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Fibrin matrices enhance the transplant and efficacy of cytotoxic stem cell therapy for post-surgical cancer

Juli R. Bagó^{a,*}, Guillaume J. Pegna^{a,*}, Onyi Okolie^a, and Shawn D. Hingtgen^{a,b,c}

^aDivision of Molecular Pharmaceutics, UNC Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, 27599-USA

^bBiomedical Research Imaging Center, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, 27599-USA

^cLineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, 27599-USA

Abstract

Tumor-homing cytotoxic stem cell (SC) therapy is a promising new approach for treating the incurable brain cancer glioblastoma (GBM). However, problems of retaining cytotoxic SCs within the post-surgical GBM resection cavity are likely to significantly limit the clinical utility of this strategy. Here, we describe a new fibrin-based transplant approach capable of increasing cytotoxic SC retention and persistence within the resection cavity, yet remaining permissive to tumorotropic migration. This fibrin-based transplant can effectively treat both solid and post-surgical human GBM in mice. Using our murine model of image-guided model of GBM resection, we discovered that suspending human mesenchymal stem cells (hMSCs) in a fibrin matrix increased initial retention in the surgical resection cavity 2-fold and prolonged persistence in the cavity 3-fold compared to conventional delivery strategies. Time-lapse motion analysis revealed that cytotoxic hMSCs in the fibrin matrix remain tumorotropic, rapidly migrating from the fibrin matrix to co-localize with cultured human GBM cells. We encapsulated hMSCs releasing the cytotoxic agent TRAIL (hMSC-sTR) in fibrin, and found hMSC-sTR/fibrin therapy reduced the viability of multiple 3-D human GBM spheroids and regressed established human GBM xenografts 3-fold in 11 days. Mimicking clinical therapy of surgically resected GBM, intra-cavity seeding of therapeutic hMSC-sTR encapsulated in fibrin reduced post-surgical GBM volumes 6-fold, increased time to recurrence 4-fold, and prolonged median survival from 15 to 36 days compared to control-treated animals. Fibrin-based SC therapy could represent a clinically compatible, viable treatment to suppress recurrence of post-surgical GBM and other lethal cancer types.

* Authors contributed equally to this study

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1. Introduction

Glioblastoma (GBM) is the most common primary brain tumor, and one of the deadliest forms of cancer^{1–3}. Invasive GBM cells escape into the non-diseased brain, making complete surgical resection impossible. Small molecule chemotherapies are unable to reach invasive GBM foci. As a result, GBM is incurable and median survival remains only 12–15 months⁴. Engineered stem cell (SC) therapies are a promising treatment strategy for GBM^{5,6}. SCs have the unique ability to seek out GBM, migrating to solid and diffuse GBM deposits. A variety of preclinical studies have shown that SCs genetically engineered with cytotoxic agents eradicate solid GBM and markedly extend survival^{5,7–12}. As a result, SC-therapy for GBM entered human patient testing where tumoricidal SCs are delivered into the walls of the surgical cavity following resection of recurrent GBMs⁸.

Despite the central role of surgical tumor resection in clinical GBM therapy and SC treatment, solid GBM models have been the mainstay of preclinical SC therapy testing⁵. We used a mouse model of GBM resection to discover that SCs directly injected in suspensions are rapidly lost from the post-surgical cavity and cytotoxic SC therapy fails to suppress the recurrence of post-surgical minimal GBM^{13,14}. We found that transplanting cytotoxic SCs in hydrogel scaffolds could restore intra-cavity SC retention (i.e.: increase the number of cells present 3 hrs post-implant) and tumor killing. Thus, there is significant interest in new matrix material that is clinically compatible and highly effective at transplanting cytotoxic SCs to eradicate residual GBM foci and delay tumor recurrence. Despite this urgent need, different matrix material for cytotoxic SC therapy is virtually unexplored.

Fibrin scaffolds, prepared by the combination of fibrinogen and thrombin proteins, were among the earliest biomaterials used to prevent bleeding and create cellular scaffolds. Fibrin is a natural biopolymer that forms a scaffold to promote cell attachment during wound healing¹⁵. Unlike slowly forming extracellular matrices, fibrin scaffolds rapidly assemble into three-dimensional branching fibers following the cleavage of fibrinogen polypeptides by activated thrombin. The biological and mechanical properties of fibrin gels have led to their extensive use in clinical patient surgery and for a variety of bioengineering applications¹⁶. TISSEEL (Baxter Healthcare Corp., Deerfield, IL.) is a clinically approved fibrin sealant that is widely used to control bleeding and stop cerebral spinal fluid leaks during surgical GBM resection in human patients¹⁷. Clinical studies have shown that TISSEEL fibrin glue is biocompatible and biostable in the brain of human patients¹⁸. Importantly, these properties have led to the use of fibrin scaffolds for tissue engineering of adipose, skin, liver, and bone tissues¹⁹, delivery of therapeutic molecule for various diseases^{19,20}, and brachytherapy treatment of brain tumors^{21–23}.

We undertook the first studies exploring a fibrin scaffold-based approach to cytotoxic SC therapy for cancer using the FDA approved fibrin sealant TISSEEL. Here, we show that packing cytotoxic SCs in fibrin matrices increased both the retention and persistence of cytotoxic SCs in the post-surgical GBM resection cavity. Yet, the fibrin scaffolds remained permissive to SC tumorigenic migration, allowing cells to rapidly exit the matrix and co-localize with GBM cells. Using different models of GBM, we found that pre-formed fibrin/cytotoxic SC patches were effective against solid GBM, while intracavity fibrin/cytotoxic

SC therapy showed significant anti-tumor effects against surgically resected orthotopic GBM xenografts. These results begin to define a new approach to cancer therapy, delivering cytotoxic SC therapies from fibrin matrices to treat both solid and surgically resected GBM.

2. Materials and Methods

2.1. Cell lines

U87, LN229, and U251 human GBM cells (American Type Culture Collection, Manassas, VA, USA) and human hMSC (Texas A&M Institute for Regenerative Medicine, TX, USA) were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/mL penicillin, 100 µg/mL streptomycin. Characterization of hMSC was performed by Texas A&M Institute for Regenerative Medicine and confirmed differentiation capacity to bone and fat as well as expression of hMSC markers was confirmed by flow cytometry.

