Development of next-generation tumor-homing induced neural stem cells to enhance treatment of metastatic cancers

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Engineered tumor-homing neural stem cells (NSCs) have shown promise in treating cancer. Recently, we transdifferentiated skin fibroblasts into human-induced NSCs (hiNSC) as personalized NSC drug carriers. Here, using a SOX2 and spheroidal culture-based reprogramming strategy, we generated a new hiNSC variant, hiNeuroS, that was genetically distinct from fibroblasts and first-generation hiNSCs and had significantly enhanced tumor-homing and antitumor properties. In vitro, hiNeuroSs demonstrated superior migration to human triple-negative breast cancer (TNBC) cells and in vivo rapidly homed to TNBC tumor foci following intracerebroventricular (ICV) infusion. In TNBC parenchymal metastasis models, ICV infusion of hiNeuroSs secreting the proapoptotic agent TRAIL (hiNeuroS-TRAIL) significantly reduced tumor burden and extended median survival. In models of TNBC leptomeningeal carcinomatosis, ICV dosing of hiNeuroS-TRAIL therapy significantly delayed the onset of tumor formation and extended survival when administered as a prophylactic treatment, as well as reduced tumor volume while prolonging survival when delivered as established tumor therapy.

INTRODUCTION

Despite advances in clinical care, metastatic brain cancer remains a major medical challenge. Breast cancer is the second most common cancer to metastasize to the brain (1). Among breast cancers, triple-negative breast cancer (TNBC) has the highest incidence of central nervous system (CNS) metastasis, affecting nearly 30% of patients (2). However, the options to treat TNBC brain metastasis are limited due to the lack of targetable surface markers on these tumor cells. Radiation treatment, surgical resection, or chemotherapy remains the standard of care (3, 4), but the median survival after diagnosis of CNS disease is less than 5 months (3, 5, 6). CNS metastases can be further subcategorized to parenchymal metastases (PMs) or leptomeningeal carcinomatosis (LC) depending on the site of tumor infiltration (7). Although the incidence rate of LC in all patients with breast cancer is only about 5% (8), the median overall survival for patients with breast cancer LC is as little as 4 weeks (7). Treatment typically consists of a multimodal approach that includes systemic or intrathecal chemotherapy and radiation, but there is no generally accepted standard of care currently available for the treatment of breast cancer LC (9). Effective therapies are urgently needed to improve outcomes for patients with TNBC CNS disease and, particularly, LC.

During the past 20 years, engineered neural stem cell (NSC)–mediated therapies have emerged as a promising therapeutic strategy against brain tumors in preclinical and clinical studies (10–13). Leveraging their innate tumor tropism, engineered NSCs have been shown to migrate through the brain, homing to tumor foci and delivering therapeutic payloads to induce effective tumor killing. Recently, the emergence of advanced cell reprogramming technology has led to the generation of induced NSCs (iNSCs). Generated by transdifferentiating somatic fibroblasts directly into NSCs without the need for a pluripotent stage (14–16), the ease of isolation and the ability to provide an autologous therapy to avoid immune rejection suggest that iNSC technology may offer advantages over other cell carriers. We previously provided the first results that showed the potential of iNSCs as tumor-homing drug carriers capable of regressing glioblastoma (GBM) xenografts (17). Using a defined set of transcription factors, we previously reprogrammed human and mouse fibroblasts into iNSCs that homed to GBM cells with a similar velocity as brain-derived NSCs and migrated through the brain, tracking invasive human brain cancer cells (17, 18). Genetically engineered iNSCs released the proapoptotic agent tumor necrosis factor–α–related apoptosis-inducing ligand (TRAIL) (hiNSCTR) at levels equal to cortical-derived NSCs and killed cocultured brain cancer cells (18). In orthotopic xenograft models, hiNSCSTR therapy reduced solid human brain cancer foci, suppressed postsurgical brain cancer, and more than doubled median survival of tumor-bearing mice (17).

Here, we report the development of a second-generation iNSC delivery platform. We provide the first evidence that a neurosphere-based culture system creates human-induced neurosphere (hiNeuroS) with a unique genetic profile and significantly enhanced antitumor capabilities. Single-cell genomic profiling revealed that hiNeuroSs were enriched for a unique set of tumor-homing gene pathways, and in vitro kinetic migration studies showed that hiNeuroSs rapidly migrate to tumor cells faster than first-generation iNSC (hiNSC). Using intracerebroventricular (ICV) delivery as a clinically compatible infusion route and mouse models of human TNBC PM, we found that hiNeuroSs populate solid metastatic brain foci at more than...
twice the level of hiNSC while persisting for over 45 days, nearly fourfold longer than first-generation cells. Real-time kinetic tracking showed that the hiNeuroS delivery of TRAIL reduced TNBC PM over fourfold, significantly extended median survival, and allowed 33% of animals to survive long term, while control and hiNSC-treated mice died within 30 days. Using a mouse model of human LC disease, prophylactic hiNeuroS therapy markedly delayed the development of TNBC LC with 50% of mice surviving over 80 days after treatment. hiNeuroS therapy also reduced the progression of established LC foci while doubling the survival of tumor-bearing animals. Together, we provide the first evidence that a novel hiNeuroS therapy may be a highly effective strategy for the treatment of metastatic brain cancer.

