereotactic injections of HB1.F3.CD NSCs (2.5×10^5, 5.0×10^5, or 1.0×10^6 NSCs injected bilaterally) into the frontal lobe. All mice had normal gait, appetite, alertness, hydration, neurological symptoms and weight during the week after NSC injection and through time to planned euthanasia. Four or 12 weeks after NSC injection, mice were transcardially perfused with 4% PFA, and blood and all major organs were harvested (brain, intestine, kidney with adrenal glands, pancreas, liver, ovaries, heart, lungs, skin, spleen and stomach). Brains and other organs of all mice appeared normal during gross examination, and no tumors or abnormal tissue masses were observed. Hematoxylin and eosin (H&E) histochemistry and human-specific mitochondrial antibody immunohistotochemistry of tissue sections through the brain and each major organ indicated these tissues appeared normal in all mice at 4 (Fig. 3A) and 12 weeks (Fig. 3B) after NSC injection, regardless of the NSC dose received. In addition, we observed no evidence of tumors or significant pathology. Typical focal gliosis and hemosiderin-laden macrophages were present at NSC injection sites. Brain tissues harvested at 4 or 12 weeks after NSC injection did not exhibit positive staining with antibody to human mitochondria, indicating that no viable NSCs were present, nor did we detect positive staining with this antibody in any major organs at either timepoint. Similar studies were carried out to evaluate NSC safety and tumorigenicity in non-tumor bearing immunocompetent adult C57BL/6J mice (n = 16) that did not receive immunosuppression. Results were similar to those observed in immunodeficient mice 4 or 12 weeks after injection of NSCs into the frontal lobe.

Tumor cells treated with HB1.F3.CD plus 5-FC prodrug therapy in vivo

We postulated that HB1.F3.CD NSCs would localize the prodrug-activating CD enzyme to invasive tumor foci (Fig. 4A). Systemically administered 5-FC would then cross the BBB and be converted to the active chemotherapeutic 5-FU at tumor sites by the CD-expressing NSCs. 5-FU is a pyrimidine analog that acts as an anti-neoplastic anti-metabolite by interfering with DNA synthesis. 5-FU and its toxic metabolites readily diffuse out of NSCs to preferentially kill surrounding, dividing tumor cells. This CD/5-FC enzyme/prodrug strategy has a large bystander effect in that expression of CD by only 2% of cells can cause significant tumor regression in mice (29). Because 5-FC alone is already approved for use in humans for CNS fungal infections, we did not perform toxicological investigations of 5-FC in mice.

Prior to in vivo experiments, we evaluated the cytoxicity of HB1.F3.CD NSCs in combination with 5-FC in co-cultures with U251 or early passage human glioma cells (PBT018) (30). Treatment with 5-FC (100 or 500 μg/ml) inhibited the growth of both the NSC-U251 (by 14.1% and 35.8%, respectively) and NSC-PBT018 (by 44.1% and 51.4% respectively) co-cultures, as compared to 5-FC only controls (Fig. S3).

We then evaluated the fate of intracranially administered HB1.F3.CD NSCs in a small cohort of glioma-bearing nude mice before or after mice completed 1 week of 5-FC

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treatment. Mice were injected with 2×10^5 U251.eGFP glioma cells into the frontal lobe on day 0, followed by caudal-lateral injections of 5×10^4 HB1.F3.CD NSCs (CM-DiI-labeled) on day 7. Mice then received daily intraperitoneal injections of 5-FC on days 11–17 (500 mg/kg twice daily for 5 days followed by 500 mg/kg once daily for 2 days) and were euthanized on day 11, prior to 5-FC treatment (Fig. 4B), or on day 21, 4 days after completion of 5-FC treatment (Fig. 4C). Examination of brains before 5-FC treatment revealed a viable glioma tumor focus (Fig. 4B); 4 days after 5-FC treatment, tumor cells were associated with interspersed NSCs (Fig. 4D, E) and showed apoptotic features and necrosis (Fig. 4C). In typical responses to treatment (depicted in figure 4), extensive areas of tumor necrosis were present, surrounded by foci of residual viable tumor. As shown by immunohistochemistry, the residual tumor cells were associated with interspersed NSCs remaining after 5-FC treatment. Although tumor necrosis is a defining feature of glioblastoma, present either as pseudopalisading foci or as geographically confluent areas, its irregular distribution in our experimental model could reflect the localization of the NSCs and the focal conversion of 5-FC to 5-FU they catalyze. Immunostaining with antibodies to markers of cell division, Ki67 and PCNA (proliferating nuclear cell antigen), showed no evidence of NSC proliferation as assessed at the time of necropsy (Fig. 4D, E).

HB1.F3.CD NSC safety and therapeutic efficacy in vivo

We conducted safety/toxicity and therapeutic efficacy IND-enabling studies in a manner as similar to the proposed clinical study as possible. HB1.F3.CD NSCs were thawed and prepared from the MCB according to the final standard operating procedures for cell preparation prior to transplantation. Ninety-six 8–10-week-old nude mice (6 males and 6 females per group; 8 groups) received stereotactic frontal lobe injections of 2×10^5 U251 glioma cells on day 0. Treatment mice were injected with NSCs ipsilateral to the tumor on day 7, and 5-FC on days 11–17 as follows: 1) NSCs (3 escalating doses, groups 1–3) in combination with 5-FC; 2) NSCs alone (3 escalating doses, groups 4–6); 3) 5-FC alone (group 7,) or 4) no treatment (group 8, n = 12) (Table 1). Two male and two female mice per group were euthanized on day 30 to measure tumor volume after treatment was completed. The remaining mice were followed for long-term survival through termination of the study at day 90. Of note, with this tumor strain and implantation dose, some mice did survive to the 90 day endpoint in all treatment groups (Table 2). To assess the safety of the treatment, we analyzed clinical event rates (defined as events such as seizures, tremors, labored breathing, signs of anorexia, bruising, dehydration, dry skin, hunched back, head tilt, gait and weight change that were secondary to treatment or tumor growth that alone or in combination warranted euthanasia), survival/time to death or event leading to euthanasia, and gross necropsy observation rates. Therapeutic efficacy was assessed on the basis of tumor volume.

During this study 30 mice failed; specifically, 28 mice were euthanized for clinical events resulting from tumor burden, one mouse was found dead having experienced tumor burden that did not lead to euthanasia, and one mouse had a clinical event attributed to surgical procedure. Fisher's Exact tests were used to compare the rates of clinical events across the treatment groups. No difference in the rate of clinical events leading to euthanasia was detected among the eight groups ($P = 0.27$); between groups receiving NSCs plus 5-FC (groups 1–3) and NSCs without 5-FC (groups 4–6) ($P = 0.80$); across the four combination dose groups (no combination therapy [groups 4–8]), three combination therapy doses [groups 1–3] [$P = 0.88$]); or between the sexes ($P = 0.51$). An omni-directional Fisher's Exact test detected no difference among the four NSC dose groups (Table 2, $P = 0.11$). The more focused single-degree of freedom Chi-squared test for trend resulted in $P = 0.06$.

