Bcl-2 Overexpression Improves Survival and Efficacy of Neural Stem Cell-Mediated Enzyme Prodrug Therapy

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Received 24 August 2017; Revised 22 February 2018; Accepted 13 March 2018; Published 20 June 2018

1. Introduction

Tumor-tropic neural stem cells (NSCs) can be engineered to localize gene therapies to invasive brain tumors. However, like other stem cell-based therapies, survival of therapeutic NSCs after transplantation is currently suboptimal. One approach to prolonging cell survival is to transiently overexpress an antiapoptotic protein within the cells prior to transplantation. Here, we investigate the utility and safety of this approach using a clinically tested, \( \nu^- \)myc immortalized, human NSC line engineered to contain the suicide gene, cytosine deaminase (CD-NSCs). We demonstrate that both adenoviral- and minicircle-driven expression of the antiapoptotic protein Bcl-2 can partially rescue CD-NSCs from transplant-associated insults. We further demonstrate that the improved CD-NSC survival afforded by transient Bcl-2 overexpression results in decreased tumor burden in an orthotopic xenograft glioma mouse model following administrations of intracerebral CD-NSCs and systemic prodrug. Importantly, no evidence of CD-NSC transformation was observed upon transient overexpression of Bcl-2. This research highlights a critical need to develop clinically relevant strategies to improve survival of therapeutic stem cell posttransplantation. We demonstrate for the first time in this disease setting that improving CD-NSC survival using Bcl-2 overexpression can significantly improve therapeutic outcomes.
are postulated to migrate to invasive tumor foci that typi-
ically elude effective distribution by traditional enzyme
expression vectors.

However, one of the overlooked challenges that may be
limiting the therapeutic potential of cell-mediated therapies
is suboptimal cell survival posttransplantation. In the case
of CD-NSC enzyme prodrug therapy, the apoptotic stimuli
encountered upon administration into the tumor resection
cavity are significant and unavoidable. Thawed cells are
placed into a stressful environment that contains poor matrix
support and high concentrations of reactive oxygen species
[6]. Studies of NSC transplantation into the brain for neuro-
logical disorders report primary NSC survival of <4–10% within
the first few days [7]. This suggests that post-
transplantation survival of the genetically modified CD-
NSC line is a critical parameter to investigate for optimal
therapeutic efficacy.

Hostile transplant environments are a challenge for many
cell therapies. One emerging strategy to address this prob-
lem is to overexpress the antiapoptotic gene, B-cell lymphoma 2
(Bcl-2). Bcl-2 is a mild protooncogene that, if transiently over-
expressed, could protect donor cells from apoptotic stimuli
during the critical 1-week window in which produgs are
administered [2] and when apoptosis is commonly observed
after transplantation [8]. In fact, the mechanism by which the
CD5/FC therapy induces apoptosis converges on Bcl-2
modulation [9], so this approach is ideally suited for this
particular NSC-mediated enzyme prodrug therapy. Thus
far, transient Bcl-2 overexpression has effectively improved
posttransplantation viability and efficacy of embryonic stem
cells [10] and mesenchymal stem cells used to treat ischemic
cardiac insults [11] and skeletal defects [12], respectively.
Transient Bcl-2 overexpression has been accomplished using
traditional nonintegrating adenoviral vectors or using min-
circle technology, which lacks any potentially inflammatory
viral and/or bacterial sequences [12].

Here, we investigate transient Bcl-2 overexpression as an
effective, safe approach to achieve prolonged posttransplan-
tation survival of CD-NSCs. One main concern is the poten-
tial risk of neoplastic transformation of implanted NSCs
given that Bcl-2 overexpression is associated with tumors of
both lymphoid and epithelial origin [13–15]. It is accepted
that Bcl-2 expression is insufficient to induce uncontrolled
cell proliferation without cooperation from a second onco-
gene [16, 17]. However, when stably coexpressed, myc and
Bcl-2 can cooperate to increase tumor incidence in a variety
of lymphoid and epithelial cell types as well as transgenic
models [18, 19]. Because CD-NSCs were immortalized using
v- myc, it is necessary to ensure that transient Bcl-2 expres-
sion does not transform CD-NSCs.

We hypothesize that transformation of CD-NSCs will not
occur upon transient Bcl-2 overexpression based on the fol-
lowing rationale. First, Bcl-2 overexpression would only tran-
siently occur during the short (<1 week) window in which
NSC survival is critical for maximum prodrug conversion
and tumor tropism. Second, v- myc expression within CD-
NSCs undergoes constitutive downregulation upon trans-
plantation, perhaps through developmental and epigenetic
mechanisms that suppress endogenous cellular myc in NSCs
during mitotic arrest [20]. Finally, even if a small fraction of
injected CD-NSCs maintains coexpression of v-myc and
Bcl-2, they would quickly succumb to the antiproliferative
chemotherapeutics generated upon administering prodruk
intended for the dividing tumor cells.

The studies presented here confirm that while CD-NSCs
are certainly susceptible to oxidative stress and anoikis, tran-
sient Bcl-2 overexpression partially rescues them. Impor-
tantly, we observed no evidence that Bcl-2 overexpression
impairs tumor tropism and prodrug expression or induces
neoplastic transformation. We also demonstrate that Bcl-2
overexpression improves both CD-NSC survival and the
therapeutic efficacy observed after one round of treatment.