RESULTS

hiNeuroS generation and characterization

The tumor-homing properties of iNSCs are the most unique and beneficial aspect of this therapy. We previously found that growing cells in clusters improves migratory capabilities (19). This suggested that a second-generation iNSC with improved functionality could be created by cluster-based culture. To create a second-generation tumor-homing iNSC, we adapted our established single factor SOX2 method to create iNSC clusters that were serially passed through multiple rounds of sphere formation and dissociation (Fig. 1A). Human fibroblasts (NHF-1) were transduced with lentivirus encoding SOX2 under the tetracycline-inducible promoter reverse tetracycline-controlled transactivator (rtTA). Cotransduction with lentiviral vectors encoding optical reporters (mCherry-firefly luciferase; LV-mC-FLuc) or the cytotoxic agent TRAIL [LV-TRAIL–green fluorescent protein (GFP)] were then used to create diagnostic or therapeutic variants. Reprogramming and sphere formation were simultaneously initiated by transferring the cells to laminin-coated flasks and culturing in ReNCell NSC growth media. Two weeks after transduction, we detected clusters of cells that continued to increase in size and number through a month of culturing (Fig. 1, B and C). Unexpectedly, imaging showed that the TRAIL spheres required more time to reach the same size and number as the diagnostic cells. However, the expression of SOX2 and the NSC marker Nestin were detected in both sphere variants (Fig. 1, D and E), suggesting the successful generation of NSC-like cells, which we termed hiNeuroS.

Previously, we and others have shown that a continuum of genetic changes occurs as fibroblasts are converted into iNSCs through the transdifferentiation (TD) process (15–17, 20). To understand the

Fig. 1. hiNeuroS generation and characterization. (A) Schematic of hiNeuroS generation process with SOX2 single-factor cell reprogramming system. (B) Bright-field (BF) and mCherry fluorescence images of hiNeuroS-mC-FLuc spheres generation process. Scale bar, 200 μm. (C) BF and GFP fluorescent images of hiNeuroS-TRAIL sphere generation process. Scale bar, 200 μm. (D) Fluorescent images showing both hiNeuroS-mC-FLuc spheres and hiNeuroS-TRAIL spheres stained for SOX2. Scale bar, 100 μm. (E) Fluorescent images showing both hiNeuroS-mC-FLuc spheres and hiNeuroS-TRAIL spheres stained for Nestin. Scale bar, 100 μm.
transcriptomic profile of cells during our sphere-based generation of hiNeuroS, we performed single-cell RNA sequencing (scRNA-seq) and real-time polymerase chain reaction (PCR) analysis. By ordering cells from different samples along a developmental trajectory, we found that, while some of the hiNeuroS cells shared a similar trajectory states with the fibroblasts, there was a unique cell group at the far end of hiNeuroS trajectory toward the latest pseudotime circled in red (Fig. 2, A and B). Pseudotemporal gene expression analysis clustered genes with similar gene expression pattern over pseudotime and showed that the genes in cluster 1 had relatively high expression levels for the most of the “time” but gradually down-regulated toward the end, while the genes in cluster 2 had the opposite expression pattern (Fig. 2C). Functional annotations revealed that cluster 1 actually contained more fibroblast-related genes, while cluster 2 contained more NSC-related genes, verifying the cell type transition from fibroblast to hiNeuroS within the cell reprogramming process. This conclusion was also confirmed by the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the two clusters, which showed that genes in cluster 1 were enriched for the GO terms related to fibroblasts, whereas those in cluster 2 were enriched for the terms related to NSCs (Fig. 2D).

We next explored transcriptional differences between hiNeuroS and the parental fibroblast at individual gene level to reveal the expression differences among several key signaling pathways not only related to NSC property but also mediating cell tumor-homing migration. We also compared the gene expression of these key biologic parameters between hiNeuroS and hiNSC to uncover differences and improvements between the original hiNSC carrier and our new sphere-based carriers. Although the expression of many genes was conserved between fibroblasts and hiNeuroS, clear and distinct transcriptional changes were present in hiNeuroS. hiNeuroS also showed transcriptional changes that were distinct from the older first-generation hiNSC, suggesting that the new neurosphere-based culture system has created a different cell product (Fig. 3, A and B). We also found that hiNSCs and hiNeuroSs all expressed classic markers of brain-derived NSCs, yet the expression patterns differed substantially between hiNSC and hiNeuroS (Fig. 3C). Tumor-homing migration is one of the most unique and critical aspects of NSC/iNSC cell therapy. When we focused our analysis on cell migration, as well as stemness and proliferation pathways, we found a list of genes associated with these pathways that were activated in hiNeuroS at high levels, but these same genes showed little or no activation in parent fibroblasts or hiNSC (Fig. 3D). Tumor-homing migration is mediated by the binding of soluble factors released from the tumor to surface receptors on NSC/iNSCs. When we analyzed the expression of surface receptor genes involved in NSC tumor tropism (11–14, 21), we found that the CXCR4, FLT1, IL6R, MET, and PLAUR were all expressed in hiNeuroS at levels 1.8- to 20-fold higher than in hiNSC or fibroblasts (Fig. 3E). These data suggest that our new neurosphere-based culture method generated hiNeuroS cells that are a genetically distinct NSC subpopulation.

**Fig. 2.** hiNeuroS cell reprogramming dynamics. (A) Single-cell trajectory analysis revealing the formation of a unique cell group toward the far end of trajectory (circled in red). (B) Bar graph showing the proportion of different cell subgroups within hiNeuroS or fibroblast samples at different pseudotime periods. (C) Pseudotemporal gene expression analysis revealing the trend of down-regulation of fibroblast-related genes and up-regulation of NSC-related genes along the trajectory. (D) GO and KEGG enrichment analyses showing that the unique cell group at the end of the trajectory had more NSC-related functions and less fibroblasts related functions.
that expresses tumor-homing pathways associated genes at levels significantly greater than hiNSC.

Together, these results suggest that our new sphere-based TD method has generated a distinct, second generation of iNSC. In comparison with the first generation (hiNSC), hiNeuroSs have a unique NSC-related gene expression profile with markedly enhanced expression of genes related to NSC tumor tropism. As this suggests that hiNeuroSs may represent a more effective anticancer therapy, we next investigated the homing and efficacy of this new cell both in vitro and in vivo.

Assessing hiNeuroS migration capability toward TNBC brain metastasis in vitro

To investigate whether the improved gene expression profile of hiNeuroS translated into enhanced tumor tropism, we performed in vitro two-dimensional coculture cell migration assays using real-time imaging to monitor cell motion over 72 hours (Fig. 4A). The fluorescent images showed that, compared to controls, hiNeuroSs directionally migrated toward tumor cells within 72 hours (Fig. 4B) but not toward normal fibroblasts (Fig. S2). When compared to hiNSCs, greater numbers of hiNeuroSs crossed the starting line at both 48 and 72 hours, with more than twice as many hiNeuroSs migrating to tumors at the later time point (Fig. 4C). We next performed single-cell motion analysis to define the direction and rate of migration of hiNeuroSs.