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We used survival analysis to compare the treatment groups based on time to failure. As described above, 30 mice failed. The remaining 66 mice were censored, that is, removed from the risk set on the day they came off study (32 mice euthanized on day 30 for volumetric analysis, 32 mice euthanized on day 90 at the end of study, 2 mice died on day 33 unrelated to study protocol). On the basis of a log-rank test, no difference was detected in survival distributions among the eight treatment groups ($P = 0.40$), between the groups receiving NSCs with 5-FC (groups 1–3) and without 5-FC (groups 4–6) ($P = 0.66$), across the four combination therapy dose groups (no combination therapy [groups 4–8], three combination therapy doses [groups 1–3] [$P = 0.65$]) or between the two sexes ($P = 0.47$). In a post hoc analysis, as a follow-up to the Chi-squared test for trend in proportions described above, we implemented a log-rank test comparing survival for low dose NSC (doses 0 and 1×10^4) with high dose NSCs (NSC doses 5×10^4 and 1×10^5). This analysis recovered a significant result (Chi-squared = 4.5, df = 1, $P = 0.03$), indicating better survival in the two higher NSC dose groups (Fig. 5A). This result should be considered preliminary, but it suggests that a threshold dose for NSCs may need to be exceeded to achieve a positive outcome.

Gross necropsy and major organ tissue evaluations revealed no significant differences among the eight groups for study-related events (tumor burden, necrosis, inflammatory response, decreased food intake; Fisher's Exact test; $P = 0.36$), procedure-related events (perfusion procedure, necropsy procedure; $P = 0.89$) or study- and procedure-related events in combination ($P = 0.29$). Further, a Fisher's Exact test indicated no significant differences in gross necropsy evaluations among the four combination therapy dose groups (no combination therapy [groups 4–8], three combination therapy doses [groups 1–3]) for study-related ($P = 0.69$), procedure-related ($P = 0.97$), study- or procedure-related ($P = 0.64$), or between groups that received 5-FC (groups 1–3, 7) or did not receive 5-FC (groups 4–6, 8) (P-values were approximately 1 for all three types of events [study-related, procedure-related, and study- and procedure-related events]). Gross necropsy findings of tumor and/or necrosis were only observed in the brain. We also tested for a trend in the rate of events across NSC doses (dose 0: no NSCs, groups 7 and 8; dose 1: 1×10^4 NSCs, groups 1 and 4; dose 2: 5×10^4 NSCs, groups 2 and 5; dose 3: 1×10^5 NSCs, groups 3 and 6), noting fewer events at higher doses (1 degree of freedom Chi-square test of trend), and a P-value of 0.07 was achieved for both the combined study-related and procedure-related events (ratio of mice experiencing events/total in each dose group: dose 0, 6/24; dose 1, 8/24; dose 2, 5/24; dose 3, 2/22; Chi-squared = 3.3) and for the study-related events alone (ratio of mice experiencing events/total in each dose group: dose 0, 2/24; dose 1, 5/24; dose 2, 2/24; dose 3, 0/22; Chi-squared = 3.2). Note that the two mice that died for reasons unrelated to study treatment did not have a necropsy examination and were not included in the analysis.

In summary, 29 of the 30 mice that experienced clinical events had events that were attributable to or were associated with tumor burden. We did not detect evidence that the conversion of 5-FC by different doses of NSC had any effect on the rate of clinical events. On the basis of the necropsy results, we did not detect differences in the rates at which animals experienced clinical events, across the treatment groups, combination treatment groups, NSC dose groups or groups receiving and not receiving 5-FC.

Therapeutic efficacy by viable brain tumor volume at day 30

Therapeutic efficacy was determined by measuring viable brain tumor volume after one round of treatment with HB1.F3.CD NSCs and 5-FC. Two male and two female mice from each group (n = 32 mice total, see Table 1 for treatment groups) were euthanized on day 30, approximately 2 weeks after the end of 5-FC treatment. Tumor volume was analyzed for 24 mice (eight omitted from analysis: one mouse had no tumor take; two mouse brains were

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processed for PCR; and five brains were not evaluable.) Images of tumor areas from mice in treatment groups 3 and 6 at day 30 are shown in Fig. 5B–E.

Tumor volume was analyzed on a natural logarithmic (*ln*) scale. Descriptive statistics for *ln* tumor volume, including means, standard deviations and sample size (n) by treatment group are presented in Table S2. Mice that received the combination therapy (groups 1–3; n=10) had significantly smaller tumors (mean *ln* tumor volume = 15.5) than mice not treated with combination therapy (groups 4–8, n=14; mean *ln* tumor volume = 16.60) using a two-sample t-test (difference in the means = −1.1; 95% confidence limits [95% cl] on the difference = −0.05, −2.11; $P = 0.041$) (Fig. 5F). In the original units (μm^3) the average tumor size of the mice receiving combination treatment was approximately one-third the average tumor size of those that did not receive combination treatment. Using linear models methodology to further examine the effect of the dose of combination therapy used, we found that the group that received the highest NSC dose (1×10^5 cells) in combination with 5-FC had a significantly lower mean *ln* tumor volume (14.5; n = 3) than did groups that did not receive combination therapy (16.6; n = 14; $P = 0.0092$). When we compared the effect of NSC treatment with and without 5-FC (groups 1–3 [mean *ln* tumor volume = 15.5, n = 10] vs. groups 4–6 [mean ln tumor volume = 16.8, n = 10]), mice that received the combination had a significantly lower mean *ln* tumor volume (difference in the means = −1.3; 95% cl for the difference in the mean *ln* tumor volumes [−0.14, −2.46]: $P = 0.03$). No difference in tumor volume was detected between groups that received NSCs (with and without 5-FC; groups 1–6) and groups that did not receive NSCs (groups 7 and 8) (95% cl=[−1.09, 0.86], $P = 0.80$) or between sexes (95% cl=[−1.32,0.63], $P = 0.47$). In summary, the mice treated with NSC/5-FC combination therapy had significantly smaller tumors than those that did not receive combination therapy, suggesting a therapeutic effect was achieved.

We collected blood samples and peripheral blood smears from mice at euthanasia to evaluate clinical chemistry and detect NSCs. No notable changes in blood urea nitrogen (Fig. 6A) or γ-glutamyl transpeptidase levels (GGT; all mice < 3 U/l) (Fig. 6B) were detected in any study mice as compared to untreated controls, indicating that NSCs did not cause kidney or liver function abnormalities, respectively. In addition, no red or white blood cell abnormalities were observed in blood smears of mice from any group at days 30 and 90 and red blood cell mass was within normal limits. No abnormal changes were noted in white blood cells, and no human NSCs were detected in peripheral blood.