2. Results

2.1. Bcl-2 Expression Constructs. Both adenoviral and mini-
circle expression vectors were utilized to transiently
overexpress Bcl-2 within CD-NSCs. After establishing opti-
mal adenoviral transduction parameters (Supplementary
Figure 1), initial Bcl-2 overexpression was observed in
66.4% of CD-NSCs as assessed by flow cytometric
analysis of Bcl-2 positive cells (Figures 1(a) and 1(c)). A
ontarget (firefly luciferase expressing) adenoviral vector
was used as control vector in all studies. A comparable level
of initial Bcl-2 overexpression (65 ± 5% of CD-NSCs) was
achieved using our eGFP-labelled minicircle construct
(Figures 1(a) and 1(b)). As controls, two other minicircle
constructs were generated containing a Bcl-2 shRNA or a
nontargeting scrambled sequence (Supplementary Figure 2).
Because the initial transfection efficiency of both control
constructs was lower (<20% of CD-NSCs), these controls
were used only during select in vitro studies.

2.2. Bcl-2 Expression Efficiency and Time Course Assessments.
While the percentage of NSCs initially overexpressing Bcl-2
was comparable between the adenoviral and minicircle
expression constructs, the rate at which they lost Bcl-2
expression differed greatly. The adenoviral construct resulted
in a gradual decline in the percentage of Bcl-2 positive NSCs,
with up to 40% of CD-NSCs Bcl-2 (+) by day 7 (Figures 1(a)
and 1(c)). In contrast, the percentage of Bcl-2 (+) CD-NSCs
quickly declined after transfection with our Bcl-2 minicircle
construct, dropping to 20% (+) by day 3 and no Bcl-2 (+) cells
by day 7 (Figures 1(a) and 1(c)). This decline in Bcl-2 expres-
sion occurred more rapidly than the decline in eGFP (+) CD-
NSCs, suggesting a short duration of Bcl-2 expression even in
minicircle-transfected CD-NSCs. Thus, both adenoviral and
minicircle expression vectors achieved transient Bcl-2 expres-
sion. Both constructs were pursued further because the opti-
mal duration for Bcl-2 overexpression is still not clear with
respect to affording increased CD-NSC survival without
compromising safety.

2.3. BCL-2 Expression Improves HB1.F3.CD NSC Survival In
Vitro. We next tested if Bcl-2 overexpression confers CD-
NSCs with a survival advantage under controlled in vitro
insults. First, cultured parental and Bcl-2-overexpressing
CD-NSCs were exposed to increasing H2O2 doses to mimic
the high-oxidative stress present upon transplantation into the brain. NSC viability was assessed both qualitatively (LIVE/DEAD imaging) and quantitatively (total cellular ATP measurements). LIVE/DEAD images show a significant loss in viable (green) parental CD-NSCs (Figure 2(a), first row) after four days of exposure to even 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), demonstrating this cell line is clearly susceptible to oxidative stress-induced damage. Similar results were observed when NSCs were modified with either the nontargeted adenovirus (Figure 2(a), third row) or the nontarget minicircle vector (Figure 2(a), fifth row). Slightly increased susceptibility to oxidative stress was apparent when NSCs were first transfected with the minicircle containing Bcl-2 shRNA (Figure 2(a), sixth row). In contrast, the cultures containing Bcl-2-overexpressing NSCs contained an increased number of viable CD-NSCs after exposure to 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (Figure 2(a); adenovirus, second row; minicircle, fourth row). In fact, adenovirus-driven Bcl-2 expression resulted in an increased number of viable CD-NSCs even after being exposed to 400 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for four days.

Quantitative ATP measurements yielded results consistent with the LIVE/DEAD imaging. Increasing \( \text{H}_2\text{O}_2 \) concentrations resulted in significantly reduced total ATP levels when applied to parental CD-NSCs (Figure 2(b)). Transducing the CD-NSCs with the control adenovirus did not rescue declining ATP levels; however, transducing with the Bcl-2 transgene was effective in maintaining total ATP at levels equal to that observed when NSCs were not exposed to \( \text{H}_2\text{O}_2 \) (Figure 2(b)). A similar, though less potent effect was observed when NSCs were not exposed to \( \text{H}_2\text{O}_2 \), an effect not observed using a scrambled shRNA sequence (Figure 2(b)).

Furthermore, Bcl-2 overexpression resulted in no significant changes in the identity or therapeutic function of CD-NSCs. The NSCs remained immunopositive for the neural stem cell marker, nestin, and immunonegative for neuronal (\( \beta \)-tubulin III) and glial (GFAP) lineage markers (Figure 2(d)). Bcl-2 overexpression had no detrimental effect on NSC tropism to tumor-conditioned media (Figure 2(c)) and no significant effect on the expression of the prodrug-converting enzymes, CD (Figure 2(e)), or carboxylesterases (Supplementary Figure 3).