2.2. Lentiviral vectors

The following lentiviral vectors were used in this study: green fluorescence protein fused to firefly luciferase (LV-GFP-FLuc), green fluorescence protein fused to Renilla luciferase (LV-GFP-RLuc), mCherry protein fused to firefly luciferase (LV-mC-FLuc), a secreted variant of TNF α -related apoptosis-inducing ligand (TRAIL, LV-sTR), and Gaussia luciferase fused to TRAIL (LV-diTR). GFP-RLuc and GFP-FLuc were constructed by amplifying the cDNA encoding Renilla luciferase or firefly luciferase using the vectors luciferase-pcDNA3 and pAC-hRLuc (Addgene) respectively. The restriction sites were incorporated in the primers, and the resulting fragment was digested with BglII and SalI, and ligated in frame in BglII/SalI digested pEGFP-C1 (Clontech, Mountain View, CA, USA). The GFP-FLuc or GFP-RLuc fragments were digested with AgeI (blunted) and SalI, and ligated into pTK402 (provided by Dr. Tal Kafri, UNC Gene Therapy Center) digested with BamHI (blunted) and XhoI to create LV-GFP-FLuc or LV-GFP-RLuc. Similarly, mCFLuc was created by amplifying the cDNA encoding firefly luciferase from luciferase-pcDNA3, ligating into BglII/SalI digested mCherry-C1 (Clontech), and ligating the mC-FLuc fragment into pTK402 LV backbone using blunt/XhoI sites. To create LV-sTR and LV-diTR, the cDNA sequence encoding sTR or diTR was PCR amplified using custom-synthesized oligonucleotide templates (Invitrogen, Carlsbad, CA, USA). The restriction sites were incorporated into the primers, the resulting fragment was digested with BamHI and XhoI, and ligated in-frame into BamHI/XhoI digested pLVX plasmid (a kind gift from Dr. Scott Magness, UNC Department of Medicine, USA). Both LV-sTR and LV-diTR have IRES-GFP (internal ribosomal entry sites-green fluorescent protein) elements in the backbone as well as CMV-driven puromycin element. All LV constructs were packaged as LV vectors in 293T/17 cells using a helper virus-free packaging system as described previously^{12,24}. GBM and hMSC were transduced with LVs at varying multiplicity of infection (MOI) by incubating virions in a culture medium containing 5 µg/ml protamine sulfate (Sigma) and cells were visualized for fluorescent protein expression by fluorescence microscopy. TISSEEL Fibrin Sealant (Baxter Healthcare Corp., Deerfield, IL, USA) was purchased from the University of North Carolina Hospitals and Clinics.

2.3. Seeding in fibrin

Fibrin patches were created under sterile conditions using Sealer and Thrombin preparations from TISSEEL Fibrin Sealant (purchased from the University of North Carolina Hospitals and Clinics). To prepare the fibrin scaffolds, eight microliters of Sealer solution (67 – 106 mg/mL fibrinogen) were mixed with 100,000–500,000 hMSCs. Eight microliters of the Thrombin (400 – 625 units/mL thrombin) preparation was added to initiate gelation and physically mixed using a micropipette tip for 30 seconds to create droplets. Fibrin patches were created by flattening the droplets through physical pressure to a thickness of approximately 1 mm. Fibrin patches loaded with cells were then cultured under standard conditions with supplemented DMEM (10% FBS, 1% P/S, 1% L-glutamine).

2.4. Scanning electron microscopy (SEM)

SEM images were captured using field emission scanning electron microscopy (FESEM JEOL 6400 F) at 15 kV and 200 kV accelerating voltage, respectively. Cell seeded samples were fixed in 10% buffered formalin for 30 min and dehydrated using a graded concentration (50–100% v/v) of ethanol. Drying of fibrin scaffolds was accomplished by immersion in hexamethyldisilazane for 15 min followed by air-drying overnight under a fume hood. Dried samples were then sputter coated with gold to observe the morphology of fibers and the attached hMSCs using FESEM.

2.5. Cell viability and TRAIL secretion in vitro

To define the viability of hMSCs in scaffolds, hMSC-GFPFLuc (1×10^5 cells/scaffold) were encapsulated in fibrin droplets as described or seeded directly into wells without encapsulation. On days 0, 2, 5 and 9 after seeding, high-resolution fluorescent images were captured using an Olympus MVX-10 microscope and bioluminescent images were captured by incubating cells in D-luciferin (1.5 $\mu\text{g}/\text{ml}$) and measuring luciferase activity using an IVIS[®] Kinetic imaging system (Perkin Elmer, Waltham, MA, USA). Cell growth was determined by quantification of the fluorescent signal intensity using NIH Image or from luciferase activity using the IVIS[®] Kinetic image analysis software and expressed as p/sec/cm²/sr. Arbitrary color bars represent standard light intensity levels (blue= lowest; red = highest). Each experiment was performed in triplicate.

To determine TRAIL secretion, hMSC-diTR (1×10^5 cells/scaffold) were seeded as describe above. On days 0, 2, 5 and 9 after seeding, equal volumes of cell culture medium containing the secreted diTR fusion proteins was collected, incubated with coelenterazine (1 $\mu\text{g}/\text{ml}$), and photon emission was determine using an IVIS[®] Kinetic imaging system (Perkin Elmer, Waltham, MA)

2.6. Time-lapse imaging and motion analysis

A 0.6% agarose mold was prepared to mimic brain tissue as described previously²⁵. 3 ml of the agarose solution was added to each well of 6-well culture plates and allowed to solidify. A 2ml aspirating pipette attached to a vacuum was used to create cavities in the agarose ~500 μm apart. hMSC-GFPRLuc embedded in fibrin were placed in one of the agarose cavities. Human U87-mCFLuc cells were seeded in the adjacent hole or the cavity was left empty and the wells were filled with media. The cell/agarose system was placed in a

VivaView live cell imaging system (Olympus) and allowed to equilibrate. Fluorescent images were captured at 10x magnification every 20 minutes for 64 hours in 6 locations per well (to monitor sufficient cell numbers). Experiments were conducted in triplicate. NIH Image was then used to generate movies and to define the migratory path of hMSCs, as well as the directionality, displacement and velocity of hMSC migration using the “Manual Tracking” and “Chemotaxis Tool” plugins.