Histopathology

There was no morphological evidence of NSCs or of tumor or pathology outside the central nervous system (Fig. 6C, D). After tumor implantation, the mouse brains showed a spectrum of appearances, ranging from rare single tumor cells to large glioma masses, which occasionally resulted in drop metastasis with involvement of the spinal leptomeninges. In the absence of tumor cells or large tumor burden, mild reactive changes, including gliosis and hemosiderin-laden macrophages, were seen at the injection sites (Fig. 6E–H). NSCs were only identified within the brain. Histopathologic evaluation of organs at day 30, including liver, kidney, adrenal glands, lung, heart, skin, spleen, pancreas, gastrointestinal tract, and reproductive organs, from mice that received HB1.F3.CD NSCs in combination with 5-FC (or without 5-FC) were similar to organs from mice that did not receive HB1.F3.CD NSCs (tumor only) (Fig. 6C, D). These findings were confirmed by immunohistochemistry studies. In conclusion, the peripheral organs, the brain and the spinal cord showed no evidence of pathology or tumorigenesis attributable to NSCs.

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Detection of HB1.F3.CD NSCs by nested PCR for *v-myc* in the brain and other organs

We used nested PCR to detect v-myc (a marker for HB1.F3.CD NSCs) to investigate how long the HB1.F3.CD NSCs remained in the brain after completion of treatment, and whether they could be detected in any organ outside the brain. Nested PCR allowed detection of v-*myc* from approximately 0.01 ng of genomic DNA purified from HB1.F3.CD NSCs (Fig. S4A). To determine the sensitivity of v-*myc* nested PCR in mouse tissues, 500 ng of DNA from naive mouse brain was added to HB1.F3.CD NSC-derived DNA used in the PCR reaction. We estimated that nested PCR can detect 1–2 NSCs when amplifying DNA from HB1.F3.CD cells directly and 10–20 NSCs when mouse tissue-derived DNA was spiked with DNA from HB1.F3.CD NSCs (assuming the DNA content in a single diploid human cell with normal karyotype is ~6 pg) (Fig. S4B). NSCs were not detectable by nested PCR in the lung, kidney, liver, spleen, peripheral blood samples or bone marrow 30–40 days after NSC administration (Fig. S5). No NSCs were detected in the brains of mice 23 days after NSC administration, with the exception of one mouse (out of 15 assayed) (Table S3; group 4, mouse 233, brain sample R1). PCR results for this mouse indicated that v-*myc* DNA was restricted to the NSC injection site and was not detected in five adjacent areas of the brain in which U251.eGFP human glioma was detected. Overall, we concluded that NSC administration at all three NSC doses, with or without combination therapy with 5-FC, was safe, non-toxic, and non-tumorigenic.

DISCUSSION

The incurable nature of malignant gliomas can be attributed in large part to their propensity to invade brain parenchyma, often spreading to distant brain regions (2, 31) NSCs are inherently tumor-tropic, and thus appear to be well suited for delivering therapeutic payloads to invasive glioma and other tumors (9, 34–38). In addition to CD, neural or mesenchymal stem cells have delivered monoclonal antibodies (e.g., trastuzumab), carboxylesterase, interferon-β, interleukins (IL-2, IL-12), thymidine kinase, and TRAIL (9, 10, 14). More recently, NSCs were used to transport the replication-competent adenoviral vector CRAd-S-pk7 to orthotopic glioma implants in mice, which significantly prolonged survival of the mice (11). Thus, stem cells can be used for tumor-specific delivery of therapeutic genes and oncolytic viruses to invasive tumors (32, 33). Research published over the last 12 years on NSC tropism to brain tumors suggests that this field has advanced sufficiently for the introduction of stem cell-based cancer therapies into clinical trials. The preclinical in vitro and in vivo studies outlined in this paper were performed to support an IND application, filed with the FDA to perform a first-in-human clinical trial of these NSCs in order to assess the safety of NSC-mediated CD/5-FC enzyme/prodrug therapy in patients with recurrent high-grade glioma.

We characterized the HB1.F3.CD NSC line to establish its genetic stability, cellular identity, therapeutic gene expression, and tumor-targeting ability. We determined that HB1.F3.CD NSCs express HLA Class I antigens but do not express detectable levels of Class II antigens (37), indicating that they are unlikely to elicit a CD4-positive helper T-cell response. They are also less likely to elicit a cytotoxic T-cell response given the ‘immune-privileged’ status of the brain, the immunosuppressed tumor microenvironment, and the standard use of dexamethasone post-operatively in brain tumor patients. In contrast to other NSC clinical trials, which aim for long-term engraftment of the transplanted cells for regenerative and cell replacement therapies (32), our anti-cancer NSC-based treatment strategy requires the NSCs to remain in the brain for only 2 weeks to produce the therapeutic effect, during which time we do not expect them to be rejected by a patient’s immune system.

We found no proto-oncogenes or tumor suppressor genes in the vicinity of the v-*myc* and CD gene insertions in HB1.F3.CD NSCs, indicating that these NSCs are not likely to be

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tumorigenic. In vivo studies corroborated that HB1.F3.CD NSCs are not tumorigenic in non-tumor bearing mice. NSCs that were viable after 5-FC treatment in orthotopic glioma mouse models did not react with markers of cell proliferation (Ki67 and PCNA), indicating the cells were not dividing. We saw no histological evidence that NSCs formed tumors in the brain or in any other major organ that we investigated, and PCR with primers for v-*myc* (a marker for HB1.F3.CD NSCs) failed to detect NSCs in peripheral organs. Other studies have also shown the HB1.F3 parental and HB1.F3.CD lines are non-tumorigenic in animal models of stroke, Parkinson's and Huntington's disease (15, 25, 37–42).

Our preclinical efficacy study, in which mice with orthotopic gliomas were treated with the combination of HB1.F3.CD NSCs and 5-FC, demonstrated a pronounced decrease in tumor volumes as compared to those in control mice. Nevertheless, residual tumor tissue was present after one round of therapy, and our histological examination revealed that all mice euthanized at day 90 had large brain tumor masses. These findings indicate that one round of NSCs and 5-FC is only transiently effective and that further optimization of the treatment regimen, including increasing the dose and/or frequency of treatment with the NSCs and prodrug, will be required in human studies.

There are no established guidelines for choosing the starting dose of NSCs for a first-in-human clinical trial. Scaling up from the average weight of a mouse brain (0.45 g) to the average human brain (1360 g) would translate, for example, from 1×10^5 NSCs administered inctracranially to mice (Table 1) to 3×10^8 NSCs in humans. When designing our pilot feasibility clinical trial, we chose to be conservative for the sake of patient safety and started with a dose of 1×10^7 NSCs, which is equivalent to 66% of the lowest dose that showed efficacy in mice. Two dose levels of NSCs are being studied in this first clinical trial. Patients with recurrent high-grade gliomas are administered the NSCs intracranially at the time of tumor resection or biopsy. Four days later, after giving the NSCs time to spread out among residual tumor and migrate to distant foci of tumor, study patients take oral 5-FC every 6 hours for 7 days. Only one round of study treatment is administered to patients in this pilot study.