### 2.4. BCL-2 Expression Improves HB1.F3.CD NSC Survival In Vivo

To determine if transient Bcl-2 overexpression can extend the time CD-NSCs remain viable \textit{in vivo}, genetically modified and parental firefly luciferase- (ffluc-) expressing CD-NSCs were injected ipsilateral to preestablished U251.DsRed glioma orthotopic xenografts. Then, longitudinal bioluminescence imaging was used to monitor the duration of NSC-derived fluc expression (Figures 3(a) and 3(b)). Despite a robust fluc signal in all groups on the day of implantation, both the parental CD-NSCs and the CD-NSCs transduced with control adenovirus had minimal signal remaining two days later (Figure 3(a), top two rows). In contrast, CD-NSCs modified to express Bcl-2 using either the adenoviral or minicircle vectors exhibited prolonged fluc signal that was still visible 4 days later (Figure 3(a), bottom two rows). Thus, NSCs exhibit prolonged viability when engineered to transiently overexpress Bcl-2, a result that becomes statistically significant by day 4 \( (p < 0.05) \) (Figure 3(b)).

To confirm that the BCL-2-overexpressing CD-NSCs are in fact more viable than the parental NSCs, three brains from both the parental and Ad.Bcl-2 group were harvested one day after implantation to immunologically assess the extent of CD-NSC survival. Representative fluorescence microscopic images of serially sectioned brains show a nestin (+) CD-NSC injection site (green) located near the Ds.
Red U251 tumor (Figure 3(c), top row). Adjacent sections were stained for both active caspase-3/7 as a marker of apoptotic cells (Figure 3(c), bottom row) and fluc as a marker for viable CD-NSCs (Figure 3(c), middle row). These images demonstrate that the vast majority of parental CD-NSCs are brightly positive for active caspase-3/7 and largely negative for fluc, suggesting they were no longer viable enough to produce this protein (intracellular degradation rate of firefly luciferase = 3 hrs [21]). In contrast, Ad.Bcl-2 CD-NSCs demonstrated intense fluc staining but negligible active caspase-3/7 staining.

2.5. Therapeutic Advantage of Bcl-2 Modified HB1.F3.CD NSC In Vivo. Interestingly, the improved CD-NSC survival did not translate to increased conversion of the 5-FC prodrug into the active effector, 5-FU (Figure 3(d)) as assessed in brains tumors harvested two days after NSC administration. Nonetheless, we utilized longitudinal bioluminescence imaging to monitor any changes in the progression of U251.eGFP.fluc glioma orthotopic xenografts if treated with either parental or Bcl-2-expressing CD-NSCs, followed by 5 consecutive days of prodrug administration (Figures 4(b) and 4(c)). In this experiment, the tumor cells were coinjected with CD-NSCs to ensure consistent intratumoral biodistribution in each experimental group. As expected, tumor progression was observed when mice received only the 5-FC prodrug. When NSCs were present to convert the prodrug into 5-FU, a noticeable qualitative decrease in tumor flux was observed 1 week after treatment (Figure 4(b)).

The decrease in tumor flux seemed to be more substantial in mice that received Bcl-2-expressing NSCs, so we calculated the average % increase in luminescence flux that occurred in each treatment group. The results at week 3 demonstrate a
significant delay in tumor progression observed when NSCs are modified to overexpress Bcl-2 using either the adenoviral or the minicircle vector (Figure 4(c)). The relative tumor burdens present within brains harvested at this 3-week time point are consistent with the noninvasive imaging results, with visibly smaller tumors present in mice that received Bcl-2-expressing NSCs (Figure 4(a)). A similar result was observed in mice that contained preestablished, patient-derived glioma cells, where the decrease in tumor flux in NSC-treated mice was more substantial when NSCs were modified to overexpress Bcl-2 (Supplementary Figure 4).

2.6. Bcl-2-Expressing HB1.F3.CD NSCs Are Nontumorigenic. Having demonstrated that Bcl-2-expressing CD-NSCs show prolonged viability, it was important to confirm that the Bcl-2 modification did not transform CD-NSCs into tumor-initiating cells. As a preliminary in vitro assessment, CD-NSCs were cultured in nonadherent agarose cultures known to induce anoikis in normal but not cancerous cells. Results show that while established tumorigenic cell lines were able to overcome a lack of integrin signaling and form proliferative colonies, neither parental nor Bcl-2-expressing CD-NSCs formed colonies (Figures 5(a) and 5(b)).
Bcl-2 overexpression did, however, significantly increase the percent of NSCs that remained viable as isolated single cells (Figures 5(a) and 5(b)). Eventually, however, even the Bcl-2-expressing NSCs died as evidenced by a sharp decline in intracellular ATP levels over 72 h of culture to reach negligible levels (data not shown). Total DNA and ATP were also measured within growing CD-NSC cultures to confirm that Bcl-2-expressing NSCs did not exhibit increased proliferation rates and still exhibit contact-inhibited growth patterns indistinguishable from that of parental NSCs (Figures 5(c) and 5(d)).

We also confirmed that Bcl-2 overexpression does not result in abnormal CD-NSC proliferation in vivo. In this experiment, the glioma line expressed DsRed, and the NSCs
expressed eGFP, but no prodrugs were administered. Brains were harvested on day 1, 7, and 14 to monitor the extent of CD-NSC proliferation when initially coinjected with DsRed glioma cells (Figures 5(e) and 5(f)). We observed that the 
Bcl-2 overexpression, particularly using the adenoviral expression vector, significantly improved the percentage of eGFP-expressing CD-NSCs still present on day 1 (60% versus 4% of parental CD-NSCs). However, the acute rejection and/or apoptosis that occurred over the subsequent 2 weeks [22] effectively eliminated most of the transplanted CD-NSCs in all groups (Figures 5(e) and 5(f)). By day 7, NSCs in all groups had ceased dividing as confirmed by negative PCNA and Ki-67 staining (data not shown).