Compared to hiNSC, hiNeuroSs migrated nearly 1.5 times as fast with more than 1.2 times the directional motion, allowing the cells to cover nearly two times the displacement of hiNSC (Fig. 4, D to F). In addition, we investigated how both hiNSCs and hiNeuroSs could have subpopulations of particularly fast migratory cells. We defined “fast migrating cells” as those that...
had a final displacement of ≥250 μm. Single-cell motion analysis and quantification revealed that 87% of hiNeuroS were “fast migrating cells” compared to only 40% of hiNSCs (Fig. 4, G and H). Together, these data suggest that hiNeuroS not only are tumoritropic but also demonstrate enhanced tumor tropism in vitro compared to hiNSCs.

Investigating the in vivo kinetics of hiNeuroS therapy following ICV infusion

In the clinical setting, ICV infusion is used to deliver chemotherapeutic agents across the blood-brain barrier (BBB) and to facilitate whole-brain delivery. To leverage the advantage of ICV route of administration, we chose to administer our cell therapy via ICV in vivo. To determine the homing and persistence of hiNeuroS therapy following ICV infusion in vivo, hiNeuroS-mC-FLuc cells were ICV-infused 1 week after TNBC tumor implantation in the brain parenchyma of mice (Fig. 5A). The fluorescent images of postmortem brain sections revealed that hiNeuroS extravasated from the ventricular system and migrated to the tumor site in as little as 3 days after ICV infusion, where the cells persisted for at least 2 weeks (Fig. 5C). Although a similar cell tumor-homing pattern was observed for hiNSC after ICV infusion (Fig. 5B and fig. S3), the hiNSC/hiNeuroS-to-tumor ratio for fluorescence intensity showed that twofold more hiNeuroS reached the tumor foci than hiNSC at all days 3, 7, and 14 (Fig. 5D). We next investigated the persistence of hiNeuroS using the same in vivo models. Serial bioluminescence imaging (BLI) showed that first-generation hiNSCs were rapidly cleared with the hiNSC signal dropping below detection within 11 days after infusion (Fig. 5, E and F). hiNeuroS showed a markedly different pattern of persistence. The quantification of the BLI suggested that the average hiNeuroS level (2.6 × 10^7) was significantly greater than hiNSCs (1.3 × 10^5) at 2 weeks after
In particular, the hiNeuroS signal increased significantly 4 days after infusion and then declined gradually with cells remaining visible through 45 days after transplant by BLI in a subset of mice (Fig. S4). Together, these results suggest that hiNeuroSs exhibit enhanced tumor homing and extended persistence compared to first-generation cell carriers. As these beneficial properties may allow hiNeuroS to achieve robust therapeutic effects, we next explored the efficacy of hiNeuroS therapy in different models of aggressive metastatic brain cancer.

Exploring the efficacy of hiNeuroS-based therapy in treating TNBC brain PM by ICV infusion

After showing that hiNeuroSs were able to rapidly populate the tumor foci following ICV infusion with long-term persistence, we next assessed the therapeutic effect of ICV-infused hiNeuroS therapy. We began by testing hiNeuroS therapy in vitro using hiNeuroS-TRAIL cells. mCherry-labeled TNBC brain metastasis tumor cells were cocultured with hiNeuroS-TRAIL cells at increasing ratios, and real-time fluorescence images were collected every 24 hours for 3 days. We found that hiNeuroS-TRAIL cells reduced the MDA-MB231-Br signal in a dose-dependent manner with the highest ratio of hiNeuroS-TRAIL to MDA-MB231-Br ratio (2:1) reducing the MDA-MB231-Br signal by 50% within 24 hours, while the lowest ratio of 1:10 showed >75% reduction in tumor cells compared to control after 72 hours (Fig. 6, A and B, and fig. S5).

To explore the efficacy of hiNeuroS-TRAIL therapy in vivo, we stereotactically implanted MDA-MB231-Br-mC-FLuc cells into the brain parenchyma of mice. Three days after tumor implantation, hiNeuroS-TRAIL, saline control, or first-generation hiNSC-TRAIL cells were infused in the contralateral ventricle. Serial tumor BLI showed that a single dose of hiNeuroS-TRAIL delivered ICV-reduced tumor volumes fourfold at day 27 after infusion and increased median survival over control mice from 38 to 52 days (HR, 0.23; 95% CI 0.05 to 0.98) (Fig. 6, C, D, and F). Thirty-three percent of the mice treated with hiNeuroS-TRAIL therapy were still alive at the end of the study (Fig. S6). In contrast, no significant tumor suppression or survival extension was observed following ICV-infused hiNSC-TRAIL therapy, suggesting that the improved genomics and persistence of hiNeuroS led to enhanced tumor kill (Fig. 6, E and G). Together, these data suggest that ICV hiNeuroS can have a significant therapeutic effect in treating TNBC brain PM.

Evaluating ICV-infused hiNeuroS-based therapy in TNBC LC

LC is one of the most lethal forms of breast-to-brain metastases, with patients with LC surviving as little as 4 weeks after diagnosis (7). In LC, metastatic tumor cells spread through the leptomeningeal space following ICV infusion. Scale bar, 100 μm. (B) Fluorescent images of brain cross sections showing the presence of hiNeuroS (red) at tumor foci (green) at different time points after ICV infusion. Scale bar, 100 μm. (C) Bar graph showing hiNeuroS-mC or hiNSC-mC to MDA-MB231-Br-GFP ratio for fluorescence intensity at different time points after ICV infusion. n = 6 technical replicates. (D) Representative BLI images showing in vivo persistence of hiNeuroS and hiNSC following ICV infusion. (F) Bar graph of ICV-infused hiNeuroS or hiNSC BLI signal in vivo over time. n = 5 for both hiNeuroS and hiNSC groups. *P < 0.05, **P < 0.01, and ***P < 0.001.