An inherent safeguard for the clinical use of HB1.F3.CD NSCs is that the CD would act as a suicide gene in the presence of 5-FC. After intracerebral administration of the NSCs, the 4 day waiting period before administration of the prodrug also allows time for the NSCs to stop dividing (by 24–48 hours after injection in preclinical studies). In the unlikely event that some NSCs would continue to divide in a patient's brain, they would be expected to be killed by the CD conversion of 5-FC to 5-FU, just as the dividing tumor cells are killed.

Clinical use of this stable, expandable, allogeneic HB1.F3.CD NSC line circumvents possible problems of stability and characterization associated with primary stem cell pools, as well as the need for continued access to sources of new cells. We hypothesize that, in patients, HB1.F3.CD NSCs injected into the tumor resection or biopsy site will localize to residual and invasive brain tumor foci and convert orally administered 5-FC to 5-FU at the tumor sites. This targeted enzyme/prodrug strategy is expected to minimize toxicity to normal tissues, potentially reducing the undesirable side effects associated with current radiation and chemotherapies (43–45), and improving the patients' quality of life. The first-inhuman clinical trial (NCT01172964) to assess the safety of NSC-mediated CD/5-FC enzyme/prodrug therapy in patients with recurrent high-grade glioma is now nearing completion, The main objectives of this study are to assess the safety and feasibility of administering these one round of NSCs intracranially to brain tumor patients and document proof-of-concept regarding NSC-mediated conversion of 5-FC to 5-FU, by measuring intracerebral drug levels, and NSC tumor-tropism, both non-invasively by MRI and at time

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of autopsy. Correlative immunologic studies will also provide valuable information about NSC immunogenicity after first exposure.

The results of this initial study will serve as the foundation for further therapeutic development for gliomas and potential applications of this approach to other invasive cancers. A phase 1 study to determine the maximum tolerated doses of these NSCs in combination with 5-FC in recurrent high-grade glioma patients who will receive repeat cycles of study treatment is planned. Simultaneous or sequential use of NSCs that have been modified to express different transgenes or combining NSC-based therapy with other anti-tumor strategies, such as radiation or immunotherapy, may also be explored in future clinical trials in order to achieve the full potential of NSC-based therapies for the treatment of cancers.

MATERIALS AND METHODS

Human HB1.F3.CD NSCs

Permission to use fetal tissue was granted to S. U. Kim (University of British Columbia, Canada) by the University of British Columbia Clinical Research Screening Committee for Studies Involving Human Subjects. Tissue was obtained from the Anatomical Pathology Department of Vancouver General Hospital. The HB1.F3 immortalized human NSC line was derived from primary cultures of fetal telencephalon (15 weeks gestation) by immortalization with an amphotropic, replication-incompetent retrovirus with the v-*myc* gene (25, 26, 39). Clones were isolated, expanded and designated as HB1 NSC lines (26, 28). One of these clones, HB1.F3, was transduced with the retroviral vector pMSCV-puro/CD, and clones were then isolated and expanded. HB1.F3.CD clone 21 was given to City of Hope under a Material Transfer Agreement.

Cytogenetics

Karyotyping and cytogenetic testing of HB1.F3.CD NSCs from the MCB (original passage 20, and additional passages 22 and 32) was performed by the City of Hope Cytogenetics Core Laboratory. Conventional cytogenetic analysis of HB1.F3.CD NSCs was performed with the BandView imaging system (Applied Spectral Imaging). Metaphase chromosome spreads were GTG-banded with trypsin by using standard procedures.

LAM-PCR and Q-PCR

LAM-PCR analysis of DNA extracted from HB1.F3.CD NSCs was used to determine the insertion site and copy number of the v-*myc* and CD genes. The analysis was performed by M. A. Harkey (Clonal Analysis Core, Core Center for Excellence in Hematology, Fred Hutchinson Cancer Research Center, Seattle, WA). Q-PCR with the standard curve method was used to further confirm the copy number of the v-*myc* and CD genes relative to the endogenous cholinergic receptor nicotinic epsilon gene (46). Q-PCR was carried out by M. K. Danks at St. Jude Children's Research Hospital, Memphis, TN.

Flow cytometry for CD expression in HB1.F3.CD cells

NSCs were treated with the Fix & Perm Cell Permeabilization kit (Invitrogen, GAS 003) for 10 min at room temperature, followed by one wash in Staining/Wash Buffer (SWB; Dulbecco's PBS without calcium and magnesium salts, supplemented with 5% FBS and 0.1 % (w/v) sodium azide), and incubated (20 min, in the dark) with anti-bCD primary antibody (BD Pharmingen, 557862). After a wash in SWB, cells were incubated (20 min, in the dark) with goat anti-mouse IgG/IgM-FITC secondary antibody (BD Pharmingen, 55598). Cells

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were then analyzed by flow cytometry (Guava EasyCyte flow cytometer, Millipore). Staining with isotype-matched antibody (BD Pharmingen, 555746) was used as a control.

NSC tumor tropism studies in vitro

Modified 96-well Boyden chamber (8-μm pore size) assays were used to assess *in vitro* tropism of NSCs to tumor cell-conditioned media, using standard methods (Chemicon kit ECM512). NSCs were detached by trypsinization, washed and resuspended in DMEM containing 5% bovine serum albumin. NSCs (3×10^4 cells/100 μl) were placed in the upper chambers, and tumor cell-conditioned medium was placed in the lower chambers (10% FCS was used as a positive control). Wells in which tumor cell-conditioned medium was placed in both the upper and lower chambers comprised the chemokinesis control. Cells were allowed to migrate for 4 hours in a cell culture incubator at 37°C and 6% CO_2. The number of migrated HB1.F3.CD cells in the lower chamber of each well was assessed with CyQuant GR fluorescent dye (Chemicon) and a fluorescence microplate reader (Molecular Devices). Assays were performed in triplicate.