2.7. Co-culture viability assays

hMSC-sTR or hMSC-GFRLuc (3×10^5) were seeded in fibrin scaffolds as described above. Human U87, Ln229, and U251 GBM cells expressing mCherry-FLuc (2×10^5 cells) were plated around the hMSCs seeded in fibrin. 3-D co-culture assays were performed to mimic *in vivo* characteristics by forming 3-D cell spheroids using the bio-assembler kit (Nano3D Biosciences, Houston, TX, USA) according to manufactures specifications. Briefly, GBM cells in a 6 well plate with an 80% of confluence, were treated overnight with 72 μ l of nanoshuttle magnetic particles. The next day, cells were detached with trypsin and plated in an ultra-low attachment 6-well plate. A magnetic driver of 6 neodymium magnets (field strength = 50 G) designed for 6-well plates were placed atop the well plate to levitate the cells to the air-liquid interface and cultured for an additional 18–24 hrs to form spheroids. Finally, the spheroids were plated adjacent to hMSC/fibrin and viability was measured by luciferase assay 48 hrs later as described previously¹². To determine the impact of reduced hMSC cell number on GBM killing, decreasing numbers of hMSC-sTR or control hMSCs were encapsulated in fibrin (1×10^2 – 1×10^4 cells/scaffold) and seeded with U87 human GBM spheroids (5×10^3 cells). Tumor cell viability was determined 24 hrs later by luciferase assay. To determine the efficacy of hMSCs engineered with different cytotoxic therapies, hMSCs expressing thymidine kinase (ThermoFisher Scientific, Waltham MA) were seeded with U87 or U251 tumor spheroids as before. Therapy was initiated by the addition of ganciclovir (GCV; UNC Hospitals) to the culture media (10 μ g/ml). GBM cell viability was assessed 72 hrs later using a luciferase-based assay.

2.8. In vivo models

Stem cell persistence—To investigate the transplant of fibrin-encapsulated stem cells in the post-surgical GBM cavity, U87-mCFLuc were harvested at 80% confluency. Nude mice (6–8 weeks of age; Charles River Laboratories, Wilmington, Massachusetts, USA) were implanted stereotactically with the U87-mCFLuc (5×10^5 cells) in the right frontal lobe 2 mm lateral to the bregma and 0.5 mm from the dura (n=12). For tumor resection, mice were immobilized on a stereotactic frame and placed under an Olympus MVX-10 microscope connected to a Hamamatsu ORCA 03G CCD camera. A midline incision was made in the skin above the skull exposing the cranium of the mouse. The intracranial xenograft was identified using mCherry fluorescence. A small portion of the skull covering the tumor was surgically removed using a bone drill and forceps and the overlying dura was gently peeled back from the cortical surface to expose the tumor. The U87-mCFLuc tumor was surgically excised using a combination of surgical dissection and aspiration under mCherry excitation. Fluorescent images of mCherry+ GBM were continuously captured to assess accuracy of image-guided surgical resection. Following tumor removal, hMSC-GFPFLuc (1×10^6 cells) were divided into two groups (n=6/group) and: 1) suspended in 10 μ l of saline and directly

injected into the walls of the cavity, or 2) seeded into the resection cavity in fibrin matrices. The skin was closed with surgical glue. hMSC levels were determined by serial bioluminescence imaging. Mice were injected intraperitoneally with D-luciferin (4.5 mg/ml in 150 μ l saline) and photon counts were measured 5 minutes after injection over 7 mins using the IVIS[®] Kinetic imager.

To determine the initial retention of hMSCs within the surgical cavity, hMSC-hMSC-GFPFLuc (1×10^6 cells) were directly injected into the walls of the surgical cavity or encapsulated in fibrin matrices and seeded into the interior of the surgical cavity. Bioluminescence imaging was performed 3 hours post-implantation to determine the levels of hMSCs retained within hours of injection, and photon emission was expressed relative to fibrin implant.

To determine the persistence of hMSC transplanted hMSCs, hMSC-GFPFLuc (1×10^6 cells) were directly injected into the walls of the surgical cavity or encapsulated in fibrin matrices and seeded into the interior of the surgical cavity. Serial bioluminescence imaging was performed 2, 4, 7, 10, 14, 21, and 28 days post-implantation.

Photon emission was determined using the IVIS[®] Kinetic imaging analysis software and expressed as p/sec/cm²/sr relative to day 0. All experimental protocols were approved by the Animal Care and Use Committees at The University of North Carolina at Chapel Hill and care of the mice was in accordance with the standards set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, USDA regulations, and the American Veterinary Medical Association.

Solid tumor therapy—Nude mice were anesthetized and U87 GBM cells (3×10^5 cells) were injected into the para-spinal space of the mouse in 100 μ l of PBS (2 independent injection sites per animal, (n=8). One week later, fibrin scaffolds bearing hMSC-sTR (n=4) or control hMSC-GFPRLuc (n=4) were surgically implanted over the established tumors. Tumor growth was determined by serial FLuc imaging and image analysis as described above, expressed as p/sec/cm²/sr relative to day 0.

Post-surgical minimal GBM therapy—To investigate the efficacy of hMSC-sTR encapsulated in fibrin for treatment of post-operative GBM, U87-mCFLuc (5×10^5 cells) were implanted into the frontal lobe of mice as described above. Established GBMs were surgically resected under image-guidance and hMSC-sTR or hMSC-GFPRLuc in fibrin were seeded into the resection cavity (n=6/group). Tumor regrowth was monitored by serial FLuc imaging as described earlier.

2.9. Tissue processing

Immediately after the last imaging session, mice were sacrificed, perfused with formalin, and brains extracted. The tissue was immediately immersed in formalin. 30 μ m coronal sections were generated using a vibrating microtome (Fisher). Sections were washed three times with PBS and visualized using a confocal microscope (Olympus). In a subset of mice, brains were isolated and incubated with or without D-luciferin, and ex vivo whole-brain bioluminescent and fluorescent imaging was performed using the IVIS[®] Kinetic system.

2.10. Statistical analysis

Data were analyzed by Student t test when comparing 2 groups and by ANOVA, followed by Dunnetts post-test when comparing greater than 2 groups. Data were expressed as mean $\pm$ SEM and differences were considered significant at $P < 0.05$. Survival times of mice groups ($n=5/\text{group}$) were compared using logrank test.