Finally, a pilot long-term tumorigenicity assessment was performed using Bcl-2-expressing CD-NSCs. We injected up to 10 times the clinically relevant human cell dose (1.0×10⁶) into the brains of nontumor-bearing immunodeficient mice. All mice had normal gait, appetite, alertness, hydration, neurological symptoms, and weight during the week after NSC injection and through time to planned euthanasia. Two months later, brain sections were examined for the presence of viable and/or proliferative NSCs. Hematoxylin and eosin (H&E) histoch...
demonstrating that Bcl-2 overexpression can safely enhance the survival of other types of therapeutic stem cells including hematopoietic stem cells [23], adipose-derived mesenchymal stem cells [12], and peripheral NSCs [24].

To transiently overexpress Bcl-2 within CD-NSCs, we tried two different nonintegrating approaches: adenoviral transduction and minicircle transfection. Both approaches are clinically relevant. If pursuing the adenoviral vector, it will be necessary to carefully evaluate the immunogenic potential and confirm the replication-deficient status of intracellular adenovirus particles [25]. We have obtained approval for use of adenovirus-driven rCE expression in our ongoing CD-NSC.hCE1m6 recurrent glioma trial (NCT02192359) [5]. Our head-to-head comparison found the adenoviral approach resulted in a longer duration in which a significant percentage of NSCs overexpressed Bcl-2. The quick decline in Bcl-2-expressing NSCs after minicircle transfection was surprising given that an expression duration of 7–14 days is more typically reported for minicircle expression vectors [26]. Perhaps, duration of minicircle-driven Bcl-2 expression may be extended if all remnant bacterial DNA was eliminated.

The major challenges of cell survival in vivo include an ischemic tissue environment, immune cell recognition, loss of ECM, and oxidative stress. Here, we demonstrate that Bcl-2 expression improves the ability of NSCs to survive amidst two of these insults in vitro. We show that Bcl-2 overexpression improves CD-NSC survival under anoxia-inducing conditions within agarose gel, without inducing colony formation indicative of transformed cells. We also demonstrate that, consistent with previous reports using other cell types [27, 28], Bcl-2 overexpression protects NSCs from the oxidative stress induced upon exposure to hydrogen peroxide. Furthermore, we report no changes in proliferation rate, therapeutic enzyme expression, trophic ability, or differentiation status of CD-NSCs upon overexpression of Bcl-2. While there have been previous reports that Bcl-2 overexpression may increase neuronal differentiation from E11.5 peripheral rat neural precursor cells [29], this was not observed for CD-NSCs. In contrast to primary NSCs, which differentiate in response to a myriad of external cues, the v-myc immortalization of CD-NSCs seems to stabilize their undifferentiated status. Bcl-2-expressing CD-NSCs survived longer than native CD-NSCs when transplanted into the brains of immunodeficient mice, as evidenced by prolonged firefly luciferase signal and significantly reduced active caspase-3 staining. We also demonstrate that improved CD-NSC survival translates to an improved therapeutic advantage in vivo. While the detected drug conversion levels did not increase, the tumor-derived luciferase signal and postharvest tumor volume measurements suggested delayed tumor progression when treated with Bcl-2-expressing NSCs.

As expected, we found no evidence that transient Bcl-2 expression transforms NSCs into tumor-initiating cells using either minicircle technology or adenoviral transduction. Though both vectors are categorized as nonintegrating, we still need to be conscious of their transformative potential if a Bcl-2 transgene were to hypothetically integrate into this v-myc immortalized CD-NSC line. Harui and coworkers demonstrated that the frequency of adenovirus integration into chromosomal DNA was around $10^{-7}$–$10^{-5}$ events per cell [30]. Estimates of episomal/plasmid integration rates are similar ($10^{-5}$) [31]. This implies that when implanting a clinical dose of 150 million CD-NSCs into patients [3], up to 150 NSCs may contain integrated DNA using either of these approaches. Thus, for cell therapies utilizing transient Bcl-2 overexpression to improve cell survival clinically, it may be prudent to incorporate a suicide gene as well. The results of this study suggest that the potential therapeutic benefit afforded by Bcl-2-mediated cell survival could outweigh the risks.

4. Conclusion

Tumor cells frequently take advantage of proteins within the apoptotic pathway to overcome a myriad of insults. We can learn from nature and manipulate this pathway within therapeutic donor cells in our efforts to combat the tumor. We provide evidence that transient overexpression of the anti-apoptotic protein, Bcl-2, within human CD-NSCs improves their resistance to transplant-associated insults. Their improved survival also translates to improved therapeutic outcomes in a xenograft orthotopic mouse model. Importantly, we observed no evidence that transient Bcl-2 overexpression transformed CD-NSCs, suggesting this approach is safe enough to merit further study. Together, these data highlight the importance of developing strategies that improve the survival of therapeutic NSCs and other cell-based therapies. These strategies will be critical to ongoing efforts to achieve improved therapeutic outcomes for glioma patients receiving NSC-mediated prodrug conversion therapies, as well as other stem cell treatments of CNS diseases.