In vivo studies

All mice were housed and treated at the City of Hope Animal Research Center. These studies were carried out in accordance with protocols approved by the City of Hope Institutional Animal Care and Use Committee (protocol #04011). The CD-expressing NSCs used for this study were derived from the HB1.F3.CD MCB and prepared according to procedures proposed for use in the clinical protocol. Any animal in distress was euthanized, consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Study design for safety and toxicity study—Ninety-six 8–10-week-old nude mice (6 males and 6 females per group; 48 male and 48 female mice total) were stereotactically implanted in the frontal lobe with human U251.eGFP glioma cells (2×10^5) on day 0. Mice were then injected with NSCs ipsilateral to the tumor on day 7, and 5-FC on days 11–17 as follows: 1) NSCs (3 escalating doses [1×10^4, 5×10^4, 1×10^5]; n = 12 mice/group; 6 male and 6 female) in combination with intraperitoneally administered 5-FC (days 11–17, 500 mg/kg twice daily for 5 days followed by 500 mg/kg once daily for 2 days); 2) NSCs alone (3 escalating doses; n = 12 mice/group; 6 male and 6 female); 3) 5-FC alone (n = 12; 6 male and 6 female) or 4) no NSCs or 5-FC (group 8, n = 12; 6 male and 6 female) (see treatment schema above Table 1 and Table 1). HB1.F3.CD NSCs used for this study were thawed and prepared from the MCB according to the final City of Hope standard operating procedures for cell preparation prior to transplantation. All mice were observed daily and scored for any debilitating signs secondary to treatment or tumor growth, including seizures, tremors, labored breathing, weight loss (20% body weight), scruffy coat, hunched posture, hypo- or hyperthermia, impaired gait, obvious illness, and inability to remain upright. The reason for euthanasia was recorded to distinguish between planned euthanasia and euthanasia due to meeting distress criteria. Thirty-two mice (4 mice per group; 2 male, 2 female) were euthanized as planned on day 30 to measure tumor volume after treatment was completed. The remaining 32 mice were followed for long-term survival through termination of the study at day 90, or were euthanized when they met euthanasia criteria.

Biodistribution studies in non-tumor bearing immunocompetent and immunodeficient mice—NSC doses included 2.5×10^5, 5.0×10^5 and 1.0×10^5 NSCs (1, 2, or 4 injections of 2.5×10^5 cells/2 μl, respectively). Cells were tracked from a depth of 2.5 mm to 2 mm over 3 min. Control mice received no NSC injections. Mice were euthanized 4 and 12 weeks after NSC injection.

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Cell and prodrug administration

Implantation of U251.eGFP glioma cells and NSCs was performed as described (36). Prior to the U251.eGFP and NSC implantation surgeries, mice were anesthetized with an intraperitoneal injection of 132 mg/kg Ketamine and 8.8 mg/kg Xylazine (0.4–0.74 ml of cocktail). All mice received a stereotactic intracranial injection of 2×10^5 human glioma U251.eGFP cells in the frontal lobe 8 days before NSC injection. The day that each mouse was administered the glioma tumor cell line was designated day 0. HB1.F3.CD NSCs were administered on day 7 via intracranial injection. Mice that received injections of NSCs subsequent to tumor administration were re-anesthetized, immobilized in the stereotactic frame, and implanted with the NSCs. NSCs were injected 0.5 mm caudal-lateral to the tumor injection site, tracked from a depth of 2.5 mm to 2.25 mm to 2.0 mm. This injection site was positioned at the tumor/brain tissue border, similar to the planned injection site into a wall of the tumor resection cavity in patients. Mice received intraperitoneal injections of 5-FC per the dosing regimen noted. Treatment of mice with 5-FC began 12 days after tumor implantation. Mice that received 5-FC were weighed daily during the period of drug administration.

Histopathological analysis

Histopathological analysis was performed by the City of Hope Pathology Core Laboratory and the Aboody laboratory according to standard protocols. Mice were perfused transcardially with 4% PFA, after which tissues were harvested, fixed, and paraffin embedded or cryosectioned.

Brain and spinal cord—Brains were 4% PFA-perfused and paraffin embedded and were microscopically examined by using 10 H&E-stained axial sections (10 μm sections, 100–150 μm apart), which allowed inspection of the cerebral cortex with the frontal injection sites, hippocampi, basal ganglia, midbrain, pons, medulla and cerebellum. In addition, 24–30 sections representing the expected tumor area were immunostained with an antibody to enhanced green fluorescent protein (eGFP; Abcam, ab290) to clearly identify the tumor cells. PFA-perfused, paraffin embedded cervico-thoracic and lumbo-sacral spinal cords were sampled in 8 H&E-stained sections at four levels, in the longitudinal and radial orientations.

Peripheral organs—Histological examination of tissue from liver, kidney, adrenal gland, lung, heart, skin, spleen, pancreas, gastrointestinal tract and reproductive organs was performed on PFA-fixed, paraffin embedded organs harvested from two mice from each group that received NSCs (groups 1–6). Six H&E stained sections on three slides at different levels through a given organ (10 μm sections, at least 100–200 μm apart) were microscopically examined. Immunohistochemistry with an antibody to eGFP was used to confirm tumor cells in selected mice. Organs, including blood and bone marrow, from the other mice from groups 3, 6, and 8 were evaluated by PCR to detect NSCs. As detailed above, histopathologic examination of brains and representative organ tissue was performed on mice euthanized at the specified timepoints (days 30 [11 days post-5-FC treatment] and 90) as well as representative tissues between days 30–90.

Blood chemistry and blood smear analysis

Blood samples and peripheral blood smears were collected from mice from each group on days 30 and 90 and sent to the University of Missouri Research Animal Diagnostic Laboratory. For each blood sample, 50 cells were counted twice from each group and from control male and female samples. One peripheral blood smear from each mouse was immunohistochemically stained for human mitochondria to confirm that no human transplanted cells were present in the peripheral blood.

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Statistical analysis

Adverse clinical events—The rates of adverse clinical events across treatment groups and sexes were compared with two tailed Fisher's Exact tests, trends in the rates of events as a function of dose were tested with a Chi-squared test of trend, and survival (time to clinical events leading to euthanasia) was compared across treatment groups with a logrank test.

Necropsy tissue—Necropsy results were categorized into three types, study-related events (tumor burden, necrosis, inflammatory response, and decreased food intake), procedure-related events (perfusion procedure, necropsy procedure) and normal animal variation. We compared rates of study-related and procedure-related events across the treatment groups and sexes with two tailed Fisher's Exact tests, and tested trends in the rates of events as a function of dose with a Chi-squared test of trend.

Volumetric brain tumor analysis—Sections (10 μm thick horizontal sections) through the entire brain were stained with an antibody to eGFP (Abcam, ab290) to identify tumor cells. Viable tumor area was calculated from 10x images of 8–20 equally spaced sections (200μm separation) from each brain, allowing uniform sampling through the tumor bed. We used Image J software to measure the eGFP-immunoreactive tumor area in each section. Delineated tumor area was verified by a certified neuropathologist at City of Hope (M. D'Apuzzo) who was blinded to the treatment groups. Tumor areas were initially measured in pixels, converted to μm^2 (1 pixel = 1.82 μm^2), and the tumor volume represented by each section was then determined by multiplying the area in μm^2 by the distance to the upper surface of the next section in the series (final volume in μm^3). These volumes were expressed as natural log values (*ln*, e-based logarithm) to facilitate further analysis. Comparisons between treatment groups and sexes were made with two-tailed two-sample t tests, and comparisons among dose levels were made using linear models methodology. All tests were done with statistical significance taken to be $p < 0.05$. All statistical analyses were performed with the statistical framework R (47).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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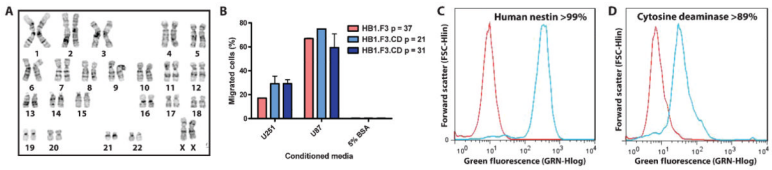