Fig. 5. hiNeuroS and hiNSC in vivo migration and persistence following ICV infusion. (A) Schematic of in vivo migration assay toward MDA-MB231-Br brain parenchymal tumor. (B) Fluorescent images of brain cross sections showing the presence of hiNeuroS (red) at tumor foci at different time points after ICV infusion. mCherry channel presented only. Scale bar, 100 μm. (C) Fluorescent images of brain cross sections showing the presence of hiNeuroS (red) at tumor foci (green) at different time points after ICV infusion. Scale bar, 100 μm. (D) Bar graph showing hiNeuroS-mC or hiNSC-mC to MB231-Br-GFP ratio for fluorescence intensity at different time points after ICV infusion. n = 6 technical replicates. (E) Representative BLI images showing in vivo persistence of hiNeuroS and hiNSC following ICV infusion. (F) Bar graph of ICV-infused hiNeuroS or hiNSC BLI signal in vivo over time. n = 5 for both hiNeuroS and hiNSC groups. *P < 0.05, **P < 0.01, and ***P < 0.001.
surrounding the brain and the spinal cord, forming multifocal tumors. There is an urgent need for treatments that improve targeting the tumors established in the leptomeninges or floating tumor cells before they can engraft. On the basis of the promising results of hiNeuroS-TRAIL therapy in models of TNBC PM, we next sought to assess the potential of ICV-delivered hiNeuroS-TRAIL therapy to treat TNBC LC. To mimic the spread of cancer cells within cerebrospinal fluid and subsequent seeding throughout the leptomeningeal space, MDA-MB231-Br-mC-FLuc cells were stereotactically infused into the cisterna magna of mice. To investigate hiNeuroS treatment of established LC tumors, we delivered therapeutic hiNeuroS-TRAIL cells or saline control via ICV infusion 3 days after tumor infusión to allow tumor foci to form (Fig. 7A). In control-treated mice, serial BLI showed that the intracisternal injection generated tumors within the brain and spinal cord, which quickly expanded more than 122- and 61-fold in the brain and spinal cord, respectively, in 17 days (Fig. 7, B and E to H). The fluorescence microscopy of postmortem tissue sections 14 days after tumor injection confirmed that tumors spread throughout multiple regions of the brain (Fig. 7C) and across all three regions of the spinal cord of control-treated animals (Fig. 7D). Animals succumbed to the aggressive and diffuse tumors 25 days after infusion (Fig. 7I). In contrast, serial imaging and postmortem tissue sections showed that ICV infusion of hiNeuroS-TRAIL cells markedly attenuated tumor progression (Fig. 7, B to D). Despite the diffuse and aggressive nature of the tumors, ICV infusion of hiNeuroS-TRAIL cells reduced the established tumor volumes 30-fold at 7 days after treatment (10 days after tumor implant) in the brain and reduced tumor in the spine below detection through 17 days after implant (Fig. 7, E to H, and fig. S7). ICV-delivered hiNeuroS-TRAIL therapy doubled survival with hiNeuroS-TRAIL–treated animals surviving a mean of 47 days (Fig. 7I).

To model prophylactic hiNeuroS-TRAIL therapy for LC, therapeutic hiNeuroS-TRAIL cells were ICV-delivered first followed by intracisternal infusion of MDA-MB231-Br–mC-FLuc cells into the cisterna magna 7 days later. Serial imaging and postmortem tissue sections showed that prophylactic ICV hiNeuroS-TRAIL cells significantly delayed tumor formation in both brain and spine (Fig. 7, B to H) and extended median survival to nearly 70 days (Fig. 7I). Within this treatment group, 33% of the animals showed no detectable signs of tumor growth in either brain or spine through 70 days after treatment (fig. S8), and 50% of the animals remained alive 3 months after infusion (Fig. 7I). Together, these data demonstrate the potential of ICV infusion of hiNeuroS-TRAIL therapy in treating or preventing TNBC LC.

DISCUSSION

In this study, we successfully developed hiNeuroS, a second generation of iNSC therapy created by direct TD of human fibroblasts using a SOX2 single-factor cell reprogramming system. In comparison with the first-generation hiNSC, hiNeuroS demonstrated a distinct genetic profile, enhanced tumor tropism, and significantly improved in vivo persistence. In therapeutic models, ICV-infused hiNeuroS therapy significantly attenuated the progression of both solid PM and LC metastatic disease.

In the past two decades, numerous studies have proved the effectiveness of using NSCs as drug delivery system to combat primary and secondary brain tumors (10, 17, 18, 22–27). While endogenous “off-the-shelf” NSCs and NSCs created by cellular reprogramming are the major two cell sources, the latter has therapeutic advantages because it opens up the possibility for personalized iNSCs transdifferentiated from a patient’s own cells, allowing for potentially reduced immunogenicity and increased persistence (11, 14). Now,
the most commonly used methods to generate iNSCs from somatic fibroblasts involve either indirect cell reprogramming with the use of classic Yamanaka reprogramming factors (OCT4, SOX2, KLF4, and c-MYC) or direct TD by introducing lineage-specific transcription factors \(^{16}\). Compared to indirect cell reprogramming, direct TD has a significantly reduced risk of teratoma formation during the cell generation process in vitro or after cell administration in vivo by circumventing the induced pluripotent stem cell stage; this makes direct TD-derived iNSC cell therapy a safer variant \(^{15}\). We have generated both the first and second generations of TD-derived iNSCs, hiNSC, and hiNeuroS through the direct TD process by using SOX2 as the sole cell reprogramming factor. While our data show that both hiNSC and hiNeuroS are positive for typical NSC markers (SOX2 and Nestin) \(^{17}\), hiNeuroSs have a substantially enhanced transcriptomic profile in terms of migration and stemness and have demonstrated better functional performance. The differential gene expression profile of hiNeuroSs, especially the genes related to stemness, compared to hiNSCs could attribute to a few reasons. First, the spheroid culture system used to generate hiNeuroSs may play a critical role in the promotion and maintenance of cell stemness in hiNeuroSs. Several studies have shown the advantages of spheroid stem cell culture system in comparison with monolayer stem cell culture system in terms of promoting the expression of genes related to not only stemness but also cell migration and proliferation \(^{28,29}\). Second, the long-term cell selection cycles during the hiNeuroS generation process may have potentially imposed selective pressure on the cell population, which contributed to the enrichment of the cells with high stemness. In addition, the different culture media used to generate hiNeuroS and hiNSC (ReNcell media versus neural induction media) may also drive the differential gene expression in stemness.