5. Materials and Methods

5.1. Cell Culture. All cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio), 1% l-glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen) and maintained at 37°C in a humidified incubator (Thermo Electron Corporation) containing 6% CO₂. When cell reached 80% confluence, they were passaged using a 0.25% trypsin/EDTA solution (Invitrogen); media were changed every 2-3 days.

5.1.1. Glioma Cell Lines. Firefly luciferase-expressing U251 and PBT-017 (U251.fluc, PBT-017.fluc) and DsRed.U251 were provided by Dr. Christine Brown. U87 human glioma cell lines were obtained from the American Type Culture Collection. U87 cells were used to generate tumor cell-conditioned media by replacing culture media with serum-free media when cells were 80% confluent, followed by a 48-hour incubation

5.1.2. Neural Stem Cell Lines. The human, v-myc immortalized, HB1.F3 NSC line was obtained from Dr. Seung Kim (University of British Columbia) [32]. Extensive characterization studies have demonstrated the HB1.F3 line is chromosomally and functionally stable, nontumorigenic,
and minimally immunogenic (HLA class II negative [1, 33]). This cell line was further transduced with lentivirus to stably express either eGFP [20] and firefly luciferase [34] and used to track stem cell distribution in vivo.

5.2. Bcl-2 Expression Constructs

5.2.1. Adenoviral Transduction. Adenovirus vectors for Bcl-2 with cytomegalovirus promoters were purchased from Vector Biolabs. For NSC transduction, NSCs were plated in 6-well plates at 90% confluence without penicillin/streptomycin, 10% FBS, 2 μg/ml protamine-sulfate (Sigma-Aldrich), and viral particles at a multiplicity of infection of 20 which was determined empirically to result in the greatest number of Bcl-2 positive cells after 24 hours (Supplementary Figure 1). After 24 hours, the transduction media was removed, and complete transduction efficiency confirmed using flow cytometry and immunohistochemistry (Supplementary Figure 1). The percent cytotoxicity following transfection was determined as 100 × (number of nontransfected adherent NSCs – number of adherent transfected NSCs)/(number of nontransfected adherent NSCs). This is an indicator of cell viability following transfection. Each transduction was carried out in triplicate and repeated at least 2 times.

5.2.2. Minicircles

(1) Construct Generation. Commercially available minicircle vector backbones in the MC-easy minicircle production kit (System Bioscience) were used to generate eGFP.Bcl-2, Bcl-2.shRNA, and NT.shRNA minicircle constructs. The vector contains a multiple cloning sites and poly(A) tail flanked by attP and attB sites for PhiC31 integrase recombination and 32x Scel sites for bacterial backbone degradation, which is kanamycin resistant.

(2) Overexpressing Minicircles. The eGFP.Bcl-2 gene insert was excised from a commercially available plasmid pEMD-Bcl-2 (EMD Biosciences) using NheI and EcoR1 restriction sites. The purified insert was subcloned into a linearized pMC.CMV.MCS-EF1-GFP-SV40PolyA minicircle parental plasmid using the multiple cloning sites. The pMC_Bcl-2:eGFP plasmid was purified and transformed into ZYCY10P3ST2 E. coli. Minicircles were generated as per manufacturer’s instructions. Minicircle and insert size were verified by performing electrophoresis on diagnostic restriction enzyme digests.

(3) Knockdown Minicircles. The short hairpin shRNA inserts were purchased from Invitrogen (Bcl-2 fwd: 5′-GAT CCA ACA TCG CCC TGT GGA TGA CCT TCA AGA GAA GTC ATC CAC AGG GCG ATG TTT TTT TG-3′; Bcl-2 rev: 5′-GTT GTA GGC GGA CAC CTA CTT AAA GTT CTC TCC AGT AGG TGT CCC CCT GCT ACA AAA AAA CTT A-3′; Non-target: Fwd: 5′-GAT CCA ATT TCT CGA ACG TGT CAC GTT TCA AGA GAA CGT GAC AGC TCC GGA GAA TTT TTT TG-3′; rev: 5′-GTT AAG AGG CTT GCA CAG TGC AAA GGT CTC TTG CAC TGT GCA AGC CTC TTA AAA AAA CTT A-3′). These inserts were used to generate Bcl-2.shRNA and NT.shRNA minicircles.

(4) Minicircle Transfection. Before transfection, 9.0 × 10⁵ NSCs were seeded into individual wells of 6-well plates. After a 24-hour incubation in growth medium without penicillin/streptomycin, the cells were exposed to DNA-Lipofectamine LTX complexes that each contained 2.5 μg of minicircle plasmid DNA/well of cells. DNA-Lipofectamine LTX complexes were made by first diluting plasmid DNA and Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) in two independent 125 μl volumes of Opti-MEM medium (Invitrogen) without serum and mixed gently. After a 5 min incubation with the Plus Reagent at room temperature, the DNA and Lipofectamine LTX in Opti-MEM were combined and incubated for an additional 5 min at room temperature to allow the DNA-Lipofectamine LTX complexes to form. The DNA-Lipofectamine LTX complexes were then added to each well containing cells and medium. The vol/wt ratios of Lipofectamine LTX/DNA are shown in Supplementary Figure 2. The cells were incubated in transfection media for an additional 24 hours before efficiency analysis. Each transfection was carried out in triplicate and repeated at least 2 times.