Fig. 1. *In vitro* characterization of the HB1.F3.CD NSC line

(A) Conventional cytogenetic analysis of HB1.F3.CD NSCs. **(B)** Tumor tropism of parental HB1.F3 and HB1.F3.CD NSCs in response to glioma cell-conditioned media at passages 21, 31 and 37 measured *in vitro*; mean ± SEM of triplicate measurements; where not visible, the error bars are contained within the graph. Data are expressed as % cells migrated, where 100% is 3×10^4 **(C)** Flow cytometry analysis of HB1.F3.CD cells immunostained with anti-human nestin (blue line) or isotype control antibody (red line). (**(D)** *E. coli* cytosine deaminase expression in HB1.F3.CD NSCs (blue line); isotype control antibody was used as control (red line).

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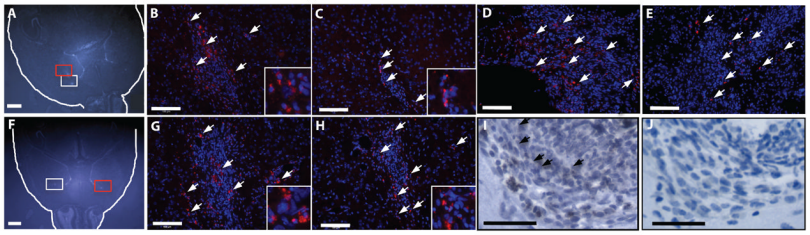

Fig. 2. HB1.F3.CD NSC tumor tropism *in vivo* in presence of dexamethasone or after radiation treatment

(A) Diagram of NSC injection (red box) caudal-lateral to tumor site (white box) or **(F)** into the hemisphere opposite the tumor site shown on 1x DAPI-stained representative brain tissue sections. Scale bar, 1 mm. **(B–C, G–H)** U251 tumors were established in the right frontal lobes of adult nude mice (n = 8). Mice received daily dexamethasone (0.2 mg/kg/day, injected intraperitoneally, started on day 7 and continued until mice were euthanized on day 14 or 17) 3 days prior to and 4 or 7 days after NSC injection. Cryosections of brains 4 days (**B, G**) and 7 days (**C, H**) after NSCs (labeled with CM Di-I, red; indicated with white arrows) were injected caudal-lateral to tumor **(B, C)** or into the hemisphere opposite to the tumor **(G, H)**. Nuclei are indicated by DAPI (blue). Insets show high power images of cryosections.. **(D, E)** C57BL/6 mice were injected with 1×10^5 syngeneic murine GL261 glioma cells into the frontal lobe. Seven days later, 1×10^5 HB1.F3.CD NSCs (CM-DiI-labeled, red) were injected caudal-lateral to tumor. No immunosuppression was given. Representative DAPI-stained brain tumor sections from mice euthanized 4 **(D)** and 15 **(E)** days after NSC administration, showing glioma (dense blue nuclei) and HB1.F3.CD NSCs (red, indicated with white arrows). Scale bars, 100 μm. **(I, J)** Ten days after U251 tumors were established in the right frontal lobes of nude mice, 10 Gy irradiation was delivered to the brain. Ten days later, HB1.F3.CD NSCs were injected into the left hemisphere. Mice were euthanized 4 days after NSC injection. Shown are paraffin-embedded brain sections stained with a polyclonal antibody to CD/DAB-Ni to identify HB1.F3.CD NSCs (**I**, NSCs indicated with black arrows) or no primary antibody/DAB-Ni control **(J)**.

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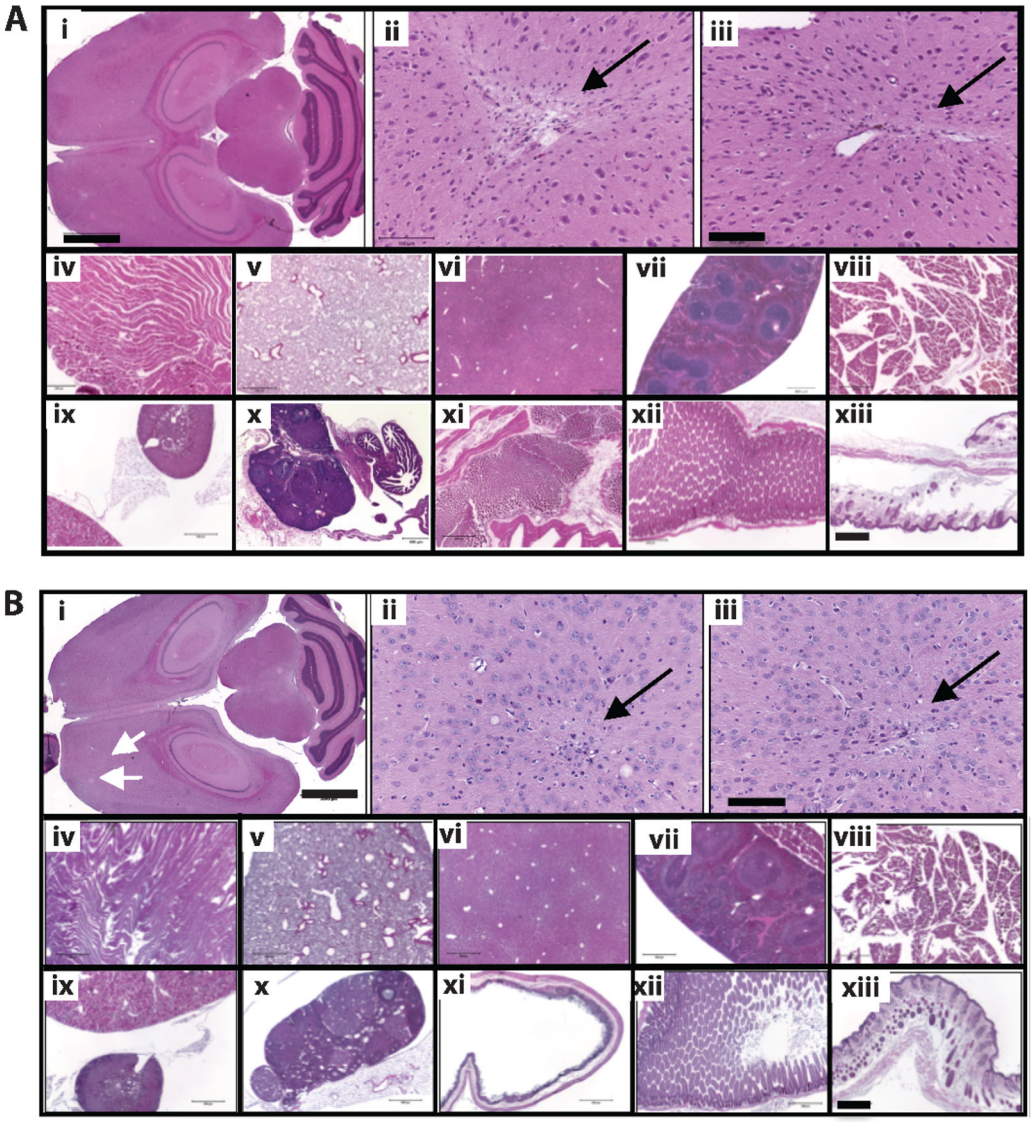