3. Results

3.1 Generation and characterization of fibrin scaffolds bearing engineered stem cells

3.1.1 Characterizing cytotoxic hMSCs encapsulated in fibrin scaffolds—To first characterize cytotoxic SCs encapsulated in fibrin scaffolds, human mesenchymal stem cells (hMSCs) engineered to express GFP and firefly luciferase (hMSC-GFPFLuc) were suspended in the fibrin component of the medical-grade fibrin sealant TISEEL (approved by the US Food and Drug Administration, STN 103980). The matrix was rapidly polymerized by addition of thrombin, encapsulating the hMSCs within the scaffold (Fig. 1A). Scanning electron microscopy showed the hMSC/fibrin matrix displayed a cross-sectional thickness of $175\text{ }\mu\text{m}$ with cells clearly attached to the fibrin (Fig. 1B). We next investigated the impact fibrin encapsulation on the growth of engineered SCs. Fluorescent and bioluminescent imaging (BLI) showed hMSC-GFPFLuc proliferated in the fibrin scaffolds 9-fold over 9 days, and there was no statistically significant difference in growth rate between encapsulated cells and cells grown in standard well plates (Fig. 1C–D). 2x magnification fluorescent images show the growth (increase in GFP signal) at a low magnification, while the 10x images show the increase in MSC number within the fibrin at a higher resolution. To validate the fluorescence images, bioluminescence imaging of the same cell populations are shown in the bottom panels. Summary graphs of these data are shown in Figure 1D and expressed relative to no scaffold controls.

An effective SC therapy must release cytotoxic agents to kill cancer cells. Scaffold architecture could dramatically alter the level of drug escaping the matrix. To investigate the impact of fibrin matrices with different architectures on SC secretion, we engineered hMSCs with a diagnostic fusion between TRAIL and Gaussia luciferase (hMSC-diTR) that we have previously utilized to detect differences in drug release between stem cell types¹². Equal numbers of the hMSC-diTR were cultured: 1) without scaffolds, 2) encapsulated within a fibrin scaffold with a droplet morphology (*droplet*), 3) encapsulated within a fibrin scaffold that was mechanically flattened after being loaded with cells (*patch: encapsulated*), or 4) seeded on the surface of a pre-fabricated flat scaffold (*patch: surface*) (Fig. 1E). BLI of media samples collected 1, 3, and 6 days post-seeding showed diTR secretion was nearly equal in all groups, suggesting the shape of the scaffold, as well as encapsulation versus surface seeding had minimal impact on hMSC-diTR drug release. Based on ability of cytotoxic SCs to efficiently grow and release cytotoxic agents from fibrin scaffolds, we investigated a new strategy where fibrin is used to increase the efficacy of cytotoxic SC therapy for cancer. The fibrin matrix is designed to retain the cells within the post-surgical cavity yet permit tumor-homing migration and robust drug release to eradicate residual post-surgical cancer foci.

3.1.2 Fibrin encapsulation increases retention and persistence of hMSCs transplanted into the GBM cavity—

Effective SC therapies must persist in the post-operative GBM resection cavity long enough and in sufficient numbers to eradicate residual GBM foci. We used our mouse models of GBM resection to investigate the retention and persistence of engineered hMSCs transplanted into the GBM cavity with fibrin encapsulation or using standard direct injection (Fig. 2A). Established intracranial human GBM xenografts were surgically debulked using image-guided micro-surgery. hMSC-mCFLuc were embedded in fibrin patches and seeded into the cavity. Alternatively, equal numbers of hMSC-mCFLuc were directly injected into the walls of the surgical cavity in a subset of animals to mimic the scaffold-free transplant that is currently used in clinical GBM patient trials. Quantitative BLI 3 hrs post-implantation showed fibrin encapsulation increased the post-operative retention of hMSCs within the resection cavity by 54% compared to standard direct injection (Fig. 2B). Fibrin encapsulation also markedly increased the persistence of the hMSCs within the post-operative cavity from 10 days to more than 28 days (Fig. 2C–D). The fibrin matrix initially allowed the hMSC-mCFLuc to proliferate in the post-operative cavity, as the BLI signal increased 2.7-fold in 7 days post-transplant before gradually declining. In contrast, hMSC-mCFLuc directly injected into the post-operative cavity were rapidly cleared from the cavity with only 19% of cells remaining at 7 days post-transplant and complete clearance by day 10 (Fig. 2C–D). *Ex vivo* whole-brain fluorescent and BLI performed 21 days post-transplant confirmed the *in vivo* BLI. Significant photon emission was detected in the resection cavity of the fibrin-encapsulated hMSC-mCFLuc group, while no BLI signal was detected in brains where hMSC-mCFLuc were directly injected (Fig. 2E). Interestingly, whole-brain cross-sectional analysis showed the hMSC-mCFLuc signal extended beyond the boarder of the resection cavity, suggesting hMSCs migrated from the cavity into the parenchyma. This was confirmed by high-resolution fluorescent imaging of tissue sections, where numerous mCherry+ hMSCs were found to line the tissue adjacent to the resection cavity in fibrin-encapsulated brains (Fig. 2F). Directly injected hMSCs were detected in tissue sections, but the few cells were confined to a small single site (Fig. 2G). Together, these data provide the first demonstration that fibrin encapsulation improves the retention and survival of engineered stem cells into the GBM resection cavity, while allowing stem cells to migrate from the matrix into the parenchyma.

3.1.3 hMSCs migrate out of fibrin scaffolds and home to GBM cells—

The tumor-selective migration of stem cells is a critical component of their efficacy against GBM^{6,26}. As such, it is vital that any scaffold delivery system permit the migration of engineered stem cells from the scaffold to colocalize with GBM. To study the migration and tumorigenic homing of hMSCs seeded in fibrin scaffolds, we developed a novel strategy that combined time-lapse motion analysis with 3-dimensional (3-D) under-agarose migration systems (outlined in Fig. 3A). In this system, a tissue culture dish is filled with a 0.6% agarose solution to mimic the composition of the brain. A cavity is created in the agarose and hMSC-GFPFLuc in fibrin scaffolds are seeded into the cavity. Human U87-mCFLuc are seeded into a second cavity 500 µm away to establish a chemotactic signaling gradient. The system is placed in an incubator microscope and kinetic images are captured every 20

minutes for 64 hrs to define the migration of hMSCs as they move out of fibrin scaffolds through the agarose matrix towards the U87-mCFLuc cells.