5.3. Bcl-2 Expression Efficiency and Time Course Assessments

5.3.1. Flow Cytometry. At select timepoints, transfected cells were resuspended in PBS before analyzing on a GuavaCyte Flow Cytometer (GuavaCyte). Transduced cells were fixed and permeabilized (Fix & Perm Cell Permeabilization kit, Invitrogen, GAS 003) and incubated 40 min with Anti-Bcl-2 (cat number 138800, Invitrogen) then 20 min with goat anti-mouse Alexa-488 before flow cytometric analysis. Transfection efficiency was determined as the number of positive NSCs/total NSCs. Histograms were generated using FlowJo (Tree Star, Ashland, OR, USA).

5.3.2. Immunohistochemistry. Standard immunological techniques were employed. Briefly, plated cells were rinsed and fixed with 4% paraformaldehyde prior to blocking for 1 hour with immunoblot. Primary antibody was applied overnight at 4°C, then after rinsing, goat anti-mouse Alexa-546 was applied. After a 4-hour incubation, the cells were rinsed, stained with DAPI (Thermo Fisher), and mounted with fluorescence mounting medium (DAKO). Cultures were imaged using a Nikon Eclipse TE2000-U microscope equipped with a SPOT RT Slider digital camera (Diagnostic Instruments). Primary antibody omission served as negative controls, and no immunoreactivity was observed.

5.4. Bioactive Effects of Bcl-2-Modified HB1.F3.CD

5.4.1. In Vitro Insult Assays. Parental and transfected NSCs were cultured for 96 hours under increasing doses of H₂O₂. Resulting viability was assessed qualitatively using LIVE/DEAD kit (Life Technologies) and quantitatively measuring absolute ATP present in culture using CellTiter-
Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions.

5.4.2. Tumor Tropism. Modified Boyden chamber chemotaxis assays were performed using 24-well cell culture plates with polycarbonate inserts (pore diameter, 8 μm) (Millipore, Billerica, MA, USA) as described previously [35]. Conditioned media from U87 glioma and 5% BSA/DMEM were added to the lower chamber of wells (500 μl/well, triplicate samples). Inserts were placed into wells, and suspensions of parental or transduced NSCs were added to the upper chamber (1 × 10^5 cells/250 μl suspended in 5% BSA/DMEM to each well). After incubation (4 h, 37 °C), cells that did not migrate were removed from the inner surface of the filter. The membrane tray was then placed in a new lower chamber containing prewarmed Accutase (Sigma-Aldrich) for 10 min at 37 °C. Detached cells in the buffer were then transferred to a V-bottom 96-well plate and centrifuged (1500 rpm, 5 min). The buffer was aspirated, and cells were lysed with cell lysis buffer. The absolute amount of DNA present in 1 × 10^5 parental or transfected/transduced NSCs were added to the upper chamber (1 × 10^5 cells suspended in 5% BSA/DMEM to each well). After incubation (4 h, 37 °C), cells that did not migrate were removed from the inner surface of the filter. The membrane tray was then placed in a new lower chamber containing prewarmed Accutase (Sigma-Aldrich) for 10 min at 37 °C. Detached cells in the buffer were then transferred to a V-bottom 96-well plate and centrifuged (1500 rpm, 5 min). The buffer was aspirated, and cells were lysed with cell lysis buffer. The absolute amount of DNA present in 1 × 10^5 NSCs and cells were lysed with cell lysis buffer. The absolute amount of DNA present in 1 × 10^5 NSCs and cells were lysed with cell lysis buffer. The absolute amount of DNA present in 1 × 10^5 NSCs and cells were lysed with cell lysis buffer.

5.4.3. Differentiation. After four days of culture, cells were fixed in 4% paraformaldehyde, then standard immunocytochemical techniques were used to visualize cell-type-specific protein expression using the following primary antibodies: nestin (MAB 5326, Millipore), β-tubulin (PRB-435, Covance), and GFAP (AB5804, Millipore).

5.4.4. Prodrug-Converting Enzyme Expression
(1) Cytosine Deaminase. One day after transfection/transduction, NSCs were fixed and permeabilized and incubated 40 min with Anti-CD (BD Pharmingen, 557,862) then 20 min with goat anti-mouse FITC (BD Pharmingen, 557,862) before flow cytometric analysis. Histograms were generated using FlowJo (Tree Star, Ashland, OR, USA).

(2) Carboxylesterase. One day after transduction, CE enzyme activity was measured by conversion of o-nitrophenyl acetate substrate to o-nitrophenol and determined by spectrophotometry at 420 nm as previously described [36].