The enhanced tumor tropism of hiNeuroS suggests its potential as a NSC-mediated drug delivery vehicle. Although the exact underlying mechanisms involved in NSC chemotaxis toward brain tumors are still being investigated, many studies have indicated that SDF-1/CXCR4, uPA/uPAR (PLAUR), VEGF/VEGFR1 (FLT1), HGF/c-Met, SCF/c-Kit, and MCP-1/CCL2 are major signaling pathways involved in NSC tumor tropism, especially toward gliomas \(^{10,12-14}\). Zhao et al.
metastasis, the current standard of care is limited to systemic chemotherapy. In treating TNBC patients with brain tumors, ICV-infused iNSC therapy is the first in human trials. In this study, however, we have made several key observations: (i) the potential role of the migratory genes specifically in hiNeuroS but not hiNSCs, including CD44 (30), CD44 (31, 32), and MMP3 (33). The up-regulation of these genes also likely contributes to the enhanced tumor tropism observed in hiNeuroS. We are continuing to explore the precise contribution of each pathway to the tumor homing process in ongoing studies.

Another key factor in the success of iNSCs is the in vivo persistence of this drug delivery vehicle, as increased persistence can significantly extend the time of cytotoxic payload production and release, thereby improving therapeutic efficacy (23, 34, 35). As potential personalized therapies, hiNeuroS and hiNSC would reduce the need of immunosuppressive drugs, which are typically used for allogeneic transplants to extend the persistence of the cells in a patient (36). In our mouse models, we showed that, while a single dose of hiNSCs could persist in the brain for around 2 weeks after ICV infusion, a single ICV infusion of hiNeuroS cells were detectable for more than 45 days. The BLI signal of hiNeuroS increases for a week following cell administration, suggesting that the hiNeuroS cells were able to proliferate for a few days in vivo. This phenomenon may be the result of cerebrospinal fluid-induced proliferation of NSCs (37). NSC proliferation-related genes that were up-regulated specifically in hiNeuroS population, such as MYC (38), may also contribute to this short-term cell proliferation after ICV infusion. Our in vitro study has shown greater hiNeuroS cell proliferation compared to hiNSC (fig. S9), but we are further characterizing the proliferative state of hiNeuroS at long-term time points in vivo. We are also studying different cyto-ablation “switches” to ablate cells, a potentially important safe mechanism as hiNeuroS being moving toward first in human trials.

ICV infusion was chosen as the preferred route of administration because it is a clinically established and extensively used CNS drug administration route with the advantages including BBB circumvention and whole brain coverage (39, 40). While ICV infusion has been primarily used to deliver chemotherapy to CNS tumors, its potential application for cell therapy delivery remains relatively unexplored (41–43). ICV-infused CAR-T cell therapies for GBM and atypical teratoid/rhabdoid tumors (ATRTs) treatment are the only studies we have found so far using ICV as cell therapy administration route to treat brain cancers (44, 45). Other studies have demonstrated the migratory but not therapeutic potential of ICV-infused stem cells for brain cancer treatment (42, 43, 46). To the best of our knowledge, we are not only the first to report the tumor homing of iNSCs toward TNBC brain PM tumor foci following ICV infusion but also the first to demonstrate a significant antitumor effect from ICV-infused iNSC therapy. In treating TNBC patients with brain metastasis, the current standard of care is limited to systemic chemotherapy, radiotherapy, and surgical resection, and no effective targeted therapy exists (5, 47). In this study, however, we have made TNBC brain PM “targetable” with our newly developed second-generation iNSC therapy. By following the chemoattractants secreted by tumors and their microenvironment (10), ICV-infused hiNeuroS cells were able to rapidly populate the TNBC brain PM tumor foci. To test the therapeutic efficacy of hiNeuroS-mediated therapy, we genetically engineered our drug delivery vehicle to secrete TRAIL, as TRAIL is generally not harmful to healthy cells (48–53). With a single dose of ICV-infused hiNeuroS-TRAIL cells, we found that we were able to significantly suppress tumor growth in the brain parenchyma and extend the median survival by 37%. Although hiNSC cells were also observed around the tumor foci following ICV infusion, no significant therapeutic effect was observed, likely due to the smaller number of hiNSCs that homed to the TNBC brain tumor and their shorter persistence in vivo.