Using this approach, time-lapse imaging showed that encapsulated hMSCs migrated out of the fibrin scaffolds and localized with co-cultured GBM cells in less than 64 hours (Fig. 3B and Suppl. Movie 1). Single cell analysis showed the migratory paths of the hMSCs were directed selectively towards the co-cultured GBM cells (Fig. 3C and Suppl. Movie 2), with few cells migrating in the opposite or lateral direction to the GBM cells (Fig. 3F). In contrast, few hMSCs migrated from the scaffold when GBM cells were not present (Fig. 3D and Suppl. Movie 3). Those cells that did exit the fibrin migrated in a random non-directed 360° pattern (Fig. 3E, F and Suppl. Movie 4). Quantitative analysis showed the presence of GBM cells increased the directionality of hMSC migration 4.6-fold, increased the displacement of hMSCs from the fibrin matrix 5-fold, and increase the velocity of hMSC migration from .32 $\mu\text{m}/\text{sec}$ to .35 $\mu\text{m}/\text{sec}$ (Fig. 3G). Together, these data suggest that fibrin scaffolds are permissive to tumoritropic hMSC migration.

3.2 Anti-tumor efficacy

3.2. Efficacy of therapeutic hMSCs in fibrin against 3-D human GBM spheroids

—The ideal cytotoxic SC therapy must release tumoricidal agents at levels sufficient to eradicate GBM cells despite fibrin encapsulation. We used a 3-D levitation cultured strategy to more accurately mimic therapy for solid GBM foci (Fig. 4A). GBM cells were labeled with magnetic material and cultured in levitation by a magnet placed over the culture dish to form 3-D human GBM spheroids. The GBM spheroids were co-cultured with hMSC-sTR or control hMSC-GFPRLuc encapsulated in fibrin and tumor killing was determined by kinetic optical imaging. BLI performed 48 hrs later revealed hMSC-sTR/fibrin reduced the viability of solid GBM spheroids by 92% (U87), 85% (LN229), and 80% (U251) compared to control-treated spheroids (Fig. 4 B–C). To determine the hMSC-sTR:GBMs ratio required to induced tumor cell death, we cultured human GBM cells with decreasing numbers of hMSC-sTR encapsulated in fibrin. Using cell viability assays, we detected statistically significant reductions in tumor viability at ratios as low as 0.1:1, however minimal killing was observed at ratios of 0.02:1 (Fig. 4D–E). To investigate fibrin-based delivery of hMSCs releasing different therapeutic agents, we performed co-culture assays utilizing hMSCs engineered with thymidine kinase (TK) enzyme/prodrug therapy. In this approach, the TK gene is inserted into hMSCs (hMSC-TK). Therapy is initiated by addition of the inactive pro-drug ganciclovir (GCV), which is converted to a toxic product by TK. This approach has been widely used in cell-based cancer therapy. As shown in figure 4F, fibrin/hMSC-TK therapy significantly reduced the viability of both U87 and U251 human GBM cells. Together, these findings suggest that 1) hMSC-sTR release cytotoxic agents from the fibrin at levels that reduce viability of human GBM spheroids, 2) there is minimum ratio of hMSC-sTR:GBM cell required for efficient tumor killing, and 3) fibrin-based transplant effectively delivers hMSCs releasing different cytotoxic agents..

3.2.2 Solid tumor therapy using cytotoxic hMSC/fibrin composites—Cytotoxic hMSC/fibrin therapy could be used to efficaciously treat solid tumors if the fibrin matrices bearing the therapeutic SCs could be implanted in close proximity to the established tumors.

To study the efficacy of fibrin-based cytotoxic hMSC therapy for solid tumors, U87-mCFLuc cells were xenografted into mice and allowed to grow for 10 days. hMSC-sTR or control hMSC-GFPRLuc were encapsulated in fibrin patches. A skin incision was made over the established tumor, and the cytotoxic hMSC/fibrin composite matrix was surgically implanted over each tumor (outlined in Fig. 4G). Serial BLI of tumor growth showed the control-treated tumor expanded rapidly, increasing in size 8-fold over the next 11 days (Fig. 4H–I). In contrast, hMSC-sTR/fibrin therapy attenuated tumor progression, resulting in tumors that were only 1.8% larger 11 days post-treatment (Fig. 4H–I). Together, these findings demonstrate that fibrin-encapsulated hMSC-sTR therapy markedly attenuates the progression of solid tumors.

3.2.3 Intracavity cytotoxic hMSC/fibrin therapy for post-surgical minimal GBM

—Surgical resection is part of the clinical standard-of-care for human GBM patients¹.

Therefore, we determined the impact of fibrin-encapsulated hMSC-sTR intracavity therapy on post-surgical minimal GBMs. Human GBM cells were xenografted into the parenchyma of mice. 1 week later, the tumors were surgically resected. Cytotoxic hMSC-sTR or control hMSC-GFPRLuc were encapsulated in fibrin patches and transplanted into the resection cavity (outlined in Fig. 5A). Intra-operative fluorescent imaging was used to reveal the location of the mCherry+ GBM, guide surgical resection, and confirm efficient seeding of hMSC-sTR/fibrin into the resection cavity (Fig. 5B). Serial BLI revealed rapid GBM recurrence in control-treated animals, with tumors increasing 110-fold in 18 days (Fig. 5C–D). In contrast, hMSC-sTR/fibrin therapy suppressed GBM recurrence 8-fold at 18 days after therapy. Survival analysis revealed the hMSC-sTR/fibrin tumor suppression allowed animals to survive more than 36 days after initial treatment (Fig. 5E). In contrast, control animals succumbed to recurrent GBMs only 15 days. Post-mortem tissue analysis revealed the presence of hMSC-sTR in the residual GBM microsatellite foci on the boarder of the GBM resection cavity (Fig. 5F). Taken together, our results strongly suggest that intracavity hMSC-sTR/fibrin therapy is a highly efficacious treatment for post-surgical minimal GBM.

4. Discussion

Thousands of patients die from brain cancer each year^{1,3}. Cytotoxic SC therapy is a promising new strategy capable of eradicating primary and invasive GBM foci in pre-clinical models^{5,6,27,28}. Developing a strategy capable of retaining cytotoxic SCs within the post-surgical cavity is essential to preventing GBM recurrence in eventual clinical trials. Fibrin is extensively used for hemostasis in human surgery, biocompatible, and an effective scaffold for regenerative medicine applications^{15,17,18,29,30}. Here, we exploit the beneficial properties of fibrin to create a new approach to cytotoxic SC therapy for GBM. We discovered that transplanting cytotoxic SCs in fibrin scaffolds significantly increased the initial retention and markedly prolonged the persistence (time to clearance) of the cells in the post-surgical GBM resection cavity. Yet, the fibrin matrix remained permissive to SC tumorotropic migration. *In vivo*, fibrin-based cytotoxic therapy reduced solid GBM, delayed post-surgical GBM recurrence, and prolonged survival of tumor bearing animals. Taken together, these findings reveal that transplanting cytotoxic SC in FDA-approved fibrin glue is a promising new strategy for treating post-surgical GBM.