5.5. Bioactive Effects of Bcl-2-Modified HB1.F3.CD NSC In Vivo
5.5.1. Tumor Xenograft Model. Athymic nude mice were anesthetized with an intraperitoneal injection of 132 mg/kg ketamine and 8.8 mg/kg xylazine. Mice were then immobilized in a stereotactic apparatus and received stereotactically guided intracranial injections of cell suspension 2 mm lateral, 0.5 mm anterior to bregma, tracked from a depth of 2.5 mm to 2.25 mm to 2.0 mm; 0.667 μl of cell suspension was injected at each level, 2 μl total. Injections were performed with a 30-gauge 5 μl Hamilton syringe over 3–5 minutes. Two minutes elapsed before moving to the next injection level to minimize backflow through the needle track. After retracting the needle, bone wax was used to occlude the burr hole, and skin was closed with skin glue. To establish glioma xenografts, mice were injected U251.DsRed human glioma cells (5 × 10^6 cells) at 1 week prior to NSC.eGFP-fluc injections. In tumor-inoculated brains, intracranial injections contained 5 × 10^6 of parental or transduced/transfected NSCs injected into either the ipsilateral or contralateral hemisphere. Buprenorphine analgesic (0.05 mg/kg) was administered subcutaneously to relieve postoperative pain. Results were obtained from 3 independent experiments that resulted in 6 mice per group. All animal protocols were approved by the City of Hope Institutional Animal Care and Use Committee. Mice were euthanized consistent with the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association when they appeared to be in discomfort or distress as judged by independent animal care personnel. Mice were housed in an AAALAC-accredited facility and were given food and water ad libitum.

5.5.2. Xenogen Imaging. For a period of 4 days, firefly luciferase-expressing NSCs were imaged in mice using a charge-coupled device camera (Xenogen IVIS-100) coupled to the Living Image acquisition and analysis software. Mice were anesthetized with isoflurane gas then received an intraperitoneal injection of D-luciferin substrate (suspended in PBS at 4.29 mg/mouse). Images were captured while the mice were anesthetized by isoflurane (1.5 L/oxygen, 4% isoflurane) and kept in an induction chamber. Light emission was measured over an integration time of 5 minutes at 12 min after injection of luciferin. To account for baseline differences across animals, each animal’s recordings were standardized to the signal measured at day 0. Cell survival curves were generated using standardized data. A drop in flux intensity was interpreted as cell death, and this was confirmed with immunostaining.

5.5.3. Immunological Tissue Analysis. Mice were harvested by CO2 asphyxiation, then brains were removed and fixed by immersion in 4% paraformaldehyde for 24 h before sinking in 30% sucrose for 48 h. The tissues were frozen in Tissue Tek OCT (Sakura Finetek Europe B.V.) and sectioned sagittally on a cryostat (Leica 17–20). Sections (10 μm thick) were collected on positively charged slides (Thermo Fisher) for immunocytochemistry. Standard immunocytochemical techniques were used to visualize cell viability using the following primary antibodies: nestin (MAB 5326, EMD Millipore), eGFP (ab2980, Abcam), and active caspase-3 (AB3623, EMD Millipore).

5.5.4. 5-FC to 5-FU Conversion In Vivo. Two days after NSC injections, mice were administered intraperitoneal 5-FC (500 mg/kg) (Sigma). 2 hours later (at the peak of 5-FU conversion), brains were harvested and quartered. The quarter containing the tumor/NSC injection was used to determine concentrations of 5-FC and 5-FU by liquid chromatography-tandem mass spectrometry LC-MS/MS. LC-MS/MS analysis was performed using a Waters Acquity HPLC system (Waters Corp.) with a Waters Quattro Premier XE Mass Spectrometer. High-performance liquid
chromatography (HPLC) separation was achieved using a Synergi Hydro-RP 4 μm 150 × 2.0 mm analytical column (Phenomenex) preceded by a Phenomenex C 18 guard column. The column temperature was maintained at 30°C, and the flow rate was 0.4 ml/minute. The mobile phase consisted of A (20 mM ammonium acetate buffer, pH 3.5) and B (acetonitrile). The following gradient program was used: 20% B (hold, 0–3 minutes), 20%–68% B (3–6 minutes), 68% B (hold, 6–6.2 minutes), 68%–20% B (6.2–6.3 minutes), 20% B (hold, 6.3–8 minutes). The total run time was 8 minutes. The electrospray ionization source of the mass spectrometer was operated in positive ion mode with a cone gas flow rate of 80 liters/hour and a desolvation gas flow of 700 liters/hour. The capillary voltage was set to 0.6 kV, and the cone and collision cell voltages were optimized to 60 V and 23 eV for camptothecin (internal standard). The source temperature was 125°C, and the desolvation temperature was 450°C. A solvent delay program was used from 0 to 4.7 minutes and from 6.1 to 8 minutes to minimize the mobile phase flow to the source.

MassLynx (Waters Corp.) version 4.1 software was used for data acquisition and processing. Positive electrospray ionization of 5-FC and 5-FU produced abundant protonated molecular ions (MH+) at m/z 587.31, 393.21, and 349.15, respectively. Fragmentation of these compounds was induced under collision-induced dissociation conditions and acidic mobile phase. The precursor → product ion combinations at m/z 587.31 → 124.14 for 5-FU and 393.21 → 349.20 for 5-FC were used in multiple-reaction monitoring mode for quantitation. Under optimized assay conditions, the retention times for 5-FC and 5-FU were 5.25 and 5.62 minutes, respectively.

5.6. Therapeutic Advantage of Bcl-2-Modified HB1.F3.CD NSC In Vivo

5.6.1. Tumor Xenograft Model. The same xenograft model described in Section 5.5.1 was utilized with the following modifications: (1) U251.eGFP,fluc was used instead of U251.DsRed, (2) dil-labeled NSCs were used instead of NSC.eGFP,fluc, and (3) a mixture of 2e5 NSCs and 2e5 tumor cells was coinjected instead of injecting the NSCs 1 week after tumor implantation.