After we determined the therapeutic potential of hiNeuroS-TRAIL to treat TNBC parenchymal brain tumors, we explored their use in the treatment of TNBC LC, a late-stage metastatic complication of TNBC where tumor cells invade to the leptomeningeal space surrounding the brain and spinal cord (54). Intrathecal chemotherapy, delivered by ICV infusion or lumbar puncture, has been one of the most important treatment modalities to treat TNBC LC. However, no intrathecal chemotherapy agent has consistently demonstrated a superior clinical outcome; moreover, intrathecal chemotherapy-associated neurotoxicity has limited its application (55, 56). In this study, we used ICV infusions to administer our hiNeuroS-TRAIL cells for the treatment of TNBC LC. Our data showed that ICV-infused hiNeuroS-TRAIL cells rapidly eliminated the tumor cells in the spine, and no tumor relapse was observed in the treatment mice until 6 days after the first death occurred in the control group. On the day of the first death in the control group, the average tumor volume in the brains of treated mice was reduced by 95%. Moreover, the median survival for the treatment group almost doubled with a single dose of ICV-infused therapy. Furthermore, ICV-delivered hiNeuroS therapy has the potential not only to treat established LC but also prevent tumor formation. Prophylactic cranial irradiation is a well-known prevention strategy for children with acute lymphoblastic leukemia (57) or patients with small-cell lung cancer (58) or aggressive lymphoma (59), which has resulted in a decrease of CNS relapse from as high as 66% to only approximately 5% (57, 60). By adopting this concept of treating tumor cells in the CNS before they can become established metastases, we also evaluated our hiNeuroS-TRAIL therapy in a prophylactic setting. We found that, with a single dose of ICV-infused hiNeuroS-TRAIL cells 7 days before tumor challenge, we were able to significantly delay the tumor progression with a 2.8-fold extension of median survival. Leptomeningeal tumor relapse was prevented in both brain and spine in one-third of the treated mice. While this significant therapeutic effect is observed with only a single dose of a single-therapeutic agent, tumor rebound was observed in some mice 2 weeks after treatment, likely due to the clearance of hiNeuroS-TRAIL cells over time. In clinic, repeated ICV drug infusion has been realized using well-tolerated devices such as the Ommaya reservoir (61). Therefore, such devices could also be used for the redosing of hiNeuroS-TRAIL cells in the clinical setting to further improve the long-term therapeutic effect of hiNeuroS-TRAIL–based therapy.

In conclusion, these studies provided strong evidence for the therapeutic potential of a second generation, single factor–reprogrammed iNSC therapy with significantly enhanced tumor-homing capability and prolonged persistence. We provide the first evidence showing
the tumor-homing capability of ICV-infused iNSCs toward TNBC brain parenchymal tumors and the significant therapeutic effect of hiNeuroS-TRAIL therapy following ICV infusion. In addition, we were also the first to show the potential of ICV-infused iNSCs to treat or prevent TNBC LC. While we have used TRAIL as the monotherapeutic agent delivered by hiNeuroS, the hiNeuroS-based delivery system can be expanded to other therapeutic cargos, such as nanoparticles, oncolytic viruses, and other gene therapies. Moreover, devices such as the Ommaya reservoir enables redosing with multiple therapeutic agents carried by hiNeuroS over time to overcome tumor resistance to a single agent. Depending on the chemokine-activator secreted by the tumor cells, hiNeuroS-based therapy has the potential to treat other types of brain cancer besides breast-to-brain metastasis, such as GBM. Our first-generation iNSCs have shown significant therapeutic effect in treating GBM with different treatment strategies (17). We believe this second-generation therapeutic system with enhanced migratory capability, persistence, and proliferating propensity could potentially further improve the therapeutic outcome in GBM models. To conclude, hiNeuroS therapy has shown remarkable promise as a future treatment for patients with TNBC CNS metastatic disease.

MATERIALS AND METHODS

Study design

This preclinical study explores the potential of developing a second-generation–induced human NSC (hiNeuroS) cancer therapy using SOX2 single factor–mediated cell reprogramming system and a neurosphere growth system. We hypothesized that, in comparison with our first-generation hiNSC, hiNeuroS had improved tumor tropism, persistence, and therapeutic efficacy against TNBC brain metastases. We used scRNA-seq and quantitative PCR (qPCR) to characterize hiNeuroS cells and their tumor-homing capabilities at the genetic level. hiNeuroS tumor-homing property was also evaluated by both in vitro and in vivo functional tests, including in vitro real-time cell migration assay and in vivo tumor-homing assay. The tumor-killing capability of hiNeuroS secreting the cytotoxic protein TRAIL was tested in two different TNBC brain metastases animal models, where hiNeuroS-TRAIL was ICV-delivered to treat TNBC parenchymal xenograft or to treat or prevent TNBC LC. TNBC brain metastases tumor volumes in both treatment and control groups were followed from treatment versus control groups. Institutional Animal Care and Use Committee guidelines were followed for all animal studies.

Cell lines

The hiTERT-immortalized human fibroblast line NHF-1 was purchased from the University of North Carolina at Chapel Hill Tissue Culture Facility. The TNBC brain metastasis cell line MDA-MB-231-Br was obtained through material transfer agreement (MTA) (T. Yoneda). MDA-MB-231-Br-GFP-NLuc (Nano Luciferase) cells or MDA-MB-231-Br-mCherry-FLuc (Firefly Luciferase) cells were generated by transducing MDA-MB-231-Br cells with LV-GFP-NLuc or LV-mCherry-FLuc, respectively. Both cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Millipore), 1% (v/v) penicillin-streptomycin (Gibco), and 0.2% (v/v) Plasmocin.

hiNSC generation

NHF-1-hTERT human fibroblasts were transdifferentiated, as described previously (17). Briefly, 1 × 10⁵ NHF-1-hTERT cells were seeded in six-well plate supplemented with 10% FBS DMEM media overnight. The next day (day 0), cells were transduced with LV-GFP-TRAIL, LV-mCherry-FLuc, or LV-GFP-NLuc for 24 hours in 2% FBS DMEM media supplemented with polybrene (8 μg/ml) (Thermo Fisher Scientific). The cells were transduced again (day 1) with a cocktail of LV-SOX2 and LV-rtTA in 2% FBS DMEM media supplemented with polybrene (8 μg/ml) for 24 hours. Before use, 1 × 10⁶ cells were seeded in T-175 flask with regular 10% FBS DMEM media overnight. Starting from day 2, cells were cultured in STEMdiff neural induction media (STEMCELL Technologies) with doxycycline (2 μg/ml; Sigma-Aldrich), and media was replenished every other day. Cells were detached with Accutase cell detachment solution (STEMCELL Technologies) for use on day 6.