In this study, we used TISSEEL as the fibrin matrix. This fibrin sealant has been extensively characterized due to its wide use in human patients to achieve hemostasis. In addition to the active ingredient fibrinogen and thrombin, the product contains aprotinin, a protease inhibitor that prevents premature breakdown of the matrix³¹. Studies have shown TISSEEL has a Young modulus of 15 kPa and tensile strength of 29 kPa³². Other fibrin matrices have been shown to be stronger and more resistant than TISSEEL, which has been attributed to the lack of fibrin-polymer crosslinking due to the absence of factor XIII from the TISSEEL formulation. However, studies have shown that TISSEEL is predicted to be fully absorbed within 14 days³¹. We utilized the TISSEEL according to manufacturer's suggestions and did not tune the fibrin matrix to alter rigidity, strength, or porosity. We found that the fibrin matrix did not interfere with the proliferation of cytotoxic SCs. Temporal analysis by both fluorescence and BLI imaging showed the rate of growth was not statistically different through 1 week between cytotoxic SCs that were grown on culture dishes or on fibrin matrices. Of equal importance, encapsulation of the cytotoxic SCs did not inhibit release of cytotoxic agents from the cells, even when the architecture of the scaffold was changed. This suggests fibrin encapsulation does not reduce the efficacy of tumoricidal SC therapy, and that it will be possible to create cytotoxic SC therapies from fibrin matrices of different shapes. Although our initial fibrin/thrombin ratios created a matrix with pores that allowed efficient drug release, this could be tuned even further by increasing or decreasing pore size by altering thrombin concentrations³³. A denser fibrin network could provide additional structure support, but reducing the pore size beyond a certain size will eventually prevent efficient drug release and tumor killing. Future studies will be required to elucidate the ideal fibrin structure for SCs engineered with different cytotoxic agents.

The ideal scaffold material for transplanting cytotoxic SCs in clinical GBM patient testing is unknown. A rapidly polymerizing matrix would shorten surgical time and eliminate the risk of physical washout of SCs. The matrix material should be biocompatible to avoid immune reaction and biostable to provide lasting structural support for the therapeutic SCs in the cavity. The material must support retention of SCs within the surgical cavity, yet allow therapeutic agents released from cytotoxic SCs to penetrate the matrix and induce killing in the GBM. Tumor-homing migration is a key advantage of SC therapy^{5,6}, and the matrix material should also permit the migration of SCs out of the matrix and into residual GBM foci. A pliable matrix would allow reshaping to determine the optimal matrix architecture that maximizes cytotoxic SC survival, drug release, and migration. Material with hemostatic properties would control post-surgical bleeding and potentially improve both SC survival and patient recovery¹⁶. Lastly, matrix material currently approved for use in human surgery would increase the clinical utility of this approach and increase the rate of translation into human patient testing.

For cytotoxic SC therapy, the transplant matrix must retain cytotoxic SCs within the surgical cavity, yet also permit tumorotropic migration to eradicate distant invasive GBM foci. To date, the "gold standard" for *in vitro* cell migration has been boyden chamber transwell assays^{9,34}. However, this method is severely limited in several ways. In particular, the transwell layout prevents serial imaging to kinetically track cell movement. Therefore, we combined under-agar assays with real-time kinetic imaging to establish a new approach to

track stem cell migration. In this approach, the use of agar allows us to establish a chemotactic gradient that more accurately mimics the *in vivo* scenario. Additionally, the ability to perform time-lapse imaging not only allows continuous visualization of cell movement over 2–3 days, but also allows determination of valuable kinetic information (directionality, displacement, and velocity of movement) that cannot be measured in transwell assays. Our imaging results showed the presence of human GBM cells drew hMSCs out of the fibrin matrix, as the SC migrated selectively to the cancer cells. This migration was directional, as few hMSCs migrated in opposite directions or lateral to the GBM. The presence of the GBM cells also increased the velocity of the hMSC migration. In the absence of GBM cells, the hMSCs remained primarily in the fibrin, slowly migrating out in a random 360-degree pattern. Although unexplored in the context of brain cancer, hMSCs are known to migrate out of fibrin matrices³⁵. Furthermore, altering the composition of the fibrin matrix significantly changes the migratory capacity of the cells. This suggests that fine-tuning the fibrin matrix could further improve the tumorotropic migration of the hMSCs to GBM cells. However, it is unclear whether altering the matrix to maximize migration will negatively impact the ability of the scaffold to retain SCs within the surgical cavity.

Retaining transplanted cytotoxic SCs within the post-surgical GBM cavity is an enormous challenge. We found that cytotoxic hMSCs within fibrin matrices increased initial cell retention 2-fold compared to traditional direct-injection methods. Additionally, more than 21-fold greater levels of hMSCs suspended in fibrin were present in the brain at day 7 when fibrin-based transplant was used, and these cells persisted markedly longer than cells delivered via direct injection. These findings were confirmed by post-mortem analysis. *Ex vivo* BLI and fluorescence imaging showed significantly greater numbers of fibrin-transplanted cells were present in the resection cavity compared to direct injection. Fluorescence analysis of brain sections revealed a significant number of fibrin-encapsulated hMSCs lined the walls of the resection cavity three weeks post-transplant, yet only a few residual cells remained in the direct injection group. These data suggest fibrin-based matrices improve the retention and persistence of cytotoxic SCs within the surgical cavity. Matrices and materials that improve cytotoxic SC retention within the GBM cavity are virtually unexplored. Previously, we found hyaluronic acid (HLA) hydrogels improve the retention of mouse neural stem cells delivered into the resection cavity¹⁴. However, the rapid gelation time of fibrin reduces the potential for cell washout and surgical time if intracavity polymerization is utilized. Additionally, the increased pliability of fibrin over the rigid HLA provides opportunities for new and potentially more effective scaffold architectures to be formed *ex vivo* or re-molding of pre-formed patches to conform to the unique architectures of different surgical cavities. Additionally, the hemostatic properties of the fibrin can aid in controlling post-operative bleeding, a benefit that cannot be achieved with the HLA. Importantly, TISSEEL is a FDA-approved matrix that is already used during human GBM patient surgery, a benefit that should expedite the translation of fibrin-based cytotoxic SC therapy into clinical trials.