Fig. 3. Safety of administering HB1.F3.CD NSCs into the brains of non-tumor-bearing nude mice

(A, B) Histological images of organs from non-tumor-bearing mice injected intracranially with 5×10^5 HB1.F3.CD NSCs and euthanized 4 weeks **(A)** and 12 weeks **(B)** after injection of NSCs. **(i–iii)** H&E-stained brain tissue sections showing normal brain tissue with focal gliosis at NSC injection sites (arrows) (Ai, ii, iii and Bi, ii, iii. Scale bars Ai and Bi, 2000 μm). Representative sections from: **(iv)** heart, **(v)** lung, **(vi)** liver, **(vii)** spleen, **(viii)** pancreas, **(ix)** kidney/adrenal, **(x)** ovary, **(xi)** stomach, **(xii)** intestine, **(xiii)** skin. Scale bars, 100 μm.

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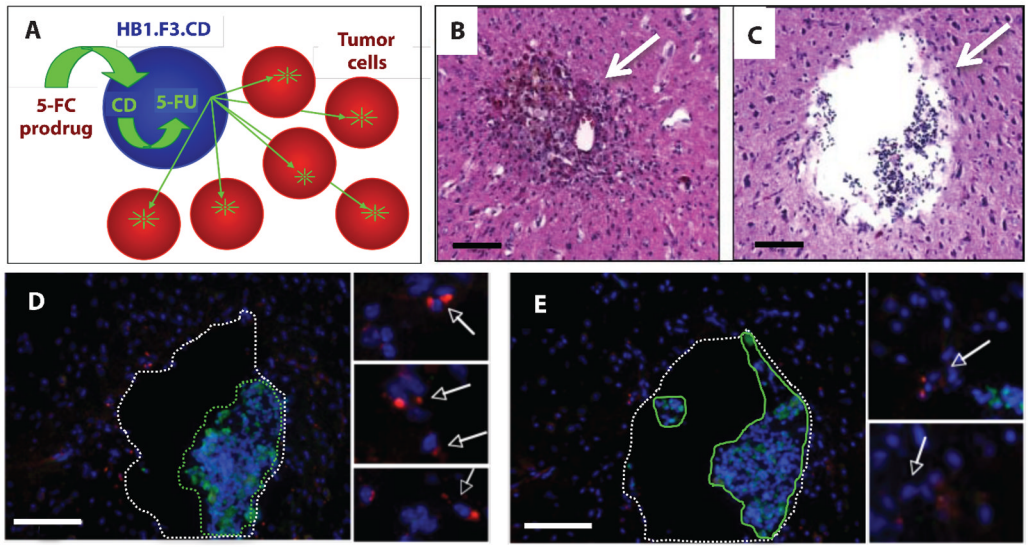

Fig. 4. NSC-mediated enzyme/prodrug therapeutic paradigm and therapeutic effect

(A) Diagram of CD-expressing NSCs localized to tumor cells, and CD conversion of 5-FC to 5-FU, which readily diffuses out of the NSCs to selectively kill surrounding, dividing tumor cells. **(B, C)** H&E-stained brain tumor sections from U251 glioma-bearing mice that received HB1.F3.CD NSCs only **(B)** or HB1.F3.CD NSCs in combination with **(C)** treatment with 5-FC. White arrows indicate tumor region. **(D, E)** Tumor sections from U251 glioma-bearing mice that showed an exemplary response to treatment with NSCs and 5-FC 4 days after completion of one week of 5-FC treatment. Original tumor area is indicated by white dotted line and residual, apoptotic appearing tumor cells are indicated by green dotted line. Fluorescence immunohistochemistry with antibodies against Ki67 **(D)** and PCNA **(E)** revealed occasional residual tumor cell proliferation (green). NSCs (CM-DiI labeled, red) remaining after 5-FC treatment were not dividing, as determined by negative immunostaining for markers of cell division Ki67 and PCNA (white arrows, high-power image insets). Scale bars, 100 μm.

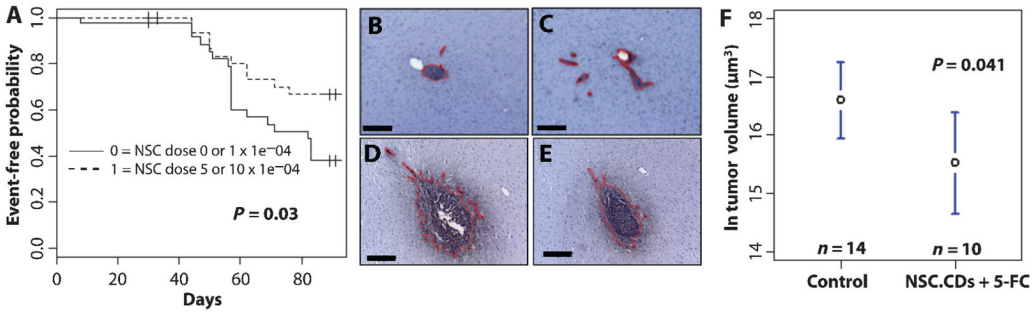

Fig. 5. Efficacy of HB1.F3.CD NSC + 5-FC combination treatment

(A) Survival curves for mice that received the indicated doses of NSCs (5×10^4 and 1×10^5 vs. no NSCs and 1×10^4). $P = 0.03$. **(B–E)** Representative eGFP-DAB-Ni-stained brain tumor sections from mice treated with the combination of NSCs and 5-FC (group 3) **(B, C)** or NSCs only (group 6) **(D, E)**. Tumor areas are outlined in red. Tumor section areas were multiplied by distance to next section through tumor to determine tumor volume. **(F)** Mean *ln* tumor volume and 95 % confidence limits of mice treated with the combination of HB1.F3.CD NSCs and 5-FC (groups 1–3) compared to control mice (groups 4–6, NSCs only; group 7, 5-FC only; group 8, tumor only) $P = 0.041$.