hiNeuroS generation

NHF-1-hTERT human fibroblasts were transduced with LV-GFP-TRAIL or LV-mCherry-FLuc, followed by the cocktail of LV-SOX2 and LV-rtTA by using the same method described above. Following transduction, 1 × 10⁵ cells were seeded in six-well plates with supplemented 10% FBS DMEM media overnight. Starting from day 2 after the initial transduction, cells were cultured in ReNcell NSC maintenance medium (Sigma-Aldrich) supplemented with epidermal growth factor (20 ng/ml) (EGF; Gemini Bio-Products), fibroblast growth factor (FGF; Sigma-Aldrich), and doxycycline (2 μg/ml; Sigma-Aldrich). EGF, FGF, and doxycycline were replenished every other day. After 1 to 2 weeks, initial spheres were collected from the media by centrifugation, resuspended to a six-well plate precoated with laminin (10 mg/ml in PBS; Sigma) overnight at 37°C, and cultured in supplemented ReNcell medium. Cells were allowed to attach to the laminin-coated plate overnight, and the following morning, nonadherent cells were washed away with PBS. Spheres were allowed to regenerate in the supplemented ReNcell medium. After floating spheres were formed again, the same sphere collection and reattachment process was repeated thrice before spheres were fully expanded for use. For in vitro and in vivo studies, spheres were broken down into a single-cell suspension with Accutase before use.

hiNeuroS immunohistochemistry staining

hiNeuroS-TRAIL or hiNeuroS-mCherry-FLuc were collected in 0.7-ml microcentrifuge tubes. Spheres were fixed with 10% neutral buffered formalin (v/v; Fisherbrand) and permeabilized in PBS-T [0.1% (v/v); Triton X-100, MilliporeSigma] with 1% (w/v) bovine serum albumin (Sigma-Aldrich). Spheres were then incubated with anti-SOX2 [1:200 (v/v); Abcam] or anti-Nestin [1:200 (v/v); MilliporeSigma] antibodies for 1 hour at room temperature. Spheres were washed with PBS thrice and then incubated with CF555 Goat Anti-Rabbit [1:1000 (v/v); Biotium] or Alexa Fluor 488 goat anti-rabbit [1:1000 (v/v); Invitrogen] secondary antibodies for 1 hour. After that, spheres were washed with PBS thrice and stained with Hoechst (Thermo Fisher Scientific) before being mounted to
microscope slides (FisherBiotech). Fluorescent images were acquired with Olympus IX71 fluorescence microscope. Images were processed using Fiji ImageJ.

**In vitro two-dimensional iNSC migration studies and analysis**

Two-chamber cell culture inserts (Ibidi) were placed in 24-well plate precoated with laminin (10 mg/ml in PBS) overnight at 37°C. A single-cell suspension of 1 × 10^4 hiNSC-mCherry-FLuc or hiNeuroS-mCherry-FLuc was seeded on the left side of the insert in supplemented neural induction media with 2% FBS or supplemented ReNcell medium with 2% FBS, respectively. The 1 × 10^3 MB-231-Br-GFP-NLuc or plain NHF-1 cells were seeded on the right side of the insert in 2% PBS DMEM. Twenty-four hours after cell seeding, inserts were removed, and wells were gently washed with PBS thrice. Then, wells were filled with supplemented neural induction media or supplemented ReNcell medium for hiNSC groups and hiNeuroS groups, respectively. Cell motion images were acquired at ×10 magnification every 2 hours with EVOS FL Auto Cell Imaging System (Life Technologies) for 72 hours. Because of the suboptimal adherence of MB231-Br cells in neural induction media or ReNcell medium, the tumor cells closest to the insert edges were disrupted during the insert removal and PBS wash steps. While most of the tumor cells remained, this disruption limited the possibility of capturing the images with both iNSCs and tumor cells present in the same 10× field of view. For cell movement tracking analysis, four beads per group were tracked. Within each bead, 10 hiNSC cells or hiNeuroS cells were randomly selected, and their cell migration motion was individually tracked over time (Fiji ImageJ). Individual cell motion tracks were plotted, and cell migration directionality, final migration displacement, total migration distance, and migration velocity were calculated as previously described (18). The number of cells that migrated across the starting line defined by the leading hiNSC cell or hiNeuroS cell in each beacon at time 0 was manually counted to determine the total number of migrating cells.

**In vitro hiNeuroS-TRAIL tumor-killing assay**

MB-231-Br-mCh-FLuc cells (5 × 10^4) were seeded in 24-well plate in 10% DMEM. The following day, a varying number of hiNeuroS-TRAIL cells were added to the wells with different hiNeuroS-TRAIL-to-MB-231-Br-mCh-FLuc ratio ranging from 20:10 to 1:10. Real-time images were collected every 2 hours with EVOS FL Auto Cell Imaging System (Life Technologies) for 72 hours. The tumor cell viability was measured by quantifying mCherry fluorescence signal with Fiji ImageJ.

**In vitro iNSC proliferation assay**

hiNSC-mCh-FLuc or hiNeuroS-mCh-FLuc cells (5 × 10^4) were seeded in 4 × 12-well plate in neural induction media or ReNcell medium, respectively. To study cell proliferation, serial BLI was performed at days 0, 1, 3, and 7. At each time point, 15 μl of d-luciferin working solution (15 mg/ml) was added to each well of one of the four 12-well plates. After 10 min, photon emission was measured using the AMI HT system. BLI data were analyzed with Aura software (Spectral Instruments Imaging).

**In vivo BLI**

To track the tumor growth of MB231-Br-mCherry-FLuc tumor growth or the persistence of hiNSC-mCherry-FLuc and hiNeuroS-mCherry-FLuc, serial BLI was performed. Mice were administered d-luciferin (3 mg per mouse in 200 μl of PBS) by intraperitoneal injection. After 10 min, photon emission was measured using the AMI HT system. BLI data were analyzed with Aura software.

**Tissue processing**

Following sacrifice, mice were perfused with intracardiac PBS and 10% formalin. Brain and/or spine were harvested and incubated in 10% formalin overnight. The next day, samples were transferred to 30% sucrose solution for 2 days before being frozen in optimal cutting temperature (OCT) media. Brain and spine samples were sectioned using Leica CM1850 cryostat at 12 and 30 μm, respectively. Sectioned tissue samples were collected on microscope slides, stained with Hoechst (Thermo Fisher Scientific), and mounted with ProLong Gold Antifade Mountant (Invitrogen).