We investigated the cytotoxic effects of fibrin-encapsulated hMSC therapy using 3-D cell culture models. In our unique approach, magnetic beads were used to create 3-D cell spheroids of different human GBM cells lines³⁶. These tumor spheroids were then

suspended adjacent to fibrin-encapsulated SCs releasing the pro-apoptotic agent TRAIL. Although 2-D cultures remain the mainstay of preclinical studies, 3-D models more accurately mimic the *in vivo* environment. We discovered that fibrin-based hMSC-sTR therapy reduced the viability of all three human GBM spheroids tested compared to control-treated spheroids, although the response was slightly varied across cell lines. We purposefully selected TRAIL because we have extensively demonstrated the anti-GBM effects of SC engineered with this secreted agent^{11,12,37–39}. This makes TRAIL ideal for use in characterizing the therapeutic efficacy of untested cell matrices such as fibrin. TRAIL induces killing by binding to death receptors present on the surface of tumor cells and triggers a well-characterized caspase-mediated apoptotic cell death⁴⁰. The fact that death receptors are present primarily on tumor cells provides high specificity for tumor killing and allows minimal toxicity to normal (non-cancerous) tissue in the brain. GBM is a heterogeneous disease and studies have demonstrated that the levels of death receptors vary in GBM cells. As the level of death receptor expression decreases, the response of GBM cells to TRAIL-induced cell death decreases. Therefore, the variability in the response of the U87, LN229, and U251 cells lines is due to differences in death receptor expression between the tumor lines^{11,41}. However, we observed that TRAIL therapy induced >80% killing in all three lines. This supports the conclusion that this approach would result in substantial tumor kill across multiple GBM cell lines. It is likely that different drug combinations will be needed to fully eradicate GBM in clinical trials. Future studies will focus on the development of fibrin-based transplant of new single-agent therapies as well as new multi-drug hMSC combination therapies.

In our study, fibrin-based transplant of cytotoxic SCs into the post-surgical GBM resection cavity suppressed GBM recurrence 5.4-fold in 3 weeks. However, more impressively, fibrin-based hMSC-sTR treatment prolonged the median survival of post-operative mice from 15 to 36 days. This suggests that transplanting cytotoxic hMSCs suspended in fibrin is an efficacious new approach for treating post-surgical GBM. Despite the robust tumor suppression and extensions in survival, the GBM still recurred. The mechanisms driving recurrence are unknown. Further development of the fibrin scaffold could improve persistence of the cytotoxic SCs by altering scaffold composition or architecture. The fibrin scaffolds could be used to deliver multi-drug hMSC therapies to target drug-resistant tumor populations. The ability of fibrin to encapsulate large numbers of cells suggests a higher initial dose of cytotoxic SCs could be delivered which could improve tumor killing, but increased dose could risk drug-related toxicities to non-cancer tissue. Fibrin itself has been extensively utilized as a drug depot, delivering numerous small molecule agents⁴². This could allow for new combination therapies delivered simultaneously from the scaffold and cytotoxic SCs. Previous studies have demonstrated the potential of fibrin-based delivery of small molecule drugs, such as temozolomide. However, similar to the clinically utilized polymer-based delivery system Gliadel, the efficacy of this approach is likely limited by reliance on the passive diffusion of the drug that is unable to reach invasive foci beyond 2cm from the resection cavity^{43,44}. The current approach uses fibrin to delivery tumoricidal SCs capable actively exiting the scaffolds and homing to distant GBM cells. This approach should improve killing of invasive tumor cells and improve treatment response, particularly for distant invasive GBM foci.

Although the results of this study are promising, there are several limitations. First, the optimal degradation rate of the fibrin matrix remains unknown. The fibrin must stabilize cytotoxic stem cells within the surgical cavity long enough to prevent surgery-induced clearance, yet must release the cells to allow accumulation in tumor foci and tumor kill. It is unclear whether a rapid degradation that allows cells to be quickly released into the brain parenchyma is more effective than a slow-degrading stable matrix. In addition, altering the matrix could influence the differentiation of the tumoricidal stem cells it is delivering, as stiffness of substrates has been shown to impact the outgrowth of neurites⁴⁵. We are actively exploring the impact of various degradation rates on cytotoxic stem cell migration, anti-tumor efficacy, and differentiation. Similarly, we are also exploring different strategies for delivering the fibrin/stem cell composition as fibrin spray is commonly used in the clinical setting and has been shown to efficiently deliver hMSCs⁴⁶. Secondly, these studies were performed using models of human GBM resection/recurrence in immune-depleted mice. This leaves the impact of the immune system unknown. Fibrin is extensively used in human patients where it is known to induce minimal immune activation. Yet, further studies exploring fibrin-based cytotoxic stem cell transplant in immune-competent hosts are required to fully define the immune activation associated with this approach and the impact this may have on treatment efficacy. Lastly, the small scale of the mouse brain leaves the question of scale-up unanswered. Large animal models of GBM resection/recurrence will be needed to determine the quantity, size, shape, and stem cell number required to effectively treat a human-scale surgical cavity in eventual human patient testing.

5. Conclusion

In conclusion, the findings of this study reveal that fibrin scaffolds are a promising new approach for intra-cavity cytotoxic SC therapy of surgically resected GBM. In this way, neurosurgeons could deliver the fibrin/cytotoxic stem cell solution onto the walls of the resection cavity following GBM removal in eventual human patient trials. The extensive clinical use of fibrin should ease the translation of these preclinical studies into patient trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2. Fibrin scaffolds improve the retention and persistence of engineered hMSCs transplanted into the postoperative surgical cavity