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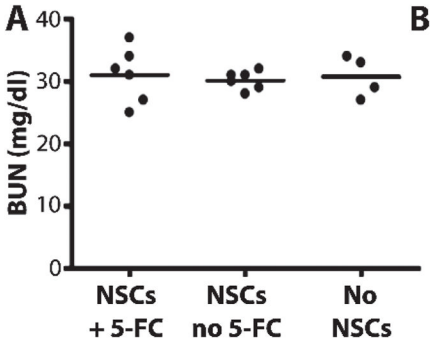

B

Comparison	*P*	Sig
NSC + 5-FC vs. NSC w/o 5-FC	0.671	No
NSC + 5-FC vs. no NSC	0.926	No
No NSC w/o 5-FC vs. no NSC	0.709	No
NSC + 5-FC vs. no NSC + 5-FC	0.891	No
NSC w/o 5-FC vs. no NSC w/o 5-FC	0.929	No

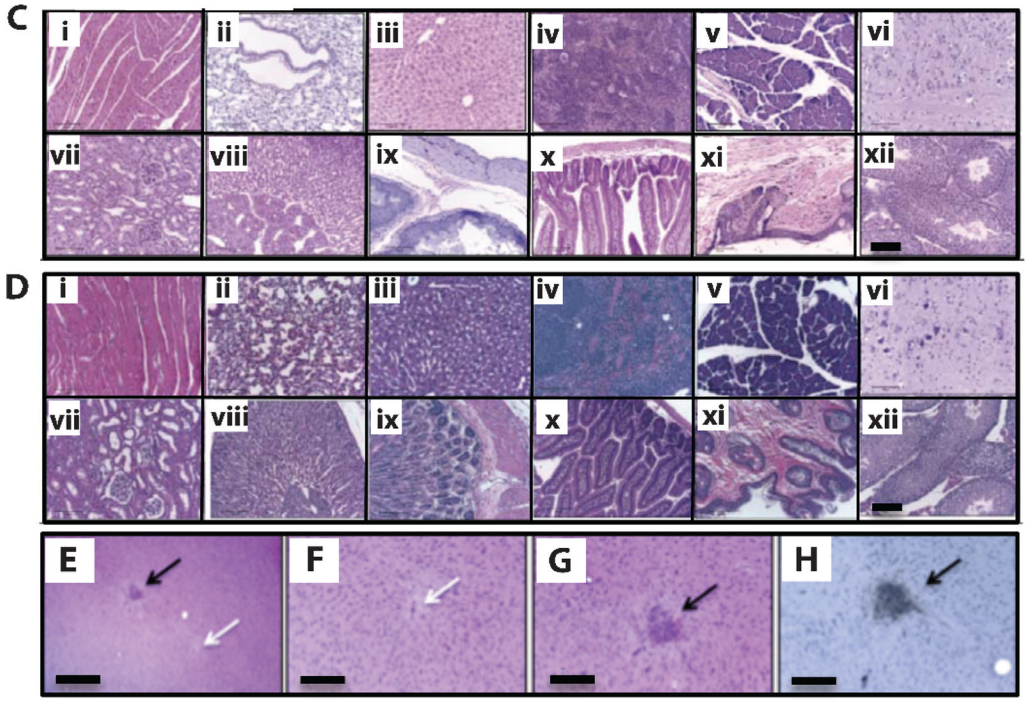

Fig. 6. HB1.F3.CD NSC safety/toxicity study data

(A) BUN and **(B**) GGT levels 90 days after tumor injection. **(C, D).** H& E-stained tissue sections from mice euthanized at day 30 that received **(C)** 1×10^5 NSCs plus 5-FC (group 3) or **(D)** 1×10^5 NSCs and no 5-FC (group 6). Images of: **(i)** heart, **(ii)** lung, **(iii)** liver, **(iv)** spleen, **(v)** pancreas, **(vi)** lumbar spinal cord (similar results for cranial, thoracic, and sacral spinal cord regions), **(vii)** kidney, **(viii)** adrenal, **(ix)** stomach, **(x)** intestine, **(xi)** skin, and **(xii)** testis (similar results for ovaries). **(E–H)** Images of brain tissue at 30 days after NSC injection; NSC injection site indicated by white arrows and tumor engraftment site indicated by black arrows. **(E–G)** Low and high power H& E-stained sections show a hypercellular focus (black arrow), consistent with tumor, measuring approximately 0.4 mm and located in the right frontal cortex, associated with hemosiderin pigment, gliosis and mild architectural distortion. Lateral to the tumor, 1.1 mm away, is a smaller focus of gliosis, consistent with the NSC injection site (white arrow), associated with hemosiderin pigment. **(H)** Brain tissue section immunohistochemically stained with an antibody to eGFP, indicating the main tumor mass and edges that infiltrate into the surrounding cortex up to 0.7 mm from the main mass, with preferential localization around blood vessels. Scale bars, 100 μm.

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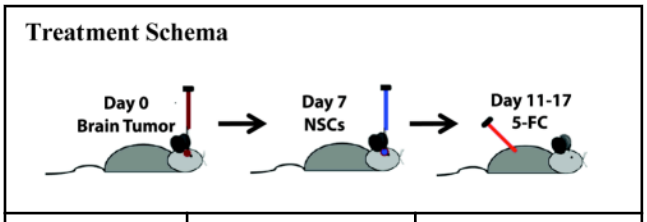
Table 1
Therapeutic paradigm for safety/efficacy IND-enabling studies

On day 0, all mice received U251 glioma injection into the frontal lobe. One week later, the NSC treatment groups received 1×10^4, 5×10^4, or 1×10^5 HB1.F3.CD NSCs caudal-lateral to tumor in same hemisphere. Four days later, the 5-FC treatment groups received 5-FC for 7 days (500 mg/kg twice a day [BID] for 5 days, then once a day [QD] for 2 days).



Treatment Group	Dose HB1.F3.CD NSCs	Dose 5-FC i.p.
1	1×10^4	500 mg/kg BID x 5 days 500 mg/kg QD x 2 days
2	5×10^4	500 mg/kg BID x 5 days 500 mg/kg QD x 2 days
3	1×10^5	500 mg/kg BID x 5 days 500 mg/kg QD x 2 days
4	1×10^4	0
5	5×10^4	0
6	1×10^5	0
7	0	500 mg/kg BID x 5 days 500 mg/kg QD x 2 days
8	0	0

n = 12 mice per group (6 males, 6 females); 96 mice total

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Table 2

Clinical events in mice

Number of mice experiencing clinical events leading to euthanasia or being euthanized on the basis of the study design (censored at day 30 or 90) by NSC dose.

	NSC Doses				
	0	**1×10^4**	**5×10^4**	**1×10^5**	**Total**
Clinical events	8	12	5	5	30
Total censored	16	12	19	19	66
Censored at day 30	8	8	8	10*	34
Censored at day 90	8	4	11	9	32

* Two mice in group 6 died unrelated to study protocol and were censored on day 33.