5.6.2. Treatment Schedule. Mice were administered 500 mg/kg 5-FC intraperitoneally 2 days after NSC tumor injections, and 5 mice per group were harvested for LC/MS/MS analysis of prodrg conversions (see Section 5.5.4). On days 5–9, mice received daily 500 mg/kg 5-FC administrations 2 days after surgery. Control mice were similarly injected with 1x PBS only.

5.6.3. Xenogen Imaging. Firefly luciferase-expressing tumor cells were imaged weekly for 3 weeks as described in Section 5.5.2. To account for baseline differences across animals, each animal’s recordings were standardized to the signal measured at day 0. A gain in flux intensity was interpreted as tumor cell growth, and this was confirmed with immunostaining.

5.6.4. Immunological Tissue Analysis. Three weeks after implantation, all brains were harvested and sectioned to visualize tumor volume as described above (see Sectio5.5.3). Standard immunocytochemical techniques were used to visualize tumor size and NSC distribution.

5.7. Tumorigenicity of Bcl-2-Modified HB1.F3.CD NSCs

5.7.1. In Vitro Colony Formation Assay. A standard soft agar colony-formation assay was used to assess cellular anchorage-independent growth in vitro. Human tumor cells (U251 glioma, 5637 bladder, and MCF7 breast, ATCC) and HB1.F3.CD NSCs (parental and Bcl-2 expressing) were encapsulated at 1e5 cells/ml within 0.5 mL 1% w/v agarose hydrogels cured in a 96-well plate. On day 0 or after culturing for 7 days in complete growth media, gels were incubated with Calcein-AM and ethidium bromide (Life Technologies) to visualize live and dead cells, respectively, then imaged using a confocal microscope (Zeiss). ImageJ software was used to count and size cells present in z-stacks compiled from 13 optical slices spaced 100 μm apart.

5.7.2. In Vitro Proliferation. NSC proliferation rates in vitro were determined by quantifying total DNA levels present in culture using the PicoGreen assay (Invitrogen) as per manufacturer’s instructions. Total ATP levels were also quantified with the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Data was obtained from 2 separate experiments involving 4–5 cultures per time point.

5.7.3. In Vivo Tumorigenicity. The same xenograft models described in Sections 5.5.1 and 5.6.1 were utilized. Brains were harvested 0, 1, 10, 21, and 60 days after NSC transplantation then sectioned as described in Section 5.5.3. Standard immunocytochemical techniques were used to visualize the number of proliferative NSCs using a PCNA primary antibody (MAB242, Chemicon). In addition, the tumorigenicity of Bcl-2-expressing NSCs was assessed by implanting 1 × 106 fluc.Bcl-2 NSCs into the brains of 3 nude mice lacking any tumor burden.

5.8. Statistical Analysis. Data are presented as mean ± SEM unless otherwise stated. Statistical significance was determined using a two-tailed Student’s t-test (*p < 0.05) unless otherwise stated.

Conflicts of Interest

All authors declare no competing interests with the exception of Dr. Aboody who declares an interest in TheraBiologics, Inc., an early stage biotechnology company focused on employing neural stem cells to treat cancer.

Authors’ Contributions

Rachael Mooney is responsible for conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing. Asma Abdul Majid is responsible for collection and assembly of data and data analysis and interpretation. Daniel Mota, Adam He, Linda Flores, Jennifer...
Covello-Batalla, Diana Machado, Joanna Gonzaga, and Soraya Aramburo are also responsible for the collection of data. Karen S. Aboody is responsible for data interpretation, financial support, and final approval of the manuscript.

Acknowledgments

This work was funded provided by STOP Cancer, The Rosalind and Arthur Gilbert Foundation, California Institute of Regenerative Medicine, the Alvarez Family Foundation, the Anthony F. & Susan M. Markel Foundation, the Daphna and Richard Ziman Family Foundation, the Ben and Catherine Ivy Foundation, the Ladies Auxiliary of the Veterans of Foreign Wars, the Accelerated Brain Cancer Cures Foundation, City of Hope, and National Institute of Health (Grants R01 CA198076, R01 FD004816-01A1, U01NS082328-01, R43 CA86768, R44 CA8678, and P30 CA033572). The authors would also like to acknowledge the City of Hope Analytical Pharmacology Core for performing the drug conversion measurements.

Supplementary Materials

Additional flow cytometry and viability quantification are provided showing the percent of NSCs that are Bcl-2 (+) after adenoviral transduction at increasing multiplicities of infection (Supplementary Figure 1). We further provide maps and electrophoretic confirmation of minicircle construct generation and flow cytometric quantification of the percent of NSCs that are eGFP (+) and Bcl-2 (+) (Supplementary Figure 2). We also provide evidence that Ad.Bcl-2 NSCs do not exhibit impaired activity of the prodrug-converting enzyme, carboxylesterase (Supplementary Figure 3). Finally, we provide additional support that Bcl-2-mediated enhancements in posttransplantation NSC viability translated to improved antitumor efficacy in a patient-derived glioma model (Supplementary Figure 4). (Supplementary Materials)

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