(A) Intra-operative images showing the seeding of engineered hMSCs encapsulated in fibrin matrices within the post-surgical cavity. Mice were immobilized on stereotactic devices (1) and an incision was made in the scalp (2). A craniotomy was performed to expose the underlying tumor (3) that was resected using image-guided microsurgery to create a resection cavity (4). Engineered hMSCs were seeded into the cavity in fibrin (5). The skin was then closed over the resection cavity (6). (B) Summary graph demonstrating that fibrin-based transplant increases the retention of hMSCs transplanted into the post-surgical cavity compared to traditional direct injection. Retention was determined by quantitative BLI to detect hMSC volumes 3 hrs after transplant. $*P < 0.05$ by Student's *t* test. (C–D) Representative images and summary data demonstrating hMSCs transplanted in the resection cavity in a fibrin matrix persist significantly longer than hMSCs delivered by direct injection. BLI images were captured longitudinally on days 0, 2, 4, 7, 10, 14, and 21 post-transplant to track the levels of transplanted hMSCs. $*P < 0.01$ by repeated measures ANOVA. (E) *Ex vivo* fluorescent and BLI imaging of brains 21 days after transplanting hMSC-mCFLuc into the resection cavity via fibrin or direct injection. A hMSC-mCFLuc signal was detected in the fibrin-transplanted animals in both the dorsal view and coronal cross-sections, but no signal was detected in the direct injection brains. (F–G) Representative fluorescent images showing the presence of hMSC-mCFLuc in the parenchyma following delivery into the resection cavity in fibrin (F) or by direct injection (G). Whole brain photo-stitched images are shown. * indicates the resection cavity. The

arrows indicate the small deposit of hMSC-mCFLuc present in the direct injection brain. Data in B and D are mean $\pm$ SEM. Scale bars in whole images in F and G are 800 μ m. Scale bars in magnified images of F and G are 100 μ m.

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Figure 3. Fibrin matrices are permissive to the tumoritropic migration of engineered hMSCs

The migration of fibrin-encapsulated stem cells to GBM cells was measured using time-lapse motion analysis and under-agar co-culture system. (A) Schematic depiction of the co-culture strategy. GFP+ stem cells were encapsulated in fibrin and seeded into one well of an agar-filled culture dish. mCherry+ U87 human GBM cells were seeded 500 μm away in an adjacent well. The cells were placed under a fluorescence microscope in an incubator and images were captured every 20 minutes for 64 hours. Image analysis software was then used to generate time-lapse movies, perform single-cell tracking, and quantify migratory direction, distance, and velocity. The imaging field is depicted by the dotted blue line. (B) Summary images showing hMSCs (green) migrate out of fibrin and home to co-cultured GBM cells (red). (C) Single-cell analysis depicting the migratory path of hMSCs from fibrin scaffolds as they home to GBM cells over time. Each line depicts the path of one cell. (D) Fluorescent images and single-cell analysis (E) showing the random motion of hMSCs in fibrin when cultured without GBM cells. (F) Rosetta graphs demonstrating the directed migration hMSCs out of fibrin scaffolds when GBM are present and the random migration of hMSCs from fibrin without GBM. (G) Summary graphs showing the directionality, displacement, and velocity of hMSCs in fibrin in the presence and absence (control) of human GBM cells. * $P < 0.05$ by Student's t test. Scale bars in B and D are 400 μm .

Figure 4. Transplanting cytotoxic hMSC/fibrin composites inhibit the progression of solid tumors

(A) The efficacy of hMSC-sTR encapsulated in fibrin for treatment of solid GBM was first determined using 3-D GBM spheroids and levitation culture. Human GBM cells were loaded with magnetic nanoparticles and seeded in 6-well plates. A magnetic driver was placed over the dish to levitate the GBM cells. Once spheroids were formed (24 hrs later), the spheroids were plated adjacent to hMSC-sTR or hMSC-GFPRLuc encapsulated in fibrin. Tumor spheroid viability was determined using FLuc BLI. (B–C) Representative images and summary graph demonstrating the significant reduction in the viability of different human GBM cell spheroids by hMSC-sTR/fibrin treatment compared to control-treated spheroids. $*P < 0.05$ by Student's *t* test. (D–E) Representative fluorescent images (D) and summary data (E) showing the viability of human GBM spheroids (red) cultured with increasing numbers of hMSC-sTR encapsulated in fibrin (green). The dotted line indicates the boarder f the fibrin matrix. $*P < 0.01$ by repeated measures ANOVA. (F) Representative images and summary data demonstrating the anti-tumor killing effects of hMSC-TK/GCV therapy (red) on U87 and U251 human tumor spheroids (green). The dotted line indicates the boarder of the fibrin matrix. $*P < 0.05$ by Student's *t* test. (G) To determine the efficacy of fibrin-based hMSC delivery on established tumors, U87-mCFLuc xenografts were created in mice. Fibrin patches were created bearing hMSC-sTR or control hMSC-GFPRLuc. An incision was made over the established tumor in mice (I), the pre-formed scaffold bearing the SCs were surgically implanted over each established tumor (2–3), and the skin incision was closed (4). (H–I) Serial bioluminescence imaging was used to follow the growth tumors in each treatment group. Significantly smaller tumor volumes were observed in hMSC-sTR-treated

tumors across multiple time points. $*P<0.01$ by repeated measures ANOVA. Data in C–F are mean $\pm$ SEM of 3 independent experiments performed in triplicate. Data in I are mean $\pm$ SEM. Scale bars in B is 400 μm .

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Figure 5. Cytotoxic hMSCs delivered into the resection cavity in fibrin delay re-growth of post-surgical residual GBM

(A) To mimic clinical fibrin-delivered stem cell therapy for surgically resected GBM, established intracranial human GBMs were surgically debulked in mice. Therapeutic stem cells were transplanted into the post-operative cavity in fibrin patches. (B) Representative images showing pre-resection mCherry+ GBMs, the post-surgical GBM cavity, and GFP+ hMSC-sTR in fibrin seeded in the resection cavity. The dotted line indicates the resection cavity. The arrowheads indicate residual GBM foci. (C-D) Representative images (C) and summary graph (D) of serial BLI showing a significant reduction in GBM regrowth in hMSC-sTR-treated animals compared to control-treated animals. Significantly smaller tumor volumes were observed in hMSC-sTR-treated tumors across multiple time points. * $P < 0.01$ by repeated measures ANOVA. (E) Kaplan-Meier survival analysis showing the survival of animals with resected GBMs treated with hMSC-sTR therapy or control hMSC-GFPRLuc. Median survival was 36 days for hMSC-sTR treated animals vs. 15 days for control-treated mice. * $P < 0.01$ by log-rank test. (F) Fluorescent images of post-mortem tissue sections showing the presence of GFP+ hMSCs (green) along the boarder of the recurrent GBM (red). Nuclei were counterstained with Hoechst (blue). Data in D are mean $\pm$ SEM. Scale bar in F is 100 μ m.