**In vivo iNSC tumor-homing studies following ICV infusion**

MB231-Br-GFP-NLuc cells (5 × 10^4) in 3 μl of PBS were implanted in the right hemisphere of the brain in athymic nude mice. One week following tumor implantation, a single-cell suspension of 5 × 10^5 hiNSC-mCherry-FLuc or hiNeuroS-mCherry-FLuc in 5-μl PBS was infused to the left ventricle of the mouse brain. The brains of two mice from each group were harvested at days 3, 7, and 14 after ICV infusion and were processed and sectioned for imaging. Once tumor was visible on tissue sectioning, every three slice was collected at thickness of 12 μm with six sections per slice. Fluorescent images were acquired using Olympus IX71 fluorescence microscopy at ×10 magnification. Representative fluorescence images at different time points were chosen from the middle slide for both groups. Images were processed using Fiji ImageJ. For fluorescence quantification and analysis, one sectioned brain sample was chosen from each of the three middle slides for each brain sample (n = 3 technical replicates for each animal). Both tumor (GFP) and hiNeuroS/hiNSC (mCherry-FLuc) fluorescence intensity were quantified, and hiNeuroS/hiNSC-to-tumor ratio for fluorescence intensity was then calculated.

**In vivo iNSC persistence studies**

A single-cell suspension of 5 × 10^4 hiNSC-mCherry-FLuc or hiNeuroS-mCherry-FLuc in 5 μl of PBS were infused into the left ventricle of athymic nude mice brain. To track the persistence of infused cells, serial BLI was performed using AMI HT system.

**ICV-infused therapy studies in TNBC PM model**

MB231-Br-mCherry-FLuc cells (5 × 10^4) in 3 μl of PBS were implanted in the right hemisphere of the brain in athymic nude mice. Three days after tumor implantation, a single-cell suspension of 5 × 10^5 hiNSC-TRAIL or hiNeuroS-TRAIL in 5 μl of PBS was infused into the left ventricle of the mouse brain. PBS (5 μl) was instead infused for the control group. To track the tumor growth, serial BLI was performed using AMI HT system. Mice were sacrificed when body weight dropped by more than 20%, and survival data were recorded.

**ICV-infused hiNeuroS therapy study in TNBC leptomeningeal metastasis model**

To establish TNBC leptomeningeal metastasis animal model, 2 × 10^6 MB231-Br-mCherry-FLuc cells in 5 μl of PBS were implanted to the cisterna magna of athymic nude mice by intracisternal injection as previously described (62). Seven days before or 3 days after
tumor implantation, a single-cell suspension of $5 \times 10^5$ hiNeuroS-TRAIL in 5 µl of PBS was infused into the left ventricle. Tumor growth in both the mouse brain and spinal cord was monitored by serial BLI using the AMI HT system. Mice were sacrificed when body weight dropped by more than 20%, and survival data were recorded. Two weeks after tumor implantation, one mouse from each group was sacrificed. Mice brain and spine were harvested, processed, and sectioned as described above. Fluorescent images were acquired using Olympus IX71 fluorescence microscopy and Olympus FV3000RS confocal microscopy. Images were processed with Olympus CellSens software.

scRNA-seq study and analysis

scRNA-seq were performed for hiNeuroS-TRAIL, hiNSC-TRAIL, and NHF-1 cells using 10x Genomics Chromium System in the University of North Carolina at Chapel Hill Advanced Analytics Core. Specifically, a single-cell suspension of different samples was prepared using Acutase cell detachment solution (STEMCELL Technologies). A total of 1777, 3510, and 4357 cells from hiNeuroS-TRAIL, hiNSC-TRAIL, and NHF-1 cells were captured. To ensure the data quality, the genes expressed in less than three cells and the cells expressing ≤ 200 genes were first filtered out from each dataset. Furthermore, the cells expressing ≥ 9000, 6500, and 6000 genes in hiNeuroS-TRAIL, hiNSC-TRAIL, and NHF-1 cells, respectively, were considered as putative doublets and excluded from the downstream analysis. To compare the three samples, the high-quality cells from the three samples were integrated together using Seurat v3 (63) to get rid of the impact of batch effect. Differentially expressed genes (DEGs) among hiNeuroS-TRAIL, hiNSC-TRAIL, and NHF-1 cells were detected using the FindMarkers function in Seurat. GO and KEGG enrichment analyses were performed for the DEGs using clusterProfiler (64). To further identify and characterize the difference in cell manifold among hiNeuroS-TRAIL, hiNSC-TRAIL, and NHF-1 cells, trajectory analysis was performed using the integrated data of the three samples in Monocle2 (65). Genes differentially expressed along the temporal trajectory were identified using differentialGeneTest function in Monocle2, with a q value threshold of 0.05.

Real-time PCR

The total RNA from hiNeuroS-mC-FLuc, hiNSC-mC-FLuc, and NHF-1 was extracted using the PureLink RNA Kit (Thermo Fisher Scientific). The expression level of selected genes was tested using Custom TaqMan Array Standard Plate (Thermo Fisher Scientific) and measured by QuantStudio 3 (Applied Biosystems). Data analysis was performed on QuantStudio (Thermo Fisher Scientific) software.

Statistical analysis

Student’s $t$ test was used when comparing two groups, while one-way analysis of variance (ANOVA) was used when comparing more than two groups. For longitudinal studies, repeated measure two-way ANOVA analysis was performed. For survival analysis, log-rank (Mantel-Cox) test was performed. Data were expressed as means ± SEM. Differences were considered significant when $P < 0.05$.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/24/eabf1526/DC1

View request a protocol for this paper from Bio-protocol.
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Development of next-generation tumor-homing induced neural stem cells to enhance treatment of metastatic cancers

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